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# Inhibition of chickpea seedling copper amine oxidases by tetraethylenepentamine

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## A B S T R A C T

Copper amine oxidases are important enzymes, which contribute to the regulation of mono- and polyamine levels. Each monomer contains one Cu(II) ion and 2,4,5-trihydroxyphenylalanine (TPQ) as cofactors. They catalyze the oxidative deamination of primary amines to aldehydes with a ping-pong mechanism consisting of a transamination. The mechanism is followed by the transfer of two electrons to molecular oxygen which is reduced to hydrogen peroxide. Inhibitors are important tools in the study of catalytic properties of copper amine oxidases and they also have a wide application in physiological research. In this study, purification of the chickpea seedling amine oxidase, was done via salting out by ammonium sulfate and dialysis, followed by DEAE-cellulose column chromatography. By using the Lineweaver - Burk plot, the $K_m$ and $V_m$ of the enzyme were found to be 3.3 mM and 0.95 mmol/min/mg, respectively. In this study, the interaction of chickpea diamino oxidase with tetraethylene- pentamine was studied. Analysis of kinetic data indicated that tetraethylenepentamine (with $K_i$=0.1 mM) inhibits the enzyme by linear mixed inhibitory effect.

**Key words:** Chickpea, Copper-containing amine oxidases**,** Tetraethylenepentamine, Linear mixed.

## INTRODUCTION

Amine oxidases (AOs) present a class of enzymes, which are divided into two main groups based on the chemical nature of the attached cofactor, including the AOs containing flavin adenine dinucleotide (FAD-AOs), and copper-containing amine oxidases ($CuAO_S$), which have a tightly bound copper ion in their molecule [1-9]. FAD-$AO_S$ and $CuAO_S$ are present in all kinds of organisms such as bacteria, yeasts, mushrooms, plants, and animals. They catalyze the oxidative deamination of mono- and polyamines (essential compounds for cell growth and proliferation) to the corresponding aldehydes and hydrogen peroxide according to the following reaction [2, 5-10]: $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$.

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Therefore, amine oxidases and products deriving from the oxidation of amines are essential in physiological processes. Inhibitors play important roles in the study of catalytic properties of AOs and can be divided into reversible and irreversible groups [11, 12]. The use of inhibitors provides substantial information to understand the catalytic mechanism of plant copper amine oxidases [3]. Mixed inhibition refers to a combination of competitive and uncompetitive inhibition. In mixed inhibition, the inhibitor binds to the free enzyme or the enzyme-substrate complex, which is a site different from the active site where the substrate binds [13, 14]. In the present study, we report on the inhibitory effect of tetraethylenepentamine on the chickpea seedling amine oxidase.

## MATERIALS AND METHODS

**Chemicals:** DEAE-cellulose, horseradish peroxidase and putrescine dihydrochloride were purchased from Sigma-Aldrich. Tetraethylenepentamine and guaiacol were purchased from Merck.

**Protein purification:** The preparation of a sufficient amount of purified chickpea amine oxidase was carried out in three steps: (a) the seeds' germination, (b) ammonium sulfate fractionation, (c) ion-exchange chromatography on diethylaminoethylcellulose. The last two steps were carried out at 4 °C.

**Germination:** Chickpea (*Cicer arietinum*) seeds were washed with 0.5% $KMnO_4$ solution to keep mold and bacteria from growing. Chickpea seeds, layered on suitable wet cotton containing trays, were watered with warm tap water (30 °C) for 2 min. This procedure was repeated every 30 min. The trays were kept at a temperature 28 °C.

**Homogenate preparation and ammonium sulfate fractionation:** After 3–6 days of germination, the seedlings were collected. Upon excision of the cotyledons, the epicotyls and roots were homogenized in a Waring blender, with an equal amount (v/w) of 0.2 M potassium phosphate, pH 7.2, containing EDTA (1 mM) and 0.1mM phenylmethylsulfonyl fluoride as protease inhibitor.

The homogenate was squeezed through a clean cotton cloth and the obtained crude extract was treated with 35% saturated ammonium sulfate. After 1h incubation, the solution was centrifuged at 13,500*g* for 30min and the pellet was discarded. The supernatant was again precipitated with 65% saturated ammonium sulfate, and after 1h incubation, a new pellet was collected by centrifugation (13,500*g* for 30 min).

The pellet was dissolved in a small volume of 10 mM potassium phosphate, pH 6.8, and dialyzed overnight against the same buffer. The dialyzed solution was frozen in liquid nitrogen and stored at -80 °C.

**Diethylaminoethyl-cellulose chromatography:** For further purification, the dialysed solution was loaded to a diethylaminoethyl (DEAE) cellulose column (Ø = 1.5 cm, *l* = 50 cm) equilibrated with 0.2 M potassium phosphate, pH 6.8, containing 0.1 mM EDTA. The column was washed with the same buffer. The enzyme was eluted from the column, while most of the impurities were retained by the column. All purification steps were carried out at 4 °C. SDS–

polyacrylamide gel electrophoresis were performed according to the Laemmli method [15]. Gels were stained for the protein with Coomassie brilliant blue G-250 and Silver Nitrate.

**Enzyme assay:** Chickpea amine oxidase activity was measured by the oxidation of guaiacol in the presence of hydrogen peroxide and horseradish peroxidase. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, 130 mM putrescine dihydrochloride as a substrate, 1 U/ml horseradish peroxidase and 5 mM guaiacol in a final volume of 1 ml. The increase in absorbance at 470 nm was recorded using a Rayleigh UV–2100 Spectrophotometer.

**Measurements of inhibitory kinetics:** The inhibitory effect of tetraethylenepentamine on chickpea amine oxidase was measured by the oxidation of guaiacol in the presence of hydrogen peroxide and horseradish peroxidase. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, 1.6-51.2 mM putrescine dihydrochloride as a substrate, 1 U/ml horseradish peroxidase, 5 mM guaiacol and 2.5 mM tetraethylenepentamine as inhibitor in a final volume of 1 ml. The decrease in absorbance at 470 nm was recorded using a UV–2100 Spectrophotometer.

## RESULTS AND DISCUSSION

The purified enzyme, obtained according to the procedure, was characterized by gel electrophoresis and spectroscopic techniques**.** Apparent molecular weights estimated, from SDS–PAGE gels, to be about 75 kDa for each consisting monomer (Figure 1). The efficiency of different amine oxidase purification steps is shown in Table 1.

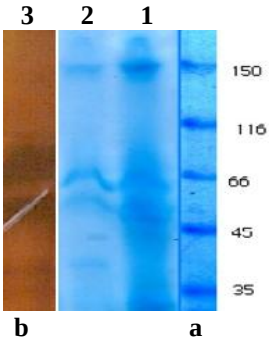


**Figure 1:** Polyacrylamide gel electrophoresis of purified chickpea seedling amine oxidase. Lane 1, molecular weight standards; lane 2, chickpea seedling amine oxidase purified by ammonium sulfate; lane 3, chickpea seedling amine oxidase purified by diethylaminoethylcellulose chromatography. b) The gel was stained for proteins with Silver Nitrate, chickpea seedling amine oxidase purified by diethylaminoethylcellulose chromatography.

**Table 1.** Characterization of the steps of the amine oxidase purification process from chickpea seedlings

| Fraction | Protein (mg/ml) | Activity (U) | Specific activity (U/mg) |
|---|---|---|---|
| Crude enzyme | 30 | 0.0364 | 0.001 |
| $(NH_4)_2SO_4$ fractionation | 8 | 0.0582 | 0.007 |
| DEAE-cellulose chromatography | 1.05 | 0.0606 | 0.057 |

We found that tetraethylenepentamine inhibited chickpea amine oxidase with the inhibition constants $K_i$ = 0.1 mM.

Several reversible and irreversible inhibitors have been described and used to investigate the structure–function relationships of plant amine oxidases [16-18]. For instance, diamino- and monoamino-ketonic compounds were synthesized and evaluated as inhibitors of pea seedling amine oxidase [19]. Noncompetitive inhibition of plant amine oxidases has also been observed with chelating agents such as cyanide and azide [20]. Moreover, diethylentriamine ($K_i$ = 72 mM) and triethylentriamine ($K_i$ = 0.57 mM) have been found to be strong noncompetitive inhibitors of pea seedling amine oxidase [21]. Pyridine carbaldoximes and alkyl pyridyl ketoximes also act as strong noncompetitive inhibitors of pea seedling amine oxidase. Finally, competitive inhibition of pea seedling amine oxidase by tetraethylenepentamine ($K_i$ = 20 µM) and pentaethylenehexamine ($K_i$ = 148 µM) were reported by Vianello et al [22].

By analyzing the Lineweaver - Burk plot, the kinetic parameters $K_m$ and $V_m$ of the purified enzyme (by using guaiacol oxidation in the presence of hydrogen peroxide, peroxidase and putrescine dihydrochloride as a substrate) were found to be 3.3 mM and 0.95 mmol/min/mg, respectively (Figure 2).

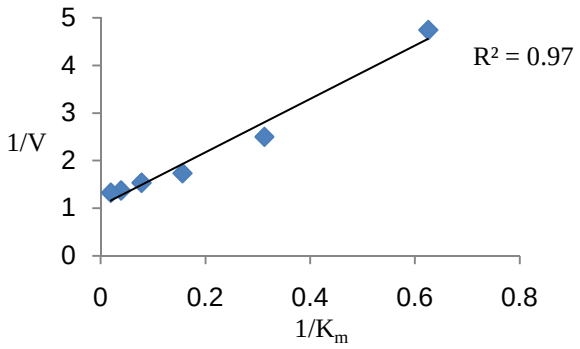


**Figure 2:** Kinetic parameters of chickpea diamine oxidase

The results showed that the presence of tetraethylenepentamine increased the $K_m$ value by 62.85 mM and reduced the $V_m$ value by 0.55 mmol/min/mg (Figure 3).

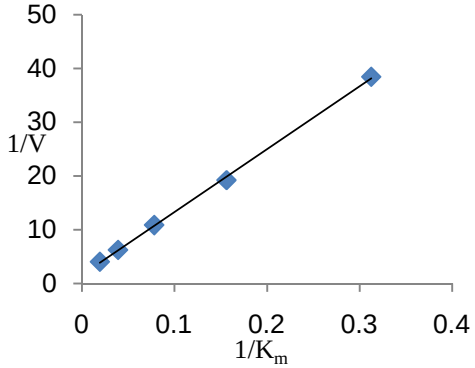


**Figure 3:** Kinetic parameters of chickpea diamine oxidase in the presence of tetraethylenepentamine

Therefore, the inhibitory effect of tetraethylenepentamine on chickpea amine oxidase is a linear mixed inhibition and could hence be applied as an effective tool for further research on the enzyme-bound copper function in chickpea amine oxidase, which aids to elucidate the structure-function relationships and its detailed physiological roles. In a linear mixed inhibition, the inhibitor can bind to the enzyme at the same time as the substrate; in other words, the binding of the inhibitor affects the substrate's binding, and vice versa. Although this type of inhibition can be reduced, it cannot overcome the increments in substrate concentration. In this case, increasing the concentration of putrescine dihydrochloride can only reduce the inhibition in a linear mixed manner. This type of inhibition generally results from an allosteric effect where the inhibitor binds to a different site on an enzyme [13, 14]. In other words, the binding of tetraethylenepentamine to this allosteric site changes the conformation (tertiary structure or three-dimensional shape) of the amine oxidase so that the affinity of the putrescine dihydrochloride for the active site is reduced.

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