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Changes in the kinetic properties of *Zea mays* NADP- malic enzyme in response to sulphur dioxide exposure

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SUMMARY

The effect of sulphur dioxide exposure on the activity of NADP- malic enzyme was studied in the leaf extracts of control and exposed *Zea mays* plants. Ten - days old plants of *Zea mays* were exposed to different concentrations of sulphur dioxide (0.8 to 23 ppm) for 4 hours in a continuous flow exposure chamber under illumination (500W tungsten bulb). The visible injury symptoms in leaves produced due to the exposure were correlated with sulphur dioxide concentration. A concentration dependent decrease in the activity of the enzyme was observed in relation to sulphur dioxide exposure. The inhibition of NADP- malic enzyme by sulphur dioxide was found to be non-competitive with a K_i value of 52.6 ppm sulphur dioxide, with respect to $NADP^+$. The enzyme showed a partial competitive inhibition by sulphur dioxide with respect to malate, whereas the inhibition was competitive with a K_i value of 15 ppm, with respect to Mg^{2+}. The relatively low K_i value with respect to Mg^{2+} demonstrates a sensitive factor for sulphur dioxide damage. The K_m values were 26.3, 142 and 51 µM for $NADP^+$, malate and Mg^{2+}, respectively.

Key words : Sulphur dioxide, *Zea mays*, Inhibition, Chlorophyll, NADP- malic enzyme

Among several air pollutants, sulphur dioxide has been reported as the most widespread phytotoxic air pollutant causing extensive injury to plants (Anuradha *et al.*, 1999; Masood *et al.*, 2001; Izrael *et al.*, 2002; Agarwal and Deepak, 2003; Xiong *et al.*, 2003; Muzika *et al.*, 2004; Wang *et al.*, 2005; Amin *et al.*, 2007; Dar *et al.*, 2008). Sulphur dioxide is known to inhibit photosynthetic carbon dioxide fixation (Masood *et al.*, 1999; Gimeno and Deltoro, 2000). However, the biochemical effects of sulphur dioxide at enzymatic level are less known. Ribulose bisphosphate carboxylase which affects the first step in carbon dioxide fixation in the C_3 pathway of photosynthesis was found to be affected by sulphur dioxide (Zeigler 1972; Masood 1987; Masood *et al.*, 1999). Zeigler (1972) first reported that inhibition of photosynthetic carbon dioxide fixation by sulphur dioxide was due to competition between carbon dioxide and sulphur products for active binding sites on the enzyme. At higher concentrations, this inhibition was reported to be non-competitive (Zeigler, 1972; Mukerji and Yang, 1974). In vitro, bisulphite was found to inhibit the activities of phosphoenolpyruvate carboxylase and malate dehydrogenase, two enzymes involved in the initial steps of C_4 photosynthesis (Osmond and Avadhani, 1970). Sulphite was also found to inhibit these enzymes (Mukerji and Yang, 1974) as well as malic enzyme (Zeigler, 1974) which decarboxylates malate to release carbon dioxide for refixation by ribulose bisphosphate carboxylase.

In our earlier paper (Amin *et al.*, 2007) on the effect of sulphur dioxide on PEP-carboxylase, it was shown that sulphur dioxide causes a competitive inhibition of PEP-carboxylase with respect to HCO_3^-, but acts non – competitively with respect to PEP and Mg^{2+}. In this paper, we report the kinetics of inhibition of NADP- malic enzyme from *Zea mays* leaves by sulphur dioxide.

MATERIALS AND METHODS

Malate and dithiothreitol were purchased from Sigma Chemical Company, USA. EDTA and magnesium chloride (AR) were purchased from Romali, India. NADP was purchased from Loba- Chemie, India. Acetone, agar and HEPES were purchased from Sisco Research Laboratories Pvt. Ltd., Bombay.

The experimental plants were raised from the seeds of *Zea mays* (Hybrid - G2 variety) in pots containing Hoagland Media (EPA, 1975) with 7 - 8 seeds/pot. The pots were wrapped with the black paper and kept in the growth chamber having controlled humidity, 25^0C temperature and 16-18 hours photoperiod. Six pots containing 10- days old plants (3-leaf stage) were fumigated with different concentrations of sulphur dioxide for 4 hours in a continuous flow exposure chamber under illumination (500W tungsten bulb). The doses of sulphur dioxide ranged from 0.8 to 23 ppm. The plants were exposed to lowest dose first and the concentration was

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increased in subsequent exposures till mild injury (MI) and severe injury (SI) appeared.

Preparation of homogenate:

10% leaf extract was obtained by thoroughly grinding leaf segments with pre - chilled mortar and pestle in a medium containing 50 mM Hepes-KOH, pH 7.8, 5 mM DTT and 0.2 mM EDTA and filtering the homogenate through a cheese cloth. The extract was then centrifuged at 10,000 rpm for 5 min and the clear supernatant was used for enzyme assays and chlorophyll estimation. All extraction procedures were performed at 4^0C.

Estimation of chlorophyll:

The chlorophyll was extracted in 80% acetone. 1 ml of the homogenate was mixed with 4 ml acetone, shaken well and kept for 1-2 hours at 4^0C. The mixture was centrifuged at 5000 rpm at 4^0C for 5 minutes. The absorbance of the supernatant was recorded at 649 nm and 665 nm.

The chlorophyll content was calculated according to the following equation (Strain *et al.*, 1971).

$$\text{Total Chl. (mg/ml)} = 6.45\ (A_{665}) + 17.72\ (A_{649})$$

Enzyme assay:

NADP - malic enzyme was assayed by the method described by Ashton *et al.* (1990). The enzyme activity was determined by following $NADP^+$ reduction spectrophotometrically at 340 nm.

The assay mixture (3 ml) contained enzyme (10-50 ml), 25 mM Hepes - KOH, pH 8.0, 5 mM malate, 0.5 mM $NADP^+$, 0.1 mM EDTA and 2 mM $MgCl_2$. The non-enzymatic reaction rates were corrected by running enzyme and substrate blanks simultaneously. The enzyme activity was assayed by measuring change in absorbance at 340 nm as a function of time.

The enzyme activity was expressed as the amount of enzyme that produces reduction of 1 m mole NADP / min/mg chlorophyll.

Kinetic studies:

In order to determine the V_{max} and K_m of the enzyme for $NADP^+$, malate and Mg^{2+} as substrate, the effect of substrate concentration on enzyme activity was studied at pH 8.0 at 25^0C. Keeping the amount of enzyme constant in the assay mixture, the concentration of $NADP^+$, malate and Mg^{2+} was increased from 20µM to 0.48 mM, 50 µM to 4mM and 25 µM to 1.0 mM, respectively. The blank was also run. The enzyme activity was determined by measuring the change in absorbance at 340 nm as a function of time.

Lineweaver – Burk plots of the reciprocal data were used to determine the kinetic parameters. The inhibitor constant, K_i was determined by using secondary plots.

RESULTS AND DISCUSSION

Exposure of plants to sulphur dioxide up to the concentration of 12 ppm did not produce any visible injury symptoms. Mild injury symptoms developed at 14.5 and 17.5 ppm sulphur dioxide concentrations. Increase in sulphur dioxide concentration to 23.0 ppm caused severe damage to plants. Sulphur dioxide at concentrations of 14.5 ppm, 17.5 ppm and 23.0 ppm producing mild to severe injuries was chosen for exposing the plants.

Effect of sulphur dioxide on the activity of NADP - malic enzyme:

NADP - malic enzyme in control and treated plants was assayed by following NADPH formation at 340 nm. The enzyme activity was determined by measuring increase in absorbance at 30 second interval up to five minutes. The enzyme gave a linear progress curve. The net change in absorbance per minute was used to calculate the enzyme activity. The results are presented in Table 1.

Table 1 : Effect of sulphur dioxide exposure on the activity of *Zea mays* NADP-malic enzyme

SO_2 concentration (ppm)	NADP-malic activity (µmole min^{-1} mg^{-1}chl)
0.0	9.63 ± 0.11
14.5	8.41 ± 0.15 (-12.66)
17.5	7.85 ± 0.09 (-18.48)
23.0	6.88 ± 0.12 (-28.55)

The enzyme activity is expressed as the amount of enzyme that produces reduction of 1 µmole of NADP+ per minute per milligram of chlorophyll

The data presented are the average of about 45 plants ± S.D

Values in brackets represent % decrease compared to control.

Inhibition of NADP – malic enzyme by sulphur dioxide with respect to $NADP^+$:

The effect of sulphur dioxide on the activity of NADP – malic enzyme at varying concentrations of $NADP^+$ is represented in Fig.1a. The data were analyzed according to Lineweaver - Burk (1934) plot (Fig. 1b). These plots clearly indicate that, with respect to $NADP^+$, K_m value of the enzyme remained constant. However, it decreased the V_{max} with increasing concentrations of sulphur dioxide. Thus, they show a noncompetitive type of inhibition against $NADP^+$. K_m for $NADP^+$ was found to be 26.3 µM. The K_i value calculated from the secondary

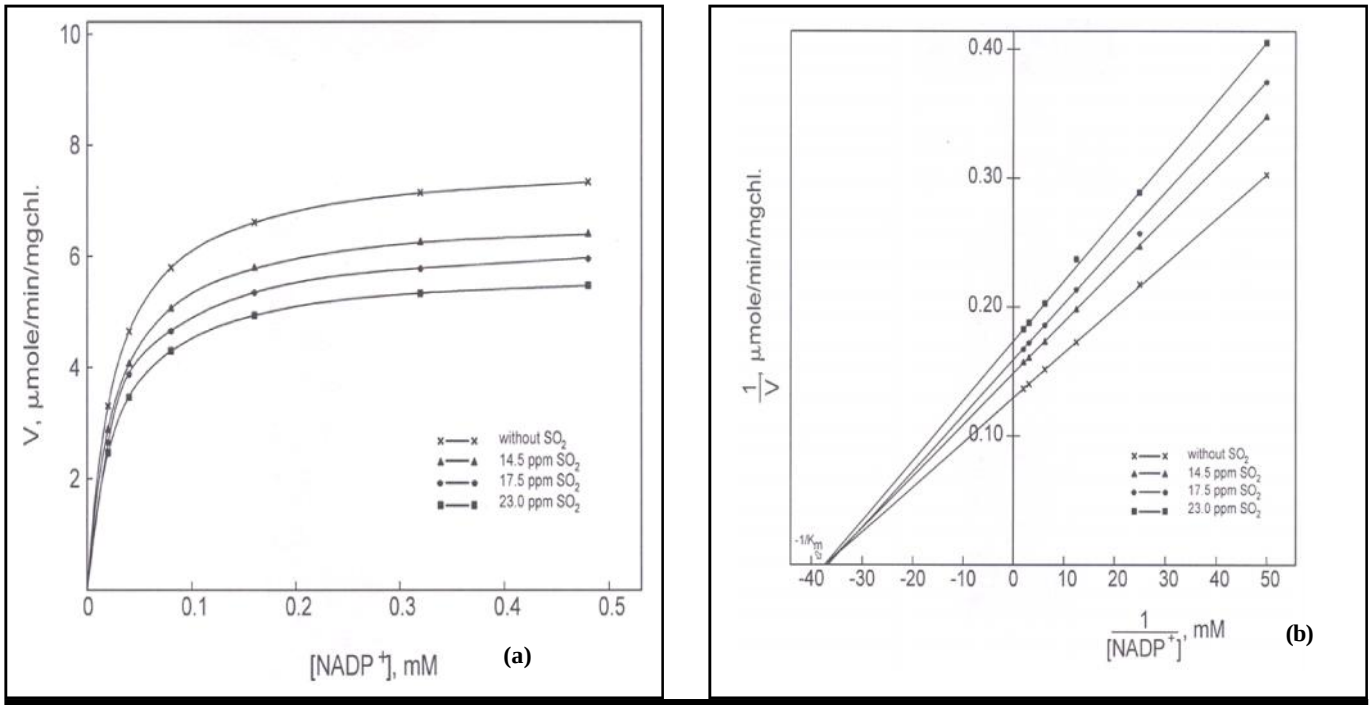

Fig. 1 : (a) Inhibition of NADP - malic enzyme by sulphur dioxide with respect to $NADP^+$ (b) Lineweaver Burk plot of Fig. a

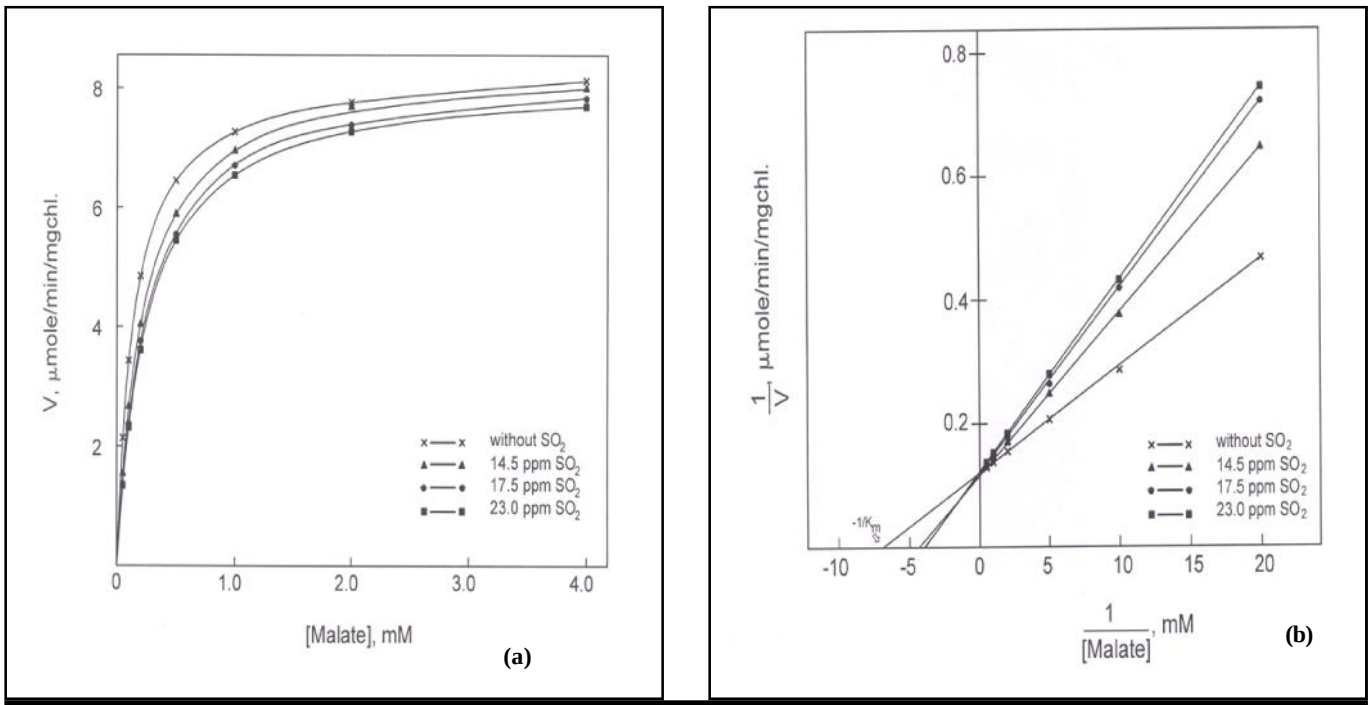

Fig. 2 : (a) Inhibition of NADP - malic enzyme by sulphur dioxide with respect to malate (b) Lineweaver Burk plot of Fig. a

plot was 52.6 ppm sulphur dioxide.

Inhibition of NADP – malic enzyme by sulphur dioxide with respect to malate:

The data for the initial velocity against the malate concentration are plotted graphically in Fig.2a. The Lineweaver - Burk (1934) plots represented in Fig. 2b indicate that, with respect to malate, a partially competitive type of inhibition was shown which did not rise with the increase of sulphur dioxide concentration from 17.5 to 23.0 ppm. The K_m for malate was found to be 142 µM.

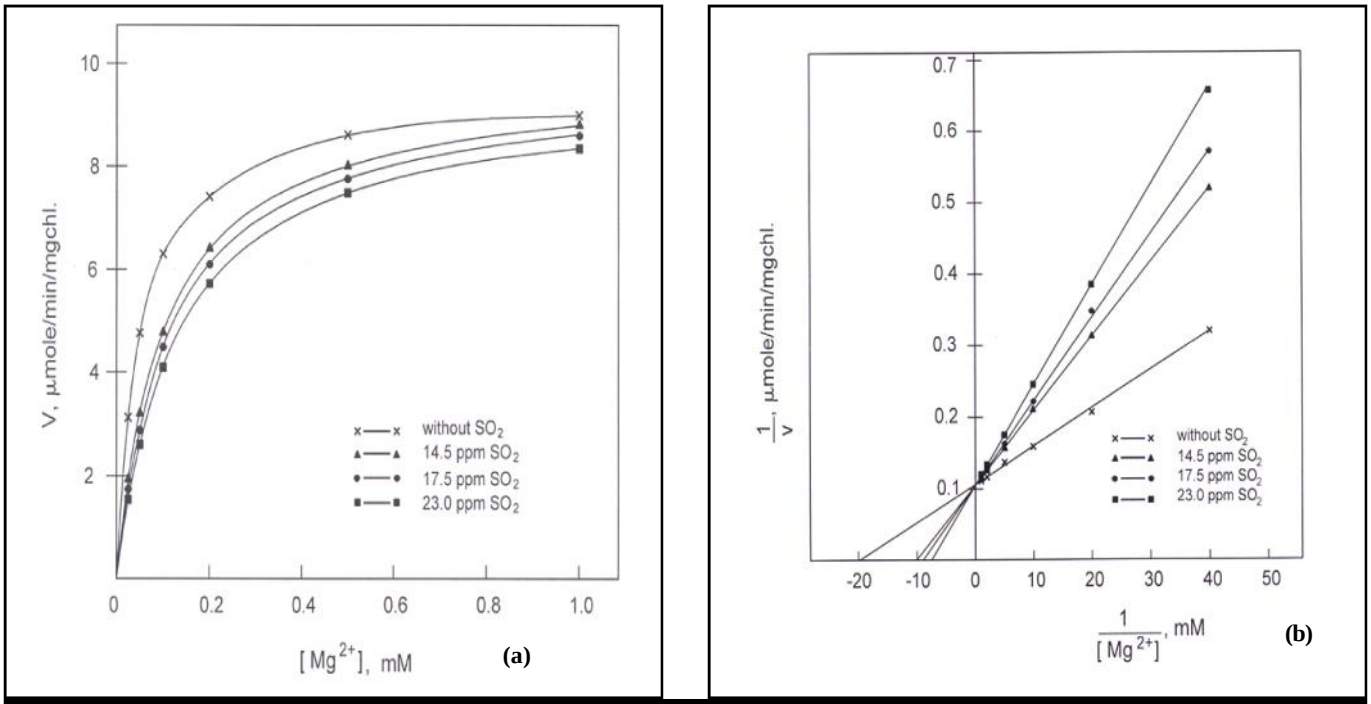

Fig. 3 : (a) Inhibition of NADP - malic enzyme by sulphur dioxide with respect to Mg^{2+} (b) Lineweaver Burk plot of Fig. a

Inhibition of NADP – malic enzyme by sulphur dioxide with respect to Mg^{2+} :

The plot between Mg^{2+} concentration and enzyme activity in control and exposed plants is shown in Fig.3a. The data was analyzed according to Lineweaver and Burk (1934) plot (Fig. 3b). These curves clearly indicate that, with respect to Mg^{2+}, increased concentration of sulphur dioxide cause increased K_m, whereas V_{max} remained constant. Hence, NADP-malic enzyme inhibited competitively by sulphur dioxide with respect to Mg^{2+}. The K_i value calculated from the secondary plot was 15.0 ppm sulphur dioxide, indicating a very sensitive point of attack. The K_m for Mg^{2+} was found to be 51 µM (Table 2).

Sulphur dioxide is known to inhibit photosynthetic carbon dioxide fixation (Pucket, 1973; Masood *et al.*, 1999; Gimeno and Deltoro, 2000; Amin *et al.*, 2007). The competitive inhibition of RUBP - carboxylase by SO_3^{2-} with respect to HCO_3^- (Zeigler, 1972) has been considered the key mechanism by which solution products of sulphur dioxide directly interfere with carbon dioxide fixation.

Table 2 : Effect of sulphur dioxide on the kinetic constants of *Zea mays* NADP-malic enzyme

Substrate	K_m, µM	K_i, ppm	Type of inhibition
$NADP^+$(20µM-0.48mM)	26.3	52.6	Non-competitive
Malate (50µM-4mM)	142	–	Partial competitive
Mg^{2+} (25µM- 1mM)	51	15.0	Competitive

Sulphur dioxide has also been found to inhibit the photosynthetic carbon dioxide fixation by competitively inhibiting the activity of PEP-carboxylase with respect to HCO_3^-. However, the inhibition is non-competitive with PEP and Mg^{2+} as substrate (Amin *et al.*, 2007).

The *Zea mays* plants showed a concentration dependent decrease the activity of NADP-malic enzyme upon exposure to sulphur dioxide. The inhibition of the enzyme by sulphite has earlier been reported by Zeigler (1974) who found that sulphite inhibits malate decarboxylation in a partially competitive way with respect to $NADP^+$ whereas the inhibition of pyruvate carboxylation with respect to NADPH is of mixed type. However, in the present study, the activity of NADP-malic enzyme was inhibited non-competitively with respect to $NADP^+$ whereas the sulphur dioxide inhibition with respect to Mg^{2+} was competitive. With respect to malate, the enzyme showed a partial competitive inhibition by sulphur dioxide. The increase in sulphur dioxide concentration beyond 17.5ppm had no effect on the K_m value of the enzyme for malate. Towards $NADP^+$, the low sensitivity is indicated by the K_i value which was found to be 52.6ppm sulphur dioxide. However, the low K_i of 15.0 ppm sulphur dioxide with respect to Mg^{2+} demonstrates a sensitive factor for sulphur dioxide damage. An optimal supply with Mg^{2+} can be used as a preventive factor.

Zeigler (1974) reported that during malate

decarboxylation, there occurs a partially competitive inhibition by CO_2 – saturated bicarbonate with respect to malate. As mentioned above, the same is true for sulphur dioxide. This indicates that both the inhibitors bind to the same site of the enzyme apart from the substrate binding site. Thus HCO_3^- and SO_2 are competing for the same site not only when HCO_3^- takes part in the reaction as found in PEP-carboxylase (Amin *et al.*, 2007), but also when it reduces the affinity of the enzyme to malate. Consequently, the competition of SO_2 for the HCO_3^- binding site may represent the first point of attack in C_4 photosynthesis.

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