The photodynamic action of UV sensitized methylene blue on the venom of Thailand cobra *Naja siamensis*

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**ABSTRACT.** The photodynamic action of UV sensitized methylene blue (MB) was studied on the venom of Thailand cobra *Naja siamensis*. The reaction mixture comprising phosphate buffer (B), methylene blue (S), and venom protein (P) was exposed to UVR (Ultra violet radiation) for 2 hours at 37°C. Stable excited species (S') were generated by photodynamic action. These in B environment produced reduced substates SH2 and photooxidised products PO in the presence of atmospheric oxygen. Increased absorbance (OD) of the reaction mixture after UVR with a corresponding loss of protein was suggested to be due to the formation of photooxidised venom products. The detoxification profile of venom protein using visible radiation in the presence of MB and oxygen appeared similar to the profile using UVR.

**KEY WORDS:** Thailand cobra, *Naja siamensis*, photosensitization, photooxidation, detoxification.

**INTRODUCTION**

Phototoxic or photoallergic type of photosensitization is commonly encountered with a variety of pharmaceutical additives and drugs, including coaltar derivatives and artificial sweeteners, such as cyclamates, sulfonamides, oral antidiabetics, etc. Photodynamic action via the formation of light-induced catalytic oxidative and reductive photon intermediates is commonly observed in drug formulations, such as riboflavin, phenothiazines, etc. Very rapid detoxification of *Clostridium botulinum* Type A toxin by photooxidation was reported using methylene blue with no loss of antigenic property (11). Nicotine in the presence of MB when exposed to UVR (Quartz, 60 Hz, 125
W, single phase) from a distance of 10 cm for 2 h did not exhibit photodynamic action. However, in visible light hydrogen acceptor from the sensitized MB molecules reacted with hydrogen donor of L-nicotine. Reoxidation of the dye was suggested in the presence of oxygen with the liberation of hydrogen peroxide, which was responsible for the irradiation and formation of photooxidised nicotine product (10). Photodynamic action of MB on β-lactoglobulin showed characteristic absorbance of OD max. at 279 nm and OD min. at 250 nm with an OD max. shift towards left and an OD min. towards right with respect to increased oxygen uptake. Absorbance increased progressively with an increase in oxygen uptake and remained stationary at max. level of oxygen uptake. Histidine and tryptophan were first shown to be photooxidised, followed by oxidation by tyrosine (9). Similar photooxidation studies were carried out by Weil and coworkers, using MB in visible light on crystalline chymotrypsin, lysozyme, and bovine insulin. Venom of Crotalus atrox was detoxified by a similar method as used by Weil and coworkers using Warburg apparatus, with the reaction mixture containing MB in a tris buffer and oxygen at 37°C and exposed to electronic radiation from visible light (2). The same procedure was used to prepare toxoid by photooxidation from various venoms (3,4). Photooxidation of the venom was carried out with the aim of removing the toxic characteristics when the antigenicity of the protein molecules was preserved.

Irradiation of Apis mellifera venom was attempted using γ Co 60 radiations to obtain a less toxic venom product, which can enhance the production of specific antivenom directed against lethal components without affecting its antigenicity (6). Detoxification of Bothrops jararaca venom, as shown by LD50 in mice, was performed by γ Co 60 radiations. UV absorption spectrum showed increase in OD with increasing irradiation doses. Antigenicity of the venom was retained up to the dose of 2000 Gy, as shown by immunoprecipitation studies (1). Agkistrodon piscivorus venom was irradiated by exposure to UV light (mercury vapour quartz, 250 uW/cm 2) from a distance of 10 cm for 1 h. and 3 h. The irradiated venom sample exhibited 3-4 fold increase in LD50 value. The loss of toxicity parallels the loss of enzymes, which include phospholipase A2, protease, and phosphodiesterase. The effect of irradiation on phospholipase A2 has been shown to be particularly sensitive by the time required for complete inactivation of the enzyme, paralleling the loss of venom toxicity (8). The mechanism of photodynamic action of the sensitizer MB on protein molecules by incident radiation involves the formation of photoperoxides (SO2) and photooxidised forms of protein (PO2) by reaction of long-lived excited species of MB with molecular oxygen. S and P in a complex form generate long-lived excited species of SP' (sensitizer protein) and in water environment generate reduced substrates of S and photooxidised form of P molecules (PO) with the liberation of hydrogen peroxide in the presence of molecular oxygen (7).

Photosensitization of venom is the process by which venom protein molecules are allowed to interact with the sensitized MB molecules by selective UV light. They undergo photooxidation, thereby causing alterations due to perturbations in the region of the UV absorption spectrum of protein molecules. UVR induced transitions in oscillating dipoles of photosensitizer molecules, thereby generating reactive oxidative and reducing photon species, which when reacted with protein molecules, cause an increase in absorption characteristics with consequent loss of protein. Sensitizer MB molecules absorbed the force field of oscillating electric dipoles to which energy was exchanged in the form of discrete irreducible quantal packets proportional to the frequency of radiation from UV source. UVR generated transitions in oscillating electric dipole that were allowed to enter the excited state, characteristic of substrates with the range of AE values in multi-component systems comprising (B), (S), and (P) molecules. While occupying a vacant position of the higher orbitals of electron acceptor, the vacant position created by MB molecules gets filled by an electron from electron donors prominently P molecules. When the reaction mixture of S, P, and B was exposed to UVR, photodynamic action generated stable excited species of S, which in B environment, produced 'reduced substrates' and photooxidised 'P' products in the presence of atmospheric oxygen.

\[
(S) + (P) \text{ in } B \xrightarrow{\text{UVR}} S' \text{'(stable excited species')} \xrightarrow{\text{SH2}} \text{reduced form of substrate} + \text{oxidised 'P' products}
\]

The reaction vessel containing sensitizer MB and venom protein samples in a phosphate buffer (Potassium phosphate, 0.05 M, pH 6.0) was exposed to ultra violet light from a UV lamp (115 volts, 60 Hz, 0.9 AMOs, Model R-52 G) at 37°C, gently stirred for 2 hours.

Compositions of the four sets of reaction mixture in a fixed volume of (3.0 ml) was as follows:

I.P (600 µg/ml on wt. basis) in 1.5 ml of B + 1.5 ml of B;
II. P in B (1.5 ml) + C (activated charcoal, 125 µl, 1.0% in B), gently stirred for 5 min following filtration under suction;

III. P in B (1.5 ml) + S (1.5 ml, 30 µg per ml = 94 µM) 2 h, gently stirred in a reaction beaker covered with aluminum foil + charcoal (125 µl, 1.0% in B), gently stirred for 5 min followed by filtration under suction;

IV. P in B (1.5 ml) + S (1.5 ml) 2 h gently stirred in a reaction vessel exposed to UVR in a chamber protected from visible light + charcoal (125 µl, 1.0% in B), gently stirred for 5 min followed by filtration under suction.

Absorbance of each reaction mixture was measured at the wavelength of 200 to 400 nm using Spectronic 2000 (B & L, USA). Protein measurements were performed using the Lowry method (5). Absorbance of colored reaction caused by reduction of phosphomolybdate-phosphotungstate salts of Folin Ciocalteau reagent (Sigma, USA) in alkaline copper reagent by tyrosine and tryptophan present in the venom protein, was measured spectrophotometrically at 660 nm using bovine serum albumin (BSA) as a standard. Protein concentration OD (min. and max.) measurements of six experiments were analysed as mean ± standard error [(m ± s.e.), n = 6, Table 1] for reactions I to IV, and ‘p’ values were obtained by comparison of reaction I with reactions II, III, and IV, based on two tailed ‘t’ test (α = 0.05).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Protein concentration [µg/ml]</th>
<th>Wavelength (nm)</th>
<th>OD (min.)</th>
<th>Wavelength (nm)</th>
<th>OD (max.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I P + B</td>
<td>154.15 ± 13.82</td>
<td>252-253</td>
<td>0.210 ± 0.012</td>
<td>278-279</td>
<td>0.337 ± 0.012</td>
</tr>
<tr>
<td>II P + B + C</td>
<td>113.90 ± 15.37</td>
<td>253-255</td>
<td>0.223 ± 0.021</td>
<td>(p &lt; 0.025)</td>
<td>(p &lt; 0.05)</td>
</tr>
<tr>
<td>III P + B + S --- C</td>
<td>110.94 ± 10.04</td>
<td>253-256</td>
<td>0.269 ± 0.025</td>
<td>(p &lt; 0.025)</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td>IV P + B + S UVR</td>
<td>75.09 ± 11.77</td>
<td>256-258</td>
<td>0.371 ± 0.012</td>
<td>(p &lt; 0.005)</td>
<td>(p &lt; 0.001)</td>
</tr>
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The profile of UV absorption spectrum of the six experiments (m ± s.e.) was shown in Figure 1 and Figure 2 as a plot of OD, and OD and relative OD versus wavelength (λ). The control reaction I of OD min. at 252-253 nm was 0.210 ± 0.012 and OD max. at 278-279 nm was 0.337 ± 0.012, when protein concentration of the reaction mixture I was 154.15 ± 13.82 µg per ml. The reaction mixture II comprised protein in buffer and charcoal treatment to obtain the value following protein consumption due to absorption in control reaction. OD min. at 253-255 nm was 0.223 ± 0.021 (p < 0.05) and OD max. at 278-280 nm was 0.278 ± 0.018 (p < 0.01), when protein concentration was 113.90 ± 15.37 µg per ml. OD at peak was decreased by 17.51 %, with a corresponding decrease in protein concentration by 26.11 %, while eliminating the color interference. Reaction III comprising venom protein sample (P) in buffer (B) and sensitizer (S) was carried out at room temperature for 2 hours in a closed light-protected container. The reaction was terminated using activated charcoal in the concentration that can remove the color index with minimal background absorption. OD min. at 253-256 nm was 0.269 ± 0.025 µg per ml (p < 0.025) and OD max. at 278-280 nm was 0.342 ± 0.025, when protein concentration was slightly decreased by 2.6 %. Reaction mixture IV was exposed at the end of the reaction, by treating in a similar way to that of the preceding reaction. OD min. at 253-258 nm was 0.278 ± 0.018 (p < 0.01), when protein concentration was 113.90 ± 15.37 µg per ml and OD max. at 278-280 nm was 0.342 ± 0.012, when the protein concentration was further decreased to 75.09 ± 11.77 µg per ml (< 0.005). The OD at peak was increased by 26.9% with a slight shift towards right (281-282 nm), when protein concentration was decreased by 32.31 % in comparison with control reaction III. The characteristics of reactions were not statistically significant except for reaction II (OD min.) and III (OD max.). UV spectrum of photooxidised reaction IV was converted in terms of protein concentration in control (I) and before exposure to UVR (III) and interpreted as a relative OD, as shown in Figure 2. Typical profiles of MB photodynamic action on Naja siamensis venom due to UVR at various reaction times monitored on Cary (USA) were shown in Figure 3. Sensitizer MB exhibited max absorption in visible region 615, 665 nm and also in 245, 290 nm region. Interference of MB due to color index with protein molecules and consequent interaction with photooxidised protein profile can be minimized by charcoal treatment. A charcoal filter was used to subtract the
color index and to obtain the photooxidised profile of venom protein following UVR. The characteristic spectrum of the control reaction and after exposure to UVR showed blunted peak and flattened decay curve. The photooxidised spectrum of venom using UV light appeared similar to the detoxified profile, using visible light in the presence of MB. Irradiation of venom protein by direct exposure to UV light in the absence of third species MB also caused an increase in OD max. proportional to the radiation time, which might be due to the direct exchange of energy with protein molecules in a buffer environment. The resulting oxidised species and quenching may cause an increase in OD unlike photooxidation in the presence of MB via formation of excited sensitized species, which may be responsible for the selective perturbation in the protein molecules by interaction with the charged groups from the amide, amino acid side chains, or from disulfide linkages. Detoxification studies on venom were performed in the presence of MB and oxygen at 37°C, using visible light in order to use the antigenic properties of photooxidised venom in the absence of toxicity for the development of titer with higher efficacy for antivenom production. A similar procedure was attempted using UV light in the presence of sensitizer MB, which also absorbs strongly in UV region with max absorbance of 245 and 290 nm. We think that direct exposure to UV radiation instead of visible light could avoid destructive effects of thermal energy, and reaction under constant stirring can utilize atmospheric oxygen to generate excited stable species of sensitizer molecules, which can consume venom protein reduced substrate, with the resultant formation of photooxidised venom products. The differences were observed in UV derivative OD spectrum, monitored on Cary (USA), between irradiated and photooxidised venom products in the presence of MB. This preliminary observation of photodynamic property would be useful to generate biologically active photooxidised products with specific alterations of pharmacological significance, based on activity related to radiation time and the comparative results of UV derivative OD profile of irradiation in the absence of sensitizer species in the reaction mixture.

**FIGURE 1.** Methylene blue induced photooxidation of *Naja siamensis* venom due to UVR. Plot of wavelength v/s O.D.
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FIGURE 2. Photooxidation of *Naja siamensis* venom by UV sensitized MB, plot of wavelength v/s OD and relative OD.

FIGURE 3. Photodynamic action of UV sensitized methylene blue on the venom of Thailand Cobra *Naja siamensis* at various exposure time.
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