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

S. AMIN, M. FAROOQ, R.K. HANS AND A. MASOOD

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°C 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
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°C.

**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°C. The mixture was centrifuged at 5000 rpm at 4°C 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 MgCl₂. 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 µmole NADP+ / min/mg chlorophyll.

**Kinetic studies:**

In order to determine the $V_{\text{max}}$ and $K_m$ of the enzyme for NADP+, malate and Mg²⁺ as substrate, the effect of substrate concentration on enzyme activity was studied at pH 8.0 at 25°C. Keeping the amount of enzyme constant in the assay mixture, the concentration of NADP+, malate and Mg²⁺ 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>
<thead>
<tr>
<th>SO₂ concentration (ppm)</th>
<th>NADP-malic activity (µmole min⁻¹·mg⁻¹·chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>9.63 ± 0.11</td>
</tr>
<tr>
<td>14.5</td>
<td>8.41 ± 0.15 (-12.66)</td>
</tr>
<tr>
<td>17.5</td>
<td>7.85 ± 0.09 (-18.48)</td>
</tr>
<tr>
<td>23.0</td>
<td>6.88 ± 0.12 (-28.55)</td>
</tr>
</tbody>
</table>

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 $V_{\text{max}}$ with increasing concentrations of sulphur dioxide. Thus, they show a noncompetitive type of inhibition against NADP⁺. $K_i$ 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.
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 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 17.5 ppm 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 52.6 ppm 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

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


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REFERENCES