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MECHANISM OF ACTION OF SALICYLIC ACID DURING DEFENSE RESPONSE OF PLANT

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ABSTRACT

Salicylic acid (SA) has been reported as an endogenous signal for the activation of certain plant defense responses, including pathogenesis related gene expression and enhanced resistance to pathogens. In our previous investigation we have observed that exogenous application of salicylic acid induced the expression of pathogenesis related -1 (PR-1) gene in okra and cowpea and enhanced resistance against *Meloidogyne incognita*. Here the interaction of SA and the rat liver microsomal membrane was investigated in the presence and absence of catalase. Our result showed that though catalase and salicylic acid have no effect on lipid peroxidation but catalase significantly increased SA induced lipid peroxidation of rat liver microsomal membrane. The result suggests that salicylic acid may form SA free radicals upon interaction with catalase. Such free radicals induce lipid peroxidation, and the products of this reaction, may activate plant defense responses.

KEYWORDS : *Salicylic acid*, Plant Defense Responses, Lipid Per Oxidation, Free Radicals, *Meloidogyne Incognita*

When plants are infected by pathogens to which they are resistant, they normally respond by expressing diverse defense mechanisms that inhibit establishment of the pathogens. In addition, uninfected parts of the plant develop greater resistance to further infection by pathogens and this has been termed systemic acquired resistance (SAR). (Mauch-Mani and Metraux, 1998). The first hint that salicylic acid (SA) might be involved in plant defense was provided by White (White, 1979) who found that injection of aspirin or SA into tobacco leaves enhanced resistance to subsequent infection tobacco mosaic virus (TMV). This treatment also induced pathogenesis related (PR) protein accumulation. It is evident that SA is an endogenous signal for the activation of certain plant defense responses, including PR-gene expression and establishment of enhanced resistance (Klessig et al., 2000) although neither PR protein nor exogenous SA induced resistance to some virus infections (Roggero and Pennazio, 1991). After TMV infection, SA accumulates to high levels at the site of infection, with a subsequent, but much smaller rise, in the uninfected systemic tissues. In tobacco, this increase paralleled the transcriptional activation of PR genes in both the inoculated and uninoculated leaves. Exogenous application of SA induced expression of the same set of nine defense related genes as were activated systemically after infection by TMV (Durner et al., 1997). The PR proteins are a group of inducible proteins whose synthesis is associated with certain types of resistance to pathogen and stress. Increased accumulation of PR proteins is associated with increased resistance to infection. Synthesis of these proteins serves as a marker for disease resistance. Their occurrence in a wide variety of plant species, including both mono and dicotyledons, suggests that they play an important role in plant disease resistance. In our previous investigations, we observed that exogenous application of SA induced expression of PR-1 gene in okra and cowpea and reduced *Meloidogyne incognita* infestation in those plants (Nandi et al., 2003). SA induced plant defense response is mediated through catalase. However, it may also acts independent of the catalase mediated pathway and the recent idea is that plant defense response may be the result of increase concentration of free radicals arising out of the enhanced rate of lipid peroxidation. To understand the exact mode of action of SA, we have studied the effect of SA on lipid peroxidation using rat liver microsomal membranes as the in vitro source of lipid bi layer.

MATERIALS AND METHODS

Reagents

Salicylic acid and catalase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other reagents were purchased from E.Mark LTD, (Bombay, India).

Preparation of Microsomes

Rat liver was perfuse with 0.6% NaCl to remove blood from the tissues and then dissected. Male albino Charles Foster strain rats (170-210 g) were used for the

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experiment. The weighed liver was homogenized in 250 mM sucrose containing 10 mM tris-HCl at pH 7.4 using a glass teflon homogenizer to prepare a 20% homogenate. The homogenate was then filtered through a double layer of cheese cloth to eliminate fat and other cell debris. All the steps were carried out at 4°C. $CaCl_2$-precipitation, followed by differential centrifugation were employed in the isolation of microsomes (Schenkman and Cinti, 1978). The filtrate obtained from the homogenate was centrifuged at 12000 x g for 10 min. To the resultant post mitochondrial supernatant (PMS) solid $CaCl_2$ (8.0 mM final concentration) was added and thoroughly mixed until homogeneity for complete aggregation of microsomes. The microsomes were sedimented by centrifugation of the $CaCl_2$-PMS mixture at 25000 x g for 15 min (L7 55 Ultracentrifuge; Beckman, Palo, Alto, CA, USA). The pellet was then washed by re suspension in an adequate volume of wash buffer (10 mM Tris-HCl, pH 7.4, containing 150 mM KCl) and resedimented at 25000 x g for 15 min. The resultant opalescent pellet was resuspended by gentle homogenization with a teflon pestle in a small volume of wash buffer to obtain the microsomal sample.

Treatments

To study the effect of SA, rat liver microsomal membranes were incubated with SA at the concentration of 10 mM. The 1.5 ml incubation mixture containing 50µl microsomal preparation, 20 µl SA and 1.43 ml of Kreb's Ringer bicarbonate solution (0.6% NaCl, 1.15% KCl, 1.22% $CaCl_2$, 2.11% KH_2PO_4, 3.82% $MgSO_4$-$7H_2O$, 1.3% $NaHCO_3$) was incubated for 2 hrs incubation at 30°C. The mixture was then centrifuged at 25000 x g for 15 min and the pellet was re dissolved in 1.5 ml washing medium from which 0.5 ml was used for malondialdehyde estimation and 1.0 ml for the conjugated diene assay. To study the effect of catalase, on SA induced lipid peroxidation, catalase (40 units) was added to the microsomal membranes prior to the addition of SA. 20 µl Cadmium chloride (10 µM) was used as a standard toxicant because it is known to increase lipid peroxidation in isolated hepatocytes. Catalase alone (40 units) was used to show whether it has any effect in lipid peroxidation or not. In another set of experiment four dilutions of catalase, ie 10, 20, 30, 40 units were used along with SA to study whether it actually interacts with SA and increased SA induced lipid peroxidation in a concentration dependent manner.

Assay of Malondialdehyde

Malondialdehyde (MDA) was estimated by the method of Buege and Aust, 1978. Briefly, 1.0 ml of microsomal sample (0.1 2.0 mg of membrane protein) was combined with 2.0 ml of trichloro acetic acid-thiobarbituric acid-HCl and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,000 x g for 10 min. The absorbance of the supernatant was determined at 535 nm against a blank that contains all the reagents minus the lipid. The MDA concentration of the sample was calculated using an extinction coefficient of 1.56 x $105M^{-1}cm^{-1}$ and expressed in terms of nmol MDA/mg protein.

Assay of Conjugated Diene

The procedure of Buege and Aust, 1978 was followed for the estimation of conjugated diene (CD). Membrane lipids were first extracted by mixing 1.0 ml microsomal sample with 5.0 ml mixture of chloroform: methanol (2:1). The mixture was then centrifuged at 1000 x g for 5 min to separate the phases. Most of the upper layer was removed by suction, and 3.0 ml of the lower chloroform layer was taken in a test tube and evaporated to dryness in a 45°C water bath. The lipid residue was dissolved in 1.5 ml of cyclohexane, and the absorbance was measured at 233 nm against a cyclohexane blank. The CD concentration of the sample was calculated from an extinction coefficient of 2.52 x $104M^{-1}cm^{-1}$ and expressed as nmol CD/mg protein.

RESULTS AND DISCUSSION

The effects of SA and catalase on the microsomal membrane MDA and CD are shown in Figure, 1. The concentration dependent activity of catalase is shown in Figure, 2. The data are reported as the mean ± SE of ten assays. As shown in Figure 1, incubation of microsomal membrane with SA does not increased CD significantly. But addition of catalase enhanced the effect of SA on lipid peroxidation significantly though catalase itself has no effect ($p < 0.05$, ANOVA). Cadmium chloride, a known toxicant that causes membrane damage by peroxidation, increased both MDA and CD significantly. Figure, 2 clearly

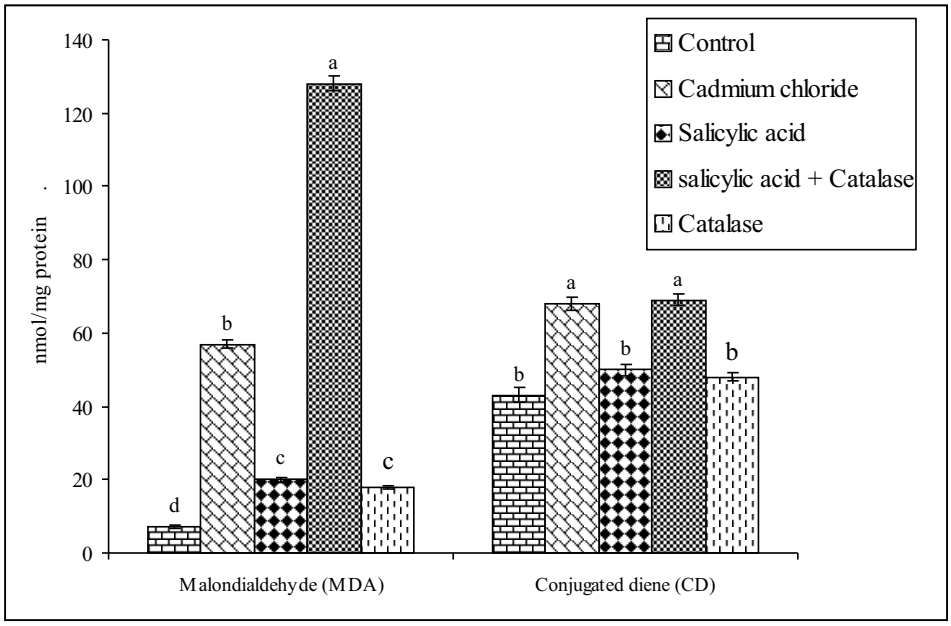

Figure 1 : Effects of SA And Catalase On Rat Liver Microsomal Membrane MDA And CD Formation

Each Bars Represents The Mean With SE of 10 Replicates

Data Were Analyzed By ANOVA ($p < 0.05$, each)

a,b,c,d = Different Small Letters In Bars Indicate Significant Difference At 5% level

shown, that the effect of SA increase with the increase in concentration of catalase.

The possible mechanism of action of SA was proposed based on the finding that SA binds with SA-binding protein (SABP) (Chen and Klessig, 1991). Sequence analysis of the purified SABP indicated that SABP is highly homologous to catalase (Chen et al., 1993). Indeed, SABP has catalase activity, as demonstrated by the ability of purified SABP to degrade H_2O_2 to H_2O and O_2. SA was found to block the SABP's catalase activity. Catalase inhibition would lead to an increase in the concentration of H_2O_2. This H_2O_2 and its derivatives could act as a secondary messenger for the expression of PR genes. However, transgenic tobacco plants with diminished catalase activity resulting from anti sense expression of the catalase gene, no increase in the constitutive expression of PR gene was observed (Chamnongpol et al., 1996). Additionally, H_2O_2 and H_2O_2 inducing chemicals were unable to induce PR protein expression in transgenic NahG plants. nahG encodes salicylate hydroxylase, an enzyme which converts SA to catechol, a compound unable to induce SAR. Although these chemicals could activate PR-1 genes in wild-type tobacco. (Neuenschwander et al., 1995). Therefore, a further mode of action of SA was proposed based on its ability to form SA free radicals upon interaction with catalase and peroxidases (Durner et al., 1997). Such free radicals may induce lipid peroxidation, and the products of this reaction, such as lipid peroxides, are potent signaling molecules in animals and possibly also in plants and may activate plant defense responses. Our results also suggested that SA interacts with catalase and induced lipid peroxidation significantly and the products of such reaction may activate plant defense responses.

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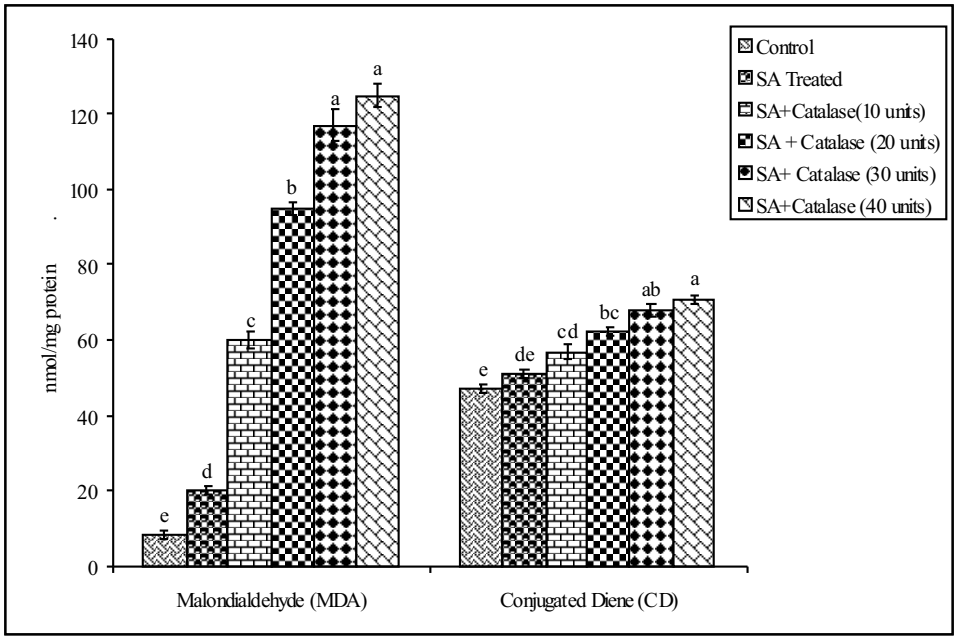

Figure 2 :Effect of Catalase At Four Different Concentrations On SA-Induced Rat Liver Microsomal Membrane MDA and CD formation

Each Bars Represents The Mean With SE of 10 Replicates Data Were Analyzed By ANOVA ($p < 0.05$, each).

a,b,c,d,e = Different Small Letters In Bars Indicate Significant Difference At 5% Level.

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