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Mechanism of Action of Acaciasides on Microsomes of *Setaria cervi*

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ABSTRACT

Cell membranes are made up of unsaturated lipids which are susceptible to oxidative damage. Oxidative damage can lead to a breakdown or even hardening (peroxidation) of lipids. It is firmly established that homogenates of most animal tissues form thiobarbituric acid reactants (peroxides) when incubated in vitro in aerobic conditions. These substances are formed by the oxidation of unsaturated fatty acids of lipid constituents of the tissue probably via hydroperoxides. Acaciaside A and B are triterpeniod saponins originally isolated from the funicles of *Acacia auriculiformis*. Acaciasides A and B contain a conjugated unsaturated system which is involved in the the damaging effects of saponins probably by resulting free radicals that labilize parasite membrane through peroxidation. Saponins have significantly enhanced membrane peroxidation in terms of both MDA and CD formation. The increased rate of peroxidation may lead to the formation of peroxyl radicals that may react with the lipid, probably by hydrogen abstraction. To further investigate the mechanism of saponins-induced membrane damage, SOD, thiourea and catalase were added to microsome membranes of *Setaria cervi* prior to the addition of saponins. It is evident that superoxide anions are probably involved in the expression of membrane damaging effect of saponins.

1. Introduction

Cell membranes are made up of unsaturated lipids which are susceptible to oxidative damage. Oxidative damage can lead to a breakdown or even hardening (peroxidation) of lipids. Lipid peroxidation alters the physiological functions of cell membranes, modify membrane properties such as membrane fluidity, permeability to different substances, and bilayer thickness and plays an important role in cellular membrane damage. Lipid peroxidation can influence the permeability of lipid membranes by increasing the dielectric constant of the membrane interior and by increasing the micro viscosity, possibly through cross-linking of lipid radicals [1]. Unsaturated lipids are easily susceptible to peroxidation [2]. Eventually, when all phospholipids are oxidized, pore formation can occur. This will allow reactive species, such as reactive oxygen and nitrogen species (RONS), to enter the cell and cause oxidative damage to intracellular macromolecules, such as DNA or proteins [3]. The presence of oxidized lipids has an immense influence on the permeability of water through bilayers. The existence of a direct relationship between lipid peroxidation and membrane leakiness has been suggested [4-6]. Increased membrane permeability caused by oxidation of lipids and membrane proteins can disrupt ion gradients, therefore altering metabolic processes [7]. Studies indicate that the phospholipid bilayer becomes more disordered as a result of the oxidation products [3].

Lipid peroxide formation can lead to membrane damage as has been shown for erythrocytes [8] and lysosomes [9]. Formation of lipid peroxides in vivo would therefore cause severe cellular damage. Under certain conditions lipid peroxides may be formed in vivo but its measurement *in vivo* has been difficult and results are controversial [10, 11]. It is firmly established that homogenates of most animal tissues form thiobarbituric acid reactants (peroxides) when incubated in vitro in aerobic conditions [10]. These substances are formed by the oxidation of unsaturated fatty acids of lipid constituents of the tissue probably via hydroperoxides.

Acaciasides A and B, two acylated triterpenoid bisglycosides isolated from the funicles of *A. auriculoformis*, contain a conjugated unsaturated system which is highly susceptible to peroxidation and known to have antihelmintic properties [12]. The saponins were found effective against both microfilariae and adult worms of *Setaria cervi* in rats [13]. An ethanol extract of the funnicles, when administered orally to dogs naturally infected with *Dirofilaria immitis* found effective against both microfilaria and adult worm [14]. The cestocidal activity of the saponins has also been reported. It was suggested that the double bonds present in nonionic surfactants form allelic radicals that may react with the molecular oxygen present in the membrane and cause lipid peroxidation [15]. This mechanism perhaps explains the increased lipid peroxidation of the sperm membrane preparation and loss of membrane integrity under the exposure to Acaciaside-B-enriched fraction of *Acacia auriculiformis* [16].

In our previous study it was observered that incorporation of acaciasides along with antibiotics gave better results on micrfilaria of *Dirofilaria immitis* both *in vitro* and in vivo [17]. In the present study we have selected the bovine filarial worm *Setaria cervi* which does not harbour the *Wolbachia* endosymbiont [18] and observed the effects of acaciasides on membrane damage in vitro. The acaciasides either cause damage to the membrane of the filarial worms or may facilitate the entry of the antibiotics that may prove to be an effective mass chemotherapeutic regimen in future. This prompted us to investigate the interaction of saponins and membrane, with *S. cervi* microsomes as our model to understand the mode of action of the saponins.

2. Experimental Methods

2.1 Preparation of Microsomes

Adult *S. cervi* were collected from the peritoneal cavity of freshly slaughtered cows at local abattoirs. The worms were then repeatedly washed with PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4) to remove blood and remnants of any host tissue. The weighed worms were homogenized in 250 mM sucrose containing 10 mM Tris – HCl, pH 7.4 with a glass homogenizer to prepare a 20% homogenate at 4 °C. The homogenate was filtered through a filter paper to eliminate cell debris. The filtrate was centrifuged at 12,000 rpm for 10 min at 4 °C. Solid $CaCl_2$ (8 mM final concentration) was added to the resultant post-mitochondrial supernatant (PMS) and thoroughly mixed until homogeneity for complete aggregation of microsomes. The microsomes were pellet down by

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centrifugation of the $CaCl_2$-PMS mixture at 25,000 X g for 15 min. The pellet was then resuspended in 0.5 mL wash buffer (10 mM Tris-HCl, pH 7.4 containing 150 mM KCl) and resedimented at 25,000 rpm for 15 min. Final pellet was reconstituted in 0.5 mL wash buffer.

2.2 Preparation of Acaciaside A and Acaciaside B

Acaciaside A and B are triterpeniod saponins originally isolated from the funicles of *Acacia auriculiformis* were respectively defined to be 3-O-[β-D-glucopyranosyl (1→6){α-L-arabinopyranosyl (1→2)}-β-D-glucopyranosyl]-21-O-{(6′ S)-2′-trans-2′,6 –dimethyl-6′-O- β-D-glucopyranosyl-2′,7′-octadienoyl} acacic acid 28-O- α-L-rhamnopyranosyl (1→6)[β-D-xylopyranosyl (1→2)]- β-D-glucopyranoside **(**1) and 3-O-[β-D-glucopyranosyl (1→6){ α-L-arabinopyranosyl (1→2)}- β-D-glucopyranosyl]-21-O-[(6′S)-2′-trans-2′, 6′-dimethyl-6′-O-{ β-D-xylopyranosyl (1→2)- β-D-glucopyranosyl}-2′,7′ - octadienoyl] acacic acid 28-O- α-L-rhamnopyranosyl (1→6) [β-D-xylopyranosyl (1→2)]- β-D-glucopyranoside (2). The structural details were elucidated by a combination of fast-atom-bombardment mass spectrometry, ^{1}H-, and ^{13}C NMR spectroscopy, and some chemical transformations [12] (Fig. 1). The mixture of acaciaside A and acaciaside B, which is water soluble, was used for testing its effects on microsome membrane preparation of *Setaria cervi*.

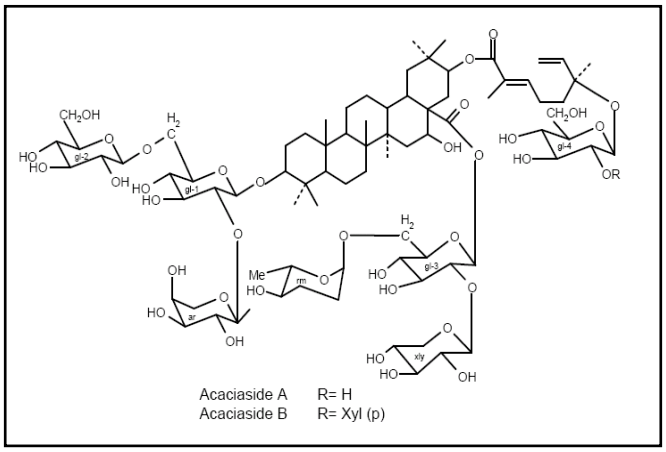

Fig. 1 Chemical structure of acaciasides A and acaciasides B

2.3 Treatments

To 50 µL microsome preparation (1 mg/mL protein), acaciasides at 1 mg/mL and Kreb's Ringer bicarbonate solution were added to a final volume of 1.5 mL. The mixture was incubated for 2h at 30 °C. The mixture was then centrifuged at 25,000 rpm for 15 min and the pellet was redissolved in 1.5 mL wash buffer from which 0.5 mL was taken for MDA assay and 1 mL for CD assay [19].

To study the effects of SOD (500 U/mL), catalase (2000 U/mL) and thiourea (1 mM), these agents were added to the membranes prior to the addition of saponins. Three dilutions of SOD (200, 400 and 500 U/mL) were used to study whether it could block the effect of saponin-induced enhanced lipid peroxidation in a concentration dependent manner. These particular doses were selected from our previous work [15, 20]. Cadmium chloride was used as a standard toxicant because it is known to increase peroxidation in isolated hepatocytes [21, 19].

2.4 Assay of Malondialdehyde (MDA)

The thiobarbituric acid (TBA) test is performed to measure the amount of thiobarbituric acid reactive substances (TBARSs) or malondialdehyde (MDA) present in the sample. MDA is generated as a degradation product from peroxidised lipids [22] and as a side product of enzymatic metabolism of thromboxanes and prostaglandins [23, 24]. The basis of the TBA method is the reaction of MDA with TBA at low pH and high temperature to form a colored complex, the MDA-TBA complex, with an absorption maximum at 532-535 nm that can be measured by visible absorption spectrophotometry [25, 26].The test works well in defined membrane systems (e.g. microsomes and liposomes), but its application to body fluids has produced a host of problems.

One mL of microsome fraction (1 mg of membrane protein) was combined with 2 mL of trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-HCl and mixed well. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. Supernatant was collected and optical density was measured at 535 nm against a blank that contains all the reagents minus lipid. The MDA concentration of the sample was calculated using an extinction coefficient of 1.56 X 10^5 $M^{-1}cm^{-1}$ [27] and expressed in terms of µM MDA/ mg membrane protein/min.

2.5 Assay of Conjugated Diene (CD)

Conjugated diene (CD) structures with a double-single-double bond (-C=C-C=C-) arrangement absorb UV light in the wavelength range 230-235 nm and can thus be detected by UV absorption spectrophotometry [28-30]. Conjugated diene measurement has successfully been used to study peroxidation in isolated lipoprotein fractions.

To one mL of microsome fraction (1 mg of membrane protein), 5 mL chloroform- methanol (2:1) mixture was mixed to extract the membrane lipids. The mixture was then centrifuged at 1000 rpm for 5 min to separate the phases. Most of the upper layer was removed by suction, and 3 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 cyclohexane, and absorbance was measured at 233 nm against a cyclohexane blank. The CD concentration of the sample was calculated using an extinction coefficient of 2.52 X 10^4 $M^{-1}cm^{-1}$ [27] and expressed in terms of µM CD/ mg membrane protein/min.

3. Results and Discussion

Incubation of microsomal membrane with saponins at 20, 50 and 100 µg/mL increased MDA formation by 12, 25 and 38%, respectively (Fig. 2) compared to control indicating that saponins enhanced lipid peroxidation (paired t-test, P <0.05). Cadmium chloride a known toxicant that causes membrane damage by peroxidation increased MDA formation by 43% (paired t-test, P <0.05, Fig. 3).

Incubation of microsomal membrane with saponins at 20, 50 and 100 µg/mL increased CD formation by 31, 51 and 52%, respectively (Fig. 4) compared to control indicating that lipid peroxidation is enhanced by saponins (paired t-test, P <0.05). Cadmium chloride increased CD formation by 58% (paired t-test, P <0.05, Fig. 5).

SOD significantly blocked the effects of saponins-induced membrane damage in a concentration dependent manner. Catalase had a minor effect (6%) on saponins-induced membrane damage and thiourea had no effect.

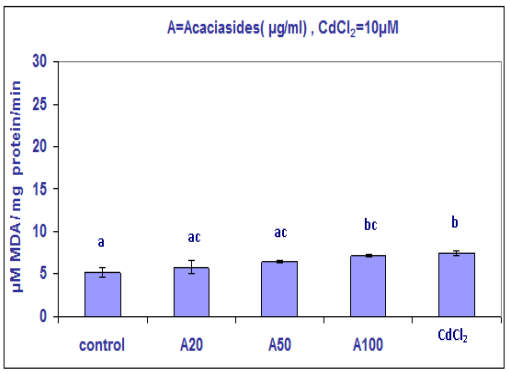

Fig. 2 Effects of acaciasides on malondialdehyde (MDA) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 20, 50 and 100 µg/mL (A20, A50 and A100 respectively) of microsomal membrane preparation. Cadmium chloride was added at a final concentration of 10 µM as positive control. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.

Nonionic surfactants are amphipathic molecules consisting of a hydrophobic (alkylated phenol derivatives, fatty acids, long-chain linear alcohols, etc.) and a hydrophilic part (generally ethylene oxide chains of various length). Due to this favorable physicochemical property, nonionic surfactants interact not only with proteins but also with membrane phospholipids to modify their structure and permeability [16]. It was suggested that the double bonds present in nonionic surfactants form allelic radicals that may react with the molecular oxygen present in the microsomal membrane forming peroxide i.e., hydroperoxide [15]. Due to the formation of hydroperoxide, peroxidation is enhanced. The conjugated unsaturated system present in Acaciasides A and B is involved in the damaging effects of saponins probably by resulting free radicals that labilize parasite membrane through peroxidation. The results clearly reveal that saponins have significantly enhanced membrane peroxidation in terms of both MDA and CD formation. The increased rate of peroxidation may lead to the formation of peroxyl radicals that may react with the lipid, probably by hydrogen abstraction. To further investigate the mechanism

of saponins-induced membrane damage, SOD, thiourea and catalase were added to membranes prior to the addition of saponins. Since SOD completely blocked the saponin-induced membrane damage, it may be suggested that superoxide anions are probably involved in the expression of membrane damaging effect of saponins. The experimental results suggest that such an interaction may also occur between the parasite membrane and saponins in vivo. Superoxide dismutase converts superoxide radicals to H_2O_2. Catalase breaks down H_2O_2 to water and oxygen. SOD and catalase constitute the first coordinated unit of defense against reactive oxygen species [31]. Since thiourea had no effect on saponin-induced membrane damage, it indicates that hydrogen peroxide is not probably involved in these reactions.

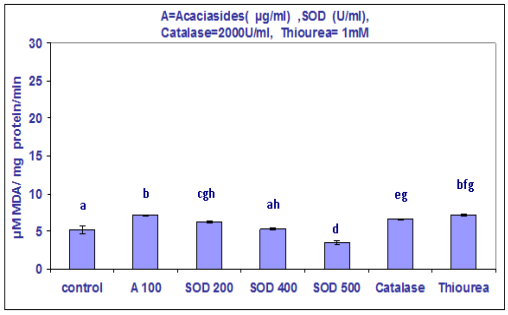

Fig. 3 Effects of acaciasides, superoxide dismutase (SOD), catalase and thiourea on malondialdehyde (MDA) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 100 µg/mL (A100) of microsomal membrane preparation. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.

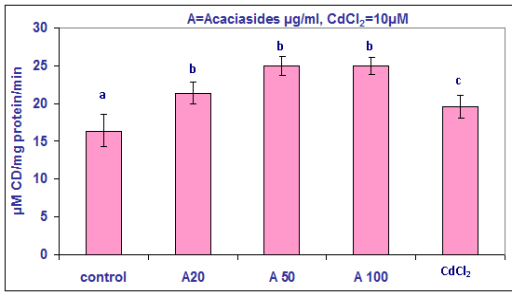

Fig. 4 Effects of acaciasides on conjugated diene (CD) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 20, 50 and 100 µg/mL (A20, A50 and A100 respectively) of microsomal membrane preparation. Cadmium chloride was added at a final concentration of 10 µM as positive control. Each bar represents the mean±S.E. (n=3).). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.

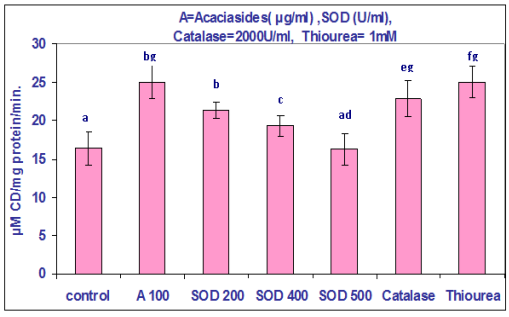

Fig. 5 Effects of acaciasides, superoxide dismutase (SOD), catalase and thiourea on conjugated diene (CD) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 100 µg/mL (A100) of microsomal membrane preparation. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts

4. Conclusion

This mechanism perhaps explains the increased lipid peroxidation of the *S. cervi* microsome preparation and loss of membrane integrity under the exposure to the mixture of acaciasides A and acaciasides B.

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