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An amine oxidase in seedlings of *Papaver somniferum* L.

A. BILKOVÁ*, L. BEZÁKOVÁ, F. BILKA and M. PŠENÁK

Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, SK-83232 Bratislava, Slovak Republic

Abstract

Amine oxidase (AO) from 4-d-old seedlings of *Papaver somniferum* L. (*Papaveraceae*) was purified (58-fold) by using ammonium sulphate precipitation and chromatography on *Sephadex G-150* and *HA-Ultrogel* columns. The most readily oxidized substrate was tyramine and other aromatic amines, while aliphatic amines cadaverine and putrescine were oxidized more slowly. Cu chelating and carbonyl reagents are the most effective inhibitors of poppy amine oxidase. Immunoblotting analysis showed cross reactivity of AO protein from poppy seedlings with polyclonal antisera against AO from pea. Obtained Mr value for AO from poppy (83 kDa) corresponds to that of copper AOs (75 - 90 kDa). These results suggest that the amine oxidase from poppy seedlings is a copper containing and tyramine specific AO.

Additional key words: aromatic amines, cadaverine, Cu-amine oxidase, immunoblotting, poppy, putrescine.

Introduction

Formation of C-N bound in alkaloids is frequently achieved by condensation of amines and aldehydes (ketones). This Mannich type condensation can proceed inter- or intra-molecularly. The carbonyl condensation unit/group can be derived from an amine group by the action of amine oxidases. Amine oxidase enzymes (AO) are widely distributed in nature. They catalyze the oxidative deamination of biogenic amines to the corresponding aldehydes, ammonia and hydrogen peroxide:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

According to the type of the prostetic group which takes part in the catalytic mechanism they can be devided into two groups: flavin-containing amine oxidases (FAD-AO, EC 1.4.3.4) and copper-containing amine oxidases (Cu-AO, EC 1.4.3.6) (Woodroofe *et al.* 1999).

Cu-amine oxidases have been isolated from different microorganisms (fungi and bacteria), plants and mammals (McIntire and Hartmann 1993, Medda *et al.* 1995). Although their exact physiological role in plants is not yet known, recent years have brought much progress in the understanding of the structure and function of these enzymes. Many plant enzymes with amine oxidase activity have been purified to homogenity and characterized, mainly from *Leguminosae* (Medda *et al.* 1995). Amine oxidases are homodimers of 70 - 95 kDa subunits. Each subunit contains Cu (II) and an organic cofactor topaquinone (TPQ) tightly bound to protein at the active-site. Copper ions are essential for the enzyme redox activity (Dooley *et al.* 1991) and for a formation of TPQ by a posttranslation modification from a tyrosinyl residue (Janes *et al.* 1990). The crystal structures of several microbial and plant Cu AOs have been reported (Parson *et al.* 1995, Kumar *et al.* 1996, Wilce *et al.* 1997, Li *et al.* 1998) and the catalytic cycle elucidated (Medda *et al.* 1999, Frébort *et al.* 2000).

Putrescine and cadaverine are intermediates in the biosynthesis of nicotine and tropane alkaloids (Hashimoto and Yamada 1994). From these aliphatic diamines aminopentanal and aminobutanal are formed by the action of amine oxidases and subsequently, by intramolecular Mannich condensation, Δ^1- pyrroline or Δ^1- pyperidine arised (Hashimoto *et al.* 1990).

(S)-reticuline biosynthetic pathway is common for all benzylisoquinoline alkaloids (Stadler *et al.* 1989). The benzylisoquinoline skeleton is formed from dopamine and 4-hydroxyphenylacetaldehyde by intermolecular Mannich

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Abbreviations: AO - amine oxidase, TO - tyramine oxidase, PSAO - amine oxidase of pea seedlings, DETC - diethyldithiocarbamate, SDS - sodium dodecyl sulphate, *HA-Ultrogel* - microcrystalline hydroxyapatite

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* Corresponding author, fax: (+421) 2 55572065, e-mail: bilkova@fpharm.uniba.sk

reaction. These condensation units are formed from tyrosine (Fig. 1) (Facchini and Bird 1998, Facchini 2001, Bilka *et al*. 2003/4). Conversion of tyrosine to carbonyl condensation unit (4-hydroxyphenylacetaldehyde) was suggested to proceed through *p*-hydroxyphenylpyruvic acid (Rueffer and Zenk 1987). Recently the amine oxidase activity was proved in *Berberis* where it is involved in the formation of carbonyl condensation unit (Hashimoto and Yamada 1994). Berberine alkaloids are formed also from (S)-reticuline. Whether this enzyme takes part also in the biosynthetic pathway of benzylisoquinoline alkaloids in opium poppy is still an unresolved problem.

Results presented in this paper indicate the presence of amine oxidase activity in the seedlings of opium poppy. The AO from *Pisum sativum* was chosen as a suitable representative of Cu-AO from plants. This enzyme can be easily purified in large amounts from the plant material (Macholán and Haubrová 1976). For comparison commercial Cu-amine oxidase from *Arthrobacter* species was also employed.

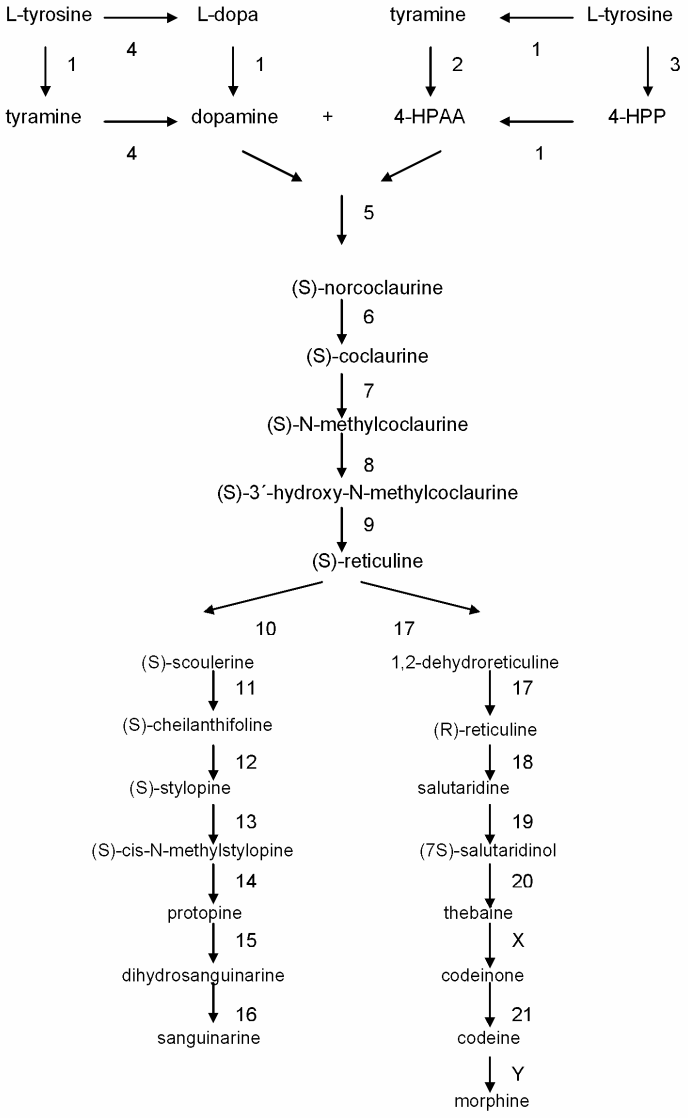

Fig. 1. Biosynthesis of morphine and sanguinarine: 1 - decarboxylase, 2) amine oxidase, 3) transaminase, 4) polyphenol oxidase, 5) (S)-norcoclaurine synthase, 6) (S)-norcoclaurine-O-methyltransferase, 7) (S)-coclaurine-N-methyltransferase, 8) (S)-N-methylcoclaurine hydrolase, 9) (S)-3′hydroxy-N-methylcoclaurine-O-methyltransferase, 10) berberine bridge enzyme, 11) (S)-chelathifoline synthase, 12) (S)-stylopine synthase, 13) (S)-stylopine-N-methyltransferase, 14) (S)-cis-N-methylstylopine hedroxylase, 15) protopine hydroxylase, 16) dihydrosanquinarine oxidase, 17) 1,2-dehydroreticuline reductase, 18) salutaridine salutaridinol-7-O-acetyltreansferase, 19) salutaridine:NADPH oxidoreductase, 20) acetylcoenzyme A: (7S)-salutaridinol-7-O-acetyltransferase, 21) (-)-codeinone reductase, X, Y) demethylases - not identified yet. (4-HPAA - 4-hydroxyphenylacetaldehyde, 4-HPP - 4-hydroxyphenylpyruvate).

Materials and methods

Seeds of *Papaver somniferum* L. and *Pisum sativum* L. were germinated on a polyurethane foam layer (thickness 10 mm) moistened with distilled water in a Petri dishes covered by a nylon cloth in the dark at 25 °C and 70 - 80 % relative humidity. On the fourth day of post-imbibition endosperms of the poppy seedlings were separated from the developing seedlings. In the case of pea seedlings endosperms were separated on the sixth day.

Amine oxidase (AO) from 6-d-old pea seedlings (55 g) was purified according to the method described elsewhere (Macholán and Haubrová 1976) with minor modifications. Rivanol treatment, fractionation on carboxymethyl-cellulose and removal of inert proteins were omitted. The crude enzyme after heat treatment was loaded onto *HA-Ultrogel* column. The proteins were eluted at a flow rate of 9 cm^3 h^{-1} by a three-step elution with the 0.1, 0.3, and 0.75 M potassium phosphate buffer (pH 7.0). The fractions with highest specific activity were pooled, concentrated by using 30-kDa membranes and stored at -20 °C. They were used as partially purified enzyme solution.

Amine oxidase from 4-d-old poppy seedlings without endosperm (65 g) was isolated by the procedures used for the pea enzyme with some modifications. Heat denaturation was omitted, because of rapid loss of activity. The dialyzed enzyme solution was loaded onto a *Sephadex G-150* column and the enzyme eluted with 0.1 M potassium phosphate buffer (pH 7.0). Active fractions were concentrated by using 30-kDa membranes to a volume 2.5 cm^3 and further purified on *HA-Ultrogel* column by the procedure used for the pea AO (PSAO). Fractions containing peak AO activity were pooled, concentrated by using 30-kDa membranes and stored in aliquots at -20 °C. They were used as partially purified enzyme solution.

The AO activity was determined spectrofluorometrically (Storer and Ferrante 1997). Tyramine, dopamine, cadaverine, putrescine, benzylamine, tryptamine, 4-phenylbutylamine and pargyline served as the substrates. The standard reaction mixture for poppy AO was composed of 0.05 cm^3 of the enzyme solution, 2.89 cm^3 5×10^{-4} M Tris-HCl buffer (pH 7.4), 0.03 cm^3 40 U cm^{-3} stock horseradish peroxidase solution and 0.015 cm^3 2 mM homovanillic acid. The standard reaction mixture for pea AO contained 0.02 cm^3 of the enzyme solution, 2.92 cm^3 5×10^{-4} M Tris-HCl buffer (pH 7.4), 0.03 cm^3 40 U cm^{-3} stock horseradish peroxidase solution and 0.015 cm^3 2 mM homovanillic acid. The standard reaction mixture for *Arthrobacter* sp. tyramine oxidase (TO) was composed of 0.005 cm^3 of the enzyme solution, 2.935 cm^3 5×10^{-4} M Tris-HCl buffer (pH 7.4), 0.03 cm^3 40 U cm^{-3} stock horseradish peroxidase solution and 0.015 cm^3 2 mM homovanillic acid. The total volume of all samples was 2.985 cm^3 and the reaction time was 10 min at 37 °C. After this time 0.015 cm^3 of 0.2 mM substrate solution (in deionized water) was added to each sample. The reactions were stopped by the addition of 0.1 cm^3 1.0 M NaOH after 35 min at 37 °C. Fluorescent products (dimers of homovanillic acid) were determinated spektrofluorometrically using an *LS-30B Perkin-Elmer* (Beaconfield, Buckinghamshire, UK) luminescence spectrophotometer set to excitation λ_{315} and to emission λ_{420}.

The protein content was determined using the method of Bradford (1976) using bovine serum albumin as a substrate. Copper ions were determined by atomic absorption using the *Perkin-Elmer 1100B* atomic absorption spectrophotometer equipped with a graphite furnace. The spectral line chosen was 324.7 nm.

To prepare polyclonal anti-AO serum partly purified AO from pea (after heat treatment) was subjected to native PAGE (Rodriquez and Flurkey 1992) and AO band was excised from the gel. The gel slide was crushed with an equal volume of phosphate buffered saline (PBS). Two rabbits were injected subcutaneously with *ca.* 200 μg of AO in 2 cm^3 of PBS. Two booster injections (200 μg of AO) were given at intervals of 1 month. The rabbits were bled on the 10^{th} day after last injection and the antisera were collected.

Denaturating SDS-PAGE was performed according to Laemmli (1970). Samples of PSAO and poppy AO in reducing conditions were prepared by boiling (100 °C, 3 min) in 0.5 volume of sample buffer [0.0625 M Tris/HCl, pH 6.8, 69 mM (2 %) SDS, 10 % glycerol, 5 % mercaptoetanol]. After electrophoresis, proteins were transferred to nitrocellulose using *Trans-Blot SD Semi-Dry Transfer Cell* (*Bio Rad*, Richmond, USA) according to manufacturers instructions. The AO was detected using immunoblot method with anti-AO serum. The second antibody reaction was carried out using swine immunoglobulines against rabbit immunoglobulines, conjugated with peroxidase. Reaction was visualised with 3,3',5,5'-tetramethylbenzidine (TMB) stabilized substrate for HRP.

Tyramine oxidase of *Arthrobacter* species, *HA-Ultrogel*, *Sephadex G-150*, homovanilic acid, horseradish peroxidase, tyramine, dopamine (3-hydroxytyramine), 4-phenylbutylamine, tryptamine, pargyline, cadaverine, putrescine, sodium diethyldithiocarbamate (DETC), sodium azide, glutathione, ß-hydroxyethylhydrazine were obtained from *Sigma*, St. Louis, USA. Swine immunoglobulines against rabbit immunoglobulines, conjugated with peroxidase (SwaR-Px, *Sevac*, Praha, CR), TMB stabilized substrate for HRP (*Promega*, Madison, USA), Trans-Blot SD Semi-Dry Transfer Cell (*Bio Rad*, Richmond, USA). All other chemicals were obtained as a pure commercial products.

Results and discussion

AO from 4-d-old poppy seedlings was purified by three-step procedure (ammonium sulphate precipitation, *Sephadex G-150* chromatography and ion-exchange chromatography on *HA-Ultrogel* column). AO from pea was purified by the same procedure with a few modifications (Table 1). The highest specific activity for enzymes from both sources was found after the final step ion-exchange chromatography on *HA-Ultrogel*. These partially purified enzymes were used in all other experiments.

Table 1. Purification of amine oxidase from poppy and pea seedlings. The substrate used to determine the activity of poppy AO was tyramine (1 μM) and of pea AO cadaverine (1 μM).

Step	Total protein [mg]		Total activity [nkat]		Specific activity [nkat mg^{-1}]		Purification [fold]	
	poppy	pea	poppy	pea	poppy	pea	poppy	pea
Crude extract	1360	3020	991	2025	0.73	0.67	1	1
70 % $(NH_4)_2SO_4$ precipitate	550	1520	890	1815	1.62	1.19	2.2	1.8
Heat denaturation	-	100	-	1325	-	13.25	-	19.8
Sephadex G-150 column	55	20	684	1120	12.44	56.00	17.0	83.6
HA-Ultrogel column	12	5	498	995	41.50	199.00	56.8	297.0

Although the substrate specifity of amine oxidases is not very high, the most readily oxidized substrate of AO from poppy seedlings was tyramine and high activity was determined also with other aromatic substrates (Table 2). Tyramine was also the most preferable substrate for enzyme from *Arthrobacter* sp. (Wouters *et al.* 1994). Pea enzyme was most active against aliphatic substrates cadaverine and putrescine and the aromatic amines appeared to be poor substrates. These results for PSAO are in good agreement with the preliminary data reported by Medda *et al.* (1995) and Peč and Frébort (1989). Aliphatic amines are the most suitable substrates for AOs from many *Leguminosae* for example *Lens esculenta*, *Onobrychis viciifolia* (Zajoncová *et al.* 1999), *Glycine max* (Vianello *et al.* 1993), *Lathyrus sativus* (Suresh *et al.* 1976), and also for enzyme from *Hyoscyamus niger*, where AO takes part in biosynthesis of tropane alkaloids (Hashimoto *et al.* 1990). As tyramine is the best substrate for AO from poppy, it indicates that this enzyme may take part in the formation of aldehyde condensation unit for the biosynthesis of (S)-reticuline in this plant.

Table 2. Substrate specificities of amine oxidases. The activities of amine oxidases from opium poppy, pea and *Arthrobacter* species were measured with various aromatic and aliphatic substrates (1 μM) and are expressed as percentages of the most efficient substrate for each enzyme. Means ± SD from three experiments.

Substrate	Poppy	Pea	*A.* species
Tyramine	100.0 ± 5.8	19.1 ± 0.6	100.0 ± 6.5
Dopamine	62.3 ± 4.2	18.4 ± 0.4	93.7 ± 5.8
4-phenylbutylamine	53.6 ± 3.5	19.3 ± 0.6	72.6 ± 5.0
Tryptamine	48.4 ± 2.8	15.7 ± 0.4	35.2 ± 2.8
Benzylamine	15.3 ± 0.9	60.5 ± 2.1	88.5 ± 4.2
Pargyline	13.7 ± 0.6	17.3 ± 0.6	14.4 ± 0.8
Cadaverine	12.0 ± 0.6	100.0 ± 6.1	15.1 ± 0.8
Putrescine	11.5 ± 0.5	96.2 ± 5.9	12.6 ± 0.5

Table 3. Inhibition [%] of amine oxidases by various compounds. Enzyme activities were measured in the presence of 3.3 μM inhibitor with 1 μM tyramine (for opium poppy and *Arthrobacter* species) or 1 μM cadaverine (for pea) as the substrate (DETC - sodium diethyldithiocarbamate). The respective activities without inhibitors were 41.5, 199.0 and 560 nkat mg^{-1} for opium poppy, pea and *Arthrobacter* species amine oxidases. Means ± SD from three experiments.

Inhibitor	Poppy	Pea	*A.* species
DETC	90.4 ± 5.2	91.2 ± 4.7	95.1 ± 5.6
β-hydroxyethylhydrazine	89.5 ± 4.9	85.5 ± 3.8	89.4 ± 4.1
NaN_3	87.8 ± 4.0	85.6 ± 4.3	82.2 ± 3.9
Glutathione	43.5 ± 2.9	38.1 ± 2.2	35.2 ± 2.2
$Na_2S_2O_3$	21.5 ± 1.2	18.2 ± 0.9	20.7 ± 1.0
$NaHSO_3$	19.3 ± 0.8	21.1 ± 0.8	19.5 ± 0.9

The amine oxidases did not show any major differences in sensitivity to inhibitors (Table 3). In all cases the strong inhibitory effect was assessed by Cu^{2+} chelating agents. The copper ligand sodium diethyldithiocarbamate (DETC) caused 90.4 - 95.1 % inhibition and sodium azide 82.2 - 87.8 % inhibition at 3.3 μM. Also the β-hydroxyethylhydrazine that react with carbonyl groups was generally inhibitory (85.5 - 89.5 % at 3.3 μM). Carbonyl group reagents that interact with the TPQ cofactor form irreversible aducts with all plant Cu-amine oxidases with concomitant loss of the catalytic activity (Padiglia *et al.* 1998). Sulfhydryl reagent glutathione inhibited the activity of all three enzymes tested, but its effect was only about one half of that of sodium diethyldithiocarbamate and sodium azide (35.2 - 43.5 %).

Redox compounds $Na_2S_2O_3$ and $NaHSO_3$ served also as inhibitors of all AOs studied. Their inhibitory effect was the lowest from all compounds tested (18.2 - 21.5 %). AO from pea and other *Leguminosae* belong to the large group of plant copper-containing AOs (Medda *et al.* 1995). Copper is present also at the active site of enzyme from *Arthrobacter* sp. (Choi *et al.* 1995). The inhibition studies suggest that AO from poppy belongs to the group of plant copper-containing AOs.

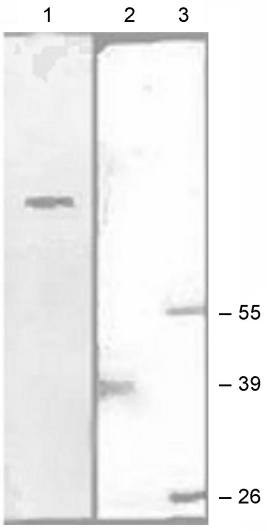

Fig. 2. Immunodetection of opium poppy AO using the polyclonal antibody anti-PSAO. 1 - opium poppy AO, 2, 3 - molecular mass standards (55 kDa - glutamate dehydrogenase, 39 kDa - aldolase, 26 kDa - triosephosphate isomerase)

The enzyme preparations used in our studies were assayed for copper content by atomic absorption spectrometry. The partly purified enzyme from pea contains 0.21 ± 0.02 μg(Cu) cm^{-3} and from poppy seedlings 0.16 ± 0.02 μg(Cu) cm^{-3}.

To find whether the polyclonal antibody anti-PSAO cross-reacts also with AO from poppy seedlings crude extract immunoblotting analysis was performed. Poppy homogenate rendered a single immunoreactive band of 83 kDa (Fig. 2). Obtained molecular mass of poppy AO corresponds to the typical subunit molecular mass of copper amine oxidases of 70 to 95 kDa (Padiglia *et al.* 1998). The most similar M_r for plant copper-AO has been reported per subunit of AO from *Thea sinensis* 81 kDa (Tsushida and Takeo 1985).

The obtained results indicate the presence of amine oxidase activity in the seedlings of opium poppy. According to substrate specificity, sensitivity to inhibitors, content of copper ions and immunoblotting analysis it appears that this enzyme is Cu-containing and tyramine specific and may be involved in (*S*)-reticuline biosynthesis.

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