Annals of Biology 30 (4) : 593-597, 2014

# Superoxide Dismutase Isozymes and their Heat Stability in Coconut (*Cocos nucifera* L.) Leaves

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## ABSTRACT

The possible isoforms of superoxide dismutase (SOD : EC 1.15.1.1) and its stability against heat treatment at various temperatures (50°C to 100°C) in the three-year old seedlings of West Coast Tall (WCT) cultivar of coconut were investigated. To identify SOD activity, crude extract from coconut leaves was subjected to native polyacrylamide gel electrophoresis followed by staining with nitroblue tetrazolium (NBT) and riboflavin. From our study, we found out 14 isoforms for superoxide dismutases in coconut. Treatment with SOD inhibitors indicated the presence of five Cu/Zn-SOD, five Mn-SOD and two Fe-SODS. In this experiment, we also found the presence of two higher molecular weight SOD isoforms, which were resistant to both the SOD inhibition treatments (Cu/Zn-SOD, Fe-SOD and Mn-SOD, Fe-SOD inhibitors). These two SOD isoforms were unstable to heat treatment and completely lost their activity by 60°C. Under sequential heat treatments, SOD specific activity decreased linearly from 6.82 to 1.88 till 80°C and increased at 100°C. This may be due to the activation of some new SOD isoforms at higher temperature. Studies on the effect of sequential heat on isoforms revealed that, out of 14 isoforms, 10 isoforms lost their activity at 80°C and only four isoforms (two each for Cu/Zn-SOD and Mn-SOD) were stable at 80°C and at 100°C, only two existing isoforms of Cu/Zn-SOD were stable but two new isoforms of Cu/Zn-SODs also reappeared.

**Key words :** Activity, coconut, heat stability, isoforms, superoxide dismutase

## INTRODUCTION

Under abiotic stress, plants undergo changes in their metabolism in order to cope up with the changing environment. One of the biochemical changes occurring when plant is subjected to biotic and abiotic stress is the production of reactive oxygen species (ROS). ROS are highly reactive and when the scavenging capacity of plant is less than the ROS production, they can seriously disrupt normal metabolism through oxidative damage of lipid, protein and nucleic acids (Halliwell and Gutteridge, 1985; Fridovitch, 1986; Davis, 1987). Plants have a number of antioxidant systems which protect them against the potential cyto-toxic effects. Antioxidant enzymes are the most important components in the scavenging system of ROS. In this antioxidant enzyme system, superoxide dismutases (SOD : EC 1.15.1.1) are ubiquitous metalloenzymes (Fridoivitch, 1975; Jackson *et al.*, 1978) that constitute the first line of defence against ROS. In living cells, SODs catalyze the dismutation of the superoxide radicals ($\tilde{O}_2$.-) into hydrogen-peroxide ($H_2O_2$) and oxygen ($O_2$) and play an important role in protecting the cells against the toxic effect of superoxide radicals produced in different cell compartments (Del Rio *et al.*, 2002).

$2\tilde{O}_2+2H \rightarrow O_2+H_2O_2$

SODs are divided into four main sub-types and are categorized based upon their cellular locations and metal-ion-binding prosthetic groups. Copper and Zink SODs (Cu/Zn-SODs) are found primarily in the cytoplasm (Vacuoles) of eukaryotic cells, manganese SODs (Mn-SODs) exist in the mitochondria of eukaryotic cells and the cytoplasm of prokaryotes, iron SODs (Fe-SODs) exist mainly in prokaryotes and the chloroplast of eukaryotic plants, and nickel SODs (Ni-SODs) are exclusively found in prokaryotes only (Jackson *et al.*, 1978; Reddy and Venkaiah, 1982; Werner *et al.*, 1992; Youn *et al.*, 1996; Pradedova *et al.*, 2009). Each type of SODs having multiple forms, Cu/Zn-SODs are sensitive to cynide (CN-) and hydrogen peroxide ($H_2O_2$), but insensitive to chloroform-ethanol ($CHCl_3$-$CH_3CH_2OH$) mixture, whereas Fe-SODs and Cu/Zn-SODs are sensitive to chloroform-ethanol ($CHCl_3$-$CH_3CH_2OH$) mixture but insensitive to $CN^-$ and Mn-SODs are insensitive to $CN^-$ and $H_2O_2$, but sensitive to $CHCl_3$-$CH_3CH_2OH$ (Fridovitch, 1975; Wang *et al.*, 2009). This selective inhibition of SODs makes it possible to distinguish the three types of SODS in crude homogenates.

The SOD pattern of various plants and some of its heat stable isoforms (Cu/Zn-SODs) has been described (Bridges and Salin, 1981; Reddy and Venkaiah, 1984; Pan and Yau, 1991; Chopra and Sabrinath, 2004). In

plant as well as in mammalian and fungal cells Cu/Zn-SOD is a major SOD (Rotilio, 1986), but plant also contains Mn-SOD and Fe- SODs in addition to Cu/Zn-SODS. According to previous thoughts, Fe-SOD was exclusively restricted to prokaryotes and some eukaryotic algae but has also been reported in several higher plant species (Bridges and Salin, 1981; Kwaitowski *et al.*, 1985; Pan and Yau, 1991; Rahnama and Ebrahimzadeh, 2006). However, in coconut some work has been done on SODs in relation to drought tolerance and lipid peroxidation (Chempakam *et al.*, 1993). But, still it is not clear which isozymes of SODs are present and their heat stability in coconut. The present study deals with the identification of SOD isoforms and their heat stability in coconut leaves.

## MATERIALS AND METHODS

The leaf samples collected from third leaf of three-year old coconut (*Cocos nucifera* L.) seedlings of WCT variety grown in poly-bags under shade net are the material for the observations. Fresh leaves (2.5 g) were cut into small pieces and ground in pre-chilled mortar in 25 ml cold 0.1 M Sodium Phosphate buffer, PVPP (1 g) β-mercaptoethanol (200 µl) and transferred into pre-chilled centrifuge tubes. The extract was centrifuged at 12000 rpm for 15 min at 4°C in refrigerated centrifuge (Hareus, Germany). Took the supernatant and re-centrifuged at 12000 rpm for 15 min at 4°C in refrigerated centrifuge (Hareus, Germany). The enzyme was partially purified from the supernatant by ammonium sulfate precipitation (final concentration 85%), and subsequent dialysis. The clear pale yellow dialysate after centrifugation was used for further enzyme assay as well as for isozymes identification. An aliquot of the enzyme extract was used to determine its protein content by the method of Lowry *et al.* (1951).

SOD activity was determined by measuring its ability to initiate photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Beauchamp and Fridovitch (1971). Non-denaturing polyacrylamide gels electrophoresis (PAGE) was carried out with 4% stacking gel and 10% separating gel using 0.1M Tris-Glysine electrode buffer (pH 8.3) at 4°C and 160 mA according to the method described by Hames and Rickwood (1990) with some modifications. To visualize the SOD activity, the gels were developed according to the method given by Beauchamp and Fridovitch (1971). The gels were incubated in dark for 20 min in a solution containing 100 ml 0.05M Tris-HCl (pH 8.2) containing riboflavin, nitroblue tetrazolium (NBT) and EDTA and illuminated the gels until the white bright bands became apparent. Detection of SOD isozymes was performed according to the methods described by Luo *et al.* (1996) with some modifications. Gels were incubated separately in 0.1% $H_2O_2$ (dissolved in 0.1 m phosphate buffer pH 7.6) and $CHCl_3$-$CH_3CH_2OH$ (0.3 : 0.5 v/v) for 30 min and then stained according to the method described above.

The effect of high temperature on the SOD activity was studied by incubating the extract in eppendorf tubes at 50°, 60°, 70°, 80° and 100°C for 10 min in water bath with occasional shaking and then cooled by keeping on ice and centrifuge it at 12000 rpm for 15 min at 4°C in refrigerated centrifuge (Hareus, Germany). After that the extract was analyzed for SOD activity and isozymes visualization according to the methods described above.

## RESULTS AND DISCUSSION

Identification of SOD isoforms was done by using native PAGE (Fig. 1). Using this method, 14 distinct SOD isoforms were detected in the coconut leaves. Two of SOD bands were of very low relative mobility than the remaining 12 SODs bands which were detected near the junction of separating gel and resolving gels, thus implying that they had very high molecular weight (Fig. 2, Lane A).

There are four distinct types of SODs : Cu/Zn-SOD, Mn-SOD, Fe-SOD and Ni-SOD (detected only in prokaryotes), which are classified according to the

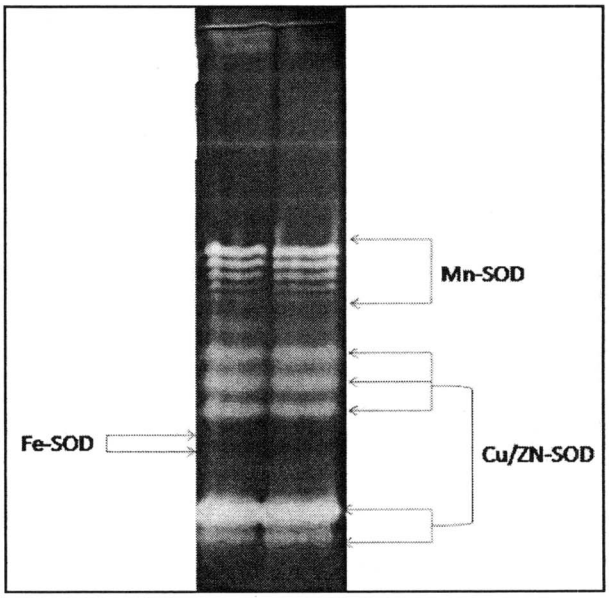


Fig. 1. Superoxide dismutase isoforms in coconut leaves.

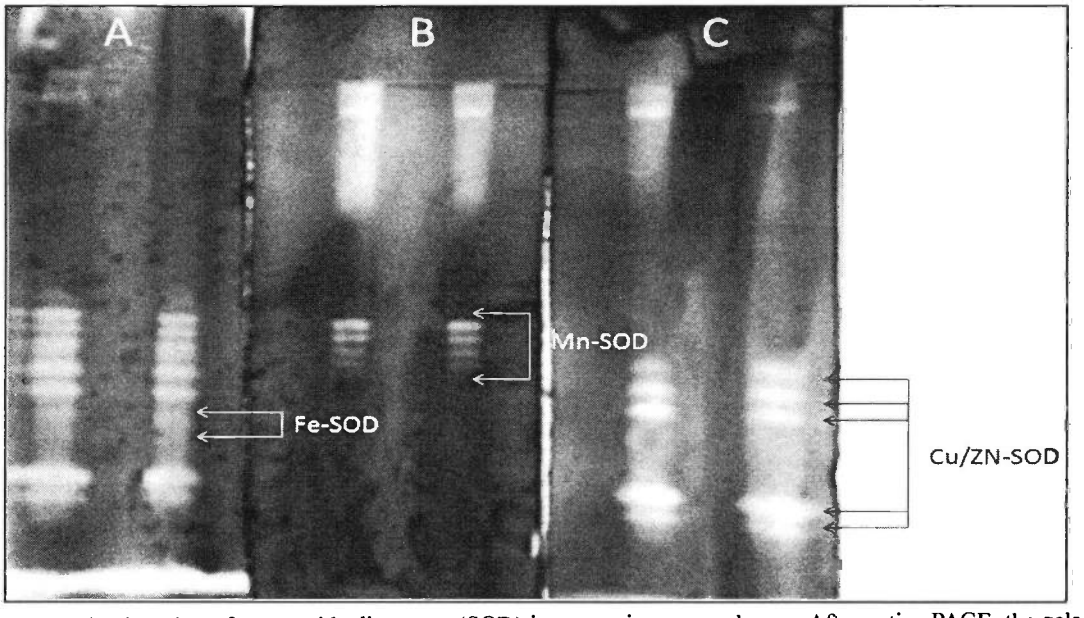


Fig. 2. Identification of superoxide dismutase (SOD) isozymes in coconut leaves. After native PAGE, the gels were given the following treatment and then they were stained with NBT reduction. Lane A : Control, untreated, B : $H_2O_2$ treatment and C : $CHCl_3$-$CH_3CH_2OH$ treatment. The direction of electrophoresis was top side cathode (-) to bottom side anode (+).

metals present at the catalytic site. Each type of SOD (except Ni-SOD) is sensitive to specific inhibitors, allowing discrimination among different forms. For determination of the SOD sub-types among SOD bands from coconut leaves, native gel was treated with different inhibitors and then stained (Fig. 3). Two very effective inhibitors, hydrogen peroxide ($H_2O_2$) and a mixture of chloroform and ethanol ($CHCl_3$-$CH_3CH_2OH$) were used to discriminate between SOD isoforms. Cu/Zn-SODs and Fe-SODs are sensitive and lose their activity with $H_2O_2$ treatment, therefore, utilization of $H_2O_2$ can identify the Mn-SOD; however, the Mn-SOD and Fe-SOD lose their activity with $CHCl_3$-$CH_3CH_2OH$ treatment. As a result Cu/Zn-SODs can be identified with $CHCl_3$-$CH_3CH_2OH$ treatment (Wang *et al.*, 2009). Although, potassium cyanide (KCN) is also a potent SOD inhibitor which can cause the inactivation of Cu/Zn-SODs and identify Mn-SODs and Fe-SODs. KCN is highly toxic, therefore, was not used for the safety purpose. In spite of that, the identification of SOD isozymes was achieved by comparison of control gel with other two treatments.

In this study, pre-treatment of the gels with $H_2O_2$ showed two high molecular weight bands and five medium molecular weight bands (Fig. 1, Lane B), while two high molecular weight bands and five low to medium molecular weight bands were visible on the gels with $CHCl_3$-$CH_3CH_2OH$ treatment (Fig. 1, Lane C). Based on the comparison of gels, we conclude that there are two Fe-SOD isozymes, five Mn-SOD isozymes, five Cu/Zn-SOD isozymes and two unknown (resistant to both the inhibitors) SOD isozymes present in coconut leaves. In higher plants, Cu/Zn-SODs are the most abundant SODs and are commonly found in the cytosol and chloroplast, Mn-SODs are mainly present in mitochondria and peroxisomes, and Fe-SODs are generally found in chloroplasts (Alscher *et al.*, 2002). Although Fe-SOD was previously considered to be not very common in higher plants, in roots of red beat (Pradedova *et al.*, 2009); carnation petals (Droillard *et al.*, 1989), and in Brassica leaves has been reported to be present in both mitochondria and peroxisomes (Droillard and Paulin, 1990) thus correlating with an observation in coconut (Fig. 1). The significant difference in SOD band width on electrophoregrame was also noticeable and it showed that Cu/Zn-SODs in native PAGE had very wide and thick bands, while Mn-SODs had medium and Fe-SODs had very thin and faint bands (Fig. 1). The similar type of results were reported in red beat roots (Pradedova *et al.*, 2009).

The thermo-stability of SOD in coconut leaves was investigated by incubating the enzyme extract at temperature ranging from 50°C to 100°C and detecting the enzymes. The SOD specific activity increased initially from 6.82 to 7.6 by 50°C and then decreased to 1.88 by 80°C but again increased at 100°C (Fig. 2). Chopra and Sabrinath (2004) observed that the SOD specific activity increased in incubation temperature above 50°C in *Chinopodium murale.*

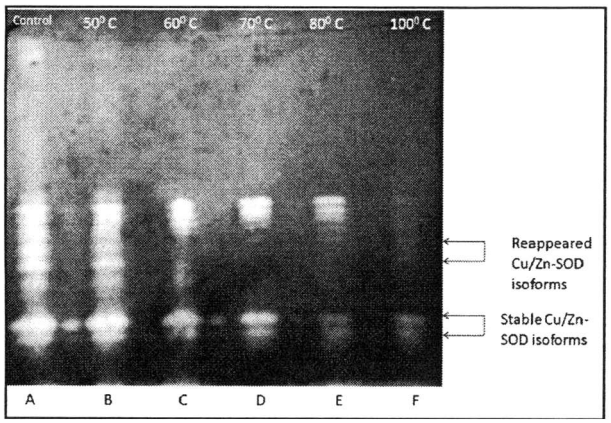


Fig. 3a. Gel showing SOD activity of coconut leaves extract incubated at different temperatures. Lane A : Control, untreated; Lane B to F : Sequential heat treated with 50°C to 100°C.

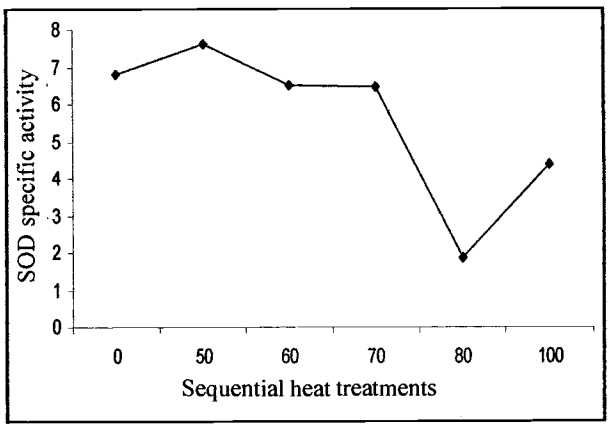


Fig. 3b. Graph showing the effect of sequential heat treatment on SOD specific activity in coconut leaves.

Similarly, sweet potato SOD showed slight enhancement in the activity at 70°C incubated for various time intervals (Lin *et al.*, 1995), while the cabbage SODs lost their activity at 70°C after 5 min (Walker *et al.*, 1987). Similar results were reported by Slaðana *et al.* (2010), Tayebeh and Hassan (2010), Khanna-Chopra *et al.* (2011), Wojciech-Pokora *et al.* (2011) and Arafet-Manaa *et al.* (2014).

When the treated samples were loaded on native PAGE gel and stained the gel for SOD activity, out of 14 isoforms, 10 isoforms lost their activity by 80°C. Only four isoforms (two each for Cu/Zn-SOD and Mn-SOD) maintained their original activity up to 80°C. At 100°C only two existing isoforms of Cu/Zn-SOD remained stable with increased band strength and two new isoforms of SODs were also detected (Fig. 2, Lane F). Chopra and Sabrinath (2004) reported the formation of new SOD isoform in *Chinopodium murale* at 60°C which was stable even at boiling temperature. The stability of the isozyme, under heat treatment, might be due to the stable confirmation of the protein due to the inter-subunit contacts or increased electrostatic steering of substrate into the active site (Lin *et al.*, 1995). The two higher molecular weight isoforms (mentioned above) were resistant to both the inhibitors but very unstable under heat treatment and completely lost their activity by 60°C (Fig. 2, Lane B). We did not get any report about these isoforms in plant kingdom. These isoforms may be containing nickel ions as reported in some bacteria (*Streptomyces* spp.). This isoform having a molecular mass approximate 60 kDa but in general, the bacterial SOD sub-units have molecular masses in the range 18-22 kDa (Youn *et al.*, 1996). Crystal structure of Ni-SODS in *Streptomyces* spp. revealed that the Ni-SOD was homohexamer consisting of four-helix-bundle sub-units (Wuerges *et al.*, 2004).

## CONCLUSION

In conclusion, in coconut leaf protein extract 14 SOD isoforms were detected with five each of Cu/Zn and Mn-SODs, two Fe-SODs and two higher molecular weight unknown isoforms. It was also observed in coconut that different isoforms reacted differently to higher temperature treatments and two new isoforms of Cu/Zn-SODs, appeared in the leaf protein extract heated at 100°C. Two isoforms of Cu/Zn-SODs were more resistant to higher temperature treatment than the other SOD isoforms. The thermo-stable isoforms of SOD may contribute towards heat tolerance of the plants and identification of higher molecular SOD isoforms may also be helpful in abiotic stress tolerance of the plants. Purification of the thermo-stable Cu/Zn-SODs, identification of unknown isoforms and their gene isolation will be carried out, which could play immense role in the genetic engineering of crops for abiotic stress tolerance.

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