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Enzyme Characteristics of Amine Oxidase in *Lyophyllum aggregatum* KÜHNEN ("Hon-shimeji")

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Amine oxidase (AO) activity was assayed in 51 species of plants. Among these plants, high AO activity was observed in *Lyophyllum aggregatum* KÜHNEN ("Hon-shimeji"); some of its properties were investigated. It was found that 96% of the AO activity was localized in the soluble fraction (100000 × *g* sup.). When β-phenylethylamine (PEA) was used as a substrate, the AO activities in mitochondrial (15000 × *g* ppt.) and microsomal fractions (100000 × *g* ppt.) had similar kinetic constants, but that in the soluble fraction was different. Based on inhibition studies, AO in Hon-shimeji could be classified into two forms. One is a metallo enzyme (containing cupric copper and pyridoxal phosphate) which is inhibited by semicarbazide and cuprizone, and the other is a flavin enzyme which is inhibited by clorgyline.

Keywords——amine oxidase; Hon-shimeji; monoamine oxidase; clorgyline; semicarbazide

Many amines are present in various kinds of plants, and the content of these amines in plant cells is higher than that in animal cells.[1] These amines may be classified into four types: aromatic amines, diamines, polyamines and aliphatic amines. Aliphatic amines are metabolized by transaminase. However, aliphatic amines, aromatic amines and polyamines are mainly oxidized by amine oxidase (AO).[1–6] Previous studies[7] have demonstrated that the diamine oxidase in pea seedlings contains copper ion, and catalyzes the oxidation of not only aliphatic diamines but also aliphatic monoamines, phenylalkylamines, histamine,[8] spermidine, agmatine, lysine and ornithine. Polyamine oxidase in barley plants is a flavin enzyme, and metabolizes spermine and spermidine.[9] In addition, tryptamine is metabolized by AO in cucumber seedlings,[10] coleoptiles in oats[11] and pea seedlings.[12] Many of these enzymes metabolize the same substrates. Therefore, to identify which type of enzyme is present in a tissue, it is essential to employ several potential substrates together with a variety of inhibitors.

In the present study, the deamination of various monoamines by AO was investigated in Hon-shimeji using several inhibitors.

Results

The Specific Activity of Amine Oxidase in Plant Homogenates

AO activity was investigated by radiometric assay[13] in 51 species of plants (some of them, with high AO activity, are listed in Table I). The most suitable substrates of this enzyme were monoamines, especially β-phenylethylamine (PEA). Higher AO activities were found in leaves than in roots of Chinese cabbage (*Brassica campestris* L. *subsp Napus Hook* fil. *et* ANDERS var. *nippo-olei fera* MAKINO), turnip cabbage (*Brassica oleracea* L. var. *gemmifera* ZENKER), spinach (*Spinacia oleracea* L.) and Chrysanthemum coronarium (*Chrysanthemum*

TABLE I. Specific Activities of Amine Oxidase in Plants

Plant species		Specific activity (cpm/10 mg tissue)			
Latin	English	5-HT	PEA	Tyr	Bz
Fungi[a]					
Hymenomycetales[b] Agaricaceae[c]					
Lyophyllum aggregatum KÜNEN	Hon-shimeji	33	2272	787	42
Agaricus bisporus Sing	Mushroom	22	2357	216	210
Lentinus edodes Sing	Cortinellus shiitake	10	334	188	71
Flammulina velutipes Sing	Enokidake	27	1977	562	67
Angiospermae[a]					
Rhoeadales[b] Cruciferae[c]					
Brassica campestris L. *subsp Rapa Hook* fil. *et* ANDERS	Turnip	65	201	20	82
Brassica campestris L. *subsp Rapa Hook* fil. *et* ANDERS (Radix)	Turnip (roots)	95	1012	241	195
Brassica oleracea L. var. *gemmifera* ZENKER	Brussel sprouts	421	1076	240	277
Labiatae[c]					
Perilla frutescens Britton	Beefsteak plant (red)	86	498	211	1224
var. *acuta* KUDA	Beefsteak plant (green)	86	259	163	405
Chrysanthemum coronarium L. var. *spatiosum* BAILEY	Chrysanthemum coronarium	439	61	0	175
Chrysanthemum coronarium L. var. *spatiosum* BAILEY (Radix)	Chrysanthemum coronarium (roots)	202	45	15	53
Rosales[b] Leguminosae[c]					
Glycine max Merrill	Soybean	4	900	25	456
Phaseolus vulgaris L.	French bean	96	898	236	361

The enzyme was assayed by the radiochemical assay method using 1 mM 5-hydroxytryptamine (5-HT), β-phenylethylamine (PEA), tyramine (Tyr) and benzylamine (Bz). The AO activity was expressed as cpm. *a*) Class; *b*) Order; *c*) Family. Nomenclature of plants in Latin from "Makino's New Illustrated Flora of Japan"[14] and in English from "Plants of the World III"[15] and "The Encyclopedia of Mushrooms".[16]

coronarium L. var. *spatiosum* BAILEY). Among the plants assayed for AO activity, higher activity was observed in the Cruciferae, Leguminosae, and especially in the Agaricaceae.

The Time Course of Enzyme Reaction in Hon-shimeji *Lyophyllum aggregatum* KÜHNEN

We examined the relation between incubation time and AO activity in the mitochondrial ($15000 \times g$ ppt.), microsomal ($100000 \times g$ ppt.) and soluble fractions ($100000 \times g$ sup.) at protein concentrations of 28.6 mg/ml, 29.9 mg/ml and 9.8 mg/ml, respectively. The enzyme reaction was linear for 30 min in each fraction.

pH Optimum

The influence of pH on AO activity in the mitochondrial, microsomal and soluble fractions is shown in Fig. 1. AO activities were measured with PEA as a substrate in 100 mM phosphate buffer (pH 5.4 to 7.6) or 100 mM Tris–HCl buffer (pH 8.0 to 9.2). The optimum pH of AO in Hon-shimeji was found to be between 6.8 and 7.0 in all fractions, and all the pH-activity curves were bell-shaped.

Subcellular Distributions of AO, Succinate Dehydrogenase and Cytochrome Oxidase Activity

The subcellular distribution of AO in Hon-shimeji was investigated by means of differential centrifugation. AO activity in Hon-shimeji was localized 3.0% in mitochondrial ($15000 \times g$ ppt.), 1.1% in microsomal ($100000 \times g$ ppt.) and 95.8% in soluble ($100000 \times g$ sup.)

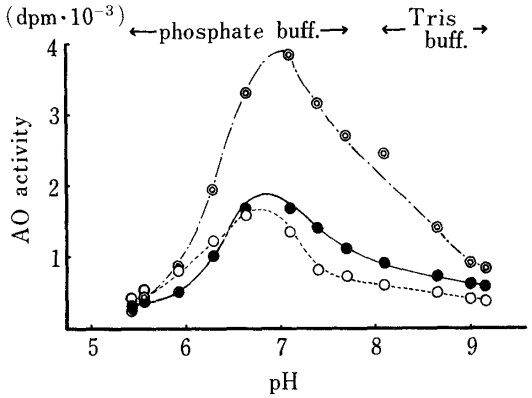

Fig. 1. The Influence of pH on AO Activity in Mitochondrial (15000 × *g* ppt.), Microsomal (100000 × *g* ppt.) and Soluble (100000 × *g* sup.) Fractions from Hon-shimeji

AO activities in mitochondrial (○---○), microsomal (●—●) and soluble fractions (◎- -◎) were measured with 0.1 mM PEA as a substrate.

TABLE II. Subcellular Distribution of AO

	Specific AO activity (pmol/mg protein/min)	% recovery of AO activity	% recovery of succinate dehydrogenase activity	% recovery of cytochrome oxidase activity
Mitochondrial fr.	1.3	3.0	80	68
Microsomal fr.	2.4	1.1	18	24
Soluble fr.	18.9	95.8	0	0

AO activity was measured with PEA (0.02 mM) as a substrate. Numerical values represent percentage recaveries of AO, succinate dehydrogenase and cytochrome oxidase activity in the fraction based on total activity of the homogenate (100%). Each value is the mean for 4 experiments.

TABLE III. Kinetic Constants of AO in Hon-shimeji

Substrate	PEA		5-HT		Tyr		Bz	
Fraction	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Mitochondrial	60.6	3.9	588	6.4	1111	2.7	1667	18.8
Microsomal	74.1	4.2	769	9.1	1176	3.9	1538	23.9
Soluble	18.5	22.1	667	23.5	1111	72.4	—	—

Kinetic constants of AO in mitochondrial (15000 × *g* ppt.), microsomal (100000 × *g* ppt.), and soluble (100000 × *g* sup.) fractions of Hon-shimeji for PEA, 5-HT, Tyr and Bz were determined. Michaelis constants and maximum velocities were calculated from Lineweaver–Burk plots. K_m values and V_{max} values are represented as μM and pmol/mg protein/min, respectively. The enzyme sources were as described in the legend to Fig. 2. Each value is the mean for 6 experiments.

fraction. When PEA was used as a substrate, specific AO activity was 1.3, 2.4, and 18.9 pmol/mg protein/min in the mitochondrial, microsomal and soluble fractions, respectively. Further, the subcellular distributions of succinate dehydrogenase and cytochrome oxidase, marker enzymes of mitochondria, were studied. Succinate dehydrogenase and cytochrome oxidase activities were mainly present in mitochondrial fraction. These enzyme activities were not detectable in soluble fraction (Table II).

Kinetic Constants of AO

Kinetic constants of Hon-shimeji AO were obtained by using Lineweaver–Burk plots (Table III). When 5-hydroxytryptamine (5-HT), tyramine (Tyr) and benzylamine (Bz) were used as substrates, Michaelis constants for a given substrate in the three fractions were almost identical, except that AO activity with Bz was not detectable in the soluble fraction. When

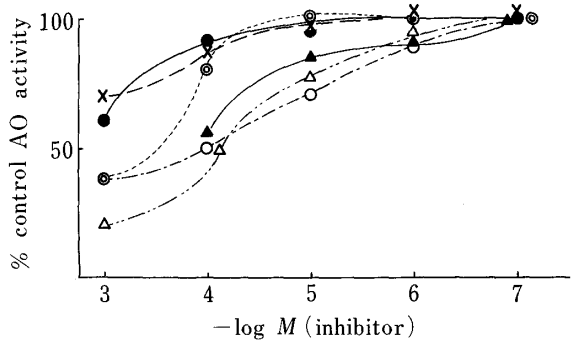

Fig. 2. Inhibition of PEA Oxidation by Clorgyline, Semicarbazide, Cuprizone, KCN, Isoniazid and Iproniazid

The mitochondrial fraction (15000 × *g* ppt.) in Hon-shimeji was preincubated at 38 °C for 30 min with clorgyline (●—●), semicarbazide (○— - —○), cuprizone (▲—▲), KCN (× --- ×), isoniazid (△---△) or iproniazid (◎---◎). Remaining activities were then measured with PEA (0.05 mM) as a substrate.

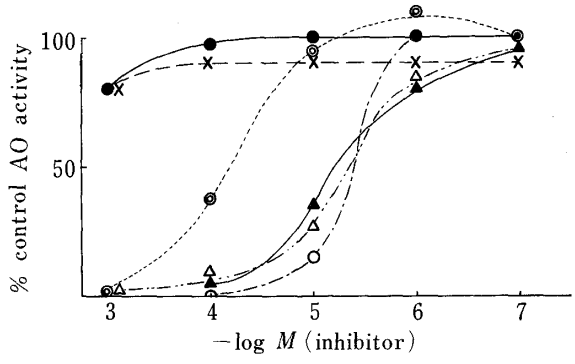

Fig. 3. Inhibition by Several Inhibitors of PEA-Oxidizing Activities in the Soluble Fraction (100000 × *g* sup.) from Hon-shimeji

Experimental conditions and symbols were the same as for Fig. 2.

PEA was used as a substrate, AO in mitochondrial and microsomal fractions showed similar kinetic constants, but that in the soluble fraction was different.

Effects of Inhibitors on AO Activities in Hon-shimeji

The effects of several inhibitors on Hon-shimeji AO were investigated. The enzyme solution was preincubated with inhibitor for 30 min at 37 °C before starting the reaction. The effects of clorgyline, semicarbazide, KCN, cuprizone, isoniazid and iproniazid on AO activity in the mitochondrial fraction are shown in Fig. 2. AO activity was inhibited 40% by 1 mM clorgyline, which is a specific inhibitor of monoamine oxidase containing flavin adenine dinucleotide (FAD) as a cofactor.[17] Further, 1 mM semicarbazide, an inhibitor of AO with the coenzyme pyridoxal phosphate,[18] and 0.1 mM cuprizone inhibited AO activity 60% and 50%, respectively. It was also inhibited by 1 mM isoniazid, iproniazid and KCN by 80%, 60% and 30%, respectively. These results suggested that AO exists in the mitochondrial fraction in two forms, a copper-containing pyridoxal enzyme and a flavin enzyme. AO in microsomal fractions had the same forms as that in the mitochondrial fraction. The AO activity in the soluble fraction (Fig. 3) was completely inhibited by 0.1 mM semicarbazide, cuprizone, isoniazid and 1 mM iproniazid, and was inhibited about 20% by 1 mM clorgyline.

Discussion

Much evidence has been reported indicating that AO in plants, *e.g.* pea seedlings[7,12,19-22] and clover seedlings,[23] contains pyridoxal phosphate and/or FAD. In 1949, Werle and von Pechmann[23] observed that AO activity in clover seedlings was increased by the addition of pyridoxal, and that hydrogen peroxide was produced during the enzyme reaction. They suggested that both pyridoxal phosphate and FAD were coenzymes of plant AO. Likewise, Goryachenkova[24] and Werle *et al.*,[25] suggested that pyridoxal phosphate and FAD are prosthetic groups of plant AO. In 1974, Percival and Purves[10] observed monoamine oxidase and diamine oxidase in cucumber (*Cucumis sativus* L.) seedlings. Yoo and Oreland[11] reported monoamine oxidase in the mitochondrial fraction of coleoptiles from oats (*Avena sativua* L.).

In the present study, AO activity was found to be higher in Hon-shimeji than in other

plants (Table I). The optimum pH of AO in Hon-shimeji was 7.0 with PEA as a substrate. In coleoptiles of oats and barley leaf, the optimum pH is 7.2 with tryptamine[11] and 4.0—4.5 with spermine[9] as substrates, respectively, and in cucumber seedlings the optimum pH is 7.0—8.7.[10] AO in Hon-shimeji was found in mitochondrial, microsomal and soluble fractions, but was mainly localized in the soluble fraction. A comparison of the kinetic constants for the amines tested shows that AO activities in mitochondrial and microsomal fractions are similar in their substrate specificity, but AO activity toward Bz was not detectable in the soluble fraction. Further, the K_m value of AO in the soluble fraction for PEA differs markedly from those of AO in the mitochondrial and microsomal fractions, indicating that the enzymic properties of AO in the soluble fraction deffer from those of AO in the mitochondrial and microsomal fractions.

AO is subject to inhibition by a variety of compounds.[18] All the AO studies thus far have shown the enzyme to be copper dependent,[19,26,27] and to be inhibited by carbonyl reagents.[12,20] In the soluble fraction of Hon-shimeji, AO activity was completely inhibited by semicarbazide (a carbonyl reagent), cuprizone, iproniazid and isoniazid, while it was inhibited about 20% by clorgyline, which is a specific inhibitor of monoamine oxidase. On the other hand, in mitochondrial and microsomal fractions, AO activity was inhibited 38 and 23%, respectively, by 1 mM clorgyline. It was also inhibited 60 and 50% by 1 mM semicarbazide and 0.1 mM cuprizone, respectively. These results suggest that at least two kinds of AO exist in Hon-shimeji. One is a copper-containing pyridoxal enzyme having properties similar to those of AO-1 or AO-3, which was separated by ion chromatography in cucumber seedling studies by Percival and Purves,[10] and was found in pea seedlings by Mann and Kenten.[21,22] The other is a flavin enzyme which is inhibited by clorgyline.

Experimental

Plant Materials——The aerial parts of *Lyophyllum aggregatum* KÜHNEN were collected in Nagano prefecture. The other plants were collected near Tokyo.

Preparation of Enzyme——Plants were homogenized with a volume of ice-cold 0.05 M phosphate buffer (pH 6.0) equal to the weight of the plant and the homogenate was used as an enzyme source. Hon-shimeji were weighed and homogenized in 0.45 M sucrose, 0.05 M phosphate buffer, pH 6.5.[28] Homogenates derived from 500 g of Hon-simeji were pooled and centrifuged at $800 \times g$ for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at $15000 \times g$ for 20 min. After centrifugation, mitochondrial pellets were suspended in the sucrose–phosphate buffer. Subsequently, in order to separate the microsomal fraction and the soluble fraction, the supernatant was centrifuged at $100000 \times g$ for 60 min. Each fraction was stored frozen at -20 °C until use for assay. The AO activity remained stable for 3 months after preparation. Protein concentrations were determined by the modified biuret method[15] with bovine serum albumin (BSA) as a standard.

Assay Procedures——AO activity was determined radiochemically as reported previously[13] with 20 μl of homogenate and 20 μl, ^{14}C-labelled amine as a substrate. All assays were carried out under an atmosphere of air, and the incubation time was 30 min at 37 °C. [1-^{14}C]Tyramine (55.3 mCi/mmol 0.01 N HCl), [7-^{14}C]benzylamine hydrochloride (55 mCi/mmol), β-[ethyl-1-^{14}C]phenylethylamine hydrochloride (50 mCi/mmol) and 5-[2-^{14}C]hydroxytryptamine binoxalate (58.5 mCi/mmol) were obtained from New England Nuclear, Boston, Massachusetts, U.S.A. and Amersham, Buckinghamshire, England. Clorgyline HCl and deprenyl HCl were kindly provided by May and Baker Ltd., England, and Dr. J. Knoll, Budapest, Hungary, respectively.

Succinate dehydrogenase activity was assayed by the method of Bonner.[29] The reaction mixture consisted of potassium succinate, 300 μmol of phosphate buffer (pH 6.0), 30 μmol of KCN and enzyme solution in a final volume of 3.0 ml. The reaction was carried out at room temperature and the decrease in optical density at 415 nm was followed.

Cytochrome oxidase activity was assayed by the method described by Smith.[30] The reaction mixture contained 100 μmol of phosphate buffer (pH 6.0), 0.08 μmol of cytochrome c and the enzyme suspension in a final volume of 2.9 ml. The reaction was carried out at room temperature and the decrease in optical density at 405 nm was followed.

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