Assay of N^1-Acetylpolyamine Oxidase Activity with N^1,N^{11}-Didansylnorspermine as the Substrate by Ion-Pair Reversed Phase High Performance Liquid Chromatography

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An assay method to measure N^1-acetylpolyamine oxidase (PAO) activity with N^1,N^{11}-didansylnorspermine (DiDNS333) as the substrate by high performance liquid chromatography (HPLC) was developed. Dansylpolyamines were synthesized, and their activity as a substrate for partially purified PAO from rat liver was evaluated. Among the dansylpolyamines, DiDNS333 was a useful substrate for the development of the PAO assay method. DiDNS333 was degraded by PAO to 1-dansylamido-3-propanal (DNS3al) and N^1-dansylnorspermidine (DNS33). As DNS3al was separated into two peaks by HPLC of the assay mixture containing aminoguanidine, determination of DNS33 was used for the development of the assay method. When the assay method was applied to inhibition studies, the DNS33 peak on the chromatogram was consistently produced, and weak interference was found in the incubation with higher concentrations of natural polyamines. This result suggested that contaminating polyamines in biological samples do not interfere with this method. When the assay method was applied to cell extract from Chinese hamster ovary cell samples, the PAO activity, even at the low level in the cells, could easily be detected with as little as 10 μg of protein, which corresponds to 1×10^5 cells. This HPLC method is a rapid, sensitive and useful assay for the measurement of PAO activity.

Key words N^1-acetylpolyamine oxidase; N^1,N^{11}-didansylnorspermine; fluorescent HPLC; polyamine; Chinese hamster ovary cell

N^1-Acetylpolyamine oxidase (PAO) catalyzes the oxidative cleavage of natural substrates, N^1-acetylspermidine and N^1-acetylspermine (AcSpm), to liberate 3-acetamidopropanal and form putrescine (Put) and spermidine (Spd), respectively, with H_2O_2 production in the conversion pathway of polyamine with an inducible enzyme, spermidine/spermine N^1-acetyltransferase.[1—3] PAO is a flavoprotein located in the peroxisomes of animal cells, and its enzyme activity is abundantly expressed in nearly all vertebrate tissues.[1] Until several years ago, PAO was believed to be a constitutive enzyme with little is known about its physiological relevance. However, changes in PAO activity have been demonstrated in tissues during postnatal development or aging[4—6] and after treatment with certain drugs[7—9] or hormones,[10—13] confirming the inducibility of the enzyme. Recently, another polyamine catabolic enzyme was cloned as spermine oxidase (SMO) which oxidizes spermine (Spm) to Spd with H_2O_2 production and is inducible when cells are treated with anticancer drugs.[14,15] In order to elucidate the specific role of these oxidative enzymes, it is important to measure the enzyme activities separately.

Most studies that assay PAO activity have measured H_2O_2 production from AcSpm as the substrate[16]; however, SMO also produces H_2O_2 with Spd production from Spm, which might interfere with the measurement of PAO activity in Spm-containing samples. The detection of H_2O_2 formation is also subject to error due to the many other cellular peroxidases that can form this product and potentially interfere with the assay. Another assay procedure for PAO was developed using radioisotope-labeled polyamines as substrates,[17] but their use was limited due to radioisotope contamination. Other procedures were cumbersome, as they involved a lengthy dansylation of natural polyamine as the substrate and separation by HPLC[18] or post-column labeling with *o*-phthalaldehyde (OPA) and separation by HPLC.[19]

A recent study of PAO substrate specificities predicted that two anionic centers and a hydrophobic region are present in the active site of PAO in addition to the cleavage site of the acetamidopropyl group and that the catalytic reaction requires a positive charge at a three- or four-methylene chain interval from the cleavage site.[20]

In the present study, previous findings regarding the active site of PAO were used to develop an assay method for PAO activity by using N^1,N^{11}-didansylnorspermine (DiDNS333) as a substrate, and this method was applied to biological samples.

MATERIALS AND METHODS

Materials Dulbecco's minimum essential medium/Ham's F12 (1 : 1) and non-essential amino acids solution were purchased from Dainippon Sumitomo Pharma (Osaka, Japan), and fetal calf serum (FBS) was purchased from Nippon Biotest Laboratories (Tokyo, Japan). Diamine oxidase from porcine kidney (0.16 units/mg), spermidine trihydrochloride, aminoguanidine bicarbonate salt and antibiotics were purchased from Sigma-Aldrich (Tokyo, Japan). Trifluoroacetic acid and 1-octaneslufonic acid sodium salt were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Spermine tetrahydrochloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Polyoxyethylene lauryl ether (Brij 35) was purchased from Nacalai Tesque (Kyoto, Japan). Cation exchange resin MCI GEL CK10S was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan).

The following compounds were prepared according to the methods described previously[21—25]: N^1-Dansylspermine tetrahydrochloride (DNS343), N^1-dansylnorspermine tetrahydrochloride (DNS333), N^1,N^{12}-didansylspermine tetrahydro-

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chloride (DiDNS343), N^1,N^{11}-didansylnorspermine tetrahydrochloride (DiDNS333), N^1-dansylspermidine trihydrochloride (DNS34), N^1-dansylnorspermidine trihydrochloride (DNS33), N^1-acetyl-N^{11}-dansylnorspermine trihydrochloride (AcDNS333), N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine dihydrochloride (MDL72527), N^1,N^{11}-diethylnorspermine tetrahydrochloride (DENSPM), N^1,N^7-dihexylnorspermidine (DH33), N^1,N^{12}-diacetylspermine dihydrochloride (DASpm), N^1-acetylspermine trihydrochloride (AcSpm), and N^1-acetylspermidine dihydrochloride (AcSpd). All other reagents and organic solvents were of commercial analytical grade.

Synthesis of 1-Dansylamido-3-propanal (DNS3al) DNS3al was synthesized according to the method of Wu *et al.*[26] with modifications. Briefly, 1-amino-3,3-diethoxypropane (1.618 ml, 10 mmol), the diethyl acetal of 3-aminopropanal, and triethylamine (1.386 ml, 10 mmol) were dissolved in 30 ml methanol. While the solution was being stirred, dansyl chloride (2.7 g, 10 mmol) dissolved in 20 ml methanol was added dropwise. The dansyl derivative was subjected to silica gel column chromatography with a solvent system of hexane : ethylacetate to obtain a single band of 1-dansylamido-3,3′-diethoxypropane to yield 3.32 g (8.50 mmol, 85.0%). 1-Dansylamido-3,3-diethoxypropane (0.87 g, 2.29 mmol) was then de-protected by acetic acid solution (acetic acid : water = 1 : 2) to yield DNS3al (0.68 g, 96.9%). The purity was confirmed by TLC and FAB-MS.

Cell Culture Chinese hamster ovary (CHO) cells were a kind gift from Professor Lo Persson (Lund University, Lund, Sweden). CHO cells were routinely grown in Dulbecco's minimum essential medium/Ham's F12 (1 : 1) containing 10% FBS, non-essential amino acids, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO_2 and 95% air. Cells were seeded at 5×10^5 cells per 10-cm diameter petri dish. After 1 d of culture, medium containing 1 mM aminoguanidine and inhibitors (25 μM MDL72527, 10 μM DENSPM) was added to the monolayers. After incubation at 37 °C, the attached cells were washed three times with phosphate buffered saline (PBS), scraped, and harvested. Cell numbers were determined by the dye exclusion method with trypan blue in a haemocytometer. The cells were centrifuged and the resulting cell pellets were sonicated in sonication buffer (0.1 M Tris–HCl (pH 9.0) containing 0.02 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA)) and used for the PAO assay.

Partial Purification of PAO from Rat Liver PAO was partially purified from rat liver as previously described.[21] Rat livers were homogenized with four volumes of 0.25 M sucrose–10 mM Tris–HCl buffer (pH 7.4) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at $600\times\boldsymbol{g}$ for 10 min. The supernatant was centrifuged at $15000\times\boldsymbol{g}$ for 10 min, then the pellet was suspended in 0.1% Triton X-100 dissolved in 10 mM Tris–HCl buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 mM DTT (buffer A). The suspension was centrifuged at $25000\times\boldsymbol{g}$ for 30 min. The supernatant was dialyzed against buffer A. Crude extracts were then used for experiments.

PAO Assay with Ac343 The PAO activity was assayed by measuring the amount of Spd from AcSpm. The incubation mixture (final volume, 100 μl) contained the enzyme solution, 0.2 mM AcSpm, 0.56 mM aminoguanidine, 0.036 mM pargyline, 0.02 mM DTT and 1 mM EDTA in 0.1 M Tris–HCl buffer (pH 9.0). Mixtures were incubated for 10 min at 37 °C, and then the reaction was stopped by the addition of 100 μl of 20% trichloroacetic acid solution. The reaction mixtures were centrifuged and analyzed by ion-exchange HPLC with an OPA post column system.[27] The HPLC conditions were as follows: column, cation exchange resin. MCI GEL CK10S, 4.6 mm$\phi\times$100 mm; elution buffer, a mixture of 12.5% (v/v) of methanol and 87.5% (v/v) of 0.28 M sodium citrate buffer, pH 5.5, containing 2.0 M sodium chloride, 0.5 ml/min; column temperature, 65 °C; post column reagent solution, 6 mM OPA in 0.4 M potassium borate buffer, pH 10.4, containing 0.2% 2-mercaptoethanol, and 0.1% Brij 35, 0.25 ml/min at 65 °C; fluorescence detection, excitation 345 nm, emission 450 nm.

PAO Assay with DiDNS333 The PAO activity was assayed with DiDNS333. The incubation mixture (final volume, 100 μl) contained the PAO enzyme solution, 0.05 mM DiDNS333, 0.56 mM aminoguanidine, 0.036 mM pargyline, 0.02 mM DTT and 1 mM EDTA in 0.1 M Tris–HCl buffer (pH 9.0). Mixtures were incubated for 10 min at 37 °C, and then the reaction was stopped by the addition of 100 μl of 20% trichloroacetic acid solution. The reaction mixtures were centrifuged and analyzed by ion-pair reversed phase (RP-HPLC). The ion-pair RP-HPLC conditions were as follows: column, TOSOH ODS-80_{TM} (4.6 mm$\phi\times$150 mm); isocratic elution solution, methanol : water (75 : 25) containing 8 mM sodium octanesulfonate and 0.1% trifluoroacetic acid; flow rate, 1 ml/min; and fluorescence detection, Ex 333 nm and Em 544 nm. Aliquots of sample solution were injected, and the determination was performed based on the peak height.

Substrate Activity of Dansylpolyamines The substrate activity of dansylpolyamines was measured by substituting the substrate with 0.2 mM dansylpolyamine in the PAO assay with DiDNS333 described above. The ion-pair RP-HPLC conditions were changed from methanol : water (75 : 25) to methanol : water (50 : 50) for the measurement of the DNS33 release from AcDNS333 that was required for their separation.

Stabilities of Dansylpolyamine with Porcine Kidney Diamine Oxidase or FBS Containing Amine Oxidase To measure the stabilities of the dansylpolyamines, the substrates were substituted with dansylpolyamine in the PAO assay with DiDNS333 without amine oxidase inhibitors. Briefly, the incubation mixture (final volume, 100 μl) contained the enzyme solution (porcine kidney diamine oxidase or FBS), 0.05 mM dansylpolyamine, 0.02 mM DTT and 1 mM EDTA in 0.1 M Tris–HCl buffer (pH 9.0).

HPLC An HPLC apparatus was assembled using two pumps (LIQUID CHROMATOGRAPH LC-$10AD_{VP}$, Shimadzu, Kyoto, Japan), a degasser (DGU-10A, Shimadzu, Kyoto, Japan), an auto injector (SIL-$10AD_{VP}$, Shimadzu, Kyoto, Japan), a column oven (CTO-$10A_{VP}$, Shimadzu, Kyoto, Japan), a flurorescence detector (RF-$10A_{XL}$, Shimadzu, Kyoto, Japan), a system controller (SCL-$10A_{VP}$, Shimadzu, Kyoto, Japan), and an integrator (CHROMATOPAC C-R8A, Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

In a recent study of the active site of PAO based on the substrate specificities, it was predicted that two anionic centers and a hydrophobic region are present in the active site of PAO in addition to the cleavage site of the acetamidopropyl group and that the catalytic reaction requires a positive charge at a three- or four-methylene chain interval from the cleavage site.[20] Based on the predicted active site of PAO, a series of dansylpolyamines were synthesized and evaluated for substrate activity against PAO partially purified from rat liver. The dansyl group was selected because Bolkenius and Seiler reported that DNS343 was degraded by PAO to produce Spd.[28] Under the assay conditions (described in Materials and Methods), the products containing a dansyl group from dansylpolyamines were identified and determined by the ion-pair RP-HPLC. The results are summarized in Table 1. The common product of assay mixtures was DNS3al, which was separated into two peaks on the HPLC chromatogram (Fig. 1). When the DNS3al was mixed with the components of the incubation mixture, the appearance of two peaks was observed in the sample mixed with aminoguanidine (data not shown). This suggested that the appearance of two peaks was caused by the interaction between aldehyde group in DNS3al and hydrazine moiety in aminoguanidine.

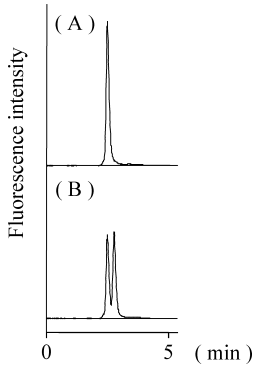
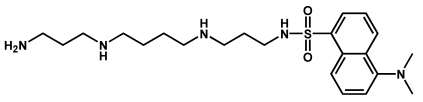
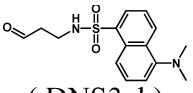
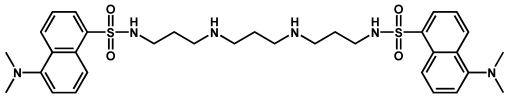
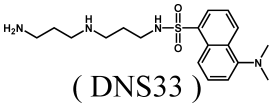
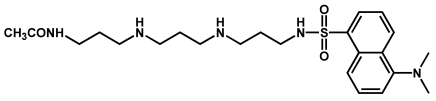
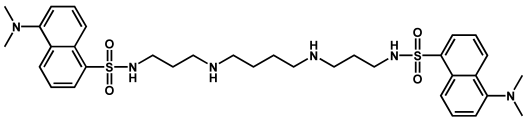
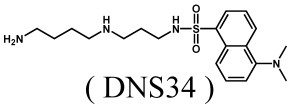
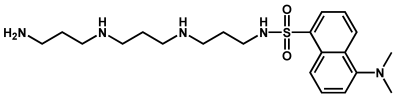
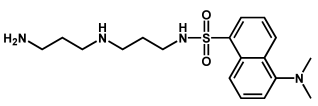


Fig. 1. Chromatograms of DNS3al in Different Solutions

The authentic DNS3al was dissolved in H_2O (A) or in the mixture containing 0.05 M Tris–HCl buffer (pH 9.0), 0.28 mM aminoguanidine, 0.018 mM pargyline, 0.01 mM DTT, 0.5 mM EDTA, 10% TCA (B).

Table 1. DNS3al Release from 0.2 mM Dansyl Polyamines by PAO from Rat Liver

Compound	Structure	Product(s)	% of DNS3al release from DNS343
DNS343		 (DNS3al)	100±1.5
DiDNS333		DNS3al &  (DNS33)	60±1.0
AcDNS333		DNS3al or DNS33	29±1.3
DiDNS343		DNS3al &  (DNS34)	24±1.1
DNS333		DNS3al	18±0.3
DNS33		Not detected	—

PAO from rat liver was incubated in the presence of 0.2 mM of the indicated dansyl polyamine as the substrate. The DNS3al was separated into two peak areas on the HPLC chromatogram. The DNS3al release was evaluated as the sum of the two peaks. The percent of DNS3al release from DNS343 was calculated, and the data are expressed as means±S.D. (n=3).

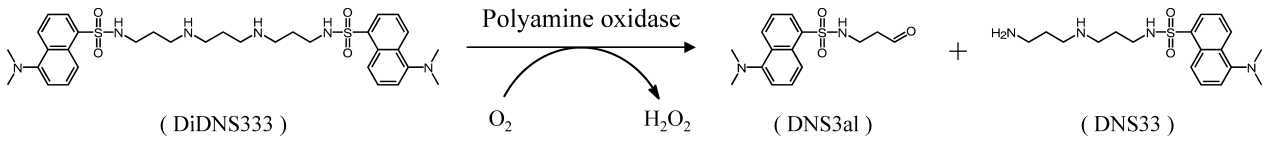


Chart 1. Degradation of DiDNS333 by PAO

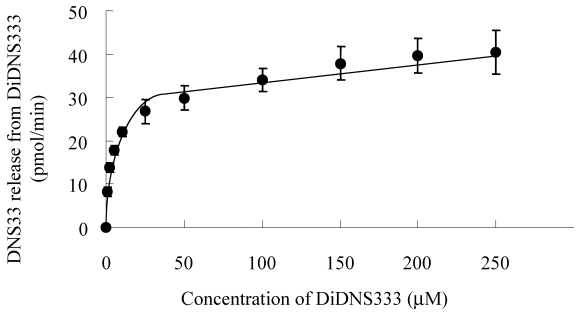


Fig. 2. Dose-Dependent Release of DNS33 from DiDNS333 with Partially Purified Rat Liver PAO

Data are expressed as mean±S.D. (n=3).

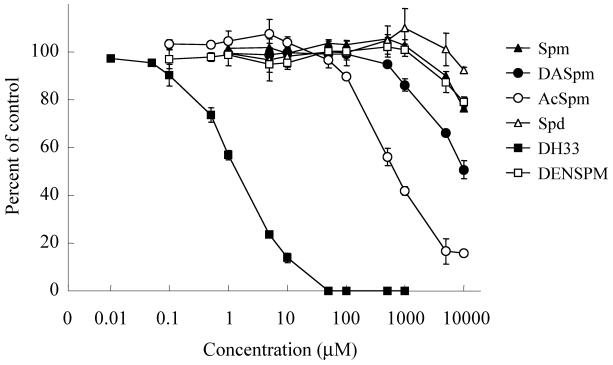


Fig. 3. Inhibition of PAO by Polyamines and Polyamine Analogs

DiDNS333 (50 μM) was incubated with rat liver PAO in the presence of the indicated concentrations of polyamines and polyamine analogs. Data are expressed as mean±S.D. (n=3).

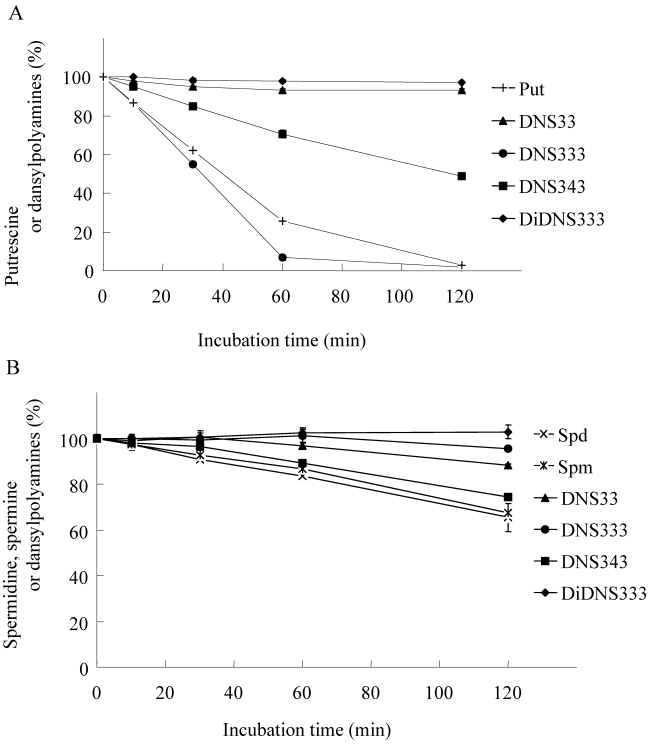


Fig. 4. Stability of Polyamines and Dansylpolyamines in the Assay Mixture without Amine Oxidase Inhibitors with Copper-Dependent Amine Oxidases

Fifty micromolars putrescine, DNS33, DNS333 or DNS343 were incubated with 0.05 units/ml of porcine kidney diamine oxidase in the incubation mixture without amine oxidase inhibitors (A). Fifty micromolars spermidine, spermine, DNS33, DNS333 or DNS343 were incubated with 20% (v/v) of the fetal bovine serum in the incubation mixture without amine oxidase inhibitors (B). These conditions were described in Materials and Methods. Data are expressed as mean±S.D. (n=3).

The other products were DNS33 from DiDNS333 or AcDNS333 and DNS34 from DiDNS343. The order of the preference of the DNS3al release evaluated by the sum of the two peaks was found to be: DNS343>DiDNS333>AcDNS333>DiDNS343>DNS333. Under the same conditions, DNS33 was not found to be efficiently oxidized. Incubation with MDL72527, an irreversible inhibitor of PAO, completely inhibited the DNS3al release from DNS343 and DNS3al and the DNS33 release from DiDNS333. Although DNS3al was the common product, the two separate peaks were not suitable to use for the development of the assay method. Thus, DiDNS333 was used for the following study.

Degradation of DiDNS333 by PAO partially purified from rat liver linearly produced DNS33 until 30 min, and the enzyme dose-dependent production of DNS33 from DiDNS333 was observed (Chart 1). Dose-dependent release of DNS33 from DiDNS333 with rat liver PAO was saturated (Fig. 2). The K_m value for DiDNS333 was 3.5 μM. A concentration of 50 μM of DiDNS333 was used for the development of the assay method. When the assay method was applied to the inhibition study with natural polyamines, Spm, Spd, AcSpm, AcSpd, DASpm, and polyamine analog, DENSPM, the production of DNS33 was inhibited at higher concentrations. As previously reported,[20] the competitive inhibitor of PAO, DH33, strongly inhibited PAO activity (Fig. 3). This result suggests that contaminating polyamines in the enzyme solution did not interfere with the assay method. Thus, the assay method was useful for the measurement of PAO activity in biological samples containing high amounts of polyamines.

Stabilities of the dansylpolyamines were assessed by incubation with different amine oxidases which are known to degrade polyamines in blood or intestinal tract (Fig. 4). Copper-dependent amine oxidase in FBS prefers Spd and Spm as the substrate. Diamine oxidase from porcine kidney prefers Put. Primary amine-containing dansylpolyamines were degraded by porcine kidney diamine oxidase and FBS; these degradations were completely prevented by the addition of aminoguanidine and pargyline (data not shown). Although DiDNS333 was not degraded in a manner similar to the previous reports with acetylpolyamines,[29] the amine oxidase inhibitors should be added to the assay mixture for the preven-

tion of DNS33 degradation.

DiDNS333 was also incubated in the assay conditions with recombinant human SMO in *Escherichia coli* extract. (The SMO/PAOh1 plasmid was a kind gift from Professor Robert A. Casero Jr., Johns Hopkins University School of Medicine, and Professor Kazuei Igarashi, Chiba University.) DNS33 was weakly produced (DNS33 release from DiDNS333 was 0.052±0.003 pmol/h/assay when Spd release from Spm was 229±17 pmol/h/assay). The DNS33 release from DiDNS333 by SMO was extremely low; therefore, it might not affect the PAO assay.

The assay method was applied to the measurement of the PAO activity in CHO cells, which have high PAO activity, as reported by Carper *et al.*[30] The quantitativities between the OPA post column labeling HPLC method with AcSpm as substrate[21] and the new method with DiDNS333 were compared with the same assay samples (Fig. 5). Spd release from AcSpm and DNS33 release from DiDNS333 were dose-dependently increased with CHO cell extracts, and well correlated with each other. The Spd production was approximately 100 times higher than DNS33 production. However, the detection limits of PAO activities in cell extracts were similar in both methods, because the detectable amount of DNS33 was more than 100 times lower than Spd. The new method also has a shorter analysis time than the OPA method (15 min *vs.* 60 min, respectively). The PAO activity, even at the low level in the cells, could easily be detected in as little as 10 μg of cell–extract protein, which corresponds to 1×10^5 cells, by the new method.

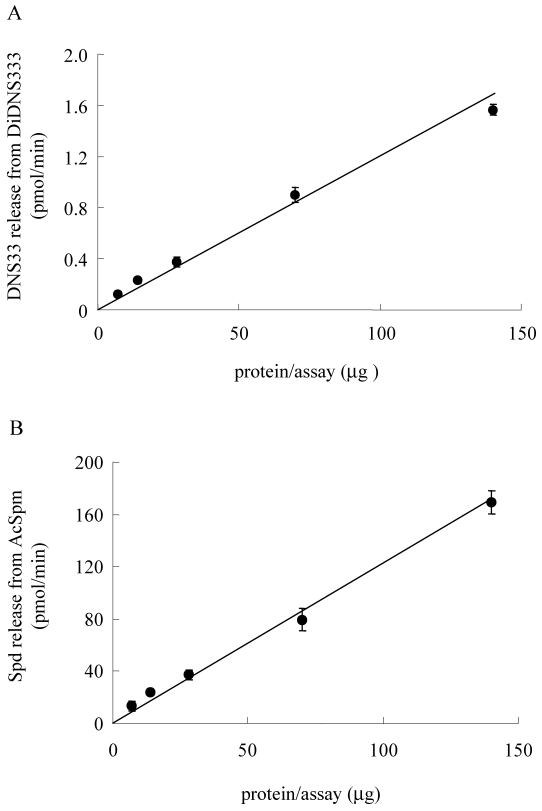


Fig. 5. Measurement of PAO Activity in CHO Cell Extracts by the New HPLC Method and OPA Post Column Labeling HPLC Method

The same extracts of CHO cells were used for comparison of the new HPLC method (A) and OPA post column labeling HPLC method (B). These conditions were described in Materials and Methods. Data are expressed as mean±S.D. (n=3).

Next, the new method was applied to CHO cell samples that were treated with DENSPM or MDL72527. One day after seeding, CHO cells were treated with 10 μM DENSPM or 25 μM MDL72527. Their growth was not different between the control and MDL72527-treated cells, but DENSPM treatment decreased their growth after 3 d (Fig. 6A).

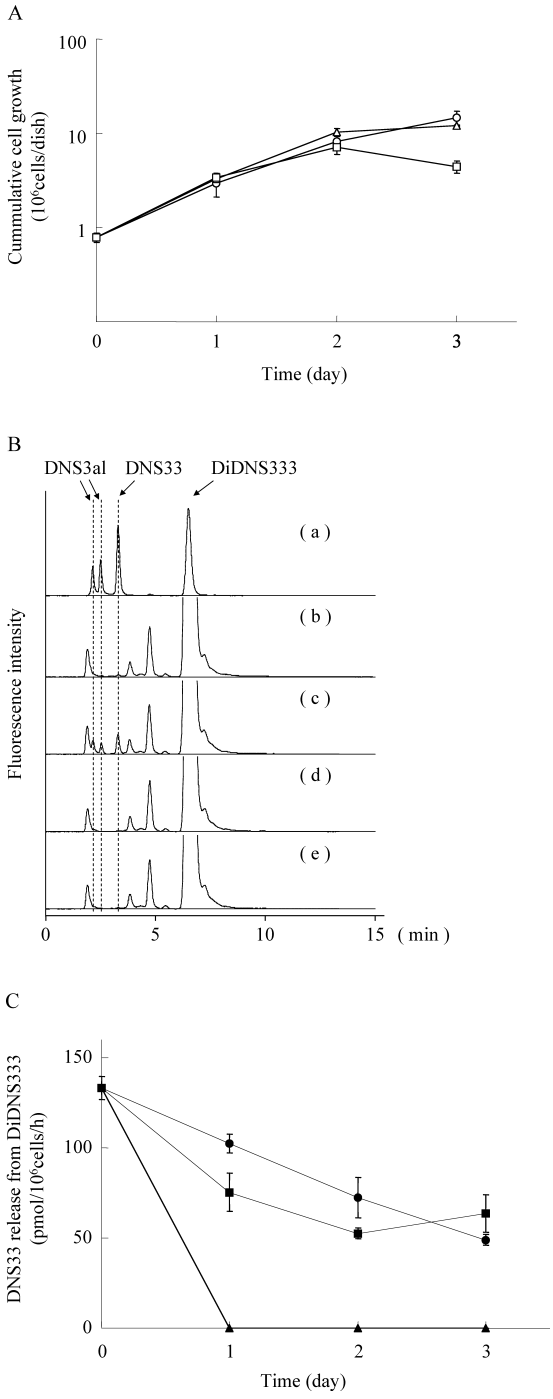


Fig. 6. Cell Growth and Polyamine Oxidase Assay in CHO Cells

(A) Cell growth. CHO cells were grown in the absence (○) or in the presence of 10 μM DENSPM (□) or 25 μM MDL72527 (△). Data are expressed as means±S.D. (n=3). (B) HPLC chromatograms of authentic standard sample and PAO-assayed cell samples. The authentic standards were dissolved in the mixture described in Fig. 1 (a) the PAO-assayed samples of control cells without (b) or with (c) 10 min incubation, and MDL72527-treated cells without (d) or with 10 min incubation (e). The assay conditions were described in Materials and Methods. (C) PAO activity in CHO cells. Cells were grown in the absence (●) or presence of 10 μM DENSPM (■) or 25 μM MDL72527 (▲). The cultured cells were used for the PAO assay. Data are expressed as means±S.D. (n=3).

The HPLC chromatograms of control cell sample and MDL72527 treated cell sample were shown in Fig. 6B. The PAO activity in the MDL72527-treated cells was significantly decreased after treatment. In the control cells PAO activity was slowly decreased during the cultures (Fig. 6C). This observation was similar to the report of Lawson *et al.*[31] In the DENSPM-treated cells, the activity decreased gradually and then increased. The increased PAO activity in DENSPM-treated cells paralleled the cell growth inhibition. Vujcic *et al.* reported[18] that K-293 cells treated with 10 μM DENSPM for 48 h exhibited a two-fold induction in PAO mRNA. There seems to be a similar response in CHO cells.

The present paper reports the development of a nonradioactive assay for PAO activity that uses HPLC and DiDNS333 as the substrate. This method is a rapid, sensitive and useful assay for the measurement of PAO activity and will contribute to PAO research in the future.

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