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Diamine Oxidase from Cultured Roots of *Hyoscyamus niger*

Its Function in Tropane Alkaloid Biosynthesis

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ABSTRACT

Diamine oxidase was partially purified from cultured roots of *Hyoscyamus niger* L. that produce considerable amounts of tropane alkaloids, and then characterized. *N*-Methylated amines inhibited the activity of the enzyme more strongly than the corresponding primary amines. *N*-Methylputrescine was the best substrate of those studied, the respective K_m values for it and for putrescine and cadaverine being 0.33, 2.85, and 6.25 millimolar. The specificity constants V_{max}/K_m for putrescine and cadaverine were 11 and 1% of the constant for *N*-methylputrescine. Marked specificity for the *N*-methylated diamine would enable the *Hyoscyamus* enzyme to function specifically in tropane alkaloid biosynthesis.

The roots of several solanaceous plants synthesize anticholinergic alkaloids such as hyoscyamine and scopolamine. The tropine moiety of these alkaloids originates from ornithine and/or arginine. In *Hyoscyamus albus*, the symmetric diamine putrescine derived from these amino acids is first converted to MP[1] by putrescine *N*-methyltransferase (EC 2.1.1.53) and then deaminated oxidatively to 4-methylaminobutanal which spontaneously cyclizes to give the *N*-methylpyrrolinium cation (Fig. 1) (12, 14, 15). This oxidative deamination is catalyzed by a DAO (EC 1.4.3.6). The subsequent enzymatic steps that lead to tropine via hygrine, however, have yet to be established.

DAOs are widespread in nature (25) and are particularly active in leguminous plants. All the plant DAOs reported so far have been dimers, several of which have been shown to contain two copper atoms and one carbonyl residue per mole of enzyme (29). Pyrroloquinoline quinone recently has been identified as the carbonyl residue of pea DAO (10). Symmetric diamines (*e.g.* putrescine and cadaverine) are good substrates for the known plant DAOs. Although the hydrogen peroxide produced by these enzymes has been suggested to function in cell wall stiffening and lignification (4), the physiological roles of plant DAOs are largely unknown.

Alkaloid-producing, cultured roots of *Hyoscyamus albus* contain both free putrescine and free MP in their cells (14). But to function specifically in alkaloid biosynthesis, the DAO in *Hyoscyamus* roots must oxidize MP more efficiently than putrescine and other diamines present. Or, these diamines must be localized in different metabolic compartments in the cells. We here characterize the DAO from cultured *Hyoscyamus* roots with emphasis on its substrate specificity.

[1] Abbreviations: MP, *N*-methylputrescine; DAO, diamine oxidase.

MATERIALS AND METHODS

Plant Materials

Roots of *Hyoscyamus niger* L. have been maintained *in vitro* in our laboratory since 1984 as described elsewhere (13). Prior to the experiments reported here, samples of these roots were transferred to 300-mL flasks containing 75 mL of auxin-free B5 medium (9) supplemented with 3% (w/v) sucrose, and were then cultured for 6 d. After being harvested with a suction filter, the roots were frozen immediately with liquid nitrogen and then homogenized in a Waring Blendor. The frozen homogenate was kept at −20°C until use.

Pea seeds (*Pisum sativum* cv Alaska) were surface-sterilized and then germinated in moistened vermiculite in the dark for 8 d. The epicotyls of the etiolated pea seedlings were excised and frozen in liquid nitrogen after which they were processed in the same way as *Hyoscyamus* roots.

All the other root, shoot and cell-suspension cultures used had been maintained in our laboratory for several years. The conditions for their culture are described elsewhere (14).

Chemicals

γ-Methylaminobutyraldehydediethylacetal was synthesized at the Takarazuka Research Center of Sumitomo Chemical Co. (Takarazuka, Japan) by the method of Mizusaki *et al.* (21). The acetal group was cleaved, after which the resulting *N*-methylpyrrolinium chloride was purified as described by Feth *et al.* (6) and then identified by ^{1}H-NMR. The MP dihydrochloride used was a gift from Professor S. Yamada, Jyosai University, Japan. The other amines used were obtained from Aldrich, Nacalai tesque (Kyoto), and Wako Pure Chemical Industries (Osaka). [1,4-^{14}C]Putrescine dihydrochloride (4.37 GBq/mmol) was purchased from Amersham International, and pig kidney DAO from Sigma.

Enzyme Assays and Protein Determination

DAO activity was determined by one of the following four methods depending on the object of the experiment.

Method 1

When MP was the substrate, the reaction product was analyzed by a modification of the method of Mizusaki *et al.*

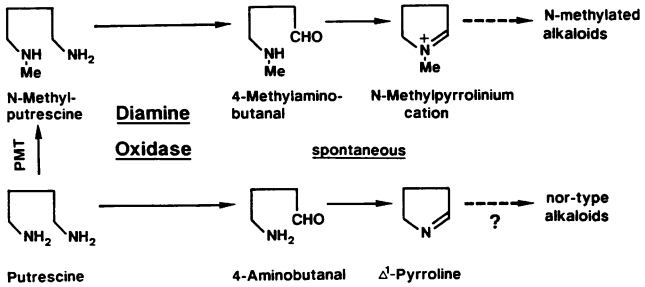

Figure 1. Early biosynthetic pathway of tropane alkaloids. Common alkaloids such as hyoscyamine and scopolamine are synthesized from putrescine via *N*-methylputrescine in *Hyoscyamus*, whereas nor-type alkaloids might be formed from putrescine via 4-aminobutanal. The diamines are deaminated oxidatively by diamine oxidase giving the respective aminoaldehydes which cyclize spontaneously. PMT, putrescine *N*-methyltransferase.

(22). The reaction mixture (total volume 50 μL) contained 100 mM potassium phosphate (pH 7.9), 2 mM MP, 0.5 mg/mL catalase, and the enzyme. After 1 h of incubation at 30°C, the reaction was stopped by the addition of 10 μL of 10% KCN solution and 100 μL of 0.0125% (v/v) collidine in $CHCl_3$. Aqueous KCN reacted quickly and quantitatively with *N*-methylpyrrolinium salt to give 1-methyl-2-cyanopyrrolidine which, after being transferred to the $CHCl_3$ phase by vigorous shaking, was analyzed by GLC. A CBP-1 capillary column (0.2 mm ϕ × 25 m; Shimadzu, Kyoto), a split ratio of 50:1 and a flame thermionic detector were used. The column temperature was 100°C and the carrier gas He was set at a flow rate of 1 mL/min. Collidine was the internal standard. The CN-derivative of the reaction product was identified as 1-methyl-2-cyanopyrrolidine by GC-MS analysis (EI mode, 70 eV). MS m/z (relative intensity) 111 (11), 109 (4), 82 (100), 67 (25).

Method 2

Enzyme activity was measured by essentially the same method described by Kusche and Lorenz (18). The [^{14}C]Δ^1-pyrroline produced by DAO from [1,4-^{14}C]putrescine (7.4 kBq, 12 mM) was extracted into the toluene phase and its radioactivity measured.

Method 3

An assay method based on the reaction of Δ^1-pyrroline with *O*-aminobenzaldehyde (16) was used during the purification of pea DAO.

Method 4

To study substrate specificity, the ammonia produced by the DAO reaction was measured enzymatically with glutamate dehydrogenase (from beef liver; Oriental Yeast Co., Tokyo) by monitoring the decrease in NADH at 339 nm (18).

When purified pea DAO preparation was assayed with MP as the substrate, methods 1 and 4 gave similar enzyme activities; whereas, with putrescine, methods 2, 3, and 4 gave similar results.

The protein concentration was determined by the method of Bradford (1) with BSA as the standard.

Purification of Diamine Oxidases

Enzymes were purified at 4°C. A homogenate of the *Hyoscyamus* roots (750 g) was suspended in 1.5 L of 100 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 5 mM EDTA, 3 mM DTT and 0.3% (w/v) ascorbate, mixed with 10% (w/v) insoluble PVP, after which it was ground thoroughly with sea sand in a mortar. The suspension was passed through a composite cheesecloth-Miracloth (Calbiochem)-cheesecloth filter and centrifuged at 11,000*g* for 30 min. The DAO in the crude extract precipitated between 20 and 40% saturation with ammonium sulfate. The pellet obtained after a second centrifugation was dialyzed overnight against 15 L of 10 mM potassium phosphate (pH 7.5) containing 1 mM DTT and 20% (v/v) glycerol (buffer A). The dialyzed enzyme solution was loaded onto a DEAE-Sepharose CL-6B column (Pharmacia; 2.6 × 27 cm) previously equilibrated with buffer A, and the column were washed with 200 mL of the same buffer. The enzyme was eluted with a linear gradient (1 L) of 0 to 0.4 M NaCl dissolved in buffer A, at a flow rate of 1.3 mL/min. Active fractions were loaded on a hydroxyapatite HTP column (BioRad; 1.6 × 10 cm) previously equilibrated with buffer A. This column was treated in steps with buffer A containing 10, 60, and 100 mM potassium phosphate (100 mL each) at a flow rate of 0.7 mL/min. Active fractions were pooled and stored as small portions at −20°C.

Subsequently, part of this partially purified DAO preparation was further purified by the FPLC system (Pharmacia) with chromatofocusing on PBE 94 (pH gradient from 7.0–4.5) followed first by hydrophobic column chromatography on Phenyl-Superose HR 10/10 and then by two cycles of gel filtration on Superose 12 (details not shown).

A crude extract was prepared from the homogenate of pea epicotyl by the procedures used for the *Hyoscyamus* enzyme. Pea enzyme activity was measured by method 3. After the crude extract had been treated with 0.4% (w/v) 6,9-diamino-2-ethoxyacridine lactate (acrinol; Nacalai tesque, Kyoto) for 10 min, the precipitates that formed were removed by centrifugation. The pea DAO in the supernatant was precipitated by 70% saturation with ammonium sulfate. The pellet obtained after centrifugation was dialyzed overnight against buffer A. The dialyzed enzyme solution was loaded onto a CM-Toyopearl 650M column (TOSOH, Tokyo), and the enzyme eluted with a linear gradient of 0 to 0.2 M NaCl dissolved in buffer A. The pea DAO in the active fractions was nearly homogeneous, as judged by SDS-PAGE, and it had a specific activity of 330 nkat/mg protein (cf. 650 nkat/mg reported for homogeneous pea DAO in ref. 10).

Crude enzyme extracts of other root, shoot and cell-suspension cultures were obtained by the procedures used for the *Hyoscyamus* enzyme.

RESULTS AND DISCUSSION

Distribution of DAO in *Hyoscyamus* Species

Root cultures of *Hyoscyamus albus* and *H. niger* produce considerable amounts of tropane alkaloids, but the alkaloid

contents in their shoot and cell-suspension cultures are very low (13). The DAO activities in root, shoot, and cell-suspension cultures of *H. albus* (measured by method 1) were 6.4, 3.2, and 16.1 pkat/g fresh weight of cells, whereas those in the cultures of *H. niger* were 7.2, 2.1, and 1.6 pkat/g fresh weight. There was no close correlation between alkaloid production and DAO activity in these *Hyoscyamus* species.

Tobacco and *Hyoscyamus* species, respectively, synthesize nicotine and tropane alkaloids by the same early biosynthetic pathway in which DAO functions to convert MP to *N*-methylpyrrolinium cation (12, 14, 15). Appreciable DAO activities have been found in tobacco tissues with low nicotine contents; callus (5, 32), leaf (32), and roots of the low-nicotine genotypes (27, 32). The biosyntheses of nicotine and tropane alkaloids apparently are not regulated directly by the DAO activities in these species.

Purification of DAO from Cultured *H. niger* Roots

DAO was partially purified from cultured roots of *Hyoscyamus niger* and its activities were followed during purification with MP or putrescine as the substrate (Table I). The activity of the DAO in the crude enzyme extract was 1.6-fold higher for MP than for putrescine. This ratio of its activity for MP and putrescine remained approximately 1.5 during purification by ammonium sulfate fractionation and column chromatographies with DEAE-Sepharose and hydroxyapatite. This indicates that a DAO with high activity for MP is a major (probably the only) type of DAO in cultured *H. niger* roots. After hydroxyapatite column chromatography the enzyme preparation had a specific activity of 544 pkat/mg protein and was used for the following characterization.

Mol Wt and pH Optimum

The mol wt of *H. niger* DAO, as determined by gel filtration on Superose 12, was 135,000 ± 11,000 (mean of 5 measurements ± SD). The purest DAO preparation obtained with the FPLC system gave a single major protein band of 66,000 on analysis by SDS-PAGE with silver staining, evidence that the enzyme is a dimer. The mol wt of the *H. niger* DAO is within the range reported for DAOs of other origin; (mol wt of dimers) 126,000 from *Vicia faba* (20), 144,000 from *Euphorbia characias* (23), 148,000 from *Lathyrus sativus* (31), 150,000 from *Trifolium subterraneum* (3), 154,000 from *Lens esculenta* (7), and 170,000 to 185,000 from *Pisum sativum* (29).

DAO activity was measured over the pH range of 6.0 to 9.0 with MP as the substrate (method 1). There was optimum activity between pH 7.5 and 8.0. At pH 7.5 potassium phosphate buffer gave activity that was about 50% higher than that with Tris-HCl buffer. The optimum pH with MP as the substrate is similar to that reported for tobacco DAO (22).

Effect of Inhibitors

The inhibiting effects of various compounds on *Hyoscyamus* DAO activity with MP as the substrate (method 1) were assessed. The copper ligand sodium diethyldithiocarbamate caused 59% inhibition at 0.1 mM. Also, the reagents that react with carbonyl groups were generally inhibitory; phenylhydrazine (69% inhibition at 0.01 mM), hydroxylamine (40% at 0.01 mM), semicarbazide (57% at 10 mM) and aminoguanidine (66% at 10 mM). Inhibition of the *Hyoscyamus* enzyme by the copper ligand and the carbonyl reagents suggests that copper and pyrroloquinoline quinone are present at the active site of this enzyme, as has been demonstrated for several DAOs (2, 28). Sulfhydryl reagents inhibited the activity of the *H. niger* enzyme only at relatively high concentrations; *p*-chloromercuribenzenesulfonic acid (91% at 1 mM) and *N*-ethylmaleimide (59% at 1 mM). At 1 mM, its activity was inhibited slightly (less than 24%) by $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$; moderately (60–80%) by $CoCl_2$, $FeSO_4$, and $NiCl_2$; and strongly (more than 90%) by $CdCl_2$, $CuCl_2$, and $HgCl_2$. KCN, which was used to stop the enzyme reaction and to modify the reaction product in method 1, inhibited DAO activity completely at 1 mM.

The inhibitory effects of several amines on the DAOs from *H. niger* roots, pea epicotyl, and pig kidney were studied (Table II). Primary amines generally inhibited pea and pig DAO activities more strongly than they did *H. niger* activity, whereas most *N*-methylated amines were stronger inhibitors of *H. niger* enzyme than of the other DAOs. When inhibition was compared for pairs of primary amines and their *N*-methylated counterpart (*e.g. n*-propylamine versus *N*-methylpropylamine), the inhibition of *H. niger* DAO activity was increased by the *N*-methylation of the inhibitor amines, whereas the inhibitions of the pea and pig DAO activities

Table I. *Purification of the Diamine Oxidase from Cultured H. niger* Roots

Enzyme activity was assayed with MP (method 1) or putrescine (Put) (method 2) as the substrate as described in "Materials and Methods."

Purification Step	Total Protein	Total Activity			Specific Activity[a] (Purification)	
		MP	Put	MP/Put		
	mg	*nkat*	*nkat*		*pkat/mg protein (-fold)*	
Crude extract	747	10.0	6.34	1.6	13	(1.0)
20–40% $(NH_4)_2SO_4$	229	12.8	9.28	1.4	56	(4.3)
DEAE-Sepharose	72.2	14.4	10.08	1.4	199	(15.3)
Hydroxyapatite	4.3	2.27	1.51	1.5	544	(41.8)

[a] MP was the substrate.

Table II. *Inhibition of Diamine Oxidases by Several Amines*

Enzyme activities were measured by method 1 in the presence of 10 mM inhibitor with 2 mM MP as the substrate. The respective activities without inhibitors were 4.18, 16.3, and 1.76 pkat for *H. niger*, pea, and pig diamine oxidases. Values are averages of two experiments.

Inhibitor	Enzyme Source		
	H. niger	Pea	Pig
	% *inhibition*		
1,3-Diaminopropane	32	70	93
N-Methyl-1,3-diaminopropane	34	55	61
n-Propylamine	28	23	48
N-Methylpropylamine	75	13	22
n-Butylamine	19	66	60
N-Methylbutylamine	70	23	19

were decreased. These three DAO activities were inhibited somewhat (less than 25%) by spermidine and spermine, but not at all by L-ornithine and DL-δ-*N*-methylornithine.

Substrate Specificity

The specificities of the three DAOs for amine substrates were established by measuring the ammonia liberated during the enzyme reactions (method 4). All the DAOs were most active against diamines with carbon lengths of 4 or 5; the monoamine *n*-butylamine was a poor substrate (Fig. 2). Of the eight amines studied, MP was the best substrate for *Hyoscyamus* DAO, and the pea and pig DAOs oxidized putrescine and cadaverine more rapidly than MP. 1,3-Diaminopropane and its *N*-methyl derivative were almost inactive with pea DAO. As expected from the proposed role of mamalian DAOs for histamine degradation (18), histamine was a good substrate for pig DAO.

MP, putrescine, and cadaverine were studied in detail for their effectiveness as substrates of DAOs. The double reciprocal plots of the concentrations of these diamines and the enzyme activities of the three DAOs all gave straight lines (not shown). There was substrate inhibition with the pea enzyme for cadaverine at more than 2 mM and with the pig enzyme for putrescine at more than 5 mM. The kinetic parameters for these diamines with the three enzymes are shown in Table III. With *H. niger* DAO, MP gave the lowest K_m value (0.33 mM) of the three substrates. This value is lower than the K_m value for MP of 0.45 mM (22) and 1.9 mM (6) reported for tobacco DAO. The pea and pig enzymes had much lower K_m values for putrescine and cadaverine (about 0.3 mM) than for MP (0.82 and 1.22 mM, respectively). Because putrescine has two aminomethylene groups that can be oxidized by DAOs in its molecule, the K_m values for a single reacting group in putrescine would be twice the K_m value of the diamine. Thus, the K_m values for the reacting group in *H. niger* DAO decreased more than 10-fold when the amino group in putrescine was a methylated one, whereas the values for the other DAOs increased 30 to 90%. The pea epicotyl and pig kidney DAOs each gave similar V_{max} values for the three diamines, but the V_{max} of *H. niger* DAO was lower with cadaverine as the substrate than with MP or putrescine. *H. niger* DAO had its highest specificity constant V_{max}/K_m for MP, the respective values for putrescine and cadaverine being only 11 and 1% that for MP, whereas both pea and pig DAOs had high V_{max}/K_m values for putrescine and cadaverine, but relatively low values for MP.

The various amines as inhibitors (Table II) or substrates (Fig. 2; Table III) clearly demonstrate that the *H. niger* enzyme has a much greater affinity for *N*-methylated than for primary diamines, unlike pea, pig, and, probably, most other DAOs. The tobacco DAO that functions in the early steps of nicotine biosynthesis has also been reported to be more active with MP than with putrescine (22). Frydman *et al.* (8) reported that *N*-ethyl-, *N*-propyl-, and *N*-butylputrescines at 0.83 mM were oxidized at rates of 25 to 76% of that of putrescine by the DAOs from pea seedling and pig kidney. Although almost no oxidation of MP by these DAOs was detected, the authors were unable to explain why the putrescine derivative with the shortest *N*-alkyl chain was not accepted as a substrate. We found that MP was oxidized by these DAOs at rates which were less than with putrescine but nevertheless considerable.

DAO is thought to have two separate subsites at its active site; a catalytic site that oxidizes an aminomethylene group, and a substrate-binding site that binds the other amino group of the substrate diamine (19). The substrate-binding subsite of *H. niger* DAO might have evolved from its ancestor DAOs by creating a cavity with a shape complementary to the *N*-methyl group of MP, which would have increased the affinity for MP because of van der Waals forces. That the V_{max} value for cadaverine is much lower than the values for putrescine and MP with *H. niger* DAO indicates that the active site of the enzyme accommodates cadaverine, the diamine with a 5-carbon chain, in an orientation that differs from that which it normally takes with diamines with a 4-carbon chain, possibly because of steric hindrance caused by the longer chain of cadaverine.

Cultured roots of *H. albus* that produce considerable amounts of tropane alkaloids have comparable cellular pools of both unconjugated putrescine and MP (14). The free putrescine content increases slightly when *H. albus* roots are actively growing, then decreases as growth slows, whereas the free MP increases markedly during the late growth phase when alkaloid biosynthesis is activated. At the onset of alkaloid biosynthesis, the free MP content is approximately 5-fold greater than the free putrescine content. That the specificity constant of *H. niger* DAO for MP is 9-fold higher than that for putrescine (Table III) is a further indication that the metabolism of putrescine to MP and thence to alkaloids predominates in alkaloid-synthesizing tissues.

It should be noted, however, that a few esters that contain nortropine (an alkamine that has a nonmethylated *N*-bridge) have been isolated in small quantities from several alkaloid-producing plants (26). Although demethylation of nicotine to nornicotine in the living plants and during the curing and drying of tobacco leaves is well documented (23), demethylation of tropane alkaloids has not been demonstrated. An alternative pathway to nor-type tropane alkaloids would start from Δ^1-pyrroline, the product of the DAO reaction with putrescine as the substrate (Fig. 1). Δ^1-Pyrroline condenses nonenzymatically with acetoacetate to give norhygrine (30). The two enzymes discovered so far in the subsequent part of the alkaloid biosynthetic pathway, tropinone reductase (17)

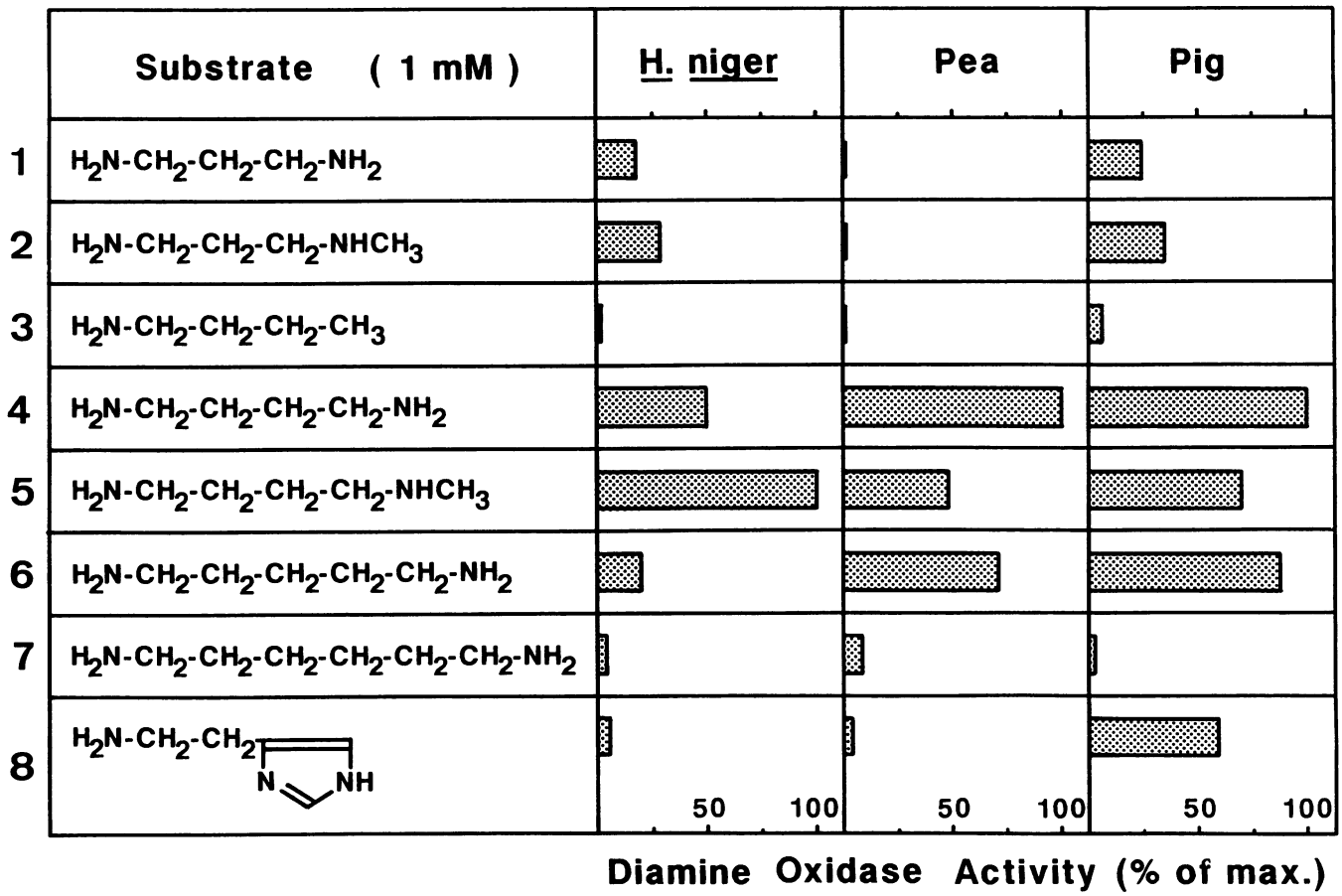

Figure 2. Substrate specificities of diamine oxidases. The activities of diamine oxidases from *H. niger* roots, pea epicotyl, and pig kidney were measured by method 4 with various amine substrates (1 mM) and are expressed as percentages of the most efficient substrate for each enzyme. Values are means of two experiments. 1, 1,3-diaminopropane; 2, *N*-methyl-1,3-diaminopropane; 3, *n*-butylamine; 4, putrescine; 5, MP; 6, cadaverine; 7, 1,6-diaminohexane; 8, histamine.

Table III. *Substrate Specificities of Diamine Oxidases*

Enzyme activities were measured by method 4 for 6, or more, concentrations of each substrate. The kinetic parameters (±SD) were calculated by the Wilkinson's statistical analysis method (33). V/K denotes specificity constants (V_{max}/K_m) expressed as percentages of the most efficient substrate for each enzyme.

Enzyme Source	Substrate								
	MP			Putrescine			Cadaverine		
	K_m	V_{max}	V/K	K_m	V_{max}	V/K	K_m	V_{max}	V/K
	mM	*pkat*	%	*mM*	*pkat*	%	*mM*	*pkat*	%
H. niger root	0.33 ± 0.03	90.4 ± 2.5	100	2.85 ± 0.37	82.7 ± 4.4	11	6.25 ± 1.26	19.6 ± 1.5	1
Pea epicotyl	1.22 ± 0.15	93.5 ± 6.2	21	0.32 ± 0.03	97.3 ± 2.2	82	0.26 ± 0.03	96.2 ± 5.5	100
Pig kidney	0.82 ± 0.06	23.3 ± 0.5	40	0.31 ± 0.02	21.9 ± 0.3	100	0.30 ± 0.01	20.5 ± 0.2	97

and hyoscyamine 6β-hydroxylase (11), act on nor-type precursors with moderate efficiencies. Other, as yet discovered, enzymes in this pathway also may accept non-*N*-methylated substrates; this would continue the metabolic flow from Δ^1-pyrroline to the nor-type alkaloids.

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