Notes

Peroxyoxalate Chemiluminescence Determination of Polyamines Using On-Line Combination of High-Performance Liquid Chromatography and an Immobilized Enzyme Column Reactor

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Polyamines such as putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm) are ubiquitous nitrogen compounds found in animals, plants and microorganisms. In plants, the polyamines play an important role in membrane stabilization, free radical scavenging and synthesis of DNA, RNA and protein.¹ The amount of polyamines in plants is informative about extent of the damage of plants by acid rain², or about the freshness of food.³ In clinical chemistry, the high urinary excretion of polyamines in cancer patients made them a candidate as tumor markers.

Consequently, many analytical methods have been developed for the sensitive determination of polyamines. In high-performance liquid chromatographic (HPLC) methods, UV detection with benzoylchloride^{2,4}, and fluorescence detection with dansyl chloride⁵, 9-fluorenylmethyl chloroformate⁶ and *o*-phthaldialdehyde⁷ have been reported. On the other hand, some methods utilizing an enzymic reaction have also been developed. In these, a flow injection analysis (FIA) method⁸ with luminol chemiluminescence (CL) detection, and HPLC methods with peroxyoxalate chemiluminescence (PO-CL)⁹ and an electrochemical detection¹⁰ are employed. Among these, HPLC method attained high sensitivity with PO-CL detection using putrescine oxidase (PUO) and diamine oxidase as immobilized enzyme column reactors (IMERs)⁹, but it did not include the separation of acetyl polyamines.

More recently, we have shown that the PO-CL method is suitable for highly sensitive determinations of hydrogen peroxide¹¹, oxidase activities¹² and substrates for oxidases.¹³ In this paper, a sensitive and selective determination of polyamines and their mono-acetyl derivatives will be described using an IMER co-immobilized with acetylpolyamine amidohydrolase

(APH) and PUO followed by an HPLC separation. Further, the method was applied to the determination of polyamines in plants to evaluate the applicability to the biological samples.

Experimental

Chemicals

Put, Cad, Spd, Spm, sodium azide and glutaraldehyde were obtained from Wako Pure Chemical Co. (Osaka, Japan). Acetyl putrescine (AcPut), acetyl cadaverine (AcCad), *N*₁-acetyl spermidine (*N*₁-AcSpd), *N*₈-acetyl spermidine (*N*₈-AcSpd) and acetyl spermine (AcSpm) used were from Sigma Chemical Co. (St. Louis, MO, USA). PUO from *pseudomonas* with 15 U/mg was obtained from Toyobo Co. (Tokyo, Japan) and APH from *mycoplasma bullata* with 12.5 U/mg was kindly donated by Kyowa Medics Co. (Tokyo, Japan). Aminopropyl-controlled pore glass (particle size, 125–177 µm; pore diameter, 500 Å; Pierce Chemicals Co., Rockford, IL, USA) was used to immobilize the enzymes. Sodium octanesulfonate (SOS) and bis(2,4,6-trichlorophenyl)oxalate (TCPO) were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and 2,4,6,8-tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine (TMP) was synthesized by our previous method.¹⁴ Water was deionized and passed through the Pure Line WL 21 P system (Yamato Scientific Co., Tokyo, Japan). The other chemicals used were of analytical reagent grade.

HPLC system

The HPLC system consists of three HPLC pumps (LC-6A, Shimadzu, Kyoto, Japan), a 7125 injector with 10-µl sample loop (Rheodyne, Cotati, CA, USA), a separation column (Asahipak ODP-50, 250×4.6 mm, i.d., 5 µm, Asahi Chemical Industry Co., Tokyo, Japan), a column

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Fig. 1 HPLC system used for the determination of polyamines. P, pump; I, injector with 10- μ l sample loop; IMER, immobilized enzyme column reactor (70 $\times$ 2 mm, i.d.); D, detector; M, mixing device; NC, noise cleaner; Rec, recorder; SC, system controller.

oven (CTO-6AS, Shimadzu), a CL detector (CLD-10A, Shimadzu), a system controller (SCL-6A, Shimadzu), a noise cleaner (Uni-1, Union Co., Gunma, Japan) and a recorder (FBR-1, Tosoh, Tokyo, Japan). A schematic diagram of the system is shown in Fig. 1. For the separation of polyamines, 0.1 M (eluent A) and 0.25 M (eluent B) Tris-HNO₃ buffer (pH 7.0), both containing 0.5 mM sodium azide and 2 mM SOS, were used as eluents. Eluent A was pumped for 22 min and then quickly changed to eluent B. Both eluents were flowed at 0.8 ml/min. A mixture of 0.4 mM TCPO and 1.0 μ M TMP in acetonitrile was used as a CL reagent and flowed at 1.0 ml/min.

Preparation of immobilized enzyme column reactor

The IMER was prepared by the glutaraldehyde method¹⁵ as follows; 100 mg of aminopropyl-controlled pore glass beads suspended in 5 ml of 2.5% glutaraldehyde aqueous solution were stirred for 1 h at room temperature and then washed three times with 5 ml of 50 mM phosphate buffer (pH 7.5). Then the glass beads were resuspended in 1 ml of the same buffer containing 5 mg of APH and PUO each, and allowed to stand for 12 h at 4°C. After removing the supernatant, the beads were washed three times with the phosphate buffer and packed in a stainless-steel column (70 $\times$ 2 mm, i.d.) by suction with a syringe. The IMER was kept in a refrigerator in 50 mM phosphate buffer (pH 7.5) until use.

Determination of polyamines in plants

Potatoes, mini-tomatoes and tomatoes were purchased from the market. As polyamines are known to be adsorbed on glass surfaces, polystyrene tubes were used to prepare and store their sample solutions. Sample plants were treated as follows for the measurement of polyamines. A sliced potato (6 g), a whole mini-tomato (ca. 16 g) and a cut tomato (ca. 20 g) were individually homogenized with 3 ml of water by a Waring blender at 15000 rpm for 5 min in an ice bath. The homogenate

spiked with standard polyamines was diluted with water to 15 ml for potato, 30 ml for mini-tomato and 40 ml for tomato. A 50- μ l of 0.5 M perchloric acid was added to a 950- μ l of diluted homogenate and mixed well; the resultant mixture was allowed to stand for 1 h in an ice bath. After centrifugation at 2000g for 10 min, a 250- μ l of supernatant was pipetted, neutralized with 15- μ l of 1.0 M NaOH and diluted with 235 μ l of eluent A. The resultant solution was passed through a membrane filter (0.45 μ m) and stored at -20°C until use. A 10- μ l portion of this solution was injected into the HPLC system.

Results and Discussion

Preparation of immobilized enzyme column reactor

At first, two types of columns immobilized with enzymes were tested: *i.e.*, an IMER co-immobilized with APH and PUO, and a tandem IMER immobilized with an individual enzyme. The CL intensity yielded by the former for each Cad, Spd, N₁-AcSpd and N₈-AcSpd was 3 times higher than the latter, and thus the IMER co-immobilized with APH and PUO was selected for further experiments. This result was similar to that obtained with cholesterol ester hydrolase and cholesterol oxidase.¹⁶ The stability of the co-immobilized IMER with APH and PUO was tentatively measured as a function of the peak height for AcPut. The peak heights were almost constant for 100 runs, and then gradually decreased; the peak height at the 250th run was about 80% of that of the initial run.

HPLC conditions

Three types of commercially available columns (ODS, C₈, aminopropyl-modified silica gel) were preliminarily examined to separate polyamines. Satisfactory separations could not be attained with these columns. Therefore, an ODP column, which was reported to give a good separation of some polyamines¹⁰, was used for further experiments with some modifications of the separation conditions. A 20 mM imidazole-HNO₃ buffer (pH 7.0), 0.2 M phosphate buffer (pH 7.7), 0.1 M Tris-HNO₃ buffer (pH 7.0) and 50 mM Veronal-HNO₃ buffer (pH 8.0) were examined as eluents. The imidazole and Veronal buffers, which are available for determining oxidase activities with PO-CL detection¹², could not separate polyamines. On the other hand, the Tris-HNO₃ buffer showed the best separation of polyamines, and gave stronger CL intensities than those with phosphate buffer. Since the effect of pH (ranging from 6.8 to 7.7) of 0.2 M Tris-HNO₃ buffer on the separation of polyamines was very small, pH 7.0 providing the maximal CL intensities was chosen. The addition of SOS to the eluent improved the separation of polyamines. The retention time was prolonged with increasing the SOS concentration from 0 to 5 mM; 2 mM SOS showing a good separation was chosen. Although 0.1 M Tris-HNO₃ buffer as an eluent showed the best separation, it took more than 150 min for the complete

Fig. 2 Chromatogram of the standard polyamines. Sample: Put, AcPut=250 pmol/injection; Cad, AcCad, Spd, N_1 -AcSpd and N_8 -AcSpd=500 pmol/injection; peaks: 1=AcPut, 2=AcCad, 3=Put, 4=Cad, 5= N_1 -AcSpd, 6= N_8 -AcSpd and 7=Spd. The arrow indicates the changing of the eluents. Sensitivity: 5.0 nAFS. Other HPLC conditions are as in the Experimental.

elution of the seven polyamines. By changing the Tris concentration instantly from 0.1 M to 0.25 M at 22 min after sample injection, the separation of polyamines was attained within 40 min. The retention times of the polyamines were as follows: 9.8 min for AcPut, 15.3 min for AcCad, 17.5 min for Put, 21.8 min for Cad, 29.5 min for N_1 -AcSpd, 32.8 min for N_8 -AcSpd and 35.5 min for Spd. The typical chromatogram of standard polyamines is shown in Fig. 2. As Spm and AcSpm were hard to convert to hydrogen peroxide because of the low specificity of PUO to Spm on the IMER, these compounds were excluded from the targets tentatively. Other polyamine oxidases should be used for the sensitive determination of Spm and AcSpm.

Since sodium azide can enhance PO-CL intensity¹⁷, the effect of its concentration on CL intensity in our system was examined; 0.5 mM sodium azide in the eluent gave the largest CL intensity without any effect on the separation of polyamines.

A mixture of TCPO and TMP as a CL reagent was used in this system because we showed that it gives an intense CL.¹² The effects of the concentrations of TCPO and TMP on CL intensities were examined. The CL intensities increased with an increase in the concentrations of both TCPO and TMP, but noise levels

also increased. The mixture of 0.5 mM TCPO and 1.0 μ M TMP giving the largest signal-to-noise ratio (S/N) was selected. The effect of flow rate of the CL reagent on CL intensity was also examined. The CL intensity increased with an increase in the flow rate from 0.6 to 1.4 ml/min; 1.0 ml/min showed the largest S/N and thus was selected. The temperature of a column oven, in which a separation column and an IMER were placed, was examined by changing the degree from 25 to 35°C. An increase in temperature had almost no effect on the separation of polyamines, but the maximum CL intensity was observed at 35°C.

Calibration curve, detection limit and precision

The good linearities of the relationships between the concentrations of polyamines and the CL intensities were obtained ($r > 0.989$) in the concentration range of 25–500 pmol for Put and AcPut, and 50–1000 pmol for Cad, AcCad, Spd, N_1 -AcSpd and N_8 -AcSpd. The equations obtained were as follows: $y = 0.127x - 0.183$ for Put, $y = 0.201x + 0.278$ for AcPut, $y = 0.042x - 0.055$ for Cad, $y = 0.030x - 0.850$ for AcCad, $y = 0.013x + 0.466$ for Spd, $y = 0.032x - 0.392$ for N_1 -AcSpd and $y = 0.017x - 0.786$ for N_8 -AcSpd. In these equations, x is the concentration of polyamine (pmol/10 μ l injection) and y is CL intensity (arbitrary unit). The detection limits were 5 pmol for Put, 2 pmol for AcPut, 15 pmol for Cad, 20 pmol for AcCad, 20 pmol for Spd, 12 pmol for N_1 -AcSpd and 25 pmol per injection for N_8 -AcSpd at S/N of 3. The sensitivity of the proposed method was 6 times higher than that of the FIA-luminol CL method⁸ with respect to Put, and comparable to that of the PO-CL method.⁹ The RSDs of CL intensities with 5 replicate measurements of 250 pmol Put and AcPut, and 500 pmol Cad, AcCad, Spd, N_1 -AcSpd and N_8 -AcSpd were 3.3, 2.8, 2.9, 4.6, 7.8, 6.6 and 7.8%, respectively.

Determination of polyamines in plants

The polyamine contents of potato, mini-tomato and tomato were determined by a standard addition method; three different amounts of standard polyamines were added to plants. Put (150–1500 nmol) and Spd (300–1500 nmol) were spiked for potato, Put (40–400 nmol) was spiked for mini-tomato and tomato. The determination of each sample was performed three times. The mean amounts of Put and Spd found in five potatoes were 121.5 ± 80.5 and 51.0 ± 24.3 nmol/g wet weight, respectively. These levels are in good agreement with those reported previously.¹⁸ In mini-tomato and tomato, only Put was detected; the amounts for each of five samples were 11.2 ± 3.4 and 8.2 ± 3.9 nmol/g wet weight, respectively. The RSDs for within-day ($n=3$), and between-day assays for a week ($n=3$) were determined for potato samples spiked with Put and Spd (125 and 250 nmol/g wet weight) as shown in Table 1. The accuracy of the method was also examined using potato samples spiked with known concentrations of standard polyamines (Table 2). The percentages of nominal were found to be $>93\%$. These results showed that the

Table 1 Between-day and within-day precisions of assays for polyamines spiked in potato

Compound	Amount added (nmol/g wet weight)	Within-day (n=3) RSD, %	Between-day (n=3) RSD, %
Put	125	5.1	7.6
	250	5.2	7.3
Spd	125	8.3	10.7
	250	5.9	6.5

Table 2 Accuracy of assay for polyamines spiked in potato

Compound	Amount added (nmol/g wet weight)	Amount found ^a (means±SD) (nmol/g wet weight)	Percentage of nominal, %
Put	125	111.5± 6.9	89.2
	250	231.3± 9.2	92.4
Spd	125	118.7±15.7	95.0
	250	236.8±27.4	94.7

a. n=4.

proposed method is suitable for the determination of polyamines. As the proposed method is simple, specific and sensitive, it should be useful for the determination of polyamines in other biological samples.

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