Purification and properties of a monoamine oxidase from

*Narcissus tazetta*

(スイセンにおけるモノアミン酸化酵素の精製と諸性質)
Purification and properties of a monoamine oxidase from 

*Narcissus tazetta*

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Chapter I

General introduction

Amines are low molecular weight organic bases which widely are distributed in plants, animals and microorganisms. They are generally divided into monoamines, diamines and polyamines, and are shown to play important roles in cellular metabolism, so that they are needed for growth, development and their metabolism that appear to be coordinated with the cell cycle (Alain and Pierre 2000). Amine production have been associated with the protective mechanisms of microorganisms against an acidic environment (Arena and Manca 2001). In higher plants and animals, biogenic amines are important nitrogenous compounds that are involved in significant physiological functions. If these compounds are consumed in high quantities, they can give rise to different alterations in the organisms (Docherty and Green 2010). These amines can be formed and degraded as a result of normal metabolic activity in organisms.

Monoamines are observed in live organs of animals, plants and fungi (Dannenburg and Liverman 1957, Gibson and Wightman 1972, Smith 1977a). In animals, monoamines modulate the activity of many neurons which is a
prerequisite for normal brain functioning (Flugge et al. 2004). Their sympathetic neurotransmitters are the catecholamines dopamine, noradrenaline and adrenaline, the indoleamine serotonin (5-hydroxytryptamine), and histamine. 5-Hydroxytryptamine is found in the stinging hairs of Mucuna pruriens, Girardinia heterophylla, Urtica spp, and probably also Luportea moroides (Bowden et al. 1954, Saxena et al. 1966, Robertson and Macfarlane 1957, Regula et al. 1972). It is considered to be one of the active principles in these stings, and is found also in the venoms of several animals (Welsh 1970). In a recent study, 5-hydroxytryptamine is a monoamine serving as a chemical messenger in diverse brain regions, as well as in blood and various other organs of mammal (Sharanappa and Adrienne 2011). The catecholamines, dopamine, norepinephrine and epinephrine, are a group of biogenic amines possessing a substituted 3, 4-dihydroxy phenyl ring that extensively exist in the animal kingdom, but they have also been detected in plants (Smith 1977b, Szopa et al. 2001). The role of catecholamines in plants is poorly documented, but it is clear that they are involved in many aspects of growth and development. The amines were proposed as precursors for various alkaloids (Guinaudeau et al. 1993, Smith 1980). They are associated with processes such as ethylene production, nitrogen
fixation, defense against herbivores, flowering, prevention of 3-indole acetic acid oxidation and gibberellins signaling (Dai et al. 1993, Kuklin and Conger 1995). Together with several neuronal and hormonal systems, monoaminergic neurons are activated by stress in vertebrates to induce various physiological and behavioral changes (McEwen 2000). In monoamines, dopamine and 5-hydroxytryptamine act a part in regulation of mood and cognitive functions, and their function is associated with a range of psychopathological conditions, particularly with mood and anxiety disorders in mammals. A lot of experimental procedures can be employed to investigate a role of monoamine neurotransmission in psychopathology (Blier and De 1998, Nutt et al. 1999). Ingestion of a mass of the tryptamines may be detrimental to humans and to some animals. For instance, there may be a connection between the incidence of myocardial fibrosis in West Africa and the high intake of plantains (bananas, Musa) in this region, as a result of the high level of 5-hydroxytryptamine in this fruit. In Australia, cattle fed on pastures (Phalaris arundinacea) develop a disease known as Phalaris staggers. This disease is probably due to the high levels of tryptamines of the pastures (Smith 1977c). Amounts of noradrenaline and dopamine are reported in venous and arterial plasma origins of animals (Bell 1988). Dopamine which consists of
about 1-5% of the total catecholamine pool within noradrenergic neurons is involved in stress response that is of paramount importance for both clinical and preclinical research (Ungless and Argilli 2010, Cabib and Puglisi-Allegra 2012). Similar to animal cells in which catecholamines stimulate glycogen mobilization, an alike role for catecholamines in the regulation of plant carbohydrate metabolism was suggested. Transgenic plants over-expressing tyrosine decarboxylase, which controls an important step of catecholamine synthesis were characterized by highly increased concentrations of norepinephrine and soluble sugars, whereas starch level was remarkably decreased. The changes indicate a positive correlation of norepinephrine with soluble sugars and a negative correlation with starch (Widrych et al. 2004).

Polyamines ubiquitously distribute in all cells. Despite the proved necessity of their presence for normal cellular growth in both prokaryotic and eukaryotic cells, their molecular functions remain unknown (Tabors 1984, Moschou et al. 2012). Depending on species, the relative intercellular concentrations of the polyamines are different, for that they can get high concentrations, up to the millimolar range (Miyamoto et al. 1993). In polyamines, putrescine, spermidine and spermine are among the major polycations in cells, together with Ca$^{2+}$ and
Mg$^{2+}$ that can bind to intracellular polyanions such as nucleic acids and ATP to modulate their function. As an important property of these specific polyamines, it is known that they are positively charged at a physiological pH and consequently they have a high affection to negatively charged cellular molecules. Therefore polyamines are very soluble in water, and they exert invigorative cation-anion interactions with macromolecules, mainly with DNA and RNA (Marczynski 1985). Thus a feature represents their best-known direct physiological role in cellular functions such as cell growth, division and differentiation (Heby 1981, Jänne et al. 1978). The polyamine content of cells is regulated by biosynthesis, degradation and transport (Igarashi et al. 1999). In bacteria, it has been known that the polyamines can modulate diverse cellular processes, including transcription and translation. However, a detailed understanding of the mechanisms remains unclear. Miscellaneous additional functions of bacterial polyamines have also been uncovered. The amines that are part of outer membranes of Gram-negative bacteria are involved in the biosynthesis of siderophores, take part in acid resistance, protect from oxygen toxicity, play a role in signaling for cellular differentiation, and are essential for plaque biofilm formation (Takatsuka and Kamio 2004, Brickman and Armstrong 1996, Foster 2004, Jung et al. 2003,
Putrescine, spermidine and spermine are formed and degraded in cells via the ODC or ADC pathway named for the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). In addition, decarboxylated S-adenosylmethionine as the donor molecule, putrescine utilization and γ-aminobutyric acid are currently known in bacteria and archaea (Foster 2004, Bowman et al. 1973, Tait 1976, Kurihara et al. 2010). In animals, polyamines have an important role in cell differentiation and proliferation, and their catabolism contributes to important physiopathological processes such as apoptosis, amine detoxification and cell signaling through both regulation of polyamine levels and their oxidatively deaminated reaction products that are mainly H$_2$O$_2$, aminoaldehydes or dialdehyde and acrolein (Averill-Bates et al. 2008, Agostinelli et al. 2009). Furthermore, polyamine synthesis is down-regulated as cells become senescent in many tissues of adults. Administration of spermidine markedly extends the lifespan of yeast, flies and their worms, and human immune cells. This treatment triggers epigenetic deacetylation of histone H3 through inhibition of histone acetyltransferase, suppressing oxidative stress and necrosis. The altered acetylation status of chromatin triggers autophagy, which is crucial for enhancing longevity (Eisenberg...
et al. 2009). In animal cells, uptake of polyamines can increase during hormonal stimulation and cell proliferation (Kakinuma et al. 1988). It has been demonstrated that polyamines can also induce programmed cell death in various animal cell types (Wallace et al. 2003, Igarashi and Kashiwagi 2010), thus indicating a bivalent function for these molecules, promoting both cell growth and cell death, likely depending on their concentration and other developmental and environmental signals (Wallace et al. 2003, Toninello et al. 2006). It has been suggested that plants had acquired a part of the polyamine biosynthetic pathway from an ancestral cyanobacterial precursor of the chloroplast (Illingworth et al. 2003). Consequently, it can be supposed that this is an ancient metabolic route in plants, which is also present in all organisms (Minguet et al. 2008). Polyamines are considered as plant growth regulating compounds; among them, cadaverine has been correlated with root growth promotion or osmotic stress mitigation in some plant species. (Cassan et al. 2009) In plants, polyamines have been implicated in many physiological processes, such as organogenesis, embryogenesis, Xoral initiation and its development, leaf senescence, fruit development and its ripening, and abiotic and biotic plant stress responses (Galston and Kaur-Sawhney 1990, Kumar et al. 1997, Walden et al. 1997, Malmberg et al. 1998, Bouchereau et al.)
The polyamines, Put, Spd, Spm, and thermospermine are major sinks of assimilated N, due to their high intracellular concentrations. The nitrogenous groups of polyamines along with their carbon scaffold are efficiently recycled and re-assimilated in various biochemical reactions. A major node of the recycling pathway is polyamine catabolism, which recycles both C and N, producing hydrogen peroxide. Polyamines are nitrogenous molecules which play a well-established role in most cellular processes during growth and development under physiological or biotic/abiotic stress conditions (Moschou et al. 2012). The complexity of polyamine metabolism and the multitude of compensatory mechanisms that are invoked to maintain polyamine homoeostasis argue that these amines are critical to cell survival (Wallace et al. 2003). The studies indicate that the intracellular levels of polyamines in plants are mostly regulated by anabolic and catabolic processes, as well as by those conjugation to hydroxycinnamic acids. Schematic representation of these processes and their interactions with other metabolic pathways is depicted in Fig.1 modified from Alcázar et al. (2010).

Amine oxidases (AOs) are found in the nature widely. AOs represent a class of enzymes that are heterogeneous in terms of structure, catalytic and
substrate-oxidation mechanisms. To classify AOs on the basis of their sensitivity towards specific inhibitors or with respect to their affinity towards particular substrates could not be satisfied adequately. Therefore a first general classification division according to the nature of the prosthetic group contained in the enzyme molecule was operated to decide. On the basis of this criterion, AOs are divided into two classes: FAD-containing AOs and Cu-containing AOs. However, in this way, there is a limit to the classification. Therefore, AOs, particularly the copper-dependent enzymes, must in some cases be subdivided in relation to their substrate specificity. Thus, FAD-AOs are also subdivided in mitochondrial monoamine oxidase (MAO) and cytosolic polyamine oxidase (PAO).

Amines are oxidatively deaminated by AOs in a reaction consuming O₂ and H₂O, and producing the corresponding aldehyde removed amine moiety, hydrogen peroxide and NH₄⁺, in stoichiometric amounts. The general equation of amine oxidation by AOs is the following equation:

(1) Cleavage at a primary amino group (terminal-oxidative reaction, catalyzed by Cu–AOs or MAOs)

\[
R-\text{CH}_2-\text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow R-\text{CHO} + \text{NH}_3 + \text{H}_2\text{O}_2
\]
(2) Cleavage at the secondary amino group (interconversion reaction, catalysed by PAOs)

\[
R_1\text{-CH}_2\text{-NH}\text{-CH}_2\text{-R}_2 + O_2 + H_2O \rightarrow R_1\text{-CHO} + R_2\text{-CH}_2\text{-NH}_2 + H_2O_2
\]

As mentioned above, one class of AOs containing FAD as prosthetic group, and in turn, ones are sub-divided into MAOs (A and B) and PAO which catalyzes the oxidation of polyamines at the secondary amino group and participates in the interconversion metabolism of polyamines. PAOs oxidize spermidine, N1-acetylspermine (and N1-acetylspermidine, but not spermine. On the other hand, in vertebrates spermine is directly oxidized by the cytosolic enzyme spermine oxidase, a flavoprotein characterized in the past as a human PAO (Wang et al. 2001) and then subsequently named SMO (Vujcic et al. 2002, Cervelli et al. 2003). Sperminie oxidation leads to the production of spermidine, 3-aminopropanal and hydrogen peroxide. While N1-acetylspermine and N1-acetylspermidine are oxidized by the peroxisomal FAD-dependent enzyme N1-acetylPAO to produce respectively spermidine and putrescine, 3-acetoaminopropanal and H2O2. These polyamine catabolic enzymes have been extensively characterized and it is well documented that both enzymes play an essential role in maintaining vertebrate polyamine homeostasis, which is

MAOs are enzymes that are tightly bound to a component of the mitochondrial outer membranes. The enzymes are classified into two forms, the MAO-A and MAO-B, based on their specificity to the substrates and specific inhibitors in animals (Abell and Kwan 2001). Both of them covalently bind to one cofactor molecule: one FAD per 63 kDa (MAO-A sub-unit) and one FAD per 57 kDa (MAO-B sub-unit). Both MAO-A and -B sequences contain the pentapeptide ‘Serine-Glycine-Glycine-Cysteine- Tyrosine’ in which the FAD cofactor is covalently bound to the cysteine (Cys) via a thioether link to one methyl of the isoalloxazine ring. In addition to the covalent FAD binding site (Cys-406 in MAO-A and Cys-397 in MAO-B), one cysteine (Cys-374) plays an important role in MAO-A catalytic activity whereas two cysteines (Cys-156 and Cys-365) are thought to be important for MAO-B activity (Wouters 1998). In the case of MAO, oxidation of the substrates is coupled to the reduction of FAD (Margherita 2001). In general, MAOs oxidize the primary amino groups of arylalkyl amines and are widely distributed in higher eukaryotes. In mammals, MAO A and B are separate gene products and exhibit 70% amino acid sequence identity. A more clear-cut
distinction is in terms of sensitivity to certain acetylenic inhibitors: MAO A is irreversibly inhibited by very low concentrations of clorgyline, but is unaffected by low concentrations of deprenyl and pargyline. The reverse is true of MAO B. Certain tissues contain only MAO-A (e.g., human placenta), others only MAO-B (e.g., blood platelets), but many tissues, like brain, contain both MAO-A and MAO-B in varying proportions. Both MAO-A and -B contain the same covalent amino acid adduct of FAD, 8a-5-cisteinyl FAD, and the peptide sequence at the flavin sites is also identical (Singer 1985, Kearney et al. 1971). Both plant PAO and human MAO-B have been recently structurally characterized by X ray crystallography (Binda et al. 1999, 2002). In this class of FAD enzymes, the flavin moiety is central to both substrate dehydrogenation and reduction of O₂ to H₂O₂. The folding of the PAO amino acid chain, relative to that of MAO-B, is crucial in defining their relative substrate binding sites and these differences account for their respective substrate diversities.

On the other class, Cu-AOs are only active on primary amino groups and contain, in addition to metal, a second organic cofactor, which reacts with semicarbazide and phenylhydrazine. This organic cofactor was identified as trihydroxyphenylalanine quinone (TPQ) (Janes et al. 1990, Klinman et al. 1991),
produced by the copper self-catalyzed posttranslational oxidation of a tyrosine residue. The catalytic reaction follows a ping-pong mechanism, consisting of enzyme reduction by the amine and re-oxidation by molecular oxygen (see Eq. (1)). Cu-AOs proteins have been implicated in leukocyte adhesion and glucose uptake, and their activities altered have been associated with inflammatory bowel disease, chronic liver disease, and diabetes (O’Sullivan et al 2004).

Cu-AOs have been found in many different organisms ranging from bacteria to mammals. In bacteria, the Cu-AOs have a well-defined role in the metabolism of primary amines as alternate sources of carbon and nitrogen to support growth. In contrast, the role of Cu-AOs in higher organisms has not been clearly established, although they have been implicated as key components in complex processes such as leukocyte trafficking involving the Cu-AO, vascular adhesion protein-1 (Salmi and Jalkanen 2002). Despite these functional differences, Cu-AOs from every known source are dimers, having subunit masses ranging from 70 to 95kDa, with 33 fully conserved residues near the catalytic site, and share fundamentally identical chemistry.

In plants, Cu-AOs have been detected in rice (Chaudhuri and Ghosh 1984), barley (Cogoni et al. 1990), maize (Suzuki and Hagiwara 1993), millet (Awal and
Hirasawa 1995), white lupine (Siepaïo and Meunier 1995), and were isolated from several sources, but only some of them, i.e. *Pisum sativum* (Kumar et al. 1996), *Lens esculenta* (Medda et al. 1995), *Triticum aestivum* (Suzuki 1996), *Euphorbia characias* (Padiglia et al. 1998) and *Vigna radiate* (Choudhary et al. 1999) have been highly purified and characterized. It is interesting to note that *Hordeum vulgare* and *Zea mays* contain both Cu-AO (Cogoni et al. 1990, Suzuki and Hagiwara 1993) and FAD-AO (Smith and Bickley 1974, Suzuki and Yanagisawa 1980). The term “SSAO”, semicarbazide-sensitive AOs, can easily be found in reference to AOs containing a carbonyl group in the cofactor. Cu-AOs usually have a similar molecular organization of their strongly interacting subunits (Klinman et al. 1991). The active site, consisting of one Cu$^{2+}$ and one TPQ, connected by a water molecule, is located inside each subunit and communicates with the solvent through a hydrophobic channel. In the last few years the structure of several Cu-AOs has been solved by X ray crystallography and cloned (Brazeau et al. 2004). Crystal structure of bovine serum AO was recently published (Lunelli et al. 2005). Under anaerobic conditions, the TPQ bond is fully cleaved in both bovine serum AO and lentil *Lens culinaris* AO, but the reaction rates are different. In lentil AO, a fast release of aldehyde occurs and the formed Cu$^{2+}$-quinolamine is
in rapid equilibrium with a radical intermediate and designated 
Cu\(^{+}\)-semiquinolamine (Bellelli \textit{et al.} 1991), while bovine serum AO forms the 
Cu\(^{2+}\)-quinolamine at a slower rate. No radical in this enzyme is detected in the 
UV/VIS spectrum, and the very low intensity signal detected in the EPR spectrum 
does not appear to be related to the catalytic reaction (Pietrangeli \textit{et al.} 2000).
These results indicate that the reaction mechanism and the role of copper have not 
yet been definitively defined (Morpurgo 2001, Pietrangeli \textit{et al.} 2003). However, 
enzymes from different sources may behave in a different way: bovine serum AO 
and lentil AO offer an example. The titration of TPQ with phenylhydrazine shows 
the binding of one mole of inhibitors per dimer in bovine serum AO, and one mole 
per subunit in plant AOs. The lower value was thought to arise from half of the site 
reactivity (Biase \textit{et al.} 1996). The reaction of oxygen appears to be different for 
plant and mammalian enzymes, i.e., copper is involved in lentil AO redox 
reactions, while bovine serum AO appears to have a structural function and/or a 
Lewis acid role (De Matteis \textit{et al.} 1999).

In this paper, I report a detailed study on the new MAO in \textit{Narcissus tazetta}. The 
objectives of this doctoral research are,

a) To find out the better method to purify MAO from the seedlings
homogeneity of *Narcissus tazetta*.

b) To characterized the properties of MAO of *Narcissus tazetta* and compare its properties with other AOs. And to examine if the enzyme is involves in flower-scent producing pathways.
Chapter II

Purification and molecular analysis of a monoamine oxidase isolated from Narcissus tazetta

Introduction

AOs have been divided into two categories depending on the cofactor, FAD or a copper-containing quinone. Enzymes containing FAD (EC.1.4.3.3) are further subdivided into MAOs and PAOs, depending on substrate specificity. In the second category, AOs (EC 1.4.3.6) are characterized by the presence of TPQ or lysyl tyrosylquinone (Mure 2004), cupric Cu-containing quinone cofactors. The copper AOs include DAO and semicarbazide-sensitive, mammalian MAO. In plants, there are two well-established types of AOs: copper-containing DAOs that act on the primary amine of many amine compounds, and flavin-containing PAOs that act function on the secondary amine of polyamines (Smith 1991, Suzuki et al. 1990). Plant DAO is one of the best known enzymes involved in amine catabolism. Spermidine and spermine are specific substrates for PAO. Additionally, MAO activity has been reported in several plants (Werle et al. 1950, Tsushida and Takeo 1985). In the plant species reported, Tsushida and
Takeo used tea leaves to purify an AO that catalyzes the oxidation reaction on monoamines including some alkylamines, ethanolamine, and benzylamine, although this last was not classified as an MAO (Tsushida and Takeo 1985). The cofactor in the tea-derived AO was not identified. Recently we reported a semicarbazide-insensitive MAO from *Avena sativa* (Zhang et al. 2012). This FAD-containing enzyme had high substrate specificity for benzylamine and 2-phenethylamine, which are oxidized to benzaldehyde and 2-phenylacetaldehyde respectively. Many aromatic and flavor compounds including fatty acid derivatives, benzenoids, and isoprenoids are found in many parts of the plant (Knudsen et al. 1993). The flavor volatiles 2-phenylacetaldehyde and 2-phenylethanol are presumably produced *in vivo* from 2-phenethylamine by an AO, dehydrogenase, or transaminase in tomatoes, although the plant genes responsible for the synthesis of the benzenoids have not yet been identified (Tieman et al. 2006). AO activity has yet to be detected in the fragrant flowers of plant species examined in our laboratory, but it has been reported that AO activity was detected in the flowers of *Narcissus tazetta* (Suzuki Y, personal communication). In this study, we purified an AO from *Narcissus tazetta* and examined its properties to determine whether it is involved in the
flower-scent-producing pathway.
Materials and Methods

Plant material

Flowering shoots of narcissus (*Narcissus tazetta* L. var. chinensis) were purchased from Kansaikako (Osaka, Japan). The shoots were used for enzyme purification.

Chemicals

The procedures of MAO purification have utilized several columns. Sepharose G-25, DEAE Sephacel and Phenyl Sepharose were obtained from GE Healthcare and hydroxylapatite was obtained from Nacalai, Japan. Lower molecular marker proteins and bovine serum albumin (BSA) were purchased from Takara, Japan. Other chemicals for pure grade were purchased from Wako, Japan.

Enzyme activity and concentrations

MAO activity at the purification steps and in the other experiments was assayed by spectrophotometric detection of the H$_2$O$_2$ produced in a reaction mixture by the modification of the quinoneimine dye method (Awal and Hirasawa 1995). The standard assay mixture contained 0.67 mM propylamine, 36 mM K-Pi buffer (pH 7.0), 0.33 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylamine (Dojin

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Laboratories, Kumamoto, Japan), 0.33 mM 4-aminoantipyrine, and 3.3 purpurogallin units of horseradish-peroxidase (Wako Pure Chemical, Osaka, Japan) and enzyme solution in a total volume of 3 mL. The reaction was initiated by the addition of the amine. The mixture was incubated at 37°C, and enzyme activity was determined by the linear increase in the absorbance at 555 nm for 5 min from initiation of the reaction.

The optimum pH of the assay mixture was determined at 37°C using acetate-NaOH buffer (pH 4.0, 4.5, 5.0, and 5.5), phosphate-KOH buffer (pH 6.0, 6.5, 7.0, and 7.5), and Tris-HCl buffer (pH 8.0, 8.5, and 9.0). Enzyme activity was expressed in katal (kat, mol s\(^{-1}\)).

**Enzyme purification**

All procedures were carried out at 4°C. Shoots of the narcissus plant (100 g) were ground in a Waring blender (MX-151S, Panasonic, Osaka) with 5 volumes of 100 mM K-Pi buffer (pH 7.0). The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 10,000 \(\times\) g for 25 min. The supernatant (500 mL) was supplemented with solid (NH\(_4\))\(_2\)SO\(_4\) to 65% saturation and incubated for 30 min. The precipitate obtained by centrifugation of the
supernatant at 10,000 × g for 25 min was dissolved in 75 mL of 20 mM K-Pi buffer (pH 7.0), yielding buffer A, and centrifuged again at 10,000 × g for 25 min. The supernatant was applied to a Sephadex G-25 column (3.5 × 35 cm) equilibrated with buffer A, and then eluted with the same buffer to remove (NH₄)₂SO₄. The eluted protein fraction (70 mL) was heated at 60°C for 5 min in plastic tubes using a metal-block heater. The heated solution was centrifuged at 12,000 × g for 10 min, and the supernatant was applied to a DEAE-Sephacel (GE Healthcare, Little Chalfont, UK) column (2.5 × 25 cm) equilibrated with buffer A and eluted with the same buffer. The active fractions were collected and applied to a hydroxyapatite (Nacalai Tesque, Kyoto, Japan) column (2.0 × 15 cm) equilibrated with buffer A and washed with the same buffer. The bound enzyme was eluted with 100 mL of a linear gradient of buffer A to 0.8 M K-Pi buffer (pH 7.0). The active fractions were pooled (20 mL) and concentrated to 4 mL using an Ultracel-100K centrifugal filter (Millipore Billerica, MA). The concentrated fraction was applied to a phenyl-Sepharose (GE Healthcare) column (1.5 × 5 cm) equilibrated with 0.8 M (NH₄)₂SO₄ in buffer A and then washed with 0.4 M (NH₄)₂SO₄ in buffer A. The bound enzyme was eluted stepwise with 100 mM and then with 50 mM (NH₄)₂SO₄ in buffer A. The active fractions eluted were
through buffer A Ultracel-100K centrifugal filter. The flowthrough fraction from the Ultracel-100K filter was applied to an Amicon Ultracel-30K centrifugal filter. The concentrated fraction was washed with buffer A on the Ultracel-30K filter by centrifugation. The purified MAO preparation was stored at −20°C in 25% (v/v) glycerol in buffer A.

The protein concentration of the pooled fraction from each purification step was estimated by the micro-assay method of Bradford (1976) with bovine serum albumin as the standard.

**Polyacrylamide gel electrophoresis**

Electrophoresis was performed under native and denatured conditions in an electrophoresis unit. Native polyacrylamide gel electrophoresis (PAGE) was carried out using a 7.5% (w/v) gel without SDS by the method described by Davis (Davis 1964). Propylamine-active staining on the native PAGE was used with the assay solution described above in “Materials and Methods” SDS-PAGE was done using a 15% (w/v) gel by the method described by Laemmli (Laemmli 1970). Full-Range Rainbow Molecular Weight Markers (GE Healthcare) were used as protein standards.
Determination of propionaldehyde

HPLC was performed to measure the propionaldehyde produced in the reacted mixture. The mixture contained 0.67 mM propylamine, 33 mM K-Pi buffer (pH 7.0), and the purified MAO solution in a total volume of 3 mL. An aliquot (100 μL) of the reaction mixture was added to 1.0 mL of 0.25 mM 4-/(N,N-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoazadizole (DBD-H; Tokyo Chemical Industry, Tokyo) in 70% acetonitrile and 0.05% trifluoroacetic acid (TFA). After 2 h, the DBD-H solution was centrifuged at 10,000 × g for 10 min, and the supernatant was injected into an HPLC sampler. Reversephase HPLC on a system composed of a pump (LC-10AD), a spectrofluorometer (RF-535, excitation 450 nm and emission 565 nm, Shimadzu Co, Kyoto, Japan), and a column of COSMOSIL C_{18}-PAQ (4.6 mm i.d. 9.25 cm; Nakarai Tesque, Kyoto, Japan) was used. The mobile phase was 70% acetonitrile and 0.05% TFA at a flow rate of 1 mL min⁻¹. The column temperature was maintained at 30°C.

Determination of molecular mass using a GS-520 column

GS-520 HQ column (Shodex Asahipak, 7.8 × 300 mm) was used to determine the molecular mass of the narcissus MAO. The column was calibrated by the
following protein standards: ovalbumin, α-glucosidase (yeast), lactate dehydrogenase (yeast), glucose oxidase (*Aspergillus niger*), and ferritin type I (horse spleen). The purified enzyme and protein standards were applied to a column equilibrated with 0.1 M K-Pi buffer (pH 7.0) containing 0.3 M NaCl and then eluted with the same buffer.

**Substrate specificity and inhibitor efficacy**

Monoamines, diamines, and polyamines were used to determine the substrate specificity of the enzyme using a standard assay mixture containing 0.66 mM of the substrate. The inhibitors of human MAOs, pargyline and clorgyline, and those of Cu-AOs, o-phenanthroline, 2,2′-bipyridyl, 2-bromoethylamine, and semicarbazide, were employed using the standard assay to assess their effects on the catalytic function of the narcissus MAO. The enzyme was pre-incubated with 1.0 or 0.1 mM of each inhibitor for 5 min before activity was measured. The $K_m$ values of some monoamines were determined from double reciprocal plots.

**Cofactor identification**

To determine copper and manganese content by atomic absorption analysis, an X5 inductive coupled plasma-mass spectrometer (ICP-MS, Bruker, Billerica,
MA) was used. The spectrophotometric nitroblue tetrazolium (NBT)/glycinate test and quinone staining on PVDF membrane after SDS-PAGE were conducted as described by Paz et al. (1991).

**Protein sequence analysis**

Amino acid sequencing of the narcissus MAO was carried out by the Edman degradation method by the Protein Sequence System (ABI Procise 491 HT, Praha, Czech) using an Immobilon 0.45 μm PVDF membrane (Millipore, Bedford, MA). The protein sample was separated by SDS-PAGE, and the protein bands were transferred to the membrane by a semi-dry electroblotting method. Two areas, corresponding to 65 kDa and 75 kDa, were excised from the membrane and analyzed individually by the Protein Sequence System.
Results and Discussion

Purification of the narcissus MAO

AO activity was detected in all organs, especially the leaves, stems, and flowers in the shoots (data not shown). The enzyme was purified from whole shoots by (NH₄)₂SO₄ fractionation, heat treatment, DEAE-Sephasel, hydroxyapatite, and phenyl-Sepharose column chromatography. Table 1 summarizes the steps involved in the purification procedures for the enzyme and the purification results when the process begins with approximately 100 g fresh weight of shoots. Minimal enzymatic activity was detected in the supernatant after the first centrifugation in the purification procedures. We postulated that small molecular material in the supernatant would disturb the enzyme assay system. In fact, when the supernatant was dialyzed against 20 mM K-Pi buffer (pH 7.0), activity was detectable. The activity of the crude extract displayed in Table 1 represents the use of the dialyzed enzyme solution in the assay method. The enzymatic activity eluted from the G-25 column was stable up to 10 min at 60°C, and approximately 67% of the protein within the desalted fraction was denatured by the heat treatment. The ensuing purification steps were accomplished using DEAE-Sephasel, hydroxyapatite, and phenyl-Sepharose.
columns. As Table 1 shows, 500 mL of homogenate, which was purified to 20% yield from the crude ground extract, contained 37.8 nkat of total MAO activity. The specific activity of the purified MAO was 81.85 nkat mg\(^{-1}\) protein, and a purification factor of 1,544-fold was obtained. The purified enzyme was applied to a gel filtration column (GS-520 HQ), and this resulted in a single peak as observed in the column-elution profile (data not shown). The enzyme was subjected to native PAGE analysis. The gel was divided into two portions after electrophoresis. One portion was stained with Coomassie Brilliant Blue G-250, and the other was stained at 37°C in enzyme-active staining (see “Materials and Methods”). A single band migrated to the same site in both areas of the gel (Fig. 3a), indicating that the enzyme was purified to homogeneity and that the active-stained band is an MAO protein.

**Determination of molecular mass**

SDS-PAGE of the phenyl-Sepharose column-purified enzyme revealed two protein bands (molecular masses of 75 kDa and 65 kDa) (Fig.3b). Many studies indicate that Cu-DAOs in plants are typically homodimers (Medda *et al.* 1995). Tea AO is also a homodimer, with a molecular mass of 80 kDa per subunit (Tsushida and Takeo 1985). The product of GS-520 HQ column chromatography
had an apparent molecular mass of 135 kDa (Fig. 4). The two bands visualized on SDS-PAGE, which represented narcissus MAO, indicated that the narcissus-derived enzyme is comprised of two different subunits, suggesting a heterodimer. There is another possibility that a 75kDa-homodimer of MAO was partially cleaved by an endogenous protease(s) during purification. However, despite the addition of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and 1 mM EDTA) to the buffers used in enzyme purification, SDS-PAGE of MAO purified by the method with inhibitors gave the same results as Fig. 3b (data not shown).

**Determination of other enzymatic parameters**

When the enzyme solution was pre-incubated for 10 min at temperatures ranging from 50 to 80°C to determine heat stability, the purified enzyme retained its activity at temperatures of 70°C, but lost activity quickly after pre-incubation at temperatures of 75°C (Fig. 2). These results indicate that purified MAO is stable at up to 70°C, which differs from the heat stability of the MAO in desalted solution after (NH₄)₂SO₄ fractionation. The difference in heat stability as between MAO in the desalted fraction and purified MAO might have been due to the proteases in the fraction. For comparison, tea AO is stable at up to 60°C.
(Tsushida and Takeo1985), not 70°C as for the narcissus MAO. Additionally, the optimum pH of the narcissus MAO was 7.0 (Fig. 5), similar to that of other Cu-AOs (Suzuki et al. 1990).

**Substrate specificity and inhibitor efficacy**

The substrate specificity of the enzyme is summarized in Table 2. The enzyme catalyzed the oxidation of aliphatic and aromatic short-chain monoamines. *N*-Propylamine was found to be the best substrate for the amines. Other short-aliphatic monoamines, ethanolamine and benzylamine, were also good substrates for the enzyme. By HPLC, propionaldehyde was detected stoichiometrically, and the mixture produced hydrogen peroxide when propylamine was incubated with the enzyme (Fig. 6). I concluded from these results that the enzyme from *Narcissus tazetta* was an MAO. The $K_m$ values for n-propylamine and benzylamine were $5.9 \times 10^{-5}$ M and $4.1 \times 10^{-4}$ M respectively. The enzyme did not catalyze the oxidation of hydroxylated benzylamines, diamines, or polyamines. It was not inhibited by 1 mM pargyline or clorgyline, both of which are potent selective inhibitors of MAOs in mammals (Table 3). Semicarbazide, an inhibitor of enzymes having a quinone cofactor, inhibited the narcissus-derived enzyme. The enzyme was also inhibited by a metal-chelating
reagent, 2, 2′-bipyridyl. In the inhibition experiments, diethyldithiocarbamate and phenylhydrazine were not available for use owing to their ability to inhibit peroxidase activity in the enzyme assay system. These results for substrate specificity and inhibitory effects indicate that narcissus MAO is comparable to tea AO (Tsushida and Takeo 1985), and is similar to semicarbazide-sensitive AOs in mammals. Although the cofactor of the tea AO was not identified, it appears to be a copper- and quinone- containing enzyme based on the effects of inhibitors. The narcissus MAO was irreversibly inhibited by 2-bromoethylamine with an inhibition constant ($K_i$) of 0.65 μM. Medda et al. indicated that 2-bromoethylamine (an irreversible inhibitor of lentil DAO) is a poor substrate and combines directly with TPQ in the enzyme (Medda et al. 1995). This strong, specific inhibitor may be useful as a probe in searching for physiological roles of narcissus MAO, especially for any involvement related to fragrant aldehydes in the narcissus flower.

**Cofactor Identification**

The copper and manganese contents of a purified MAO from *Narcissus tazetta* were measured by an ICP-MS. The concentrations of copper and manganese in the purified enzyme were estimated to be 54.37 ppb (Cu) and 2.61 ppb.
(Mn)/0.3419 μM of the protein. Then, the narcissus MAO was found to be a copper-containing protein having 2.45 atoms of copper per mole of protein. AOs have been divided into two categories depending on their cofactor, FAD or Cu plus quinone. The narcissus MAO, the latter type of AO, appears to contain two copper atoms in a narcissus MAO molecule. In addition, the Mn content of the protein was calculated to be 0.14 atom of manganese per mole of protein. In general, Cu-AOs contain one copper atom per subunit (Paz et al. 1991). Fenugreek Cu-AO contains Mn, and its contents is 0.2 moles per mole of native enzyme as determined by atomic absorption spectrometry (Šebela et al. 1997). When manganese ions were added to the reaction mixture for MAO, enzyme activity was accelerated to 122% (Table 3), but the catalytic importance of manganese ions is obscure.

The NBT/glycinate method was used as a specific stain to detect quinoproteins. Narcissus MAO protein after SDS-PAGE was electroblotted onto a PVDF membrane. Figure 8, panel A displays staining of the mammalian quinoprotein, the purified narcissus MAO (lane 2), bovine serum AO (lane 3), and pig kidney AO (lane 4). Panel B in Figure 8 shows the electroblot seen in panel A counterstained red for protein with Ponceau S. The MAO consists of a
heterodimer containing 75-kDa and 65-kDa subunits (Fig. 3b). As panel B (lane 2) shows two bands were stained by NTB/glycinate in the same 65 kDa and 75 kDa positions. These results indicate that the 65 kDa and 75 kDa subunits were quinoproteins. Based on the other copper AOs, the quinone in the narcissus MAO may be TPQ (Suzuki et al. 2009). The oxidized form of the AOs has a distinctive pink color at about 470-490 nm due to the presence of TPQ. The absorbance at 490 nm was 0.29 in lentil DAO solution (6.9 x 10^{-5} M enzyme c) (Medda et al. 1995) and 0.05 in tea MAO solution (8.6 x 10^{-5} M) (Tsushida and Takeo 1985). The spectrum of pink was shifted to that of yellow at 346, 432, and 462 after the addition of putrescine (Medda et al. 1995). No obvious spectrum at about 470-490 nm was detectable in the narcissus enzyme solution (6.8 x 10^{-7} M). The amount of 0.092 mg of the purified enzyme must have been too little for detection of absorption spectrum. In the future, large amounts of enzyme will be necessary to examine electric charge of copper by electron paramagnetic resonance.

**Amino acid sequencing of narcissus MAO**

The N-terminal amino acid sequence of the two subunits of the narcissus MAO was determined by the Edman degradation method. Two identified 13 residues
from each subunit of narcissus MAO were neither identical to partial sequences of any protein reported elsewhere nor similar to any amino acid sequence of leguminous DAOs or gramineous PAOs. While the N-terminal 13 residues of the 65 kDa subunit of the MAO showed fair homology (77% identity) to a predicted protein of AO from barley (Matsumoto et al. 2011), the 75 kDa subunit had no homology anywhere with the barley AO (Fig. 7). A comparison of the quinone-containing sequences with the gene-encoded sequences indicated that the precursor of the cofactor was a specific Tyr residue occurring in a highly conserved sequence: Asn-Tyr (TPQ)-Asp/Glu-Tyr (Jones et al. 1992). Researchers should examine hereafter the sequencing of the two monomers to determine whether narcissus MAO has two different monomers containing TPQ.

In mammals, three main functions have been postulated concerning the biological importance of Cu-AOs: to remove biologically active molecules such as primary amines; to form corresponding aldehydes that in some cases are known to affect cell differentiation, proliferation, and survival dramatically; and to produce hydrogen peroxide as a signal molecule rather than a harmful side product on-site (Boyce et al. 2009). In prokaryotes and fungi, AOs allow for the use of primary amines as sole sources of nitrogen for growth. In Aspergillus
*niger*, two AOs were induced in cells in a growth medium containing benzylamine as nitrogen source (Schilling and Lerch 1995). In plants, enzymes containing DAOs are important to the synthesis of alkaloids, cell-wall formation, and wound healing (Medda et al. 2009), but no physiological role of plant MAOs has yet been clearly defined. In a previous paper, a FAD-containing MAO (EC.1.4.3.4) purified from oat seedlings was suggested to have a catabolic role in the oxidation of phenethylamine, resulting in the decarboxylation of phenylalanine, which results in the hydrolysis of storage proteins in oat grain during germination (Zhang et al. 2011). The narcissus MAO has broad substrate specificity for short-chained aliphatic and aromatic monoamines and exerts no activity on any diamines or polyamines. MAO, a key enzyme in the rice sl-mutant, oxidizes tryptamine, which is biosynthesized from tryptophan by tryptophan decarboxylase (Ueno et al. 2003). Tryptamine-induced leaf lesions were significantly suppressed in the presence of semicarbazide, but narcissus MAO, despite having similar inhibitory properties, did not oxidize tryptamine (Table 2). Tea AO, similar to narcissus AO, is thought to have a role in the catabolism of ethylamine as a main component of the leaves (Tsushida and Takeo 1984), but there is no report on monoamines in the narcissus species. The
MAO may also be involved in the catabolism of short-chained aliphatic and aromatic monoamines in the shoots. Many volatile compounds containing some benzenoids have been isolated from a fragrant flower of the genus *Narcissus* (Dobson *et al.* 1997), but the narcissus MAO does not appear to be involved in the synthesis of the flavor benzenoids 2-phenylactaldehyde and 2-phenylethanol, because it was a lower affinity for phenethylamine (Table 2). I intend to conduct further studies of endogenous substrates of MAOs in plant species.
Chapter III

General discussion

The heterogeneous superfamily of AOs includes enzymes found in all living systems, ranging from prokaryotes to eukaryotes (Agostinelli et al. 2004). These enzymes are involved in cellular and extracellular metabolism of amines (monoamines, diamines and polyamines) whose oxidation may generate other biologically active substances like aldehydes, ammonia, and hydrogen peroxide. In prokaryotes and fungi, AOs allow using primary amines as sole sources of nitrogen for growth. In Aspergillus niger, two AOs were induced in cells in a growth medium containing benzylamine as nitrogen source (Schilling and Lerch 1995), whereas the regulatory mechanisms of AOs have not yet understood in the metabolism of amines in plants.

MAO appears to be ubiquitously expressed in higher eukaryotic organisms. MAO-like activity has been reported in the Ascarida galli, Nippostrongylus braziliensis (Mishra et al. 1983; Agarwal et al. 1985), cockroach (Subhashni et al. 1983), fish, birds, amphibians, echinoderms, carp (Kinemuchi et al. 1983; Yoshino et al. 1984), ring-dove (Hall et al. 1985) and frog (Kobayashi et al. 1981).
MAOs are found in all mammals where MAOs have been looked for, and the enzymes are bound to the outer mitochondrial membrane and have a FAD molecule covalently bound to the protein via an 8α-thioether linkage to a cysteinyI residue (Kearney et al. 1971). In plants, MAO that was purified from oat, like mammal and other MAOs, is a FAD-containing MAO (EC.1.4.3.4). In this report, I have successfully purified it from *Narcissus tazetta* and examined its properties, especially determined copper as cofactor in Narcissus MAO.

In plants, the enzymes containing DAOs were important for the synthesis of alkaloid, cell wall formation and wound healing (Medda et al. 2009). On the other hand, a physiological role of plant MAO has not yet been determined clearly. As far as we know, MAO-A and MAO-B catalyze the oxidation of the principle biogenic amines are serotonin, adrenaline, epinephrine, dopamine and tyramine (Waldmeier 1987). It was not similar with MAO-A and MAO-B of mammals’ on substrate specificity. The amines catalyzed by narcissus MAO were aliphatic and aromatic short-chain monoamines. The classification of copper-containing amine oxidases (Cu-AOs) including mammalian, microbial, and plant AOs takes into account their origins and substrate specificities. The enzymes of plant origin especially are subjected to intensive studies of the interface of biochemistry and
plant physiology since play key roles in essential metabolic pathway in plants (Bachrach, 1985). Cu-AOs have been shown to contain a covalently bound cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), that derives from the modification of an endogenous tyrosine residue (Janes et al. 1990). In addition to TPQ, Cu-AOs also contain a single copper ion in the active site that is involved in the biogenesis of TPQ as well as the catalytic cycle involving oxidation of primary amine substrates. Thus, Cu-AOs contain both an inorganic and organic cofactor, and, in particular, a detailed understanding of the nature of the interaction of molecular oxygen with the cofactors is likely to shed new light on oxygen activation in biology.

The enzyme was also inhibited by a metal-chelating reagent, 2,2’-bipyridyl. Using an ICP-MS, narcissus MAO was found to be a copper-containing protein having 2.45 atoms of copper per one molecule of enzyme protein. It can be considered that the enzyme is a Cu-AO. Medda et al. (1995) indicated that 2-bromoethylamine, the irreversible inhibitor to lentil DAO, is poor substrate and combines directly with TPQ in the enzyme. The results by NBT/glycinate method which was used as a specific stain for quinoprotein show that each 75kDa and 65kDa subunit of narcissus MAO has a quinone, respectively.
The quinone in narcissus MAO may be TPQ, comparing with other copper AOs (Suzuki et al. 2009).

In fact, Cu-AOs generally play a nutritional role in microorganisms, utilizing primary amines as nitrogen or carbon sources, and in mammals are involved in detoxification and metabolic and vascular diseases. In mammals, three main functions have been put forward concerning the biological importance of Cu-AOs, namely, (1): to remove biologically active molecules like primary amines, (2): the formation of the corresponding aldehydes that in some case were found to affect dramatically cell differentiation, proliferation and survival, (3): the on-site production of hydrogen peroxide as a signal molecule rather than a harmful side product (Boyce et al. 2009).

Although physiological roles of Cu-AOs in plants is not precisely known, they are implicated in wound healing, detoxification, and cell growth by regulating intracellular diamine and polyamine levels. The aldehyde produces may have a key role in the biosynthesis of some alkaloids and the hydrogen peroxide released may be involved in the formation of lignin and suberin. The strong and specific inhibitor may be useful for a probe as searching physiological roles on narcissus MAO, especially doing if the enzyme involves any fragrant aldehydes in flower of
narcissus.
Summary

Semicarbazide-sensitive amine oxidase activity was detected in *Narcissus tazetta*. The enzyme was purified to homogeneity by the criterion of native polyacrylamide gel electrophoresis (PAGE) with DEAE-Sephacel, hydroxyapatite, and phenyl-Sepharose columns. The molecular mass of the enzyme, determined using a GS-520 HQ column, was estimated to be 135 kDa. SDS-PAGE yielded two bands of 75 kDa and 65 kDa. The enzyme, which had catalytic activity for some aliphatic and aromatic monoamines, belongs to a class of monoamine oxidases (MAOs). The $K_m$ value for n-propylamine was $5.9 \times 10^{-5}$ M. A substrate analog, 2-bromoethylamine, inhibited enzyme activity. Redox-cycling staining detected a quinone in the MAO protein. By inductively coupled plasma mass analysis, it was determined that there were 2.44 moles of copper atoms per mole of the enzyme. Protein sequence analysis revealed that there was no identity between two N-terminal residues of the 75 kDa and 65 kDa proteins of narcissus MAO.
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**Table 1.** Summary of the Purification of Monoamine Oxidase from Narcissus Shoots

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<tr>
<th>Purification Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (nKat)</th>
<th>Specific activity (nKat mg(^{-1}) prot.)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<td>Crude extract</td>
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<td>718</td>
<td>37.8</td>
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<tr>
<td>(NH(_4))(_2)SO(_4) precipitate</td>
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<td>728</td>
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<td>Heat treatment</td>
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<td>Substrate</td>
<td>Relative activity (%)</td>
<td>Km (M)</td>
<td>$k(s^{-1})$</td>
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<td>------------</td>
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<tr>
<td>n-Propylamine</td>
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<td>n-Butylamine</td>
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<td>Ethylamine</td>
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<td>$1.3 \times 10^{-4}$</td>
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<td>Benzylamine</td>
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<td>$4.1 \times 10^{-4}$</td>
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<td>n-Pentylamine</td>
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Table 3 Effects of inhibitors and manganese chloride on narcissus MAO activity

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<tr>
<th>Inhibitor</th>
<th>Concentrations (mM)</th>
<th>Inhibition (%)</th>
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<td>Semicarbazide</td>
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<td>100</td>
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<tr>
<td></td>
<td>0.001</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
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<tr>
<td>2-Bromoethylamine</td>
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<td>71</td>
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<tr>
<td></td>
<td>0.0005</td>
<td>42</td>
</tr>
<tr>
<td>2,2’-Bipyridyl</td>
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<td>0.05</td>
<td>105</td>
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</table>
Fig. 1 Polyamine metabolism and interaction with other metabolic routes. Biosynthetic pathways for polyamines and related metabolites are indicated by continuous lines. Dashed lines show the formation of putrescine-derived alkaloids, polyamine conjugation and catabolic processes.

Numbers refer to the following enzymes: 1 nitrate reductase, 2 nitrite reductase, 3 nitrogenase, 4 glutamine synthetase, 5 glutamate synthase, 6 glutamate N-acetyltransferase, 7 acetylglutamate kinase, 8 N-acetyl-β-phosphate reductase, 9 acetylornithine transaminase, 10 acetylornithine deacetylase, 11 ornithine-carbamoyl transferase, 12 arginosuccinate synthase, 13 arginosuccinate lyase, 14 arginine decarboxylase, 15 agmatine iminohydrolase, 16 N-carbamoylputrescine amidohydrolase, 17 ornithine decarboxylase, 18 SAM synthetase, 19 SAM decarboxylase, 20 spermidine synthase, 21 spermine
synthase, 22 thermospermine synthase, 23 glutamate decarboxylase, 24 diamine oxidase, 25 putrescine hydroxycinnamoyl transferase, 26 polyamine oxidase, 27 _-aminobutyrate aminotransferase, 28 succinic semialdehyde dehydrogenase, 29 arginase, 30 ornithine aminotransferase, 31 nitric oxide synthase, 32 ACC synthase, 33 ACC oxidase, 34 nitrate reductase, 35 lysine decarboxylase.
Fig. 2. Thermal inactivation curve of narcissus monoamine oxidase.

The purified monoamine oxidase solution (●) or the desalted solution after ammonium sulfate precipitation (▲) was incubated at the specified temperature for 10min and each activity was measured. For other details, see Materials and Methods.
Fig. 3. Polyacrylamide gel electrophoresis of narcissus monoamine oxidase. (a) SDS-free gel of narcissus monoamine oxidase. L; Coomassie-stained SDS-free gel, R; N-propylamine-positive spot on SDS-free gel, (b) SDS-gel of narcissus monoamine oxidase. L; Marker, R; Coomassie-stained SDS-gel. For other details, see Materials and Methods.
**Fig. 4.** Molecular mass determination of narcissus monoamine oxidase.

Gel filtration (GS-520 HQ, HPLC) column; 1 ovalbumin (45kDa), 2 α-glucosidase from yeast (52 kDa), 3 lactate dehydrogenase from yeast (140 kDa), 4 glucose oxidase from *Aspergillus nigar* (186 kDa), 5 ferritin type I from horse spleen (440 kDa).

▲ narcissus monoamine oxidase (135 kDa). For other details, see Materials and Methods.
Fig. 5. Effect of pH on narcissus monoamine oxidase activity.
Fig. 6. Detection of propionaldehyde as DBD-H derivative using HPLC.

A: authentic propionaldehyde solution, B: the reacted mixture with n-propylamine as substrate, C: before reacting, 2-bromoethylamine (0.1 mM) was added into the mixture. The arrows are peak of propionaldehyde-DBD-H. For other details, see Materials and Methods.
**Fig. 7.** Comparison of the amino acid sequence of narcissus monoamine oxidase with homologous sequence of barley predicted amine oxidase. N-terminal narcissus MAO was sequenced by the Edman degradation method. NCBI GenBank accession number for barley is BAJ85075. Amino acids of barley AO identical to that of narcissus MAO are shade in gray. For other details, see Materials and Methods.
Fig. 8. NBT/glycine staining of quinoproteins on an SDS-polyacrylamine gel electrophoresis electroblot. A and B; lane 1, marker; lane 2, purified narcissus monoamine oxidase; lane 3, bovine serum amine oxidase; lane 4, pig kidney diamine oxidase. The electroblot was first stained with NBT/glycine (A) and then counterstained with Ponceau (B). For other details, see Materials and Methods.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADC</td>
<td>arginine decarboxylase</td>
</tr>
<tr>
<td>AO</td>
<td>amine oxidase</td>
</tr>
<tr>
<td>Cu-AO</td>
<td>Cu-containing amine oxidase</td>
</tr>
<tr>
<td>DAO</td>
<td>diamine oxidase</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidases</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
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<tr>
<td>PAO</td>
<td>polyamine oxidase</td>
</tr>
<tr>
<td>SAMDC</td>
<td>S-adenosylmethioninamine decarboxylase</td>
</tr>
<tr>
<td>SAO</td>
<td>serum amine oxidase</td>
</tr>
<tr>
<td>SMO</td>
<td>spermine oxidase</td>
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<tr>
<td>SS AO</td>
<td>semicarbazide-sensitive amine oxidase</td>
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<td>-------</td>
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<tr>
<td>TPQ</td>
<td>trihydroxyphenylalanine quinone</td>
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