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Competitive inhibition of copper amine oxidases by vitamin B hydrochloride in chickpea

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A B S T R A C T

Copper amine oxidases (CAOs) catalyse the oxidative de-amination of biogenic amines which are ubiquitous compounds essential for cell growth and proliferation. The enzymes are homodimers containing both topaquinone and a Cu(II) ions as cofactors at the active site of each subunit. After extraction and purification of chickpea (*cicer arietinum*) amine oxidase by chromatoghraphy, K_m and V_{max} of the enzyme were determined to be 3.3 mM and 0.95 mmol/min/mg, respectively, using a Lineweaver-Burk plot. In this study, the interaction of chickpea diamino oxidase with vitamin B hydrochloride was studied. Vitamin B hydrochloride (Thiamin) by K_i=8 mM acted as competitive inhibitor of the enzyme**.**

Key words: Chickpea, Copper-containing amine oxidases**,** Vitamin B hydrochloride, Competitive inhibitor.

INTRODUCTION

Amine oxidases (AOs) are a superfamily of oxidoreductase enzymes which catalyze the oxidative deamination of biogenic amines, including mono-, di- and polyamines by the two electron reduction of O_2. Aldehyde, ammonia, and H_2O_2 are produced in the reaction occurring with a ping-pong mechanism that can be divided into two half reactions: the first, referred to as “reductive half reaction,” involves the oxidation of amine to aldehyde and the formation of a reduced form of TPQ cofactor:

$$E_{ox} + \text{R-CH2\_NH3} \rightarrow E_{red} + \text{R-CHO}$$

The second half reaction, known as "oxidative half reaction" involves the reoxidation of the enzyme with the contemporarily release of ammonia and H_2O_2 [1-33]:

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$$E_{red} + O_2 + H_2O \rightarrow E_{ox} + NH_3 + H_2O_2$$

These oxidation products inhibit the growth of bacteria and could also interfere with the growth of cancer cells, which are known to proliferate rapidly. A considerable decrease in tumor cells was observed when amine oxidase was incubated with various amines [34]. Also a series of pharmacological studies from Masini et al. indicate that diamino oxidase (histaminases) of pea seedlings counteracts cardiac anaphylaxis and pulmonary asthma-like reactions to aerosolized allergen in sensitized guinea pigs [8].

These enzymes are widely distributed in organisms ranging from bacteria, yeasts, plants and mammals [1, 4-9, 12, 18-35]. CAOs show heterogeneity in structure as well as the differences in substrate and inhibitor specificity and mechanisms of oxidation [8, 19]. Two main types of these enzymes are (i) copper-containing amine oxidases and (ii) flavin-containing amine oxidases [2, 3, 8, 15, 16, 23, 35, 36-39]. The X-ray structures, available for CAOs of different sources are homodimers of subunit sizes about 70–95 kDa in which each subunit contains one Cu(II) and one 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor formed from a conserved tyrosine residue in a post-translational event [4, 8-29, 33, 37-41]. The present data on the enzyme substrate specificity to different substrates vary widely and are different for enzyme preparations from different organisms. The best substrates for all CAOs are 1,4-diaminobutane (putrescine) and 1,5 diaminopentane (cadaverine) [6, 35, 39, 42, 43].

Though the biological role of CAOs in plants is not yet known, the activity of these enzymes helps regulate several processes in plants due to the production of product H_2O_2 which affects plant germination, seedling establishment, and root growth. Hydrogen peroxide production is also associated with cell wall maturation and lignifications during growth, as well as wound-healing and the reinforcement of cell walls during cellular stress due to pathogenic attack. [6, 8, 15, 19, 25-28, 44-47]. Inhibitors play important roles in the study of catalytic properties of AOs. Both CAOs and PAOs show their own spectrum of inhibitors, which reflect the structure and composition of the active sites [48-50]. In case of chickpea seedling enzyme as a representative of plant CAOs, the active site copper atom is coordinated by three histidine side chains and two water molecules in an approximately square pyramidal arrangement and TPQ is located in the core of the enzyme at the end of a well defined substrate-access channel next to a CuII [10, 21, 51]. TPQ binds with copper in the inactive form of the enzyme but not with the active form [52]. Inhibitors can be divided into reversible, irreversible, and mechanism-based inactivator groups [53]. Two main types of reversible inhibitors are recognized, competitive and non- competitive. In the present study the inhibitory effect of Vitamin B hidrochloride on chickpea seedling amine oxidase is reported.

MATERIALS AND METHODS

Chemicals: DEAE-cellulose, horseradish peroxidase and putrescine dihydrochloride were purchased from Sigma-Aldrich In addition to vitamin B hydrochloride (2-[3-[(4-

Amino-2-methyl-pyrimidin-5-yl)methyl]-4-methyl-thiazol-5-yl] ethanol) and guaiacol, purchased from Merck.

Germination: Chickpea (*Cicer arietinum*) seeds were washed with 0.5% KMnO4 solution to keep mould and bacteria from growing. Chickpea seeds, layered on cotton in suitable dishes, were watered with warm tap water (30°C) for 2 min. This procedure was repeated every 30 min and the dishes containing the seeds were kept at a temperature around 28°C.

Protein purification: The preparation of a sufficient amount of purified chickpea amine oxidase was carried out in three steps as described previously by Talaei et al. [54].

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 a protease inhibitor.

The homogenate was squeezed through a clean cotton cloth and the obtained crude extract was treated with 35% saturated ammonium sulfate. After incubation for 1h, 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. 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 was performed using the Laemmli method [55]. 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 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 UV–2100 Spectrophotometer.

Measurements of inhibitory kinetics: Vitamin B hydrochlorie inhibitory effect 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 as substrate, 1 U/ml horseradish peroxidase, 5 mM guaiacol and 0-20 mM vitamin B hydrochloride as inhibitor in a final volume of 1 ml. The decrease in absorbance at 470 nm was recorded using a UV–2100 Spectrophotometer.

RESULTS

The purified enzyme, obtained according to the procedure, was characterized by gel electrophoresis and spectroscopic techniques. Apparent molecular weights were estimated, from SDS– PAGE gels, to be about 75 kDa for each consisting monomer. The efficiency of different amine oxidase purification steps were reported in a previous work [54]. We also found that vitamin B hydrochloride inhibited chickpea amine oxidase with the inhibition constant K_i=8 mM.

Several reversible and irreversible inhibitors have been described and used to investigate the structure–function relationships of plant amine oxidases [49, 56, 57]. Diamino- and monoamino-ketonic compounds were synthesized and evaluated as inhibitors of pea seedling amine oxidase. 1,5-Diamino-3-pentanone is the most powerful competitive inhibitor of the enzyme [58]. Other competitive inhibitors of AOs are some alkaloids, bivalent transition metal complexes with diamines, and monoamine cysteamine [2, 53]. Competitive inhibitors of *Trigonella foenum-graecum* (FGAO) are 1,5 diamino-3-pentanone, 1,4-diamino-2- butanone (K_i ~0.02 *mM*), aminoguanidine, cinchonine, L-lobeline, β-aminopropionitrile, and pargyline [59]. Some physiologically important derivatives of 4,5-dihydroimidazole are competitive inhibitors of pea (*Pisum sativum*) diamine oxidase (PSAO) [60]. A series of *N,N´*-bis(2-pyridinylmethyl) diamines was synthesized and characterized for their competitive inhibition effects toward plant copper-containing amine oxidase and polyamine oxidase. Kinetic measurements with PSAO and oat (*Avena sativa*) polyamine oxidase demonstrated reversible binding of the compounds at the active sites of the enzymes as they were almost exclusively competitive inhibitors with K_i values ranging from 10^{-5} to 10^{-3} M. The studied *N,N´*-bis(2-pyridinylmethyl) diamines are characterized by the presence of terminal aromatic rings, which seem to fit the hydrophobic pocket of plant amine oxidases [42] (Figure 1).

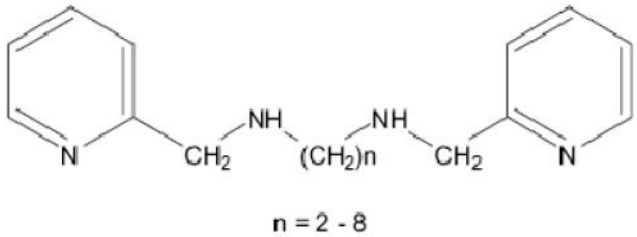

Figure 1: Chemical structures of the studied competitive inhibitors of plant amine oxidases

All these compounds show strong affinities for binding to the TPQ cofactor as normal substrates, but their complexes are hydrolyzed very slowly at a rate about 1% of that of putrescine [61].

Although K_m and V_{max} as kinetic parameters of purified enzyme were reported in our previously published work [54], here, the results related to its inhibition in the presence of vitamin B hydrochloride revealed an increment in the K_m. Furthermore, V_m value improved

as 0.95 mmol/min/mg, without varying in the presence of different concentrations of inhibitors (Figure 2).

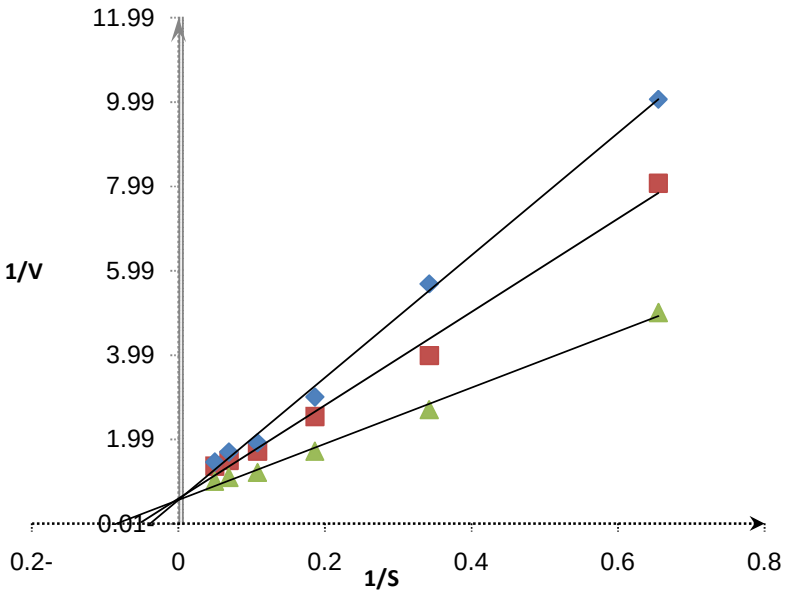

Figure 2: Kinetic parameters of chickpea diamine oxidase in the presence of Vitamin B hydrochloride. Inhibitor concentration used: 0 (▲), 10 mM (■), 20 mM (♦).

Therefore, the inhibitory effect of vitamin B hydrochloride on chickpea amine oxidase is a competitive inhibition and could hence be applied as an effective tool for further research on the enzyme-bound TPQ cofactor function in chickpea amine oxidase, which aids to elucidate the structure-function relationships and its detailed physiological roles. Vitamin B hydrochloride showed strong affinity for binding to the TPQ cofactor as normal substrates (Figure 3).

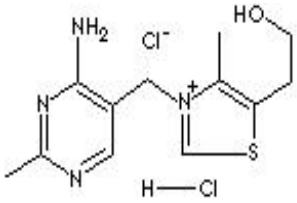

Figure 3: Chemical structures of vitamin B hydrochloride

Due to structural similarity of vitamin B hydrochloride with other plant copper amine oxidases inhibitors, for example, *N,N´*-bis(2-pyridinylmethyl) diamines, the competitive

inhibitory potency vitamin B hydrochloride improved in chickpea amine oxidase. In a competitive inhibition, the inhibitor binds to the enzyme on the same site as the substrate. The enzyme is unable to convert the inhibitor into product, and thus serves as a block against binding to the substrate. When the inhibitor is removed enzyme activity can be restored. Since increasing the concentration of the substrate can overcome inhibition, V_{max} is the same in the presence and absence of the inhibitor. In this case, at low concentrations of putrescine dihydrochloride, vitamin B hydrochloride inhibitory effect was more as compared to high concentrations.

Conflict of Interest: Author has no financial or any non-financial competing interests.

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