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Screening of the occurrence of copper amine oxidases in *Fabaceae* plants

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Abstract

Aim of this work was to find the best source for obtaining high amount of copper amine oxidase (EC 1.4.3.6) that can be further used for analytical or industrial applications. The study focused on plant enzymes, because they occur in much higher content in the starting material than the enzymes from other sources, have higher specific activity and are also more thermostable. Presence of the amine oxidase was tested in extracts from 4 to 7-d-old seedlings of thirty-four various *Fabaceae* plants. Amine oxidases from nine selected plants were purified by general method involving ammonium sulfate fractionation, controlled heat denaturation, and three chromatographic steps. Kinetic properties of the amine oxidases purified were tested with a wide range of substrates and inhibitors and were found to be very similar. Best purification yield, and total and specific activities were obtained for the enzyme from grass pea (*Lathyrus sativus*) throughout all purification steps. Hence, the grass pea extract was chosen as a suitable candidate for massive production of the amine oxidase.

Additional key words: *Lathyrus*, *Coronilla*, *Lens*, *Lupinus*, *Medicago*, *Melilotus*, *Onobrychis*, *Pisum*, *Tetragonolobus*, topa quinone, *Trigonella*, *Vicia*.

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Abbreviations: GPAO - amine oxidase from *Lathyrus sativus* (grass pea); MAAO1 - amine oxidase from annual *Melilotus alba* (white sweet clover); MAAO2 - amine oxidase from biennial *Melilotus alba*; MOAO - amine oxidase from *Melilotus officinalis* (common melilot); MSAO - amine oxidase from *Medicago sativa* (alfalfa purple medica); PAAO - amine oxidase from *Pisum sativum* convar. *speciosum* (field pea); SPAO - amine oxidase from *Lathyrus odoratus* (sweet pea); TCAO - amine oxidase from *Trigonella coerulea*; TFAO - amine oxidase from *Trigonella foenum-graecum* (fenugreek).

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Introduction

The enzymes that catalyze oxidative deamination of a number of biogenic amines to the corresponding aldehydes, hydrogen peroxide and ammonia have been found in bacteria, fungi, plants and animals. There are two main classes of such enzymes: copper-containing amine oxidases (EC 1.4.3.6) which oxidize a wide range of primary amines (McIntire and Hartmann 1992, Medda *et al.* 1996) and FAD-containing amine oxidases (EC 1.4.3.4) that are restricted to polyamines spermidine and spermine (Smith 1985). In plants, high contents of copper-containing diamine oxidases have been found in germinating seeds of *Fabaceae* (Medda *et al.* 1995), whereas FAD-containing polyamine oxidases have been found in *Gramineae*. Enzymes of both types act in various physiological processes related to polyamine degradation (Frederico and Angelini 1991). Putrescine, spermidine and spermine show growth stimulating effect, stabilize cell walls and prolong the period of senescence. Products of putrescine and cadaverine oxidation, 4-aminobutanal cycling to Δ^1-pyrrolline and 5-aminopentanal cycling to Δ^1-piperideine, respectively, are the precursors for biosynthesis of alkaloids (McIntire and Hartmann 1992).

Concerning the copper-containing amine oxidases of *Fabaceae*, the enzymes from pea (*Pisum sativum*) and lentil (*Lens esculenta*) seedlings have been comprehensively studied so far (Medda *et al.* 1995). Copper and the organic cofactor topa quinone (Janes *et al.* 1992) have been found to mediate the amine oxidation following a ping-pong mechanism (Hartmann and Klinman 1991). Just very recently, the crystal structure of the pea enzyme has been solved (Kumar *et al.* 1996).

Amine oxidases have very broad substrate specificity and can oxidize wide range of compounds bearing primary aminogroup as recently shown for the enzyme from lentil (Medda *et al.* 1996). Concerning the practical use of these enzymes, the amine oxidase from pea seedlings has been used to construct amperometric biosensor based on the immobilized enzyme (Toul and Macholán 1975, Macholán and Slanina 1991, Wimmerová and Macholán 1996) for the assay of substrates and inhibitors. Amine oxidase has been used to detect histamine in meat products as a freshness control (Karube *et al.* 1980, Ohashi *et al.* 1994, Alam *et al.* 1995). The enzyme can be also used for large-scale preparation of aldehydes as recently demonstrated for the production of vanillin from vanillylamine using the amine oxidase from *Aspergillus niger* (Yoshida *et al.* 1997).

Looking for the most suitable source for massive production of amine oxidase usable for practical applications, we focused on plant enzymes which occur in much higher content in the starting material than the enzymes from other sources, are more thermostable, and have the highest specific activity.

Materials and methods

Chemicals: 2-Hydroxyputrescine (Macholán 1965), 3-hydroxycadaverine (Macholán 1972), *E*-2-butene-1,4-diamine (Macholán *et al.* 1975), *Z*-2-butene-1,4-diamine (Peč *et al.* 1991), and 2-butanone-1,4-diamine and 3-pentanone-1,5-diamine (Macholán

1965, 1974) were synthesized. Crystalline hydroxyapatite was prepared by the procedure of Mazin and Sulimova (1975) and mixed with a *Celite® 545* (*Fluka*, Buchs, Switzerland) in a volume ratio 1:1, before used as a chromatographic sorbent. All other chemicals were commercial products of analytical purity grade.

Plant material: Seeds of *Lathyrus odoratus* L. cv. Spencer carmine were purchased from *Semena* (Veleliby u Nymburka, Czech Republic). Seeds of *Medicago sativa* L. cv. Europe, *Pisum sativum* L. cv. Miracle from Kelvedon, *Lens esculenta* Moench. cv. Lenka, *Pisum sativum* L. convar. *speciosum* (Dierb.), and *Onobrychis viciifolia* Scop. cv. Višňovského were purchased from *Oseva* (Brno, Czech Republic). Seeds of *Lupinus albus* L. cv. Start, *Vicia pannonica* Crantz.cv. Dětěnická Panonská, *Vicia sativa* L. cv. Ebena and *Vicia villosa* Roth. cv. Viola were obtained from *Agritec* (Šumperk, Czech Republic). Seeds of *Coronilla varia* L. cv. Eroza, *Lathyrus sativus* L. cv. Newbred Troubsko, annual *Melilotus alba* Medik. cv. TB-9, biennial *Melilotus alba* medik. cv. Krajová, *Melilotus officinalis* (L.) Pall. (M: 1) (wild from Southern Moravia), *Trigonella coerulea* (L.) Sér. (M: 3) (wild from Southern Moravia), *Trigonella foenum-graecum* L. (M: 1), and *Vicia peregrina* L. (wild from Southern Moravia) were obtained from the Research Institute of Forage Crops (Troubsko u Brna, Czech Republic). Seeds of *Lathyrus amphicarpos* L., ssp. *quadrimarginatus* Bory et Chaub., *Lathyrus annuus* L., *Lathyrus aphaca* L. (G, M: 1), *Lathyrus articulatus* L., *Lathyrus clymenum* L. (G, M: 1), *Lathyrus digitatus* (M. Bieb.) Fiori, *Lathyrus gorgoni* Parl., *Lathyrus hierosolymitanus* Boiss., *Lathyrus latifolius* L. (G, M: 2), *Lathyrus laxiflorus* (Desf.) O. Kuntze, *Lathyrus neurolobus* Boiss. et Heldr., *Lathyrus ochrus* (L.) DC., *Lathyrus paranensis* Burk., *Lathyrus sylvestris* L., and *Lathyrus tingitanus* L. (G, M: 1) were obtained from the Institute of Plant Genetics (Gatersleben, Germany).

The seeds were soaked in distilled water for 24 h, transferred onto a *Perlite EP AGRO* (*Perlit*, Šenov u Nového Jičína, Czech Republic) layer, irrigated with tap water and germinated for up to 12 d at 23 °C in the dark. Amine oxidase activity and content of proteins were assayed daily in extracts prepared by homogenization of 10 g portions of seedlings in 20 cm^3 of 0.1 M potassium phosphate buffer, pH 7.0.

Amine oxidase activity and protein assay: Amine oxidase activity in crude extracts was determined by a spectrophotometric method with *E*-2-butene-1,4-diamine as the substrate (Macholán *et al.* 1975). This method is based on a detection of the aldehyde produced in the amine oxidase reaction and thus it is not affected by the presence of catalase in crude extracts like other commonly used methods based on the hydrogen peroxide detection. The reaction mixture (1.3 cm^3) contained 0.1 M potassium phosphate buffer, pH 7.0, catalase (25 μg) and 3.3 mM *E*-2-butene-1,4-diamine. The reaction was started by the addition of 0.2 cm^3 enzyme solution, incubated at 30 °C for 10 min and stopped by adding 1 cm^3 of Ehrlich´s reagent. The reaction mixture was incubated at 50 °C for 30 min, and then chilled on an ice bath before reading the absorbance of produced pyrrole at 563 nm. Concentration of protein was determined according to Bradford (1976) with bovine serum albumin as a standard.

Immunoblotting of plant seedling extracts. SDS-PAGE (Laemmli 1970) was performed on a slab polyacrylamide gel (12.5 %), then the proteins were transferred onto a *Immobilon*™ 0.45 μm polyvinylidene difluoride membrane (*Millipore*, Bedford, USA) in 0.025 M Tris - 0.15 M glycine buffer (pH 8.5) containing 20 % of methanol (10 h, 50 mA). The blots were then blocked with 50 cm^3 of 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS) containing 3 % gelatin for 2 h at laboratory temperature with reciprocal shaking and washed twice with 50 cm^3 of TBS containing 0.05 % Tween 20 (TTBS) for 10 min. The membranes were then incubated with the suitable concentration of primary rabbit antibody against *Aspergillus niger* AKU 3302 amine oxidase AO-II (Frébort *et al.* 1996) (250-fold diluted) in 50 cm^3 of TBS containing 1 % gelatin for 2 h and washed twice with 50 cm^3 of TTBS for 10 min. Then, the membranes were incubated with protein A - horseradish peroxidase (5000-fold diluted) (*Bio-Rad*, Hercules, USA) in 50 cm^3 of TBS - 1 % gelatin for 2 h, washed twice with 50 cm^3 of TTBS and once with 50 cm^3 of TBS for 10 min each and stained in the mixture of 50 cm^3 of TBS and 10 cm^3 of cold ethanol containing 0.03 g of 4-chloro-1-naphthol and 0.03 cm^3 of hydrogen peroxide for suitable time period. The stained membranes were washed with distilled water and photocopied. Following prestained calibration proteins (*Bio-Rad*) with the indicated molecular mass were used as references: lysozyme (16 kDa), soybean trypsin inhibitor (27 kDa), carbonic anhydrase (35 kDa), ovalbumin (57 kDa), bovine serum albumin (87 kDa), and phosphorylase *b* (105 kDa).

Purification of amine oxidases: For preparation of amine oxidases from plant material we used a purification method based on the procedures of Cogoni *et al.* (1989) and Padiglia *et al.* (1991). All purification procedures were performed at 0 - 5 °C and the buffers used contained 1 μM Cu(II). Seedlings (1 kg), germinated in the dark for 4 - 7 d, were homogenized for 10 min by a *Moulinex* hand blender in 2 dm^3 of 0.1 M potassium phosphate buffer, pH 7.0. Crude homogenate was filtered through a nylon mesh cloth and centrifuged at 5000 *g* for 60 min. The precipitate was discarded and the supernatant was fractioned with 20 - 30 % saturated ammonium sulfate, stirred for 30 min and centrifuged at 5000 *g* for 60 min. The supernatant obtained was further fractioned with 55 - 70 % saturated ammonium sulfate, stirred for 30 min and centrifuged at 5000 *g* for 60 min. The precipitate was then collected, suspended in 40 cm^3 of 0.1 M potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight. The dialyzed sample was rapidly heated up to 60 °C and kept for 5 min with stirring. The solution was cooled down to 4 °C on a water-ice bath, centrifuged at 15 000 *g* for 30 min and dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.0. The dialyzed sample was loaded onto a *DEAE-cellulose SH-23* (*Fluka*, Buchs, Switzerland) column (2.5 cm i.d. × 20 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. During loading and washing with the above buffer, the eluted solution of $A_{280} > 0.4$ was collected. This solution, was then applied directly onto a hydroxyapatite column (2.5 cm i.d. × 20 cm), equilibrated with 20 mM potassium phosphate, pH 7.0. The column was washed with the same buffer until A_{280} of the eluted solution decreased below 0.05. After initial application of 0.1 M potassium phosphate buffer to remove some contaminants, pH 7.0, the

amine oxidases were eluted by 0.3 M potassium phosphate buffer, pH 7.0. Fractions with highest amine oxidase activity were pooled, dialyzed overnight against 20 mM potassium phosphate buffer, pH 7.0, and concentrated in an ultrafiltration cell (*Amicon*, Danvers, USA) equipped with an *XM 50* filter. Finally, the enzyme was submitted to size-exclusion chromatography on a *Sephacryl S-300 HR* (*Pharmacia Biotech*, Uppsala, Sweden) column (2.5 cm i.d. × 50 cm) and eluted with 20 mM potassium phosphate buffer, pH 7.0, at a flow rate of 1.25 cm^3 min^{-1}. Fractions with highest enzymatic activity were pooled and concentrated by ultrafiltration as described above.

SDS-PAGE of purified enzymes: SDS-PAGE (Laemmli 1970) was performed on a slab polyacrylamide gel (10 %), the samples were heated with sampling buffer at 100 °C for 7 min prior to electrophoresis. As molecular mass markers, the LMW electrophoresis calibration kit (*Pharmacia Biotech*, Uppsala, Sweden) was used: Phosphorylase *b* (94 kDa), bovine serum albumin (63 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa). The gel was stained with Coomassie Brilliant Blue G-250.

Protein sequence analysis: Highly purified amine oxidases from *Lathyrus articulatus* and *L. clymenum* were obtained by following the general purification procedure up to heat denaturation as described above. Then the enzyme samples (nanomolar amounts) were further purified by SDS PAGE and electroblotted onto a *Immobilon* 0.45 μm polyvinylidene difluoride membrane (*Millipore*) in 0.025 M Tris - 0.15 M glycine buffer (pH 8.5) containing 20 % of methanol (10 h, 50 mA). The membrane parts containing the amine oxidase protein band at around 75 kDa were cut and used for N-terminal sequencing performed by automated Edman degradation on a *Model 476A Protein Sequencer* (*Applied Biosystems*, Foster City, USA).

Kinetic measurements. During the purification, the amine oxidase activity was determined using a coupled reaction with horseradish peroxidase and guaiacol (Frébort *et al.* 1989), with putrescine as a substrate. Measurements of substrate specificity and inhibitions were carried out in a 1 cm cell (thermostated to 30 °C) on a *Lambda 11* spectrophotometer, equipped with a Peltier-thermostatable single position cell holder and a magnetic stirrer (*Perkin-Elmer*, Überlingen, Germany). The reaction mixture (1.7 cm^3) contained 0.1 M potassium phosphate buffer, pH 7.0, 0.5 mM guaiacol, peroxidase (10 nkat), amine oxidase and inhibitor (optional). The enzyme was usually preincubated for 5 - 15 min with the respective inhibitor in the reaction mixture, then the reaction was started by injecting 0.05 cm^3 of putrescine (final concentration 0.1 - 0.5 mM, or 2.5 mM for saturation) and the increase in absorption at 436 nm was recorded for 3 min. Kinetic constants were calculated from initial rates using the program *GraFit 3.0* (*Erithacus Software Ltd.*) obtained from *Sigma Chemicals* (St. Louis, USA). Determination of the pH optimum was carried out in 0.1 M potassium phosphate buffers pH 5.8 - 8.0.

Results and discussion

Presence of the amine oxidase activity was tested in extracts from 7-d-old etiolated seedlings of 34 species of *Fabaceae* family. The highest activity per plant wet mass, over 2 nkat g^{-1}, was found in *Lathyrus sativus*. The activity was also high in some other *Lathyrus* species, *Trigonella foenum graecum*, *Pisum sativum* convar. *speciosum* and *Vicia peregrina*. These plants showed also relatively high specific activity of the amine oxidase in crude extract of the seedlings. On the other hand,

Table 1. Comparison of the amine oxidase activity in crude extracts of *Fabaceae* plants. Activities were measured by the dehydroputrescine method (Macholán *et al.* 1975) unless stated otherwise.

Plant	Activity per plant mass [nkat g^{-1} (f.m.)]	Specific activity [μkat g^{-1} (protein)]
Coronilla varia	0.00	0.00
Lathyrus amphicarpos	0.42	1.00
Lathyrus annuus	0.75	1.63
Lathyrus aphaca	0.50	0.63
Lathyrus articulatus	1.40	0.76
Lathyrus clymenum	1.54	1.34
Lathyrus digitatus	0.03	0.03
Lathyrus gorgoni	0.38	0.56
Lathyrus hierosolymitanus	0.16	0.24
Lathyrus latifolius	0.24	0.47
Lathyrus laxiflorus	0.11	0.34
Lathyrus neurolobus	1.07	1.07
Lathyrus ochrus	0.52	0.68
Lathyrus odoratus	1.30	0.69
Lathyrus paranensis	0.03	0.06
Lathyrus sativus	2.13	0.91
Lathyrus sylvestris	0.89	1.78
Lathyrus tingitanus	0.02	0.03
Lens esculenta	0.21	0.05
Lupinus albus	0.17	0.04
Medicago sativa	0.14	0.05
Melilotus alba annual	0.27	0.18
Melilotus alba biennial	0.25	0.15
Melilotus officinalis	0.24	0.17
Onobrychis viciifolia	0.48	0.19
Pisum sativum	0.23	0.41
Pisum sativum convar. *speciosum*	0.82	0.56
Tetragonolobus purpureus	0.04	0.01
Trigonella coerulea	0.23	0.14
Trigonella foenum-graecum	0.78	0.52
Vicia pannonica	0.31	0.29
Vicia peregrina	0.91	0.47
Vicia sativa	0.10	0.01
Vicia villosa	0.22	0.13

very low activities were found in *Coronilla varia*, *Lathyrus digitatus*, *L. tingitanus*, *Tetragonolobus purpureus* and *Vicia sativa* (Table 1).

Western blotting of some crude extracts and partially purified enzymes with the antibody against copper amine oxidase from the fungus *Aspergillus niger*, revealed that the plant enzymes studied share only little homology with the fungal enzyme. Despite that the staining was very faint, the samples from *Lathyrus odoratus*, *L. sativus*, *Medicago sativa*, *Melilotus officinalis*, *M. alba*, *Onobrychis viciifolia*, *Pisum sativum* convar. *speciosum*, *Trigonella coerulea*, *T. foenum-graecum*, *Vicia pannonica*, *V. peregrina*, and *V. villosa* gave positive staining of a band around 75 kDa on SDS-PAGE (not shown), which fits to the subunit size of copper amine oxidases (Frébort and Adachi 1995). Extracts from *Coronilla varia*, *Lupinus albus*, *Tetragonolobus purpureus*, and *Vicia sativa* did not show any staining. The faint level of staining accounts most likely for the fact that the amino acid sequence similarity of plant (pea or lentil seedling) and fungal amine oxidases is very low, only about 27 %, although structurally important residues are mostly conserved (Frébort and Adachi 1995).

Enzymes from following nine plants with higher amine oxidase content were chosen for further study: *Lathyrus odoratus* (SPAO), *L. sativus* (GPAO), *Medicago sativa* (MSAO), annual and biennial *Melilotus alba* (MAAO1 and MAAO2), *M. officinalis* (MOAO), *Pisum sativum* convar. *speciosum* (PAAO), *Trigonella coreluea* (TCAO) and *T. foenum graecum* (TFAO). The maximum activity in *Medicago sativa*, both *Melilotus alba*, *M. officinalis*, and *Pisum sativum* convar. *speciosum* was observed between 4 - 5 d of germination and in *Lathyrus odoratus*, *L. sativus*, *Trigonella coerulea*, and *T. foenum graecum* between 6 - 7 d of germination. After homogenization of seedlings removed of roots, the crude homogenate was fractioned with ammonium sulfate. Seedlings of *Medicago sativa*, *Melilotus alba*, and *M. officinalis* were very small, thus they were homogenized as whole without removing the roots. Suitable concentration of ammonium sulfate to remove the contaminating protein was chosen within the range 20 - 30 % and for precipitation of isolated amine oxidases in the range 55 - 70 % saturation (MAAO1, MAAO2 and MOAO, 30 and 55 %; PAAO and TFAO, 30 and 65 %; GPAO and SPAO, 25 and 65 %; MSAO, 20 and 70 %; TCAO, 30 and 70 %).

The purification grade after ammonium sulfate precipitation was only about 1.5 - 2-fold for most of the enzymes. The main advantage of this procedure, however, was a substantial volume reduction, necessary for the following heat denaturation step. All amine oxidases had good thermal stability at 65 °C. After short heating at 60 °C for 5 min, the total protein content decreased 1.3 - 1.6-fold in case of MSAO, MAAO1, MAAO2 and MOAO, and for the others there was more than 2-fold decrease, while total activity remained almost unchanged (Fig. 1). The amine oxidase content was further enriched by ion-exchange chromatography. The amine oxidases did not bind to *DEAE-cellulose* equilibrated with 20 mM potassium phosphate buffer, pH 7.0, while majority of contaminating ballast proteins was removed by binding to the ion-exchanger. The loss of total activity in this purification step was 10 - 20 % for GPAO, MAAO1, PAAO, SPAO and TFAO, but for MAAO2, MOAO, MSAO and TCAO it was much higher, about 60 - 80 %. Subsequently, the eluted solution was

directly loaded onto a hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The enzymes with amine oxidase activity were eluted from

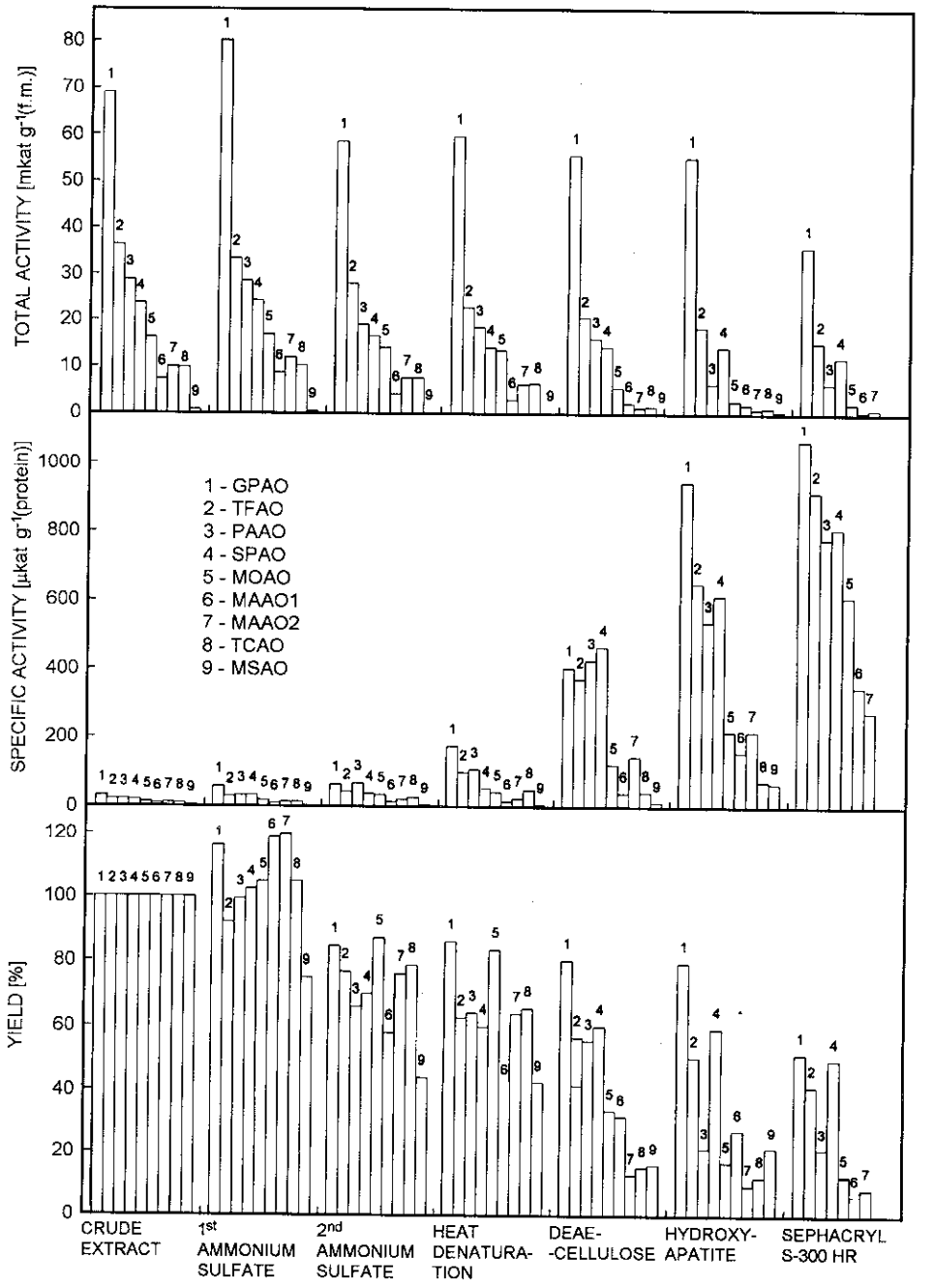

Fig. 1. Course of purification of amine oxidase from *Fabaceae* seedlings.

the column by 0.75 M potassium phosphate buffer, pH 7.0. Remaining impurities, mainly proteins of lower molecular mass, were at last removed by size-exclusion chromatography on a *Sephacryl S-300 HR*. The use of a single equilibrating buffer, 20 mM potassium phosphate, pH 7.0, was one of the advantages for the above three chromatographic steps in the present method.

Isolations of MSAO and TCAO had to be stopped after the hydroxyapatite due to limited amount of the enzymes remaining. These enzymes showed the specific activity only 64 and 71 μkat g^{-1}(protein) with putrescine as a substrate, respectively, and were far from being purified (SDS-PAGE not shown). The yield of the enzymes studied was in the range of 7 - 22 % and purification grade from 46 to 91-fold, except for MSAO, 261-fold (Fig. 1). Final preparations of MAAO1, MAAO2, MOAO and PAAO had the specific activities of 364, 274, 609 and 780 μkat g^{-1}(protein), respectively, and showed about 50 % purity on SDS-PAGE. Other enzymes were purified to electrophoretic homogeneity (Fig. 2). The specific activities for GPAO, SPAO and TFAO were 1071, 810 and 916 μkat g^{-1}(protein), respectively, with putrescine as the substrate.

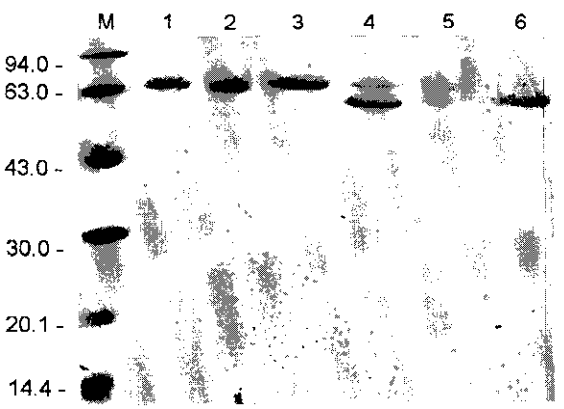

Fig. 2. SDS-PAGE of some purified and partially purified amine oxidases: M - molecular mass markers, 1 - SPAO (2.8 μg), 2 - GPAO (1.4 μg), 3 - TFAO (2.7 μg), 4 - PAAO (2.4 μg), 5 - MAAO1 (1.4 μg), and 6 - MAAO2 (2.3 μg). The gel was stained with Coomassie Brilliant Blue *G-250*.

Amine oxidases from *Lathyrus articulatus* and *L. clymenum* were purified by the procedure shown above up to heat denaturation, then further purified in nanomolar amounts by SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. The blots were used for N-terminal sequencing by automated Edman degradation that revealed the sequences VTPLHFQHPLDPLTK and VTPLHKQHPLDPLTKEEFLA for the amine oxidases from *L. articulatus* and *L. clymenum*, respectively. Further cycles were unreadable. The sequences are almost identical to those for amine oxidases from *L. sativus* and *L. odoratus* obtained recently (Šebela *et al.* 1998) and for other amine oxidases from *Fabaceae* plants (Rossi *et al.* 1992, Tipping and McPherson 1995, Zajoncová *et al.* 1997, Šebela *et al.* 1997).

Table 2. Substrate specificity of *Fabaceae* amine oxidases. Activities were measured with 2.5 mM substrates using coupled reaction with horseradish peroxidase and guaiacol in 0.1 M potassium phosphate buffer, pH 7.0. The rate of putrescine oxidation was arbitrarily taken as 100 % (*p*-dim.-benzylamine = *p*-dimethylaminomethyl-benzylamine, N-1-naph.diamine = N-1-naphthyl-ethylenediamine).

Substrate	Relative reaction rate [%]								
	PAAO	MAAO1	MAAO2	MOAO	MSAO	TCAO	TFAO	GPAO	SPAO
Cadaverine	112	114	113	111	110	123	105	113	120
Putrescine	100	100	100	100	100	100	100	100	100
Agmatine	25	22	21	43	26	20	44	32	27
Spermidine	43	32	30	42	32	44	31	26	27
2-hydroxyputrescine	15	24	27	11	14	13	27	18	17
Z-2-butene-1,4-diamine	20	37	33	21	11	21	16	16	16
E-2-butene-1,4-diamine	17	13	17	21	15	11	14	16	16
p-dim.-benzylamine	18	17	14	15	14	16	11	14	15
Tyramine	3	4	7	9	7	7	9	6	5
Histamine	4	7	6	5	2	4	4	3	4
Ethylenediamine	3	0	0	4	2	1	3	0	2
Benzylamine	0	0	0	2	1	1	2	2	2
n-hexylamine	2	0	0	5	1	0	1	2	0
Spermine	5	4	3	5	5	3	2	2	2
N-1-naph.diamine	2	2	5	5	1	5	4	3	3
Tryptamine	3	6	9	6	9	5	9	5	3
2-phenylethylamine	4	8	8	12	3	4	10	6	4
Dopamine	3	6	10	8	2	4	4	2	3
L-arginine	3	0	0	0	0	1	1	0	0

Table 3. Kinetic parameters K_m [mM] and V_{lim} [μkat g^{-1}(protein)] of studied amine oxidases with the best substrates. Activities were measured with 0.1 - 2.0 mM substrates using coupled reaction with horseradish peroxidase and guaiacol in 0.1 M potassium phosphate buffer, pH 7.0. Michaelis constants and limitation velocities were calculated from initial rates using GraFit 3.0. MAAO1, MAAO2, MSAO and TCAO were not purified to homogeneity, apparent V_{lim} values.

Substrate		PAAO	MAAO1	MAAO2	MOAO	MSAO	TCAO	TFAO	GPAO	SPAO
Putrescine	K_m	0.25	0.29	0.27	0.26	0.38	0.25	0.30	0.29	0.30
	V_{lim}	780	364	274	609	64	71	916	1070	807
Cadaverine	K_m	0.08	0.15	0.12	0.13	0.14	0.08	0.12	0.12	0.13
	V_{lim}	874	415	310	676	70	87	962	1209	968
Agmatine	K_m	0.27	0.29	0.31	0.36	0.40	0.15	0.45	0.25	0.50
	V_{lim}	195	80	58	262	17	14	403	342	218
Spermidine	K_m	1.54	1.43	1.43	1.18	0.91	0.78	0.95	1.45	1.67
	V_{lim}	335	116	82	256	20	31	284	278	218
E-2-butene	K_m	0.10	0.15	0.18	0.13	0.18	0.10	0.20	0.09	0.10
-1,4-diamine	V_{lim}	55	47	47	128	10	8	128	171	129
2-Hydroxy	K_m	0.74	0.37	0.39	0.37	0.48	0.25	0.59	0.63	0.67
-putrescine	V_{lim}	117	87	74	67	9	9	247	193	137

Number of compounds containing primary amino group of general formula RCH_2NH_2 was tested as substrates (Table 2). As well as other copper-containing amine oxidases (McIntire and Hartmann 1992), all enzymes studied showed broad substrate specificity. In accordance with the enzymes of plant origin (Medda *et al.* 1995), diamines and polyamines were readily converted. Monoamines were also oxidized, but at considerably lower rate. The best substrates were the aliphatic diamines cadaverine and putrescine, polyamines agmatine and spermidine, 2-hydroxyputrescine and both 2-butene-1,4-diamines. Ethylenediamine, histamine, spermine, *n*-hexylamine and aromatic amines were oxidized at much lower rates. Table 3 shows the kinetic constants K_m and V_{lim} for the best substrates putrescine, cadaverine, agmatine, spermidine, *E*-2-butene-1,4-diamine and 2-hydroxyputrescine. Values of K_m of single substrates are very similar for all studied amine oxidases. The lowest K_m was determined for cadaverine in the range 0.08 - 0.15 mM and the highest K_m for spermidine about 1.2 - 1.7 mM.

Table 4. Inhibition character and inhibition constants K_i [mM] of typical inhibitors of the amine oxidases studied (CI - character of inhibition, C - competitive inhibition, NC - noncompetitive inhibition). Activities were measured with putrescine as a substrate (0.1 - 0.5 mM) using coupled reaction with horseradish peroxidase and guaiacol in 0.1 M potassium phosphate buffer, pH 7.0, after preincubation (time in minutes is in parentheses) with the inhibitor necessary to reach steady state as indicated. Inhibition constants were calculated from initial rates using GraFit 3.0.

Inhibitors		PAAO	MAAO1	MAAO2	MOAO	MSAO	TCAO	TFAO	GPAO	SPAO
Substrate analogues										
1,5-Diamino-	K_i	0.025	0.048	0.050	0.040	0.048	0.060	0.020	0.026	0.026
3-pentanone	CI	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]
1,4-Diamino-	K_i	0.17	0.038	0.038	0.075	0.110	0.035	0.025	0.040	0.030
2-butanone	CI	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[5]	C[5]	C[5]
Copper chelators										
Diethylene-	K_i	11.5	8.75	9.25	8.75	15	7	5.7	16	14
triamine	CI	NC[5]	NC[5]	NC[5]	NC[5]	NC[5]	NC[5]	NC[5]	NC[5]	NC[5]
o-Phenanthroline	K_i	56	15	22	25	25	61	17	16	14
	CI	NC[10]	NC[10]	NC[10]	NC[10]	NC[5]	NC[5]	NC[10]	NC[5]	NC[5]
2,2′-Bipyridyl	K_i	95	67.5	72.5	50	117.5	130	89	50	75
	CI	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]
Alkaloids										
Cinchonine	K_i	125	400	400	350	670	340	210	150	120
	CI	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]
L-Lobeline	K_i	160	100	100	90	75	120	150	180	200
	CI	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]	C[0]
Other										
Aminoguanidine	K_i	1.7	3.4	3.4	4.2	6.3	2.1	1.1	0.5	1.0
	CI	C[15]	C[10]	C[10]	C[10]	C[15]	C[10]	C[15]	C[15]	C[15]
Acetone oxime	K_i	2600	430	350	480	1500	1240	2800	700	450
	CI	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[10]	NC[15]	NC[15]

The enzymes were inhibited by substrate analogues, copper chelators and some alkaloids (Table 4). For all studied enzymes, the same type and similar strength of the inhibition by particular inhibitors were found. Substrate analogs, 3-pentanone-1,5-diamine and 2-butanone-1,4-diamine ($K_i \sim 10^{-8}$ M), were the most potent reversible and competitive inhibitors of all amine oxidases studied. These amines inhibit pea seedling amine oxidase as well (Skyvová and Macholán 1970) being oxidized at a very low rate compared to usual substrates. Copper chelators were all non-competitive inhibitors with K_i values of 10^{-5} M as observed earlier for the pea enzyme (Hill and Mann 1962, Peč and Frébort 1992). The alkaloids cinchonine and L-lobeline were competitive inhibitors with K_i values ranging from 0.075 to 0.67 mM. Similarly to the amine oxidase from pea seedlings (Luhová *et al.* 1996), this inhibition may be caused by binding to a hydrophobic site located close to the cofactor and thus blocking the active site.

To conclude the screening, it has been revealed that various *Fabaceae* plants can be used as rich sources for obtaining higher amounts of amine oxidase. Reasonably pure enzyme preparation reaching about 50 % purity can be rapidly obtained (in three days) from *Lathyrus odoratus*, *L. sativus*, *Pisum sativum* convar. *speciosum,* and *Trigonella foenum-graecum*, by two step ammonium fractionation, heat denaturation and chromatography on DEAE-cellulose. The enzymes from *Lathyrus odoratus*, *L. sativus* and *Trigonella foenum-graecum* can be further purified by chromatography on hydroxyapatite and Sephacryl S-300 HR to complete homogeneity. From the point of view of purification yield and quantity, as the best candidate for large scale purification of these three plant sources comes out the seedlings of *Lathyrus sativus*.

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