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EVIDENCE FOR SULPHITE OXIDASE ACTIVITY IN SPINACH LEAVES

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; chloroplast; hepatic sulphite oxidase; pH dependency; radical scavengers; temperature dependency; *Thiobacilli* sulphite oxidase; thylakoid.

Abstract—The present paper provides evidence that spinach chloroplasts possess a sulphite oxidase activity coupled with oxygen consumption and reduction of ferricyanide. This activity is associated with thylakoids and solubilized by non-ionic biological detergents. The pH and temperature dependencies of sulphite oxidase activity solubilized by Triton X-100 from spinach thylakoids were consistent with those of an intrinsic membrane protein. This isolated activity was insensitive towards radical scavengers (mannitol, mannose and fructose) and catalase, and was inhibited only with very high concentrations of superoxide dismutase. Thus, observed sulphite oxidation was not induced through the photosynthetic electron transport system, but achieved via a thylakoid membrane enzymic system showing a sulphite oxidase activity. Kinetic parameters of thylakoid sulphite oxidase were measured and compared with those of other sulphite oxidases.

INTRODUCTION

Sulphite metabolism by higher plants has been widely studied, particularly in the case of SO_2 fumigation. Early data [1] indicated that high concentrations of SO_4^{2-} were present in SO_2-treated alfalfa and sugar beet. This fact was supported by $^{35}SO_2$ experiments with spinach [2], soybean [3, 4] and bean [5]. It has been found [6, 7] that SO_2 was rapidly metabolized into SO_4^{2-} in the light and in the dark and it has been established [3, 4] that light was effective in SO_2 incorporation through stomatal aperture and on SO_2 oxidation to SO_4^{2-}. The site and nature of this oxidation are still obscure and, up to now, there is no evidence that a specific sulphite oxidase might be involved [8]. However, there are indications that chloroplasts could perform sulphite oxidation: SO_4^{2-} accumulation is reported in chloroplasts [5, 9]. The inner envelope membrane is permeable to sulphite via the phosphate translocator [6, 10–13] and a sulphite binding site is present in the thylakoids [14, 15]. Dittrich *et al.* [16] have suggested that chloroplasts could be the site of both detoxification and toxic effects of SO_2. Asada and Kiso [17] have shown that spinach chloroplasts induced light-dependent aerobic oxidation of sulphite via the electron transport chain, but recent results [18–21] have shown that SO_3^{2-} oxidation was effected by intact chloroplasts from wheat and spinach in the light and in the dark, and should not, therefore, be ascribed only to this non-enzymic aerobic oxidation. On the contrary, these results suggested that an enzymic system in chloroplasts and containing sulphite oxidase activity could be involved in sulphite metabolism. The goal of this paper is to present results describing the presence of sulphite oxidase activity in spinach leaves.

RESULTS

Distribution of sulphite oxidase

To determine the presence of sulphite oxidase activity in spinach leaves, tissues were homogenized with hypotonic buffer (0.05 M phosphate, pH 7.8), and filtered crude extract, supernatant and resuspended pellet after centrifugation were assayed for sulphite oxidase activity. Intact chloroplasts and purified thylakoids were prepared from foliar tissues homogenized with isotonic buffer (0.05 M phosphate, 0.4 M sorbitol, pH 7.8). Sulphite oxidase was assayed as reported in Experimental, following either the reduction of ferricyanide or the associated oxygen uptake. As reported elsewhere [22], it was observed that ferricyanide was 10 times more effective than oxygen as an electron acceptor.

It was found that crude extracts of spinach leaves contained an enzymic system which catalyses the reduction of ferricyanide by sulphite at the rate of 75 nmol min^{-1} mg^{-1} chlorophyll. Activity largely sedimented in the 27 000 *g* pellet, indicating that sulphite oxidase was bound to membranes (Table 1). The activity was associated with the fractions containing intact chloroplasts on Percoll gradient (results not shown).

When chloroplasts were isolated, purified and broken, sulphite oxidase activity was found in the pellet fraction (Table 1), confirming that the enzyme was associated with membranes, and more precisely with chloroplast membranes. The precise localization in the thylakoids was determined by isolating and purifying this fraction.

The possibility that the enzyme is membrane associated was confirmed by experiments directed towards the solubilization of sulphite oxidase. The extraction of

Table 1. Distribution of sulphite oxidase activity in spinach leaves

Tissue	Fraction	Activity (% of total ± SD)
Leaf extract	Supernatant	10 (± 8)
	27 000 *g* pellet	90 (± 8)
	Total	100
Broken purified chloroplast	Stroma	5 (± 2)
	Thylakoid	95 (± 2)
	Total	100

the 27 000 *g* pellet with water and with neutral buffers failed to solubilize the enzyme entirely. Only in the presence of detergents was the enzyme solubilized (Table 2). Furthermore, it appeared that non-ionic biological detergents had the best efficacy. As it was verified that sorbitol was not able to solubilize sulphite oxidase activity, 0.4 M sorbitol was preferred to sucrose in the preparation of intact chloroplasts.

All other results related here refer to sulphite oxidase activity determined in thylakoids dispersed in 1% Triton X-100. In this case, activity in thylakoids was about 10 times that in crude spinach extract in regard to chlorophyll concentration.

Kinetic, temperature and pH dependences of enzyme activity of thylakoids

The effect of varying substrate concentrations (sulphite concentration between 15 and 800 μM) on velocity was studied, following the reduction of ferricyanide in 0.1 M Tris–HCl buffer (pH 8.5) at 30°. The kinetic constants were calculated from the least-squares-fitted linear Lineweaver–Burk. The apparent K_m for sulphite was calculated to be 35 μM and the maximal velocity 1710 nmol min^{-1} mg^{-1} chlorophyll.

The effect of temperature on the oxygen consumption, coupled with sulphite oxidase activity, is presented in Fig. 1. The optimum temperature was about 50°. The Arrhenius relationship ($\ln(V_{max})$ versus $1/T$) is linear over the temperature range 25–55° (Fig. 2). The apparent energy of activation E_a was calculated to be 56 kJ mol^{-1}. The thermodynamic parameter $\Delta H^{\ddagger}$ was equal to 54 kJ mol^{-1} at 30°. To calculate $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$, we need the activity expressed in sec^{-1}. Since we do not know the enzyme concentration, we cannot calculate the activity in sec^{-1} and, therefore, $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$ in J mol^{-1} and J mol^{-1} K^{-1}, respectively [23].

Thylakoids were stored at − 80° in 50 mM phosphate buffer with or without 50% (v/v) glycerol. No more activity was detectable in glycerol samples after glycerol removal and thylakoid dispersion in Triton X-100. In the case of samples kept in the presence of phosphate buffer, there was no appreciable loss of activity upon thawing. Furthermore, spinach thylakoids stored at − 20° presented a constant sulphite oxidase activity for more than three months. On the other hand, activity was largely diminished by boiling: 30% lost for 2 min and 86% for 15 min.

Table 2. Solubilization of sulphite oxidase activity from 27 000 *g* pellet of spinach leaves extracts

Nature of extractant	Supernatant activity recovered* (% of maximal activity)
None	0
0.4 M Sorbitol	0
0.4 M Sucrose	15
5 M NaCl	0
5% Brij 25	24
1% SDS	31
5% SDS	23
5% Na deoxycholate	nd†
2.5% *n*-Octyl β-D-glucopyranoside	41
1% Triton X-100	63
2.5% Triton X-100	61
5% Triton X-100	100

*Activity was measured in the supernatant of centrifugation after stirring pellet in the presence of extractant during 2 hr.

† No spectrophotometric determination could be realized in the presence of 5% Na deoxycholate.

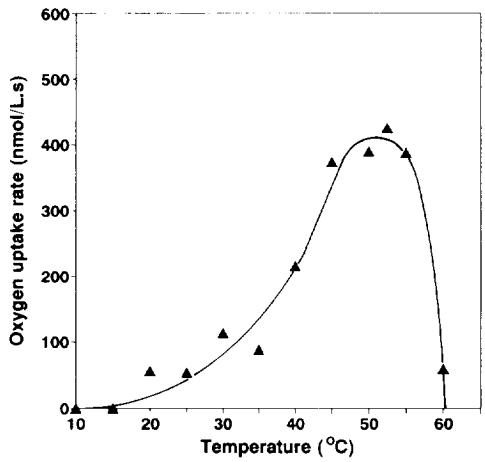

Fig. 1. Temperature dependence of oxygen consumption due to chloroplastic sulphite oxidase. The thylakoid concentration in the reaction mixture corresponded to *ca* 0.05 g chlorophyll l^{-1}. Experiments were done in triplicate (mean s.d.: 48 nmol l^{-1} s^{-1}).

The effect of pH on the sulphite oxidase activity of thylakoids was investigated using citrate–phosphate buffer for the range pH 4–6, phosphate buffer for the range pH 7–8 and Tris–HCl over pH 8 (Fig. 3). At pH 8, the same oxygen consumption rate was obtained in 0.1 M phosphate buffer and 0.1 M Tris–HCl: 142.9 and 152.4 units mg^{-1} chlorophyll, respectively. From the pH profile obtained, we have calculated the optimum pH value and the apparent p*K* values of the enzyme (defined as pH corresponding to apparent $V_{max}/2$). These are listed in Table 3.

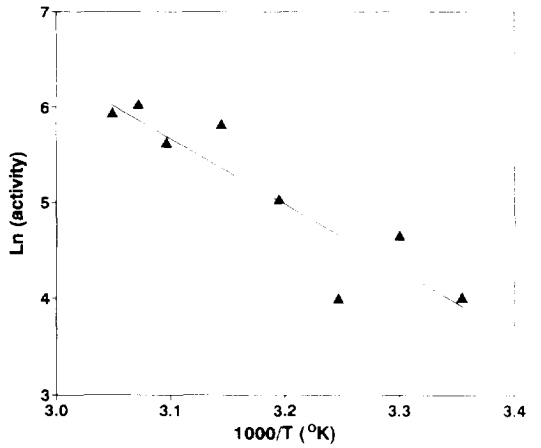

Fig. 2. Arrhenius relationship of Napierian logarithm of sulphite oxidase activity as a function of reciprocal absolute temperature. Thermodynamic parameters were calculated from the slope of this plot ($y = -6.768x + 26.64$; $r = -0.910$). Activity was expressed in units mg^{-1} chlorophyll; units as defined in Experimental.

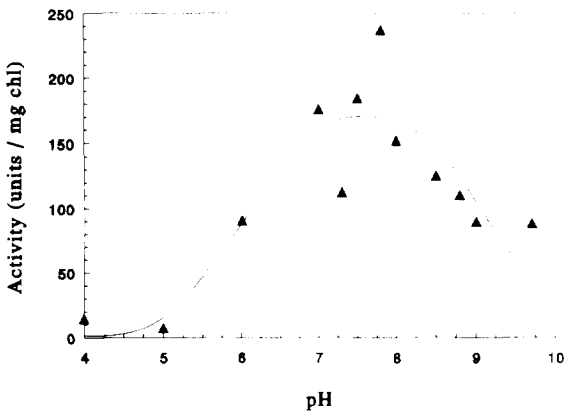

Fig. 3. pH dependence of sulphite oxidase activity. The thylakoid concentration in the reaction mixture corresponded to *ca* 0.05 g chlorophyll l^{-1}. Buffer was citrate–phosphate (0.07 M–0.09 M) below pH 7, 0.1 M phosphate from pH 7–8 and 0.1 M Tris–HCl over pH 8. Experiments were done in triplicate (mean s.d.: 49 units mg^{-1} chlorophyll).

Action of different compounds on sulphite oxidase activity

Recently, Miszalski and Ziegler [24] have suggested that, in plants, oxidation of SO_3^{2-} could be initiated (i) by superoxide anion formed on the reduction side of the electron transport system in chloroplasts, (ii) by free radicals as $OH^{\cdot}$ or univalent oxidation of SO_3^{2-} in $SO_3^{\cdot-}$, or (iii) by H_2O_2. In order to check that sulphite oxidation observed in complex reaction mixtures such as spinach thylakoids dispersed in Triton X-100 was not due to the action of $O_2^{\cdot-}$, H_2O_2 or free radicals, we have determined the activity of sulphite oxidase extracted from thylakoids in the presence of various concentrations of mannitol, fructose and mannose, which are known as

Table 3. pK and optimum pH values of sulphite oxidase activity of spinach thylakoids

$pK_{1\,obs}$	6.0
$pK_{2\,obs}$	9.2
pH_{opt}	7.6
pK_{ES1}	6.0
pK_{ES2}	9.1
V_{app} (units mg^{-1} chl.)	172
V_{theor}	180

The observed pK (pK_{obs}), optimum pH (pH_{opt}) and the optimum apparent rate (V_{app}) values were determined from experimental results given in Fig. 3. The values of theoretical pK_{ES} and V were calculated according to ref. [23] by fitting data to equation: $V_{app} = V \times [H^+] \times K_{ES1}/(K_{ES1} \times [H^+]) + [H^+]^2 + (K_{ES1} \times K_{ES2})$.

potent free radical scavengers [17], in the presence of catalase or in the presence of superoxide dismutase, which catalyses the dismutation of $O_2^{\cdot-}$ to H_2O_2 [25]. The effect of these inhibitors was analysed both on ferricyanide reduction and oxygen consumption associated with sulphite oxidase activity. In the case of radical scavengers, no inhibition of sulphite oxidase was observed at the concentrations used (Table 4). On the contrary, the addition of mannitol led to a slight activator effect. The addition of catalase to the reaction mixture was without effect (Table 5). If H_2O_2 could oxidize SO_3^{2-}, the dismutation of H_2O_2 with catalase would lead to a slowing down of sulphite oxidation. Thus, it seems that H_2O_2 was not able to oxidize SO_3^{2-} to SO_4^{2-}, and our previous observation was confirmed [18]. Superoxide dismutase inhibited sulphite oxidase activity only at high concentrations (above 65 U ml^{-1}, Table 5).

The inhibitory effect of the reaction product, sulphate, was also investigated. The concentrations needed to achieve 50% inhibition of oxygen reduction and ferricyanide reduction were 0.15 and 0.33 M sulphate, respectively.

Sulphite oxidase purification

Applying the procedures described for hepatic sulphite oxidase [22, 26] to the purification of spinach sulphite oxidase was unsuccessful. The procedure was adapted from the purification of sulphite oxidase of *Thiobacillus* [27, 28]. The thylakoids dispersed in 0.05 M phosphate buffer (pH 7.8) containing 1% of Triton X-100 were treated with crystalline $(NH_4)_2SO_4$. The fraction which precipitated at 50% $(NH_4)_2SO_4$ was dissolved in 0.02 M phosphate buffer (pH 7.4) and dialysed at 5° against the same buffer for 3 to 6 hr. Dialysed extract was applied to a DEAE-cellulose column equilibrated with 0.02 M phosphate (pH 7.4). Sulphite oxidase activity always ran with the protein front and was collected in approximately the same volume as had been applied to the column. The DEAE eluate was mixed with calcium phosphate gel (4 mg mg^{-1} protein) and stirred for 30 min. The gel was collected by centrifugation, and the sulphite oxidase was

Table 4. Effect of various concentrations of mannitol, mannose and fructose on sulphite oxidation by spinach thylakoids dispersed in Triton X-100

Carbohydrate concentration (mM)	Relative sulphite oxidase activity*		
	Mannitol	Mannose	Fructose
0	100	100	100
10	138	93	96
20	132	92	104
40	127	91	105

*Average of measurements from both assays (reduction of ferricyanide and oxygen uptake method). Mean real activity without effectors was 87 nmol $min^{-1} mg^{-1}$ chlorophyll determined with oxygen uptake method (thylakoid concentration: 0.05 g chlorophyll l^{-1}) and 780 nmol $min^{-1} mg^{-1}$ chlorophyll determined with ferricyanide reduction (thylakoid concentration: 0.04 g chlorophyll l^{-1}).

Table 5. Effect of superoxide dismutase and catalase on sulfite oxidase activity of spinach thylakoids

Treatment	Concentration (units ml^{-1})*	Relative sulfite oxidase activity†
None		100
Catalase	625	98
	1250	87
	2500	100
	10000	99
Superoxide dismutase	16	103
	33	78
	65	117
	81	62
	162	73
	325	46

*Enzyme concentrations were expressed in units (as defined in Experimental) per ml of reaction mixture.

†Average of measurements from both assays (reduction of ferricyanide and oxygen uptake method). Experimental conditions and real activity observed without effectors were as related in Table 4.

eluted with 0.15 M phosphate (pH 7.4). The protein preparation thus obtained has an activity 50 times higher than in crude spinach extract. It was submitted to SDS-PAGE. Four major protein bands were detected (M_r 65, 53, 36 and 33 kDa), indicating possibly the presence of contaminating bands. On the other hand, sulphite oxidase might be made up of different subunits. Attempts at specifically staining sulphite oxidase activity after PAGE were not successful as reported elsewhere [27, 28]. The pI value is *ca* 6.6.

DISCUSSION

The results presented here show that spinach chloroplasts possess a sulphite oxidase activity coupled with an oxygen consumption and a reduction of ferricyanide. This activity is associated with thylakoids and is solubilized by non-ionic biological detergents. These properties are those of an intrinsic membrane protein.

The results obtained for the pH and temperature dependency of spinach sulphite oxidase activity were consistent also with the real existence of a chloroplastic protein, taking into account that experiments have been carried out on crude extracts and not on purified enzyme. The optimum temperature of 50° appeared high, but, in general, an enzyme is more heat stable in crude cell-free preparations containing a high concentration of other proteins, provided that no proteases are present [23]. The value of the energy of activation that has been calculated (56 kJ mol^{-1}) is slightly lower than the one of hepatic sulphite oxidase (71.2 kJ mol^{-1}, [29]) , but is compatible with the activation energy of spinach enzymes. Since the difference between the observed pK values is greater than 3, the observed pK and V_{max} are close to the true values [23]. However, it is well known that it is very difficult to assign an experimental pK value to the reactive group of an amino acid. For example, the value of the pH of the imidazolium of histidine ranges from 5 to 7 and that for the sulphydryl group of cysteine ranges from 8 to 11. The pK values observed with sulphite oxidase of spinach thylakoids could refer to these groups.

The effect of all inhibitors used proves that the sulphite oxidation we have observed was not induced through the electron transport system in chloroplasts. Asada and Kiso [17] reported that several sugars and alcohols, especially mannitol, work as scavengers for radicals produced during the aerobic chain oxidation of sulphite by illuminated chloroplasts. However, in our experiments, mannitol, mannose and fructose had no significant effect on the rate of oxygen uptake or ferricyanide reduction. It was also observed [17] that spinach superoxide dismutase was a very potent inhibitor for the oxidation of sulphite (100% inhibition with 16 U ml^{-1}) and proposed that a superoxide anion formed by the univalent reduction of oxygen by illuminated chloroplasts is the initiator of sulphite oxidation. We observed that bovine erythrocyte superoxide dismutase inhibited the activity of spinach thylakoid sulphite oxidase solubilized by Triton X-100 only at high concentration (50% inhibition with 325 U ml^{-1}). In the same way, Miszalski and Ziegler [24] observed in their experiments that superoxide dismutase (1 to 50 U ml^{-1}) did not much change sulphite oxidation due to a free radical producing system and they hypothesized that in their *in vitro* system, superoxide anion was not the initiator of the aerobic oxidation of sulphite. The insensitivity of sulphite oxidase towards catalase was reported earlier [22] and these observations confirm that H_2O_2 is not a reactant for the sulphite oxidation in chloroplasts [17, 18]. All these results confirm the presence in spinach thylakoid membranes of an enzymic system containing sulphite oxidase activity.

Several sulphite oxidases were characterized in animals and bacteria, but spinach sulphite oxidase could not be strictly compared with one of them with respect to its kinetic parameters. In the literature, the apparent K_m for sulphite obtained for an enzyme compared to another were very dispersed. For example, it was calculated to be 20 or 40 μM for *Thiobacillus novellus* according to the electron acceptor used [30, 31], 140 μM for hepatic sulphite oxidase [22] and 580 μM for *T. ferrooxidans* [28]. Contrary to sulphite oxidase isolated from *T. novellus* [30] and *T. thioparus* [27], spinach sulphite oxidase is better coupled with ferricyanide than cytochrome c (data not shown) and can also function directly with oxygen. Perhaps, it is for this reason that we failed to observe a high inhibitory effect of sulphate on sulphite oxidase activity. Lyric and Suzuki [27] have reported that the sulphate inhibition was competitive with respect to cytochrome c in *T. thioparus* and that the lack of sulphate inhibition observed in the case of *T. novellus* enzyme was due to the use of ferricyanide rather than cytochrome c as the electron acceptor. We also failed to observe an inhibitory effect of phosphate: at pH 8, the same oxygen consumption rate was obtained in 0.1 M phosphate buffer and 0.1 M Tris–HCl. A strong phosphate inhibition was observed with *Thiobacilli* [27, 30] and it was assumed that this inhibition resulted from the structural similarity between phosphate and the reaction product sulphate. In the case of hepatic sulphite oxidase, it was observed [22] that the reduction of cytochrome c by sulphite oxidase was sensitive to sulphate and phosphate, while these compounds had no effects on the reduction of oxygen.

The behaviour of spinach sulphite oxidase during the purification procedure shows that it differs greatly from hepatic sulphite oxidase. This fact seems to confirm the similarity of spinach sulphite oxidase with sulphite oxidase from bacteria.

EXPERIMENTAL

Plant material and tissue extracts. Spinach leaves (*Spinacia oleracea*) were purchased from a local market. Extracts were prepd by homogenizing tissues with 5 vol. of 0.05 M NaPi, (pH 7.8) in a Waring Blendor. The extract was filtered through a bolting cloth (30 μm pore size). The filtered suspension was centrifuged (10 min at 27 000 *g*, JA 20 rotor, Beckman) and the pellet resuspended in NaPi buffer (25 ml ml^{-1} of pellet). Each fr. (filtrate, supernatant and resuspended pellet) was assayed for sulphite oxidase activity. For carrying out solubilization studies, 27 000 *g* pellets of chloroplast extracts were dispersed in 10 ml NaPi buffer containing various compounds as specified in results. After 2 hr at 5°, the tubes were centrifuged and the activity of sulphite oxidase in the supernatant was assayed.

Intact chloroplasts and purified thylakoids were prepared from a crude spinach homogenate using an isolation medium containing 0.05 M NaPi buffer and 0.4 M sorbitol (pH 7.8). After filtration, the suspension was centrifuged at 1500 *g* for 10 min. The pellet was resuspended in the isolation medium and layered on the top of a discontinuous percoll gradient: 70%, 40%, 20% [32]. After centrifugation (45 min, 15 000 *g*, Centrikon T2060 ultracentrifuge Kontron), intact plastids were recovered at the interface of the 40 and 70% Percoll layers. In order to eliminate Percoll adhering to the plastids, the suspensions were washed (× 2) with 100 ml isolation medium and pelleted by centrifugation at 6000 *g* for 10 min. These isolated intact chloroplasts could be broken by an osmotic shock (phosphate buffer alone) and thylakoids were sepd by a final centrifugation (27 000 *g* for 10 min).

Thylakoids were prepd from the same homogenate of spinach leaves as above. The bolting cloth filtrate was first centrifuged at 1090 *g* for 1 min and the pellet discarded. A second centrifugation (at 5110 *g* for 2 min) led to a chloroplastic pellet, which was submitted to an osmotic shock. Finally, thylakoids were recovered by centrifugation at 27 000 *g* for 10 min. This method was adopted to prepare rapidly a large stock of thylakoids. After their prepn, thylakoids were kept at − 25° and thawed when necessary. To determine sulphite oxidase activity in thylakoids, aliquots were dispersed in NaPi buffer containing 1% Triton X-100 (chlorophyll concn: 0.2–0.3 g l^{-1}). The procedure was identical to that described previously.

Protein concn was determined by the BCA protein assay reagent [33] and chlorophyll content after extraction in 90% MeOH [34].

Enzymic assays. Sulphite oxidase was assayed according to ref. [22] following the reduction of ferricyanide at 420 nm (DU-70 Beckman spectrophotometer). Enzymic extracts were added to cuvettes containing 0.4 mM Na_2SO_3, 0.4 mM $K_3Fe(CN)_6$, 0.1 mM EDTA and 0.1 M Tris–HCl buffer (pH 8.5). The final reaction vol. was 2.5 ml and the temp. was 30°. A blank was performed by omitting sulphite in the reaction medium and was subtracted from the assay. One unit of enzyme activity was defined as the amount of enzyme catalysing the disappearance of 1 nmol substrate min^{-1} at 30° under standard conditions.

Oxidation of sulphite was also followed from O_2 consumption using a Clark-type oxygen electrode connected to a recorder with a suitable amplification. The reaction mixt. consisted of 0.1 M Tris–HCl buffer (pH 8.5), 0.1 mM EDTA, 1 mM Na_2SO_3 and enzymic extract in a final vol. of 1.9 ml at 30°. After the concn of O_2 in the reaction vessel reached 615 μM by aeration, the reaction was started by adding Na_2SO_3. As observed in ref. [29], a transient rapid initial rate was observed, followed by a slower but linear rate during the assay time (10 min). This linear rate was taken as a measure of the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme catalysing the disappearance of 1 nmol sulphite min^{-1} at 30°, taking into account that the reduction of 1 nmol O_2 allowed the oxidation of 2 nmol SO_3^{2-}. O_2 consumption observed by omitting sulphite was subtracted from the assay. The effect of temp. on sulphite oxidase activity was assayed following the associated O_2 uptake. As the rate of O_2 consumption diminished with time above 35°, it was calculated between 0.5 and 2 min.

Polyacrylamide gel electrophoresis. The purity of enzyme was checked by PhastSystem electrophoresis (Pharmacia) with PhastGel Gradient 8–25 (continuous 8–25% gradient polyacrylamide gel) under denaturating conditions and by PhastSystem isoelectric focusing with PhastGel IEF 3–9 (gel covering the pH range 3–9). Proteins were detected by Coomassie blue staining.

Chemicals. All chemicals were of analyt. grade and all reagents were prepd in Milli-Q H_2O. Superoxide dismutase (from bovine erythrocytes) and catalase (from bovine liver) were purchased from Sigma. The definition of unit of catalase activity was that of Sigma and the one of superoxide dismutase activity as defined in ref. [25].

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