Superoxide Dismutase and Sulfite Oxidation

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DDTC, SOD, Sulfite Oxidation

50 U SOD in 1 ml or 10 mM DDTC do not much change sulfite oxidation due to the free radicals procuding system xanthin–xanthin oxidase. Sulfite oxidation due to the activity of this free radicals producing system proceeds probably as the univalent oxidation of sulfite. The role of SOD in SO_2 resistance mechanism in plants and the role of DDTC as SO_2 oxidation effect stimulator is discussed.

Introduction

Numerous studies on the physiological and biochemical effect of SO_2 on plants have been made in the recent years. Sulfur in form of SO_3^{2-} is toxic to plants, in contrast to SO_4^{2-}. There exist several systems in plant leaves that can mediate the SO_3^{2-} oxidation and therefore rouse detoxification [1]. Oxidation of SO_3^{2-} can be initiated by any system that induces univalent reduction of oxygen or univalent oxidation of SO_3^{2-}. A superoxide anion can be the initiator of the oxidation of SO_3^{2-}.

It is known that SO_2 is less toxic in light than in the dark for plants having similar light and dark uptake pattern [2]. The decreased injury in the light is apparently caused by light induced detoxification of SO_2 reactants. The predominant site for SO_2 oxidation are green plastids. Superoxide anion formed on the reduction side of the electron transport system in chloroplasts is the initiator of the aerobic oxidation. The SO_2 oxidation is much stronger in green protoplasts than in protoplasts isolated from etiolated plants [3]. This reaction is inhibited by DCMU [4, 5].

The oxidation of sulfite proceeds as a chain reaction. The photooxidation of SO_2 is powerfully inhibited by SOD, an enzyme that decomposes free oxygen radicals. Probably sulfite oxidation in light is initiated through the above mentioned univalent reduction of oxygen, although these observations do not provide direct evidence whether superoxide radicals are the initiators or only active intermediates propagating the chain reaction [5]. Quite contradictory to this facts [6] found that high SOD activity is correlated with resistance against SO_2 and an inhibition of SOD decreased the SO_2 resistance. In addition, longer-lasting SO_2 application increases SOD activity, possibly in response to increased free radical production. Similar results were obtained by [7]. The role of SOD in SO_2 resistance does not seem to be quite clear. In studies on SO_2 effects on plant metabolism DDTC was used as SO_2 injury stimulator [8]. According to [6] and [9] DDTC is an inhibitor of SOD. But [8] observed the same or less injury in tomato plants treated with DDTC compared to plants not treated with DDTC and both exposed to SO_2.

The aim of a present work was to study the role of SOD in the resistance mechanism of plants to SO_2 toxicity and the influence of DDTC on this process.

Methods

Free radicals producing system (X–Ox)

Free radicals generated by the catalytic action of xanthine oxidase operating on its substrates were used to initiate oxidation of sulfite. The reaction was performed as described by [10] at 25 °C in a medium containing 0.2 M phosphate buffer, pH 7.8, xanthine oxidase 0.05 U ml^{-1}, and 0.375 mM xanthine. The reaction was started with xanthine.

Abbreviations: DDTC, diethyldithiocarbamate; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); SOD, superoxide dismutase (EC 1.15.1.1); tricine, (N-trishydroxymethyl)-methylglycine; X-Ox, xanthine and xanthine oxidase.

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SOD activity and $O_2^{\cdot -}$ level measurements

For measuring the SOD activity according to [10] in the presence of different effectors 1 U of enzyme into 2 ml buffer solution was added before the reaction was started with xanthine. The activity was tested in the presence of 2 mM sulfite or 10 mM KCN or DDTC in the range from 0.0 to 10 mM or without these effectors. Reaction was stopped after 30 min with addition of sulfanylamide to the concentration 30 mM and HCl to the concentration of about 5%. The level of $O_2^{\cdot -}$ which was not dismuted with SOD was determined with radical-induced oxidation of 0.5 mM hydroxylamine to nitrate. Hydroxylamine was added before reaction was started with xanthine. For nitrate determination naphthylethylendiamine to the concentration of 0.25 mM was added and extinction was measured at 540 nm. The SOD activity was determined from a standard curve. The same method was used to estimate the level of $O_2^{\cdot -}$ in experiments without addition of SOD.

Sulfite determination

Aerobic oxidation of sulfite with X–Ox system was performed as described above. The sulfite concentration was measured using a colour reaction with fuchsin as described by [11] with the following modifications. To 1 ml free radicals producing system in 0.1 M tricine buffer pH 7.8 with 0.375 mM xanthine and 0.05 U ml^{-1} xanthine oxidase with 1.0 mM sulfite, SOD to the concentration 50 U ml^{-1} or DDTC to the concentration 10 mM was added. As the control a sample without xanthine oxidase was used. After 1–22 min 50 µl of this reaction mixture were added to 1 ml of mercuric chloride solution saturated at 25 °C 20 µl of formaldehyde and 100 µl of saturated fuchsin in conc. HCl. Extinction at 550 nm was measured after 5 min and sulfite concentration was determined by comparison with a standard curve. A separate curve was plotted for experiments with DDTC.

For other experiments DTNB according to [12] was used for the determination of the sulfite concentration. For aerobic sulfite oxidation the X–Ox system in 0.2 M phosphate buffer pH 7.8 was used as described above. Initial sulfite concentration was 0.2 mM and its concentration was determined 10, 20 or 30 min after the addition of 1 ml reaction medium to 20 µl 100 mM DTNB suspension. Directly after that the absorption at 412 nm was measured and sulfite concentration determined by comparing with the standard curve. In one experiment the reaction was started with SOD addition to the concentration 7 U ml^{-1} without the free radicals producing system. In other experiments hydroxylamine to the concentration 0.375 mM or SOD to the concentration 1 to 50 U ml^{-1} were added before reaction was started with xanthine. In control experiments sulfite oxidation without xanthine was performed. In the case without xanthine the reaction was started with xanthine oxidase. In one experiment in which sulfite oxidation without X–Ox system in the presence of SOD was measured, reaction was started with sulfite addition.

Xanthine and xanthine oxidase were purchased from Boehringer, all other chemicals from Sigma.

Results

The data presented in Fig. 1 show that with increasing DDTC concentration in the range from 0.1 to 10 mM in the presence of 1 U of SOD in the reaction medium the dismutation level of $O_2^{\cdot -}$ expressed as SOD activity rose.

As shown in Fig. 2 the dismutation activity due to SOD can be strongly inhibited in the presence of KCN. The dismutation activity in this case is similar to the one in the free radicals producing system without SOD with or without KCN. In the presence of 10 mM DDTC the dismutation activity rose about 5 times. The addition of DDTC to SOD inhibited with KCN shows the dismutation activity at the level 3.5 times more than 1 U SOD alone. 10 mM DDTC allows dismutation activity at the level of about 4.5 U of SOD. The dismutation activity of SOD can be increased after addition of

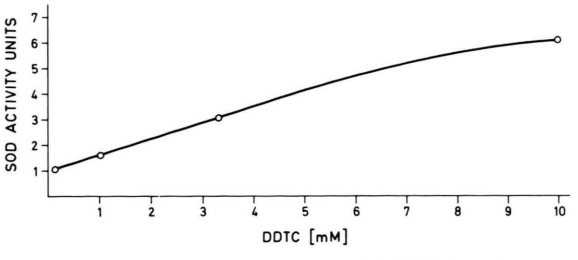

Fig. 1. $O_2^{\cdot -}$ dismutation activity of 1 U SOD in the presence of DDTC in a concentration range 0.0–10.0 mM.

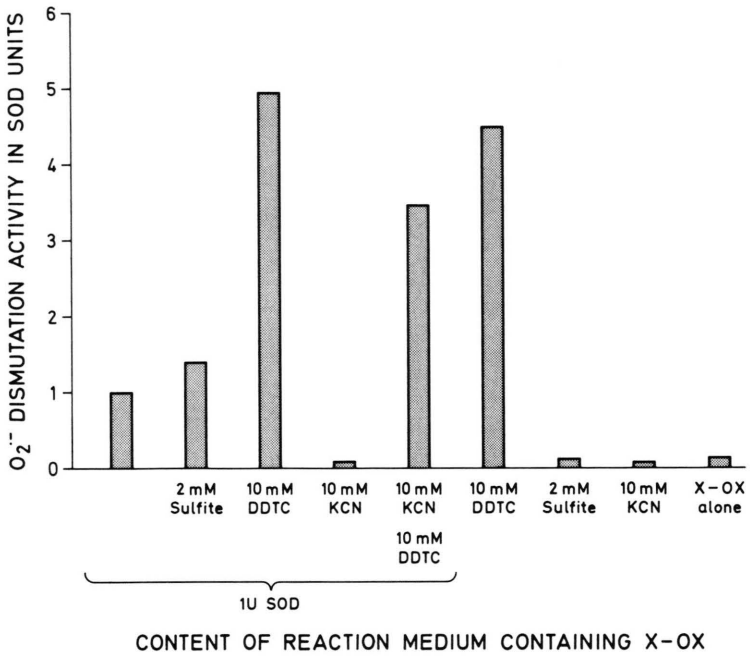

Fig. 2. $O_2^{\cdot-}$ dismutation activity (in SOD units) in 2 ml 0.2 M phosphate buffer pH 7.8 in the presence of: SOD, sulfite, DDTC or KCN.

2 mM sulfite to 1 U of SOD. 2 mM sulfite alone do not lower $O_2^{\cdot-}$ level.

To examine wheather sulfite oxidation in the reaction with $O_2^{\cdot-}$ decreases in the presence of SOD and DDTC, the sulfite level was measured in the reaction medium containing the free radicals producing system. As shown in Fig. 3 SOD at high concentration of 50 U ml^{-1} or 10 mM DDTC do not change much sulfite oxidation. As a control experiment the sulfite oxidation was carried out in a medium without xanthine oxidase. The sulfite level was not much changed during 22 min of reaction. This experiment was done using parafuchsin colour reaction.

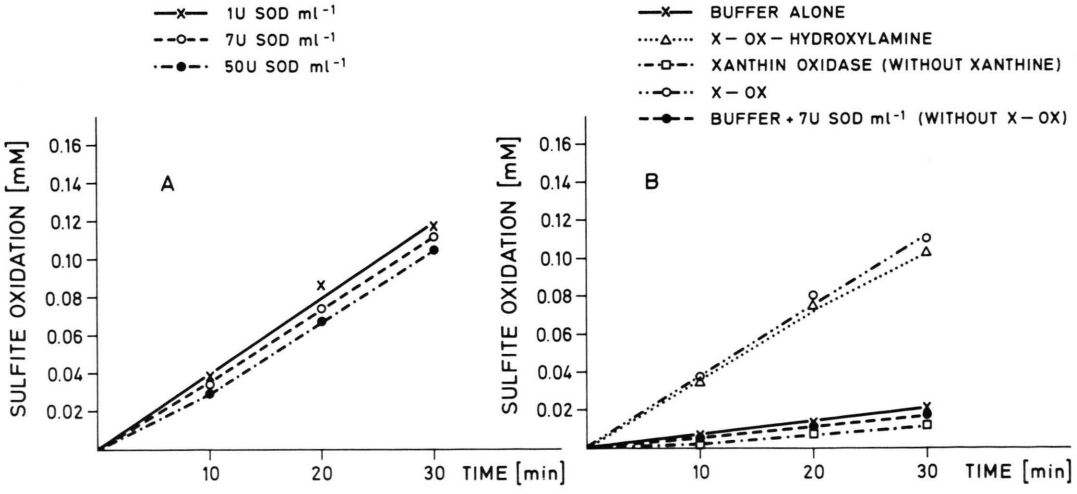

Fig. 3. Sulfite oxidation in 0.1 M tricine buffer pH 7.8 with a free radicals producing system (X–Ox) measured by fuchsin colour reaction in the presence of 10 mM DDTC or 50 U ml^{-1} SOD. Initial sulfite concentration was 1 mM. Control sample was without xanthine oxidase.

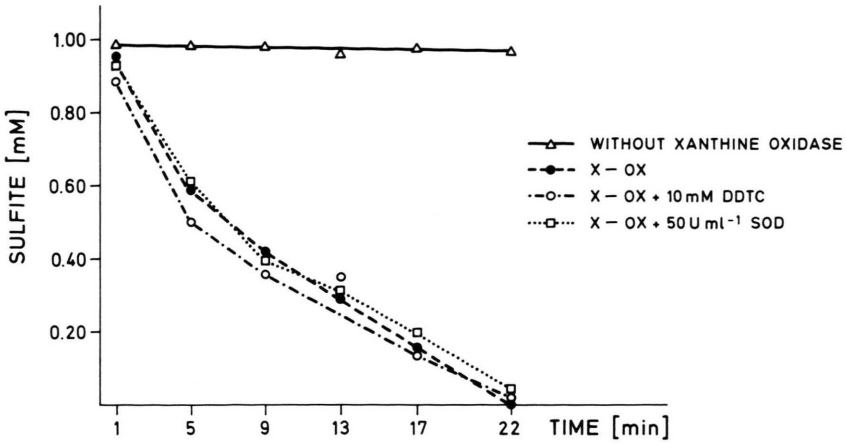

Fig. 4. Sulfite oxidation in 0.2 M phosphate buffer pH 7.8 with free radicals producing system (X–Ox) measured by DTNB in the presence of SOD concentration in the range 1–50 U ml^{-1} or in the presence of 0.5 mM hydroxylamine. As control samples sulfite oxidation in the presence of xanthine oxidase or 7 U SOD in 1 ml or in buffer alone were measured. Initial sulfite concentration was 0.12 mM.

Similar experiments in the presence of different concentrations of SOD were carried out using the reaction with DTNB. In Fig. 4 it is shown that 1–50 U ml^{-1} of SOD does not change strongly sulfite oxidation. The addition of 0.5 mM hydroxylamine also does not change sulfite oxidation. The presence of SOD without the free radicals producing system shows very low sulfite oxidation, similar to that with not complete free radicals producing system without xanthine. The sulfite oxidation in such a reaction medium was similar to that in buffer alone.

Discussion

As it was shown in Fig. 1 increasing concentrations of DDTC in the range from 0.1 to 10 mM rise the dismutation activity of $O_2^{\cdot -}$. There are some possibilities to explain these results: DDTC can affect the free radicals producing system, or can act as radical scavenger. These results do not support previous data from the literature pointing to a decrease in the activity of SOD due to DDTC action in plants [6], but according to [13] DDTC inhibits only Cu-SOD. It was assumed that DDTC decreases SOD activity because of its well-known copper chelating action. Our experiments show (Fig. 1) that if, as reported in the literature, inhibition of SOD takes place in our experiment, it must at the same time have a strong scavenger effect. According to [6] DDTC should increase the inhibiting effect of SO_2 because of SOD inhibition. But [8] in the experiments with tomato plants have not obtained such results. We suppose that if DDTC acts as radical scavenger it can inhibit the synthesis of SOD because of the substrate ($O_2^{\cdot -}$) lowering effect. Due to this DDTC action SO_2 cannot be sufficiently oxidized.

The results shown in Fig. 2 could indeed show that DDTC can act as a radical scavenger. The $O_2^{\cdot -}$ dismutation is higher in the presence of 1 U SOD and 10 mM DDTC together in the reaction medium than in the presence of SOD or DDTC alone. As expected, 2 mM sulfite can lower the $O_2^{\cdot -}$ level. Addition of 10 mM KCN stops the dismutation activity of SOD and does not change a lot the dismutation due to simultaneously added DDTC. These results are difficult to comment because as reported by [14], xanthine oxidase can be inhibited with respect to the oxidation of purines by treatment with cyanide. According to these results one could expect that the dismutation of $O_2^{\cdot -}$ due to DDTC goes parallel to that due to SOD. The data presented in Fig. 3 show that high rate of sulfite oxidation in the medium is possible, when X–Ox system is active. Processes which generate reactive radicals initiate chains and thus catalyze the oxidation of sulfite.

The aerobic oxidation of sulfite proceeds by a free radical chain reaction which may be initiated by the process initiating either the univalent oxidation of sulfite or the univalent reduction of oxygen. According to the experiments shown in Fig. 3 it can be assumed that both SOD at the concentration to 50 U in 1 ml and 10 mM DDTC do not change sulfite oxidation. As we suggested above, DDTC can decrease the $O_2^{\cdot -}$ level, so the effect can be comparable with SOD. Because of un-

changed sulfite oxidation in the presence of DDTC this experiment allows to suppose that 10 mM DDTC does not change the function of our X–Ox system as we have previously supposed. In this way we support the idea that DDTC can act in our experiments as a radical scavenger. But in a medium-containing X–Ox system sulfite can be probably oxidized on some other way, maybe by a direct univalent oxidation or reaction with H_2O_2 and not due to the reaction with $O_2^{\cdot -}$. Similar experiments as presented above were conducted using the DTNB colour reaction for measuring sulfite concentration. These experiments were done with lower initial sulfite concentration (0.12 mM). The addition of SOD shows no significant effect on sulfite oxidation in the range 1–50 U ml^{-1} reaction medium (Fig. 4a). As we know, SOD should be effective when oxidation of sulfite is performed due to the reaction with $O_2^{\cdot -}$. Despite this we suppose sulfite oxidation in this experiment is not due to the reaction with $O_2^{\cdot -}$. The nature of the sulfite-O_2 chain reaction was described by [15]:

$$X + SO_3^{--} \rightarrow X^- + SO_3^{\cdot -} \quad (1)$$

$$SO_3^{\cdot -} + O_2 \rightarrow SO_3 + O_2^{\cdot -} \quad (2)$$

$$O_2^{\cdot -} + H^+ \rightarrow HO_2^{\cdot} \quad (3)$$

$$HO_2^{\cdot} + SO_3^{--} \rightarrow HO_2^- + SO_3^{\cdot -} \text{ (etc.)}. \quad (4)$$

We suggest that sulfite can be detoxified in reaction medium with $O_2^{\cdot -}$ started from the reaction (3) (*e.g.* in green plastids in light – SOD-sensible) or by $SO_3^{\cdot -}$ started from the reaction (1) (*e.g.* xanthine–xanthine oxidase system or with H_2O_2 – SOD-insensible).

The presence of hydroxylamine does not change sulfite oxidation to a significant measure. This makes us sure that in previous experiments (Fig. 1, Fig. 2) sulfite oxidation cannot be changed to a greater degree in the presence of hydroxylamine reaction with $O_2^{\cdot -}$.

As shown in Fig. 4b sulfite cannot be oxidized in the presence of SOD alone. There are no data in literature that SOD can directly reduce the level of SO_2. But as mentioned, there is evidence that SOD activity correlates with SO_2 resistance. Many authors supposed that each system that induces univalent reduction of oxygen can detoxify SO_2. We suppose that the higher are the natural (native) possibilities of the plant tissue of producing $O_2^{\cdot -}$ the higher SOD level is needed. We suggest that in this way the SOD level correlates with SO_2 resistance. The induction of high SOD activity after long-term fumigation with SO_2 reported by [6], [7] and [16] might be a secondary effect, maybe due to the production of $O_2^{\cdot -}$ in reaction with $SO_3^{\cdot -}$.

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[1] R. MacLeod, W. Farkas, I. Fridovich, and P. Handler, J. Biol. Chem. **236,** 1841 (1961).
[2] D. M. Olszyk and D. T. Tingey, Plant Physiol. **74,** 999 (1984).
[3] Z. Miszalski and H. Ziegler, in preparation.
[4] W. Libera, H. Ziegler, and I. Ziegler, Planta **109,** 269 (1973).
[5] K. Asada and K. Kiso, Eur. J. Biochem. **33,** 253 (1973).
[6] K. Tanaka and K. Sugahara, Plant Cell Physiol. **21,** 601 (1980).
[7] H. J. Jäger, H. J. Weigel, and L. Grünhage, Europ. J. Forest Pathol. **16,** 98 (1986).
[8] D. M. Olszyk and D. T. Tingey, Plant Physiol. **77,** 935 (1985).
[9] K. Asada, M. Takanashi, and M. Nagate, Agr. Biol. Chem. **38,** 471 (1974).
[10] E. F. Elstner and A. Heupel, Anal. Biochem. **70,** 616 (1976).
[11] W. M. Grant, Anal. Chem. **19,** 345 (1947).
[12] R. E. Humphrey, W. Melbourne, and W. Hinze, Anal. Chem. **42,** 698 (1970).
[13] M. Scarpa, P. Viglino, F. Momo, F. Bracco, L. Battistin, and A. Rigo, J. Biochem. Biophys. Meth. **22,** 135 (1991).
[14] I. Fridovich and P. Handler, J. Biol. Chem. **231,** 899 (1958).
[15] I. Fridovich and P. Handler, J. Biol. Chem. **235,** 1835 (1960).
[16] H. Schulz, Biochem. Physiol. Pflanzen **181,** 241 (1986).