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Full Length Research Paper

Purification and characterization of amine oxidase from *Vigna mungo* L. seedlings

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Amine oxidases (AO) are a group of enzymes that catalyze oxidative deamination of various amines and thus are of potential use in analytical applications. Amine oxidase from five-day-old *Vigna mungo* L. seedlings (VAO) was purified using ammonium sulfate fractionation and Q-Sepharose chromatography to 544 purification folds with 65% yield. VAO apparently is a homodimer with denatured molecular weight of 73 kDa. This enzyme is relatively stable in a pH range of 6.0 to 8.0 and at temperature below 40°C with a complete activity loss upon storage at pH 4.0 or temperature over 60°C (1 h). Kinetics studies of VAO with putrescine, cadaverine, histamine, and tyramine showed k_{cat}/K_m values of 2.54×10^7, 6.73×10^6, 2.65×10^5, and 3.31×10^3 M^{-1} s^{-1}, respectively, with undetectable catalytic activity toward tryptamine. VAO was partially inhibited by ethylenediaminetetraacetic acid (EDTA) and completely inhibited by phenylhydrazine, suggesting it is likely a member of copper-containing AO family.

Key words: Amine oxidase, cadaverine, histamine, putrescine, tyramine, tryptamine, *Vigna mungo* L.

INTRODUCTION

Amine oxidases (AO, histamine:oxygen oxidoreductase, E.C.1.4.3.22) are a group of enzymes that oxidize diamines, such as histamine, and also some primary monoamines, with little or no activity towards secondary and tertiary amines (Chang et al., 2008). These copper-containing, glycosylated enzymes usually are homodimers of 70 to 95 kDa subunit, depending on their sources (Medda et al., 1995). Each subunit contains one tightly bound Cu(II) and an organic prosthetic group identified as 2,4,5-trihydroxyphenylalaninequinone, also known as TOPA-quinone (TPQ) (Janes et al., 1990). The TPQ cofactor is obtained from post-translational modification of a conserved tyrosine (Janes et al., 1990), and can react with carbonyl-group modifying reagents such as phenylhydrazine forming irreversible adducts which results in the loss of AO catalytic activity (Padiglia et al., 1998b). These enzymes are widely distributed in mammals, plants, as well as microorganisms, both prokaryotic and eukaryotic.

In prokaryotes and lower eukaryotes, AOs play roles in utilization of amines as the sources of carbon and nitrogen necessary for cell growth (Cohen, 1998). In higher eukaryotes, however, these enzymes are involved

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Abbreviations: AO, Amine oxidase; **BSAO,** bovine serum amine oxidase; **HRP,** horseradish peroxidase; **LCAO,** *Lathyrus cicera* amine oxidase; **PSAO,** *Pisum sativum* amine oxidase; **SBAO,** amine oxidase from soybean seedlings; **TPQ,** 2,4,5-trihydroxyphenylalaninequinone; **VAO,** amine oxidase from *Vigna mungo* L. seedlings.

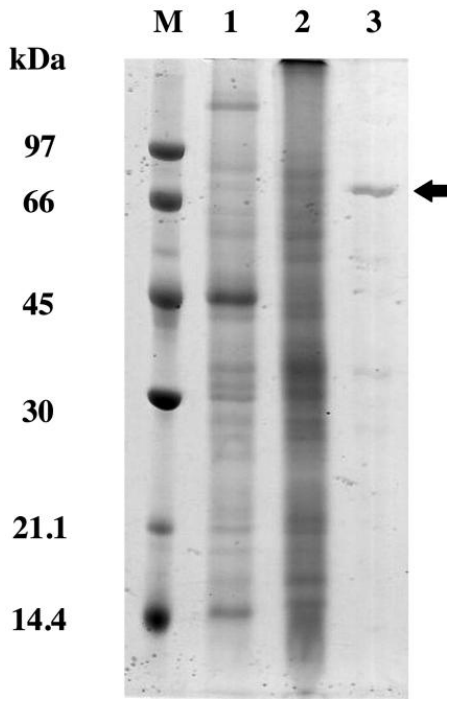

Figure 1. Coomassie-stained SDS-PAGE showing samples from various steps of VAO purification. Shown in lanes 1 to 3 are crude extract from *V. mungo* L. (300 µg), dialyzed fraction after 30 to 70% ammonium sulfate fractionation (350 µg), and purified VAO from Q-Sepharose Fast Flow chromatography (6.25 µg), respectively. Low molecular weight markers (GE Healthcare Life Science) are in lane M. The purified VAO band indicated with an arrow is of at least 80% homogeneity.

in broader cellular functions, including detoxification, wound and resistance responses, secondary metabolism, cell growth, development, and defense (Fontecave and Eklund, 1995; Cona et al., 2006).

AOs catalyze oxidative deamination of various amines via a two-step mechanism (Morpurgo, 2001):

Step 1: Reduction of AO by substrate

$$E_{ox} + R\text{-}CH_2\text{-}NH_3^+ \rightarrow E_{red}\text{-}NH_3^+ + R\text{-}CHO$$

Step 2: Reoxidation of AO using molecular oxygen

$$E_{red}\text{-}NH_3^+ + O_2 \rightarrow E_{ox} + NH_4^+ + H_2O_2$$

This generates a corresponding aldehyde from the amine substrate, as well as ammonium ion and hydrogen peroxide detectable with quantitatively analytical systems. These make AO an attractive system for analyses of various amines. Nevertheless, the only commercially available AO from porcine kidney has lower specific activity than enzymes from plants, especially those belonging to the leguminous family (Cohen, 1998; Wimmerova et al., 1999). AO activity is found in many plants such as *Glycine max* (Vianello et al., 1993), *Pisum sativum* (Vianello et al., 1999), *Onobrychis viciifolia* (Zajoncova et al., 1997), *Trigonella foenum-graecum* (Luhova et al., 1995), *Euphorbia characias* (Padiglia et al., 1998a), *Cicer arietinum* (Rea et al., 1998), *Triticum aestivum* (Suzuki, 1996), and *Papaver somniferum* L. (Bilkova et al., 2005). Among the many species of legumes in Thailand, we have isolated and characterized AO from *Vigna mungo* L. (black bean) seedlings as a potential enzyme for amine analysis applications.

MATERIALS AND METHODS

All chemicals and reagents used were of analytical grade. *V. mungo* L. seeds were obtained from local market. Horseradish peroxidase, cadaverine, putrescine, tyramine, and 3,3'-diaminobenzidine were the products of Sigma-Aldrich, USA. Tryptamine and 4-aminoantipyrine were products of Fluka, USA. Phenol was purchased from Merck, USA, and dithiothreitol (DTT) was from Research Organic, USA.

Purification of AO from *V. mungo* L. seedlings

Seeds of *V. mungo* L. were soaked in distilled water overnight and germinated on moist vermiculites in the dark for the first day. After that, the germinated seeds were exposed to light and cultivated. AO activity was then followed up to eight days. For purification of AO from *V. mungo* L. seedlings (VAO), the 5-day-old seedlings were collected and washed with distilled water and their cotyledons were removed.

The washed *V. mungo* L. seedlings were homogenized with an equal amount (w/v) of 50 mM potassium phosphate buffer, (pH 7.0) using 450 W blender (Moulinex, France). Crude extracts were obtained by squeezing the homogenates through four layers of muslin cloth and centrifuged at 20,000 g for 20 min at 4°C to remove insoluble debris. Ammonium sulfate was added to the soluble fraction to 30% (w/v) saturation. After 1 h incubation at 4°C, the suspension was centrifuged at 20,000 *g* for 20 min to discard precipitated materials. The supernatant was subsequently precipitated at 70% (w/v) ammonium sulfate saturation. After 1 h incubation, the new pellet was collected with centrifugation at 20,000 *g* for 20 min. The collected pellet was dissolved in a small volume of 50 mM potassium phosphate buffer (pH 8.0), dialyzed 3 times against the same buffer, and kept at -20°C for storage.

The dialyzed solution was applied to a column (2.5 x 10 cm) of 40 ml packed Q-Sepharose Fast Flow (GE Healthcare Life Science, USA) pre-equilibrated with 200 ml of 50 mM potassium phosphate buffer (pH 8.0). The column was washed with 50 mM potassium phosphate buffer, pH 7.6 to remove unbound molecules. Then, VAO was eluted from the column using 50 mM potassium phosphate buffer, pH 6.6. All fractions containing AO activity were collected and concentrated using Amicon Ultra-15 centrifugal filter units with 10 kDa MWCO (Millipore, USA). The purification results are summarized in Table 1 while sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of samples is shown in Figure 1.

AO activity assay

The method used for AO activity measurements was modified from

peroxidase-coupled assay (Trinder, 1969). Cadaverine was used as a substrate for AO reaction. The released H_2O_2 was then used as a substrate for a coupled peroxidase reaction catalyzed by horseradish peroxidase (HRP).

$$\text{Cadaverine} + O_2 \xrightarrow{\text{AO}} \text{Piperidine} + H_2O_2 + NH_3$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{HRP}} \text{Quinoneimine dye} + 4H_2O$$

The standard reaction mixture contained 2 mM phenol, 2 mM of 4-aminoantipyrine, 5 U/ml HRP, and 1 mM of cadaverine in 0.1 M potassium phosphate buffer at pH 7.0. The reaction was incubated at 25°C for 10 min, while the resulting quinoneimine was continuously monitored at 510 nm using UV-2501PC spectrophotometer (Shimadzu, Japan).

One katal (kat) of VAO is defined as the amount of the enzyme that produces 1 mole of hydrogen peroxide (equivalent to a half mole of quinoneimine adduct) per second under the standard condition (25°C, pH 7.0) using 1 mM cadaverine as a substrate. Specific activity of VAO is reported in nkat/mg protein.

Protein determination and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentration was determined based on Bradford method (Bradford, 1976) using Coomassie Plus® Protein Assay Reagent Kits (Pierce Biotechnology, USA) with BSA as a protein standard. The concentrated, purified VAO in SDS loading buffer (0.1 M DTT, 2% (w/v) SDS, 0.08 M Tris pH 6.8, 10% (v/v) glycerol and 0.004% (w/v) bromophenol blue) was heated at 95°C for 5 min for purity assessment using SDS polyacrylamide gel electrophoresis (12.5%T, 2.67%C) according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250.

For AO-activity staining, equal amounts of VAO were electrophoresed with slight modification. An aliquot of VAO was completely denatured as described previously, while another aliquot was partially denatured with similar SDS loading buffer but in the absence of DTT and with no heating. SDS was removed from the electrophoresed SDS gel for VAO renaturation by soaking the gel in 2.5% (v/v) Triton X-100 for 1 h and washed with excess distilled water. The gel was stained based on AO activity by a method modified from peroxidase-coupled assay (Trinder, 1969), using activity-staining solution that contained 0.002% (w/v) 3,3'-diaminobenzidine as a chromogen, 1 U/ml of HRP, 10 µM of cadaverine as a substrate in 0.1 M potassium phosphate buffer, pH 7.0. The gel was incubated in the activity-staining solution in the dark at room temperature for 30 min to allow for color development, and subsequently soaked in distilled water to stop the reaction. A duplicate gel was also made in parallel for Coomassie staining.

Copper determination

The presence of copper ion in VAO sample was determined with graphite furnace atomic absorption spectrophotometer Analyst 600 (Perkin Elmer, USA), using Copper PerkinElmer Pure AS Calibration Standard to construct a standard curve covering the range of 0 to 25 µg/l. The spectral line of 324.8 nm was selected for copper detection.

Effects of ethylenediaminetetraacetic acid (EDTA) and phenylhydrazine on VAO activity

The effects of ethylenediaminetetraacetic acid (EDTA), a metal chelating agent, as well as phenylhydrazine, a carbonyl-group modifying reagent, on VAO activity were investigated. An aliquot of VAO was dialyzed twice against 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM EDTA (1:1000 volume ratios). Activity of VAO, after dialysis against EDTA, was measured in the standard assay reaction, in comparison with VAO dialyzed against 50 mM potassium phosphate buffer, pH 8.0 without EDTA. In addition, the EDTA-treated VAO was dialyzed against 50 mM potassium phosphate buffer, pH 8.0 without EDTA and then incubated with 100 mM $CuSO_4$ for 10 min at 25°C. Aliquots (20 µl) of the pre-incubated VAO were then tested with the standard AO assay to determine the effect of Cu^{2+}. For determination of the effect of phenylhydrazine, VAO was pre-incubated with different concentrations (1 to 1000 nM) of phenylhydrazine for 5 min at 25°C prior to the AO activity assay.

Determination of pH and thermal stability of VAO

Aliquots of VAO were pre-incubated in buffer solutions of different pH values in the range of 4 to 9 for 1 h at 25°C. The buffer solutions were mixtures of 0.1 M each of sodium acetate, potassium phosphate, and Tris-HCl, with the required pH values adjusted with either 0.1 N HCl or 0.1 N NaOH before making up to the final volume. A 20 µl aliquot of the pre-incubated VAO was used to initiate the reaction using the standard assay described previously. To determine thermal stability of VAO, a similar protocol was applied with aliquots of VAO pre-incubated in 0.1 M potassium phosphate buffer pH 7.0 at various temperatures ranging from 30 to 60°C for 1 h. The standard reaction mixture was also pre-incubated for 10 min at 25°C prior to reaction initiation by addition of 20 µl of the pre-incubated VAO.

Kinetics of VAO

Steady-state kinetic parameters of VAO were determined using continuous assay for the AO activity in the presence of varying concentrations of substrate: putrescine (0 to 50 µM), cadaverine (0 to 100 µM), histamine (0 to 2.0 mM), tyramine (0 to 5.0 mM), or tryptamine (0 to 1.0 mM) under the standard conditions. About 25 µg of VAO were used in each assay reaction. Kinetic parameters were determined using Prism (GraphPad, USA) version 5 employing a non-linear least square fit of the data to the Michaelis-Menten equation. Each data point of the curves was assayed in triplicate and the mean ± SD values were calculated. The K_m value for each substrate was calculated from curve fitting to the Michaelis-Menten equation using the Prism program.

RESULTS AND DISCUSSION

Purification of VAO

V. mungo L. seeds were germinated and cultivated. Extracts from the seedlings were assayed for AO activity everyday up to eight days of growth. After germination, AO activity rose to its maximum on the fifth day of cultivation, followed with a sharp decrease on subsequent days. Very little AO activity was found in cotyledons, compared with the rest of the seedlings. Generally, AO is mainly found in plant tissues undergoing lignification and extensive wall stiffening events, which suggests its involvement in cell wall strengthening and rigidity (Cona et al., 2006).

Table 1. Purification table of amine oxidase from *V. mungo* L. seedlings.

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)
Crude extract	1.788	44.01	0.025	100	1.00
30–70% ammonium sulfate fractionation	262.7	35.84	0.136	81.4	5.54
Q Sepharose FF chromatography	2.130	28.51	13.4	64.8	544

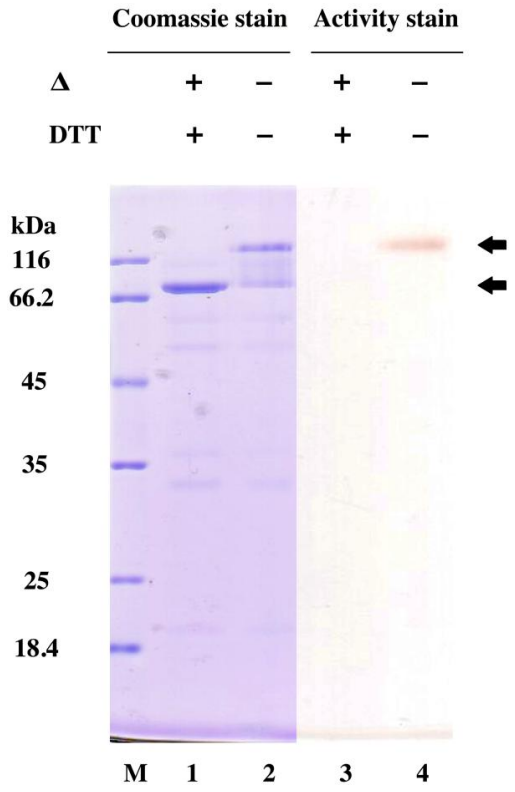

Figure 2. Coomassie- and activity-stained SDS-PAGE of VAO. VAO samples were completely or partially denatured in SDS-loading buffers with DTT and 95°C treatment (lanes 1 and 3) or without DTT and no heating (lanes 2 and 4) prior to electrophoresis. The gel was cut in half for Coomassie BBR-250 (lanes M, 1, and 2) and AO activity (lanes 3 and 4) staining. Low molecular weight markers (Fermentas) are in lane M. The two VAO forms are pointed with arrows. Only the upper band could regain AO activity upon removal of SDS, suggesting this form resemble its native form.

Cotyledons serve as food storage organs that provide proteins, starch, and lipids for utilization by the seedlings during non-photosynthetic growth after germination (Taiz and Zeiger, 2002). Once the storage foods are depleted, cotyledons have other roles in photosynthesis despite no growth and development occurring in cotyledon part. This might explain the lack of AO activity in cotyledon. Moreover, the cotyledons contain a lot of enzymes responsible for breaking down the storage foods during germination. Thus, cotyledons should be excised from seedlings before preparation of crude extract to reduce impurities, especially proteases that might affect the overall AO yield. Similar procedures are reported in the preparation of AOs from soybean (Vianello et al., 1993), pea (Vianello et al., 1999), and poppy seedlings (Bilkova et al., 2005).

Five-day-old, cotyledonless *V. mungo* L. seedlings was homogenized for extraction of VAO. Total activity of 44.01 nkat (Table 1) was obtained from 50 g seedlings. The crude extract was precipitated with 30% ammonium sulfate to remove the undesired proteins and other impurities such as pigments, lipids and phenolic compounds by centrifugation. The 30% supernatant, which contained AO activity, was subsequently fractionated with 70% ammonium sulfate.

After centrifugation, the supernatant was discarded and the 30 to 70% precipitates were dissolved in and dialyzed against 50 mM potassium phosphate buffer, pH 8.0. Roughly, 81% yield of AO activity with 5.5-folds increase in specific activity was obtained. The dialyzed solution was loaded into Q-Sepharose fast flow column pre-equilibrated with 50 mM potassium phosphate buffer, pH 8.0. The major impurities were washed out while VAO bound to the column. Once no A_{280} of protein impurities was detected in the wash, VAO was eluted out by lowering the pH to 6.6. This step could significantly remove the majority of contaminated proteins and increased the purification fold to 544 with approximately 65% recovery yield (Table 1). The collected VAO was concentrated and divided into aliquots for –20°C storage. The purity of VAO in each step of purification was monitored by SDS-PAGE as shown in Figure 1.

Characterization of VAO

A major stained band with an estimated mobility of a 73 kDa protein was visible on the Coomassie-stained SDS-PAGE (Figures 1 and 2), which reflected the monomeric size of VAO. We were able to detect AO activity on native-PAGE gel using our chromogenic coupling assay reagents which displayed a broad color band co-localized with one of the multiple bands stained with Coomassie dye. The AO-activity staining method was then applied to verify the presence of VAO band in SDS-PAGE. The result shows that VAO could regain AO activity when SDS was removed from the gel, provided that the VAO

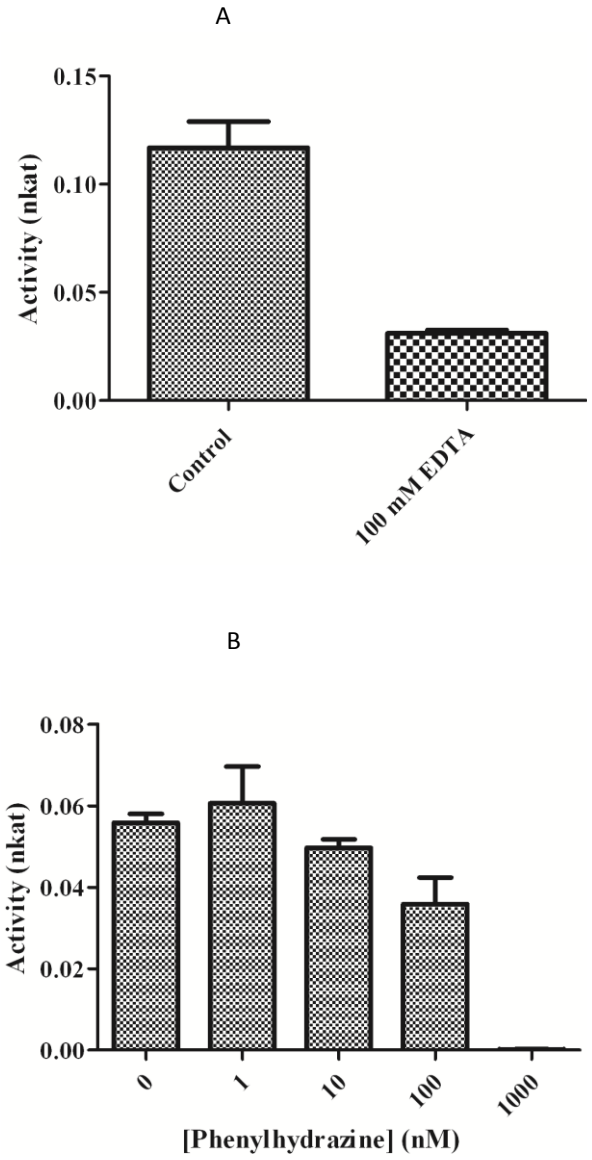

Figure 3. Inhibition of VAO activity upon treatment with either EDTA or phenylhydrazine. VAO samples were treated with (a) EDTA or (b) phenylhydrazine prior to AO activity assay. Reduction of VAO activity by the pre-treatment with metal ion chelator or the carbonyl-group modifying reagent resembles the profile of copper-containing AOs. Data shown were mean values of triplicate measurements.

sample was only partially denatured in the absence of DTT and with no heating (Figure 2). Inclusion of either DTT or heat treatment in the sample preparation prevented the activity regain. The apparent size of the activity-stained band, however, was larger than the 116 kDa MW standard and co-migrated with the larger of the two bands stained with Coomassie dye (Figure 2, lanes 2 and 4). These suggested that VAO may form a homodimer of 73 kDa subunits in its functional, native state.

Copper content and inhibition studies of VAO

The purified VAO sample was found to contain 5.08 ± 0.1 μg/l copper ion as determined by atomic absorption spectrophotometry. Since there have been several reports on plant AOs containing copper ion as well as TPQ essential for their catalytic activities, EDTA and phenylhydrazine were used to probe whether VAO showed similar properties (Figure 3). EDTA, a metal chelating agent at 100mM caused AO activity reduction to 24.5% in comparison with the control under the standard conditions (Figure 3a). This loss of VAO activity, however, was not regained upon pre-incubation with Cu^{2+} ion.

We also found that phenylhydrazine could completely inhibit VAO at 1 μM (Figure 3b), suggesting there was a reactive carbonyl group crucial for VAO catalysis. The VAO inhibition results were similar to inhibition studies of copper-containing AOs from other plants (Zajoncova et al., 1997; Bilkova et al., 2005). Together with the detection of copper in the purified VAO sample, these suggested that VAO might be a copper-containing AO. Additional studies are needed, however, to conclusively verify this.

Stability of VAO

Thermal stability of VAO was examined upon storage at various temperatures for 1 h (Figure 4a). The activity of VAO was found stable up to 40°C and dropped dramatically to approximately 60% upon 1 h incubation at 50°C. The enzyme was almost completely inactivated when stored at 60°C for 1 h. The effect of pH on the stability of VAO was also studied (Figure 4b). VAO activity decreased slightly when it was stored in a pH range of 6.0 to 8.0 for 1 h at 25°C. The activity, however, dropped to 50% at pH 4.5 and completely diminished at pH 4. This behavior was similar to that of AO from soybean seedlings (SBAO), which entirely lost its activity upon storage at pH 4 (Vianello et al., 1993). Nevertheless, SBAO was totally inactive at pH above 8.0, while 75% of VAO activity was still retained at pH 9.0. Our findings show that even though SBAO was more stable in mildly acidic environment (pH 5 to 7), VAO appeared more robust in a moderately alkaline solution.

Kinetic parameters of VAO

Five biogenic amines with different structures were used as substrates of VAO for steady-state kinetics study. These included heterocyclic (histamine and tryptamine), aromatic (tyramine), and aliphatic (putrescine and

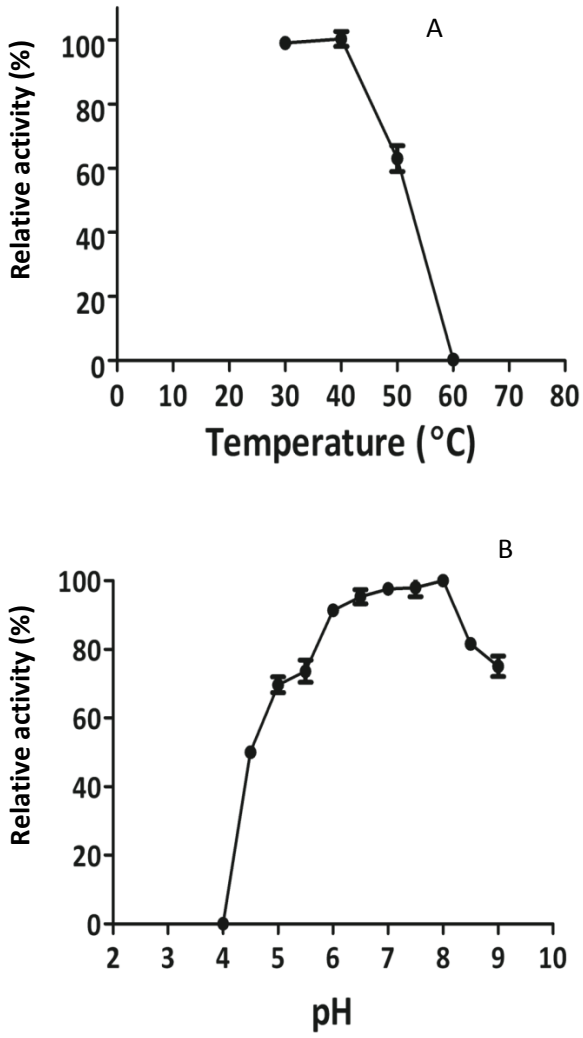

Figure 4. Thermal and pH stability of VAO. VAO samples were pre-incubated in (a) potassium phosphate buffer pH 7.0 at various temperatures ranging from 30 to 60°C or in (b) the buffer mixtures pH 4 to 9 at 25°C for 1 h prior to AO activity assay. Data shown were mean values of triplicate measurements. The maximum activity (100% relative activity) was 43 or 25 pkat for thermal or pH stability measurement, respectively.

cadaverine) primary amines. The modified peroxidase-coupled assay was developed to indirectly measure AO activity via oxidation of 4-aminoantipyrine (yellow color) and phenol by H_2O_2, which was generated from oxidative deamination of the amine substrate, to quinoneimine adduct (pink color) spectrophotometrically detectable at 510 nm. The coupling components (phenol, 4-aminoantipyrine, and HRP) were added in excess to the reaction to ensure that the rate of AO reaction was not limited by that of the coupling reaction.

Kinetic parameters of VAO towards the amine substrates are summarized in Table 2. Assuming a monomeric size of VAO of 73 kDa to estimate its molar concentration used in the assay, catalytic rate constants k_{cat} for those amines could be calculated, together with the specificity constants k_{cat}/K_m. The ranges of catalytic rates and K_m values of VAO were comparable with those of AOs from other sources such as *Lathyrus cicera* (LCAO), *Pisum sativum* (PSAO), and bovine serum AO (BSAO) (De Matteis et al., 1999; Pietrangeli et al., 2007).

The k_{cat}/K_m values of VAO for cadaverine and putrescine were 25 to 96 folds over that of histamine and greater than 2000 folds over that of tyramine. VAO activity towards tryptamine was not detectable up to 1.0 mM concentration in the reaction. These suggested that small and aliphatic amines may be the most preferable substrates of VAO. Heterocyclic and aromatic amines, represented by histamine, tyramine, and tryptamine, showed lower substrate specificity for VAO. These were similar to the results reported for AOs from other plants such as LCAO and PSAO (Pietrangeli et al., 2007) but significantly different from AO from animal source likes BSAO (De Matteis et al., 1999). While VAO, LCAO, and PSAO prefer putrescine over histamine and tyramine, BSAO apparently has very poor catalytic activity towards putrescine. Interestingly, BSAO displays mixed preferences towards both aromatic and aliphatic amine substrates with most preferred k_{cat}/K_m values for spermidine, spermine, and 4-aminomethylpyridine ranging from 1.1×10^4 to 1.8×10^5 $M^{-1}s^{-1}$ and those least preferred putrescine, histamine, and tyramine ranging from 1.3 to 1.3×10^2 $M^{-1}s^{-1}$ (Pietrangeli et al., 2007). These observations may reflect the different roles of AOs in metabolic pathways between plants and animals.

Conclusion

Among several species of legumes in Thailand, we have purified and characterized AO from *V. mungo* L. (black bean) seedlings. Kinetics data suggested this VAO prefer aliphatic amines putrescine and cadaverine over aromatic or heterocyclic substrates histamine, tyramine, and tryptamine with substantial selectivity. Together with its relative stability in the neutral and alkaline pH, the facts that VAO can withstand temperature up to 40°C and has its catalytic rates in the range of 5 to 130 s^{-1} with catalytic efficiencies k_{cat}/K_m ranging 10^3 to 10^7 M^{-1} s^{-1} make this enzyme appealing for various applications including those for analytical amine detections.

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Table 2. Steady-state kinetic parameters of VAO.

Substrate	Kinetic parameters			
	K_m (μM)	V_{max} (pkat)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Putrescine	5.16±0.589	44.7±1.11	131±3.25	2.54×10^{7}
Cadaverine	13.2±1.73	30.4±1.11	88.7±3.27	6.73×10^{6}
Histamine	213±13.5	18.9±0.370	56.5±1.09	2.65×10^{5}
Tyramine	1410±760	1.56±0.270	4.68±0.831	3.31×10^{3}
Tryptamine	N/D[a]	N/D[a]	N/D[a]	N/D[a]

[a], No significant activity was detected up to 1.0 mM tryptamine.

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