Research Article

Enhancing Role of Proline in Scavenging Free Radicals by Antioxidative Defence System during Stress in Black Gram and Cluster Bean

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ABSTRACT

Plants often face the challenge of severe environmental stresses that exert adverse effects on plant growth and development. Stress induces the production of reactive oxygen species. Under normal circumstances, concentrations of oxygen radicals remain low because of the activity of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidise Proline accumulates in high amount, in addition to the activities of enzymes in several plants under stress. This accumulation of proline has been shown to protect plants against damage by reactive oxygen species. The high capability of proline to quench singlet oxygen and hydroxy radicals can be well understood by its chemical properties. Proline contributes to stabilizing sub-cellular structures like membranes and proteins and scavenging free radicals under stress conditions. These free radicals are scavenged by low molecular weight antioxidative metabolites e.g., glutathione, ascorbic acid, α-tocopherol and antioxidative enzymes e.g., catalase, ascorbate peroxidase and superoxide dismutase. Accumulation of proline has been suggested to contribute to stress tolerance by acting as the molecular chaperons and maintains the protein integrity and enhancing the activities of different enzymes. Proline also acts as an antioxidant suggesting its role as free radical scavenger. Therefore, in the present study, antioxidant enzymes were superimposed with proline and made to dock with the free radicals, and the interaction energy values were compared with normal antioxidant enzymes. Superimposition of proline showed enhanced interaction energy.

Keywords: Superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, antioxidants, free radicals, molecular docking, proline.

INTRODUCTION

Plants are subjected to various types of environmental stresses such as salinity, water deficit, temperature extremes, toxic metal ion concentration and UV radiations, throughout their life cycle. These environmental factors control the growth and productivity of plants to varying degrees, depending upon the severity of stress. One of the stress response in the plants is the stimulated production of reactive oxygen species like $OH\cdot$, $O_2\cdot$, H_2O_2 etc. These species cause considerable damage through peroxidation of membrane lipid components and also through direct oxidation and interaction with various macromolecules like proteins, lipids and nucleic acids Low ROS concentration participates in signal transduction mechanism. These radicals are scavenge by low molecular weight antioxidative enzymes like catalase, ascorbate peroxidase and superoxide dismutase. Cells have adapted different mechanisms to keep the radicals level in check [1]. However, under different stress conditions the free radical generation exceeds the overall cellular antioxidative potential leading to oxidative stress, which contributes to adverse effects on plant growth. Ali *et al.*, [2] reported that the exogenous proline applied as spray treatment at seedling and/or at vegetative stage of *Zea mays* resulted in an enhanced growth under water deficient environment. Proline applied as pre-sowing and seed soaking treatment alleviated the adverse effects generated by drought stress in *Triticum aestivum* resulting in an enhanced growth and yield characteristics [3].

MATERIALS AND METHODS

The Protein Data Bank (PDB) is a repository for the 3-D structural data of proteins from where antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were retrieved[4]. Molecular structures of free radicals like superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical and reactive nitrogen species (RNS) such as nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$) were retrieved from Chemspider[5]. The retrieved structures of enzymes were analyzed using RasMol which is a molecular graphics program intended for the structural visualization of proteins[6]. 3-D structures of proteins are provided by the UniProtKB/Swiss-Prot database[7]. Binding sites of proteins are associated with structural pockets and cavities. CastP provides identification and measurements of surface accessible pockets as well as interior inaccessible cavities, for proteins[8]. It also measures the number of mouth openings, area of the openings and circumference of mouth and surfaces for each pocket. It provides measured parameters for pockets, cavities and mouth openings, as well as listing of wall atoms and mouth atoms for each pocket. TMHMM server is used to predict transmembrane helices in proteins[9]. Each selected amino

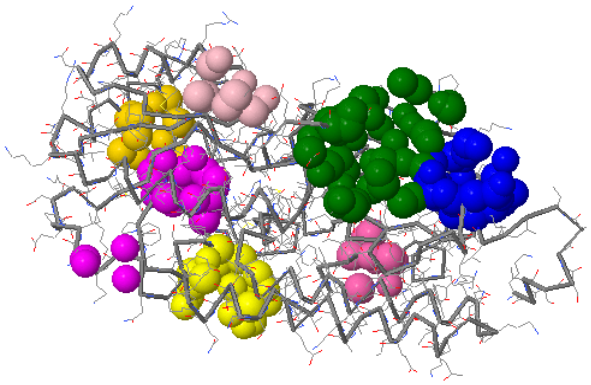
acid sequence is subjected to transmembrane topology analysis using TMHMM prediction server. Hex is an interactive protein docking and molecular superposition program, written by Ritchie[10]. Hex understands protein structures in PDB format. Using Hex software, protein-ligand docking is possible. In this docking one molecule (always protein) acts as receptor and the free radicals as ligand.

RESULTS

In order to find out the best effective interaction of free radicals with antioxidant enzymes, Hex docking was carried out and their binding affinities were calculated using free energy simulations. In this docking, radical molecules such as super oxide, hydrogen peroxide, hydroxy radical and hydroperoxy radical, nitric oxide and peroxynitrite acted as ligand molecules which formed the targets of the enzymes to inhibit their function (Fig.3).

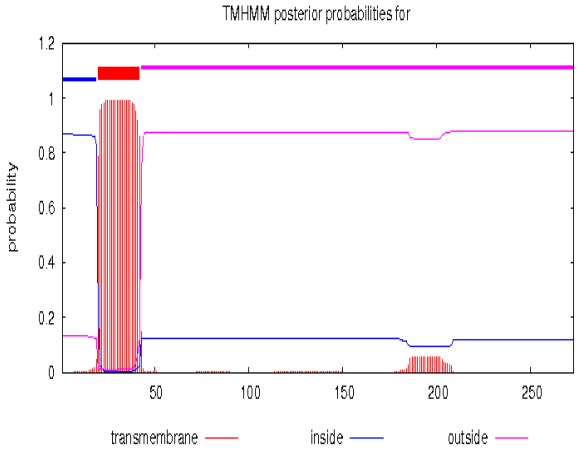
An important characteristic feature of an ideal receptor molecule is the occurrence of binding sites on its surface. All antioxidant enzymes showed ligand binding sites having different area and different volume to accommodate specific ligand for the entry and interaction (Fig.1).

Figure 1: Ligand binding sites of guaiacol peroxidise enzyme



When the ligand molecule is docked with the receptor, one of the above binding sites preferably the larger one functions as the active binding site. In the active binding site, the inner wall of the binding site cavity is lined with amino acid residues which directly interact with the atoms of the ligand molecule. During molecular docking, amino acids play an important role by interacting with the ligand molecule. The active site is the collection of aminoacid residues. All atoms in the enzyme lying within a specific distance of 5 Å were considered. The union of all such atoms was considered as a collective force belonging to the active site. In addition to this, an electrostatic force was used to distinguish between the actual site and the other smaller cluster of sites.In each active binding site, the type of amino acids varies. All antioxidant enzymes were checked for antigenicity by using TMHMM prediction server (Fig.2).

Figure 2: Topology prediction of guaiacol peroxidase



Catalase

Docking catalase receptor with free radicals was carried out and the e values were tabulated (Table.1). In this study, super oxide showed a maximum e-value (-401.30) followed by hydrogen peroxide (-320.00), hydroxy radical (-201.69) and hydroperoxy radical (-201.08) of nitric oxide (-103.95) and peroxynitrite (-104.60) (Table.1). In this study, the order of the ability of free radical scavanging activity of catalase was super oxide > hydrogen peroxide > hydroxy radical > hydroperoxy radical > nitric oxide > peroxynitrite > the influence of proline can be witnessed by the super imposition of proline with catalase primary structure. The superimposed structure of catalase, when docked with the free radicals, showed a high range of e negative values ranging from -304.76 to -602.23(Table-2) whereas the primary structure of catalase without proline after docking showed e value from -103.95 to -401.30 only(Table-1). From the above, it is concluded that the superimposition of proline has enhanced the free radical scavanging activity of catalase.

Guaiacol peroxidase (GPX)

Guaiacol peroxidase is a heme containing enzyme molecule. All the six free radicals acted as the targets for the enzyme guaiacol peroxidase (GPX), to inhibit their function. Docking results of guaiacol peroxidase (GPX) receptor with free radicals was tabulated (Table.1).The influence of proline can be witnessed by the super imposition of proline with guaiacol peroxidase. The structure of guaiacol peroxdase, after superimposition of proline, when docked with the free radicals,showed a high range of e negative values ranging from -205.63 to -503.14 (Table-2) , while the primary structure, guaiacol peroxidase after docking showed only e value from -145.16 to -303.19 Table-1). In this study, the order of the free radical scavanging activity of proline superimposed guaiacol peroxidase was hydrogen peroxide (-503.14) > hydroxy radical (-405.14 > super oxide (-402.05) > hydroperoxy radical (-356.05) > nitric oxide (-345.56) > peroxynitrite (-205.63) (Table-2). From the above, it is concluded that the superimposition of proline has enhanced the free radical scavanging activity of guaiacol peroxidase. Proline has

played an important role in stress tolerance by way of free radical scavenging.

Ascorbate peroxidase (APX),

The efficiency of the complex between the free radicals and the receptor molecule was identified via docking and the inhibition nature of the ascorbate peroxidase (APX), and their binding affinities were calculated using free energy simulations.

The e values of docking results of ascorbate peroxidase (APX) with free radicals were tabulated (Table.1).The influence of proline can be witnessed by the super imposition of proline with ascorbate peroxidase (APX). The secondary structure of ascorbate peroxidise, after superimposition of proline, when docked with the free radicals,showed a high range of e negative values ranging from -203.65 to – 600.02(Table-2 while the primary structure, ascorbate peroxidase (APX),after docking showed only e value from-198.50 to -500.01(Table-1) . In this study, the order of the free radical scavanging activity of proline super imposed ascorbate peroxidase,was peroxynitrite (Fig.3) showed a maximum e-value(-600.02)followed by super oxide (-425.09), hydrogen peroxide(-403.65), nitric oxide (403.56) hydroxy radicals(-369.90) and hydroperoxy radical (-203.23) (Table.2). From the above, it is concluded that the superimposition of proline has helped ascorbate peroxidase in scavenging free radicals very effectively and is playing an important role in stress tolerance by way of free radical scavenging.

Monodehydroascorbate reductase (MDHAR)

Monodehydroascorbate reductase is an FAD enzyme showing increased activity under environmental stresses. Docking results of monodehydroascorbate reductase receptor with free radicals were tabulated (Table1.).

Table 1: showing e negative values of docking between free radicals and enzymic antioxidants receptor molecules

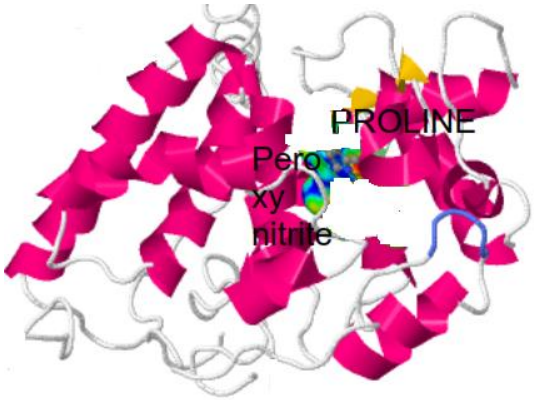
S.No	Free radicals	catalase (CAT)	guaiacol peroxidase (GPX),	ascorbate peroxidase (APX),	Mono dehydroascorbate reductase (MDHAR),	dehydroascorbate reductase (DHAR)	glutathione reductase (GR)	superoxide dismutase
1	Super oxide	-401.30	-202.05	-329.00	-357.00	-395.00	-390.02	-580.00
2	Hydrogen peroxide	-320.00	-303.19	-309.96	-300.04	-259.13	-492.94	-385.04
3	Hydroxy radical	-201.69	-203.13	-257.10	-103.06	-216.05	-390.93	-360.03
4	Hydroperoxy radical	-201.08	-157.05	-198.50	-156.07	-150.08	-275.09	-295.05
5	Nitric oxide	-103.95	-145.16	-307.03	-249.00	-180.94	-100.81	-210.01
6	Peroxy nitrite	-104.60	-200.00	-500.01	-270.04	-106.05	-105.67	-115.07

Table 2: showing e negative values of docking between free radicals and enzymic antioxidants receptor molecules superimposed with proline

S.No	Free radicals	catalase (CAT) with Proline	guaiacol peroxidase (GPX), with Proline	ascorbate peroxidase (APX), with Proline	Monodehydroascorbate reductase (MDHAR), with Proline	dehydroascorbate reductase (DHAR),with Proline	glutathione reductase (GR)with Proline	superoxide dismutase
1	Super oxide	-602.23	-402.05	-425.09	-455.00	-465.02	-459.01	-690.10
2	Hydrogen peroxide	-500.01	-503.14	-403.56	-405.34	-450.53	-502.04	-535.06
3	Hydroxy radicals	-405.60	-405.14	-365.90	-203.46	-326.45	-400.03	-461.02
4	Hydroperoxy radical	-204.78	-356.05	-203.65	-256.37	-256.48	-345.59	-390.02
5	Nitric oxide	-403.45	-345.56	-403.23	-345.70	-190.04	-200.61	-200.11
6	Peroxy nitrite	-304.76	-205.63	-600.02	-378.54	-200.65	-365.01	-219.08

Among the amino acid residues, proline is playing an important role in the binding as an attractive force and deciding factor in alleviating the free radicals. The influence of proline can be witnessed by the super imposition of proline with monodehydroascorbate reductase. The secondary structure of monodehydroascorbate reductase, after superimposition of proline, when docked with the free radicals, showed a high range of e negative values ranging from -203.46to -455.00)(Table-2)

Figure 3: Molecular docking of ascorbate peroxidase (APX) with peroxy nitrite



while the primary structure, monodehydroascorbate reductase after docking showed only e value from -103.06 to -357.00 only(Table1). In this study, the order of the free radical scavanging activity of proline super imposed monodehydroascorbate reductase, super oxide showed a maximum e-value (-455.00) followed by hydrogen peroxide(-405.34), peroxy nitrite (-378.54), nitric oxide (-345.70) hydroperoxy radicals (-256.37) and hydroxy radicals (-203.46).From the above, it is concluded that the superimposition of proline was very effective in scavenging free radicals. Proline is playing an important role in stress tolerance by way of free radical scavenging.

Dehydroascorbate reductase (DHAR),

The secondary structure of dehydroascorbate reductase after superimposition of proline, when docked with the free radicals, showed a high range of e negative values ranging from -190.04 to -465.02 (Table 2) , while the primary structure, dehydroascorbate reductase after docking showed only e value from -106.05 to-395.00 (Table1)

In this study, the order of the free radical scavanging activity of proline super imposed dehydroascorbate reductase, was super oxide showed a maximum e-value(-465.02) followed by hydrogen peroxide(-450.53), Hydroxy radicals(-326.45) and hydroperoxy radical(-256.48) peroxy nitrite(-200.65), nitric oxide (-190.04) (Table.2). From the above, it is concluded that the superimposition of proline was very effective in scavenging free radicals. Proline, is playing an important role in stress tolerance by way of free radical scavenging.

Glutathione reductase (GR)

The increased activity of glutathione reductase is observed under environmental stresses. In chloroplast, glutathione reductase is involved in detoxification of H2O2. A correlation between the oxidative stress resistance and activity of GR is a common occurrence.

The influence of proline can be witnessed by the super imposition of proline with glutathione reductase. The secondary structure of glutathione reductase after superimposition of proline, when docked with the free radicals, showed a high range of e negative values ranging from -200.61 to – 502.04(Table2) while the primary structure, glutathione reductase after docking showed only e value from -100.81 to -492.94 (Table1). In this study, the order of the free radical scavanging activity of proline super imposed glutathione reductase was hydrogen peroxide (-502.04), followed by super oxide (-459.01), Hydroxy radicals (-400.03) peroxynitrite (-365.01), hydroperoxy radical (-345.59) and nitric oxide (-200.61). From the above, it is concluded that the superimposition of proline was very effective in scavenging free radicals at the time of stress.

Table 3: showing e negative values of docking between free radicals and enzymic antioxidants receptor molecules with proline

S.No	Free radicals	Monodehydroascorbate reductase (MDHAR), with Proline	dehydroascorbate reductase (DHAR),with Proline	glutathione reductase (GR)with Proline	superoxide dismutase
1	Super oxide	-455.00	-465.02	-459.01	-690.10
2	Hydrogen peroxide	-405.34	-450.53	-502.04	-535.06
3	Hydroxy radicals	-203.46	-326.45	-400.03	-461.02
4	Hydroperoxy radical	-256.37	-256.48	-345.59	-390.02
5	Nitric oxide	-345.70	-190.04	-200.61	-200.11
6	Peroxy nitrite	-378.54	-200.65	-365.01	-219.08

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) plays central role in defense against oxidative stress. SOD activity has been reported to increase in plants exposed to various environmental stresses, including drought and metal toxicity. Increased activity of SOD is often correlated with increased tolerance of the plant against environmental stresses. Superoxide dismutase is located in cytoplasm, chloroplast, peroxisome and mitochondria.

Among the amino acid residues, proline is playing an important role in the binding site as an attractive force and deciding factor in alleviating the free radical. The influence of proline can be witnessed by the super imposition of proline with superoxide dismutase. The secondary structure of superoxide dismutase after superimposition of proline, when docked with the free radicals, showed a high range of e negative values ranging from -219.08 to – 690. 10) (Table2) while the primary structure, superoxide dismutase after docking showed only e value from (-115.07 to -580.00 (Table1). In this study, super oxide showed a maximum e-value (-690.10)followed by hydrogen peroxide(-535.06), Hydroxy radicals(-461.02) hydroperoxy radical (-390.02) , and Peroxy nitrite(-219.08) , nitric oxide (-200.11) (Table.3). In this study, the order of the free radical scavanging activity of superoxide dismutase was super oxide > hydrogen peroxide > Hydroxy radicals > hydroperoxy radical > nitric oxide > Peroxy nitrite (Table2). From the above, it is concluded that the superimposition of proline is very effective in scavenging.

DISCUSSION

Stress induces the production of reactive oxygen species. Under normal circumstances, concentrations of oxygen radicals remain low because of the activity of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidase. Under stress, accumulation of proline occurs, in addition to the activities of enzymes. Proline accumulates in high amount in several plants under stress. This accumulation of proline has been shown to protect plants against damage by free radicals. Therefore, the chemical reactivity of proline with free radicals is to be discussed, with the aim to understand the molecular mechanism of the protective effect of proline. The high capability of proline to quench super oxide and hydroxy radicals can be well understood by its chemical properties. Proline reacts with OH• under hydrogen abstraction by forming the most stable radical, which in plants under stress could be well understood by its property to scavenge free radicals [11].

The phenomenon of proline accumulation is known to occur under water deficit, salinity, low temperature, heavy metal exposure and UV radiations, etc. Apart from acting as osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals and buffering cellular redox potential under stress conditions. These free radicals are scavenged by low molecular weight antioxidative enzymes e.g., catalase, ascorbate peroxidase and superoxide dismutase. Accumulation of proline has been suggested to contribute to stress tolerance in many ways. As proline acts as the molecular chaperons, it is able to maintain the protein integrity and enhancing the activities of different enzymes. Numerous studies have reported proline as an antioxidant suggesting its role as free radical scavenger and singlet oxygen quencher. Stress, in general, is known to alter plant-water relations which may affect water uptake, ascent of sap, stomatal functioning and retardation of chlorophyll biosynthesis and ultimately result in decreased photosynthesis. Decrease in leaf water potential is also associated with stress. Exogenously applied proline maintained turgidity in leaves of barley and wheat undergoing stress excessive levels of ROS result in oxidative damage to plants, e.g., nucleic acid damage, oxidation of proteins and lipids and degradation of chlorophyll pigments[12].

Exogenous application of proline resulted in increase of its endogenous levels that antagonized the toxic effects of Selenium by improving the growth of seedlings. The damage caused by stress was reduced significantly with simultaneous increase in the activities of enzymatic and non- enzymatic antioxidants. Exogenous proline application besides enhancing the activity of antioxidative enzymes (CAT, POX and SOD) is also known to enhance the activity of other enzymes. Nitrogenase activity in drought-stressed soybean nodules was significantly enhanced when proline (an osmolyte) was applied exogenously. It is well-established that stress results in increased proline accumulation in root nodules[13].

A large body of data suggests a positive correlation between proline accumulation and plant stress. Proline, an amino acid, plays a highly beneficial role in plants exposed to various stress conditions. Besides acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule. Review of the literature indicates that a stressful environment results in an overproduction of proline in plants which in turn imparts stress tolerance by maintaining cell turgor or osmotic balance; stabilizing membranes thereby preventing electrolyte leakage; and bringing concentrations of free radicals within normal ranges, thus preventing oxidative burst in plants. Reports indicate enhanced stress tolerance when proline is supplied exogenously at low concentrations. However, some reports indicate toxic effects of proline when supplied exogenously at higher concentrations. In this article, we review and discuss the effects of exogenous proline on plants exposed to various abiotic stresses. Numerous examples of successful application of exogenous proline to improve stress tolerance are presented. The roles played by exogenous proline under varying environments have been critically examined and reviewed[12].

Reports indicate that proline is responsible for scavenging the ROS and other free radicals. Proline, when applied exogenously to roots of Arabidopsis, resulted in a reduced level of ROS, indicating the ROS scavenging potential of proline[14]. Hoque *et al*., [13]reported that the activities of antioxidative enzymes viz. catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) were significantly enhanced when proline was applied exogenously in tobacco suspension cultures exposed to salinity stress.

CONCLUSION

From the above, it is concluded that the super imposition of proline over antioxidant enzymes, has increased the activity of enzymes and increased the docking energy considerably. In docking super oxide with enzymes, catalase, MDHAR, DHAR and super oxide dismutase, exhibited effective interaction with high e negative values. Hydrogen peroxide showed best interaction with guaiacol peroxidase and glutathione reductase and super oxide dismutase, hydoxy radical interacted well with catalase, guaiacol peroxidise, glutathione reductase and super oxide dismutase. Peroxy nitrite was highly interactive with ascorbate peroxidise. The response of other free radicals with various enzymes was average or less than average.

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