peat deposits in the central Kerala coastal region. Whether the subsidence of these beds to depths of 50 m was due to progressive transgression of the sea and sea-level changes, and how far such subsidence is aided by tectonics need focused attention. Further work is in progress in this direction.

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Superoxide dismutase activity in haemocytes and haemolymph of *Bombyx mori* following bacterial infection

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The haemocytes and haemolymph of the silkworm *Bombyx mori* L. were analysed for their superoxide dismutase (SOD) activity following *Bacillus subtilis* inoculation using a photochemical assay system consisting of methionine, riboflavin, and p-nitro blue tetrazolium. The SOD assay had a maximum activity at pH 7.8 with light exposure optima of 15 min. The optimum concentration of the crude enzyme source for the assay was 80–90 μg equivalent protein of haemocytes and 248–284 μg protein of plasma. The enzyme activity was ca. 5-fold higher in the haemocytes than the plasma, and it maintained a plateau throughout the test period (10 min to 9 h) in both fractions in healthy condition. However, upon infection after an initial burst (10 min), the SOD activity sharply declined at 30 min and then significantly enhanced in larval haemolymph from 1 h post-inoculation. In haemocytes, on the other hand, infection caused a depression in SOD activity except during 1–3 h, when a transient enhancement was noticed. This implies that modulation of superoxide anion generation is an early response as part of the defensive process against pathogen invasion. Also, the haemolymph and haemocytes trigger such response differentially by which this deleterious flux of superoxide is scavenged in its turn by the SOD enzyme.

MOLECULAR oxygen is an essential element of life, yet as a result of incomplete reduction of oxygen to water, reactive oxygen species (ROS) are generated in all aerobes. Most ROS are generated as superoxide anions (O_2^-), and are rapidly dismutated either non-enzymatically or enzymatically by the action of superoxide dismutase (SOD) to hydrogen peroxide and oxygen[1]. Pathogen-derived generation of O_2^- by NADPH oxidase in mammalian neutrophils is a common feature[2]. In fact, the bactericidal effect of polymorphonuclear leucocytes depends on their superoxide generative capacity[3,4], and biosynthesis of SOD is mainly controlled by increased intracellular

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fluxes of O_2^- in numerous micro-organisms[5] as well as in higher organisms[6,7]. A few reports are available in this regard in insects. Generation of O_2^- in the haemolymph plasma of the lepidopteran insect *Pseudeletia separata*[8] and high SOD activity in the larval haemocytes of the cabbage looper *Trichoplusia ni*[9] have been reported. This finding leads to the prediction that, analogous to the mammalian phagocytic action, insects produce a deleterious flux of superoxide in their haemolymph prior to phagocytosis and encapsulation. However, no study has been undertaken till date on the O_2^- generation nor has the SOD activity in haemocytes and haemolymph of insects been challenged with a pathogenic bacteria.

Here we report on the activity of SOD in haemocytes and plasma of healthy larvae of the silkworm *Bombyx mori* as well as those infected with a pathogenic bacteria *Bacillus subtilis*.

Laboratory colonies of the indigenous, multivoltine silkworm *B. mori*, race Nistari, were reared at room temperature (28 ± 1°C) and natural photoperiod. Larvae were fed on mulberry leaves ad lib. Fifth instar larvae (4-day-old, weighing 1.5 ± 0.19 g) were used in this experiment.

Cultures of *B. subtilis* (procured from the Institute of Microbial Technology, Chandigarh, India) pathogenic to the silkworm were maintained on 1.5% nutrient agar medium at 30°C in a bacteriological incubator (REMI, Model: RI-6A). The bacteria were used in mid-logarithmic phase at a titre of 6.75×10^5 cfu ml^{-1}.

An aliquot of 3 µl *B. subtilis* (ca 2.025×10^3 cfu) was inoculated to larvae (50 µl Hamilton microsyringe (Reno, NV) with a 26 guage 0.5″ needle) and 500 µl haemolymph pooled from 4 to 5 larvae at 10 min, 30 min, 1, 2, 3, 6 and 9 h post-inoculation (pi) on ice in phenyl thiourea-coated eppendorf tubes from both treated and healthy larvae, separately. The tubes were microfuged at 900 *g* to gently pellet the haemocytes and have a cell-free plasma. The latter was removed with a Pasteur pipette and diluted with an equal volume of cold 50 mM $NaPO_4$ buffer (pH 7.0). The haemocytes were resuspended in 500 µl of the same buffer and cells were gently disrupted using a glass pestle in the microfuge tubes. The diluted cell-free plasma and the haemocyte homogenate were then re-centrifuged at 12,000 *g* for 10 min at 4°C to obtain the cytosolic supernatant.

The ability of SOD (EC 1.15.1.1) to inhibit the reduction of nitro blue tetrazolium (NBT) by O_2^- generated photochemically was measured according to the method of Beauchamp and Fridovich[10], with slight modifications. 2.5 ml of the assay mixture consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin and 0.1 mM EDTA with 80–100 µl enzyme extract.

Riboflavin was added last and the tubes of uniform thickness were placed 30 cm below a light source consisting of two 15 W fluorescent lamps and the reaction allowed to run for 15 min, during which time the development of colour was found to be linear. The reaction was stopped by switching-off the light and the tubes were covered with a black cloth. The absorbance at 560 nm was read in a Shimadzu 160-A spectrophotometer. A non-irradiated reaction mixture which was run in parallel did not develop colour and served as control. There was no measurable effect of the diffused room light. The reaction mixture lacking enzyme developed the maximum colour and this decreased with increasing volume of enzyme extract added. The per cent inhibition at 560 nm was plotted as a function of volume of enzyme extract in the reaction mixture. The pH, duration of light exposure and enzyme concentration were determined with diluted haemolymph plasma (1 : 1 with extraction buffer $NaPO_4^-$ pH 7.0).

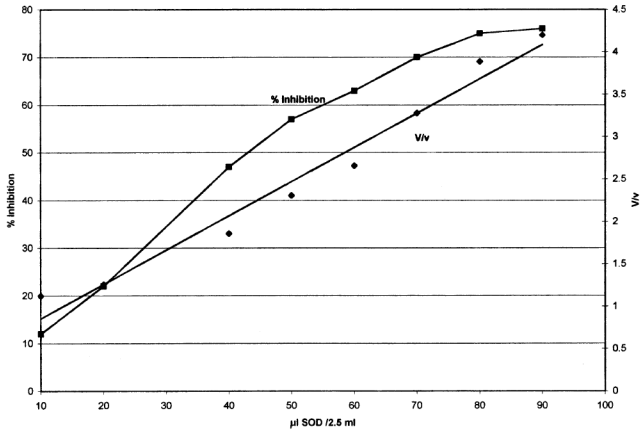

Figure 1. Inhibition of NBT photoreduction versus the concentration of crude SOD from *B. mori* haemolymph. Per cent inhibition is not linear with SOD concentration. Linearity ($r = 0.973$) is obtained by plotting V/v ratio against SOD concentration, V and v representing the rate of the reaction in absence and presence of enzyme respectively. Linearity between [SOD] and V/v was also confirmed with haemocyte homogenates.

One unit of SOD activity is defined as the amount that inhibits the NBT photoreduction by 50% and thus activity of SOD is quantitated on the basis of the per cent inhibition it caused[10]. The relationship between per cent inhibition and the amount of SOD was not linear, however, Asada *et al.*[11] using crystalline spinach SOD established a linear relationship between SOD concentration and the V/v ratio, fitting the equation:

$$V/v = 1 + K' [SOD]. \quad (1)$$

Equation (1) can be rewritten as:

$$K' [SOD] = (V/v) - 1, \quad (2)$$

where V and v represent the rate of the reaction in the absence and presence of SOD respectively, and K' corresponds to the slope of the line. This linear relationship ($r = 0.973$) was also observed in our assay system (Figure 1).

Therefore eq. (2) on the basis of linearity can again be rewritten as:

$$K'[SOD] = (V/v) - 0.973. \quad (3)$$

According to the consideration of one enzyme unit here at 50% inhibition of the assay reaction, the product $K'[SOD]$ equals unity. Therefore, in this study, the SOD activity was determined directly from the V/v ratio according to the equation:

$$\text{SOD units ml}^{-1} = [(V/v) - 0.973]\ (\text{dilution factor}). \quad (4)$$

SOD is a metalloenzyme that functions as a double-edged sword, for it converts O_2^- to H_2O_2, which is readily degraded to the very reactive $^{\cdot}OH$ via the Fenton reaction. This enzyme also protects the cells by dismutating superoxide free radicals (O_2^-) generated in different cellular loci to hydrogen peroxide and molecular oxygen[5,12]. We assayed the SOD activity photochemically using methionine, riboflavin and NBT[10]. The optimization of exposure to light, pH of the assay buffer and quantity of enzyme protein was done using haemolymph (cell-free plasma) of the silkworm larvae as these factors are reported to be varied in different animal and plant systems during SOD assay[13,14]. An exposure of 15 min to light (Figure 2 *a*), a pH of 7.8 (Figure 2 *b*) and an enzyme protein of 248 µg corresponding to 70 µl crude enzyme source (Figure 2 *c*) per 2.5 ml of assay mixture were optimum for our system and we maintained these conditions in subsequent experiments. Superoxide generation in larval haemolymph has been studied earlier in the lepidopteran *Pseudaletia separata* by Arakawa[8,15]. However, no study has been made on the quantitation of SOD in insects. This study first quantitates the SOD activity in *B. mori* haemocytes and haemolymph on infection by a pathogenic bacteria, *B. subtilis*. In our system, the pattern of SOD activities was similar in haemocytes and haemolymphs of non-inoculated (healthy) larvae and maintained a plateau throughout the test periods (Figures 3 and 4). However, presence of substantial amounts of SOD in both haemocytes and haemolymph plasma suggests that O_2^- scavenging enzymes are present in both fractions. In the haemocytes, at 10 min post-inoculation (pi) a sharp difference ($p < 0.01$) between activity in healthy haemocyte fractions and infected haemocyte fractions was observed (Figure 3). In infected haemocytes the activity was only 4270 units ml^{-1}, whereas in healthy haemocytes it was 9192 units ml^{-1}. Evidently, initially upon infection there was a depression of activity of the enzyme; however, at 30 min pi there was a sharp increase in SOD activity in infected cells reaching normal healthy control values, whereupon again at 1 h pi it fell sharply to 3401 units ml^{-1}. At 2 h pi the SOD activity peaked in haemocytes (12,704 units ml^{-1}) and then gradually declined till 6 h pi, where it fell below normal values. Thereafter, there was again an increase in its activity in infected cells at 9 h pi (8043 units ml^{-1}), though it was less than the healthy control values. Superoxide generation as revealed by NBT reduction showed a similar pattern (unpublished data). Membrane stimulation of phagocytic leukocytes results in a series of metabolic reactions known as the respiratory burst[16,17]. The reactions start with an increased consumption of oxygen by the phagocytes, followed by the one-electron reduction of oxygen molecules to superoxide anions (O_2^-). Superoxide is then readily converted into peroxide (H_2O_2), and eventually also hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$) is formed. These four active oxygen species can act as killing agents by themselves or in combination

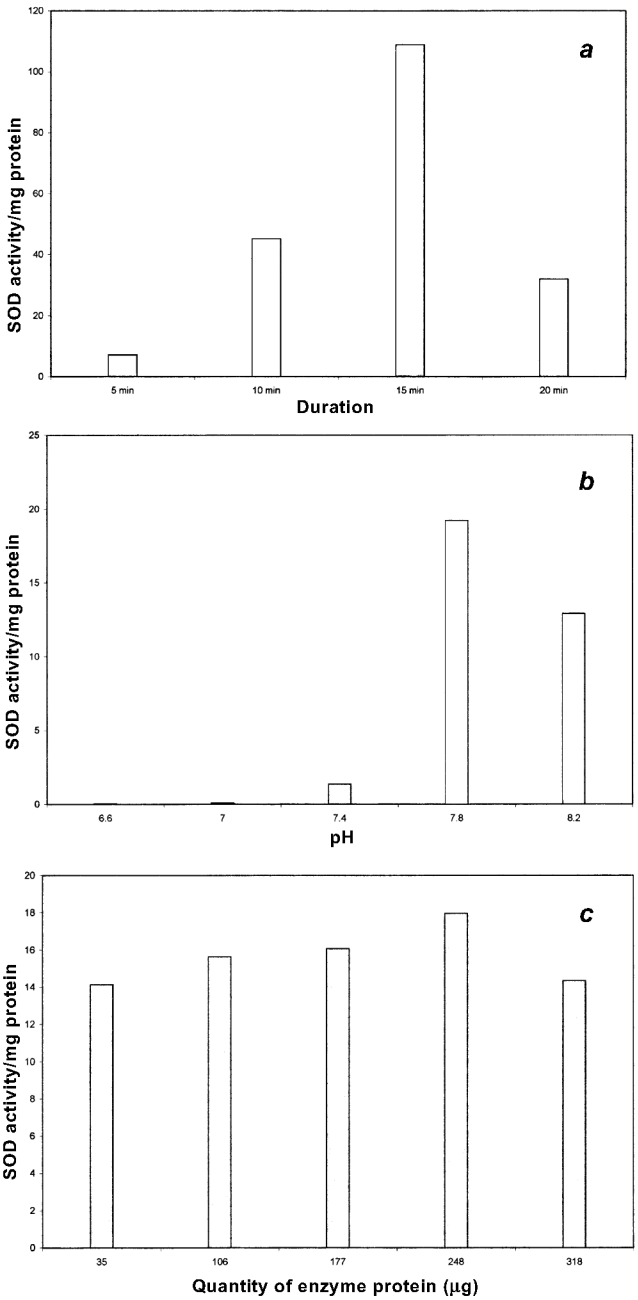

Figure 2. Optimization of (*a*) duration of exposure to light, (*b*) pH of the assay buffer system and (*c*) quantity of enzyme protein required for assay of SOD activity in the insect system.

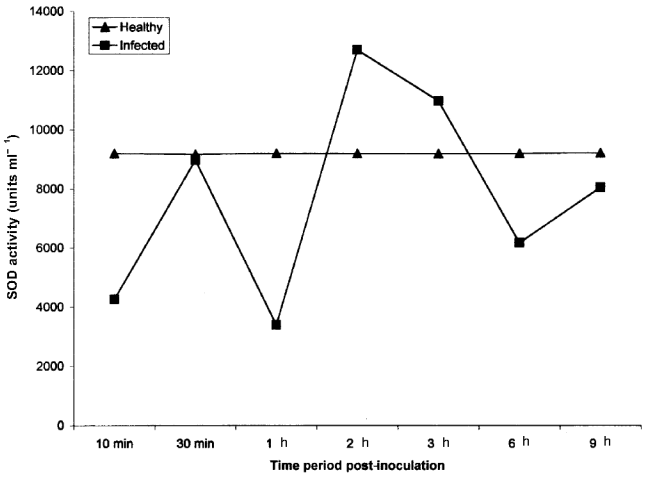

Figure 3. SOD activity in haemocytes of *B. mori* infected with *B. subtilis* (mean of $n = 3$).

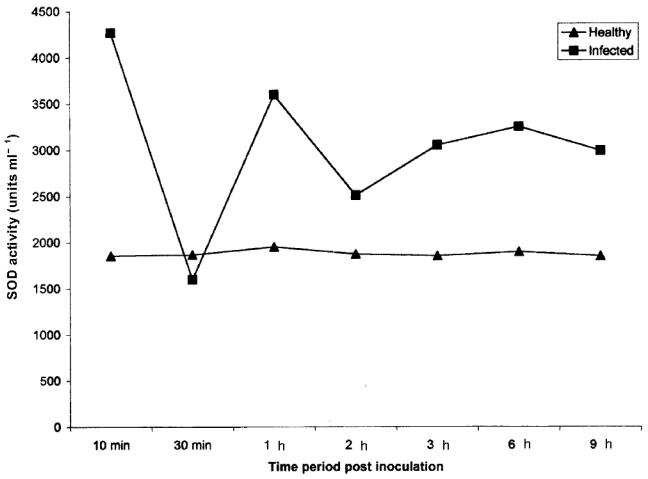

Figure 4. SOD activity in the cell-free plasma of *B. mori* infected with *B. subtilis* (mean of $n = 3$).

with (lysosomal) enzymes; the microbicidal effect of H_2O_2, for instance, is greatly enhanced by its participation in the myeloperoxidase–H_2O_2–halide system. Since the haemocytes are involved in the defensive process, it is expected that in the 'physiological race' between the host cells and the pathogen, a process of suppression and resurgence of enzyme activity may occur depending on the respiratory burst that takes place upon infection. The haemocytes of the pond snail *Lymnaea stagnalis* have also been shown to generate reactive forms of oxygen upon stimulation[18].

In the cell-free plasma on the other hand, there was a reverse picture (Figure 4) with high activity in infected plasma (4270 units ml^{-1}) which, though was the same as that in the haemocytes, was far more than the normal values found in healthy plasma at 10 min pi (1855 units ml^{-1}, $P < 0.01$). Thereafter the SOD activity fell to 1599 units ml^{-1} at 30 min marginally below the normal healthy values. The activity again peaked to 3599 units ml^{-1} and then declined to 2509 units ml^{-1}, though it remained higher than the normal values in healthy plasma. Then a gradual increase in activity was noticed, which fell slightly at 9 h pi. An insect body cavity filled with haemolymph is the final place to resist pathogens that have invaded surface barriers. Antibacterial mechanisms such as lysozymes and antibacterial protein systems are present in the haemolymph. The higher activity following infection in plasma points to the presence of this enzyme in the haemolymph which readily responds to pathogen invasion, and the respiratory burst that occurs in the haemocytes. In mammals, superoxide production by leukocytes is widely known to be essential for bacterial killing[19]. Indeed, the SOD activities were grossly a biphasic response in inoculated haemocytes and cell-free haemolymph plasma, and a fluctuating pattern was tracked all along the probing period. Such type of biphasic response of SOD has also been reported from a number of plant systems stimulated by pathogen attack[20]. Surprisingly, though the endogenous level of SOD activity was 5-fold lower in the haemolymph than the haemocytes, it enhanced maximally upon pathogen attack. Superoxide production (in bursts) in the insect haemolymph supernatant and by haemocytes is quite possibly a defensive mechanism against non-self invaders. There is a possibility based on the work of Ahmad *et al.*[9], that lepidopteran haemolymphs phagocytic cells produce bursts of superoxide. The chief defensive cells are the plasmatocytes and the granular cells in *B. mori*, which take part in phagocytosis, encapsulation and nodulation reactions in response to bacterial infection[21]. Thus, the present work also substantiates this observation. The cells are the seat of generation of superoxide and perhaps they have the mechanisms to control the deleterious flux of superoxide both in haemocytes and haemolymph. However, further studies are needed to elucidate the function of superoxide anions and their controlling enzyme systems in the defensive process in insects.

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