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Presence of sulphite oxidase in yam leaves

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ABSTRACT: Yam chloroplast possesses sulphite oxidase activity when coupled with ferricyanide reduction and oxygen consumption. This activity is associated with thylakoid and is solubilized using non-ionic biological detergent. pH and temperature dependence of the enzyme indicate that it is an intrinsic membrane protein. This enzyme is insensitive to radical scavengers (mannitol, mannose and fructose) and catalase but is inhibited by high concentration of superoxide dismutase. Sulphite oxidation is not induced by photosynthetic electron transport system but is achieved by membrane bound sulphite oxidase activity. The kinetic parameters of the enzyme were compared with those of other sulphite oxidases.

Key Words: Yam plant (*Dioscorea rotundata*); Yam leaves; Sulphite metabolism; Sulphite oxidase.

Introduction

Sulphite metabolism by higher plants has been widely studied with respect to SO_2 fumigation. Early information showed that higher concentration of SO_4^{2-} were present in sulphite treated alfalfa and sugar beet (1) which was later confirmed by studies $^{35}SO_2$ treated spinach (2), soybean (3,4) and beans (5). It was reported (6) that SO_2 was rapidly converted to SO_4^{2-} in the light and darkness and it was also reported that light was more effective in the incorporation through stromal aperture and on SO_2 oxidation to SO_4^{2-}. The site and nature of this oxidation is still unknown and there is no evidence up till now that specific sulphite oxidase is involved (7). However, there are indications that chloroplasts could carry out SO_2 oxidation because of SO_4^{2-} accumulation in the chloroplasts (5). The inner membrane is permeable to sulphite through the sulphate translocation (8-9), and a sulphite binding site is present in the thylakoid (10, 11). It has been suggested that chloroplast could be the site of detoxification of toxic effect of SO_2 (12). Asada and Kise (13) have shown that spinach chloroplast induced light dependent aerobic oxidation of SO_2 through electron transport chain. However, recent results showed that sulphite ocidation (14) was effected by intact chloroplasts from wheat and spinach in the light and in the dark, and that this sulphite metabolism should not be ascribed only to non enzyme oxidation. However, these results suggest that an enzyme system in the chloroplast containing sulphite oxidase (EC 1.8.3.1) activity could be involved in sulphite metabolism.

This paper reports for the first time the presence of sulphite oxidase activity in white yam plant.

Materials and Methods

Plant Materials:

White yam (*Dioscorea rotundata*) leaves were obtained from the stems of the yam plant growing in the author's garden. Chemicals and reagents were of analytical grade and obtained from Sigma except Sephadex G series which were the product of Pharmacia Fine Chemicals.

Extraction:

Yam leaves (300g) were homogenized in 100cm^3 of 0.05M sodium phosphate buffer pH 7.8 in a warring domestic blender. The extract was filtered through several layers of muslin cloth and the filtrate was centrifuged for 10 min at 27,000g. The pellet was suspended in the phosphate buffer (30 cm^3 of NaPi to 1.0cm^3 of pellet). The filtrate, supernatant and the pellet was each assayed for sulphite oxidase activity. Solubilization studies were carried out by dispersing the pellets of the chloroplast extracts in 20.0cm^3 of phosphate buffer containing various compounds shown in Table 2. After 2 h incubation at 5°C, the tubes were centrifuged and the activity of sulphite oxidase in the supernatant was determined. The intact chloroplasts and purified thylakoids were prepared from crude yam homogenate using isolation medium containing 50 mM phosphate buffer and 0.4M sorbitol pH 7.8 according to Jolivet et al (15).

Protein concentration:

Protein content was determined using dye-binding method (16) and chlorophyll content was determined after extraction in 90% methanol (17).

Enzyme assay:

Sulphite oxidase activity was assayed by following the reduction of ferricyanide at 420 nm (18) using Unican SP 1800 spectrophotometer. The final volume of the reaction mixture was 2.5 cm^3 and the reaction was carried out at 30°C. A blank was prepared by omission of sulphite in the reaction mixture. Enzyme activity was also assayed by following oxygen consumption using Clark-type oxygen electrode (19). Effect of temperature on sulphite oxidase activity was assayed in the temperature range of 22°C to 60°C by following O_2 uptake (15).

Polyacrylamide gel electrophoresis was carried out as in ref. (20).

Effect of pH on enzyme activity:

Sulphite oxidase activity was assayed at different pH value, ranging from 4 to 9.5 using phosphate buffer (pH 4 to 7) and Tris-HCl from 7 to 9.5.

Results and Discussion

Distribution of sulphite oxidase:

Leaves of yams were homogenized in 50 mM phosphate buffer pH 7.8. The filtered extract, supernatant and pellet were assayed for sulphite oxidase activity using ferricyanide reduction method. Intact chloroplast and purified thylakoids were also prepared from leaf tissues homogenized with isotonic 50 mM phosphate buffer containing 0.4M sorbitol (pH 7.8). It was found that ferricyanide method was about 10 times more effective than O_2 uptake as electron acceptor. In this study therefore, ferricyanide method was preferred to O_2 uptake in most assays. Similar observation was also reported by Cohen and Fridevich (18). It was found that crude extract of yam leaves contained enzymic system that can affect the reduction of ferricyanide by sulphite at the rate of 80 nkat mg-1 chlorophyll. The activity was largely found in the pellet sedimenting after centrifugating at 27,000g and this indicates that sulphite oxidase activity is membrane bound (Table 1). When chloroplasts were isolated, purified and broken, Sulphite oxidase activity was

found in the pellet fraction (Table 1). showingthat the enzyme is associated with the chloroplast membrane. To confirm that the enzyme is membrane bound, experiments were directed towards solubilization of sulphite oxidase. The 27,000g pellet was extracted with water and neutral buffer but this failed to solubilize the enzyme. However only in the presence of detergents was the enzyme extracted. The most effective being non-ionic biological detergent. Triton X-100 (5%) (Table 2). As sorbitol is not able to solubilize the enzyme, it was used to prepared the intact chloroplast instead of sucrose (15). The activity obtained with Triton X-100 treated thylakoid was 10 times higher than that of crude yam extract. These results show that the yam leaf chloroplasts possess a sulphite oxidase activity which is associated with thylakoid and is solubilized by non-ionic biological detergent. These properties are those of an intrinsic membrane protein. The effect of sulphite concentration on enzyme activity showed that the enzyme obeyed Michaelis-Menten equation with apparent Km value of 40 uM and Vmax of 2250 nkat mg^{-1} chlorophyll. Several sulphite oxidase have been characterized in animals and bacteria and their Km values varied greatly. For example, the Km values reported vary from 20 µM to 40 µM for *Thiobacillus novellus* according to electron acceptor used (21), 140 µM for hepatic enzyme (18) and 580 µM for *Thiobacillus ferrooxidans* (23). The Km obtained for the yam enzyme is similar to that of *T. novellus* (40 µM) and higher than that of spinach enzyme (15) but lower than those obtained for hepatic enzyme (18) and *T. ferrooxidans* (23).

Table 1: Distribution of sulphite oxidase activity in yam leaves

Tissue	Fraction	Activity (nkat mg^{-1} chloroph)
Leaf extract	Supernatant	80.0
	27,000g pellet	720.0
Broken purified chloroplast	Stroma	48.0
	Thylakoid	750.0

Table 2: Effect of superoxide dismutase and catalase on sulphite oxidase activity.

Treatment	Concentration of Enzyme (units cm^{-3})	Sulphite oxidase activity (nkat mg^{-1} chloro)
None	-	820.0
Catalase	600	811.8
	900	812.0
	1200	820.0
	5000	811.9
Superoxide dismutase	16	820.0
	32	640.0
	85	451.0
	160	459.0
	320	328.0

Activity of the enzyme was assayed using ferricyanide reduction.

Effect of temperature

The optimum temperature found for the enzyme is 50°C with activation energy of 54.0 KJ mol^{-1}. The Arrhenius plot of In Vmax versus 1/T is linear over the temperature range of 25°C to 55°C (Fig. 1). The optimum temperature of 50°C is high. However, enzyme is more heat stable in crude free cell preparation containing high concentrations of other proteins, provided no proteases are present (24). The value of energy of activation calculated from Fig. 1 is lower than that of hepatic sulphite oxidase (71.2 KJ mol^{-1}) (19) but similar to activation energy reported for spinach sulphite oxidase (15). The thylakoid yam enzyme is stable at -80°C in 50 mM phosphate buffer without 50% glycerol and no appreciable activity was lost on thawing. However, thylakoid stored at -20°C retained its sulphite activity over 4 months. No activity was obtained in glycerol sample after the removal of glycerol by dialysis. Activity of the yam enzyme was decreased by boiling. 35% of the activity being lost for 2min and 80% for 15 min. This yam sulphite oxidase behaviour is comparable to that of spinach enzyme (15) which lost 86% of its activity when boiled for 15 min.

Table 3: Solubilization of sulphite oxidase from pellet of yam leaf extract.

Nature of extractant	Supernatant activity (nkat mg^{-1} chlorophyll)
None	0
0.4 M sorbitol	0
0.4 M sucrose	80
5.0 M NaCl	0
5% Brij 25	160
1% SDS	216
5% SDS	190
5% Na deoxycholate	0
2.5% n-Octyl-β-D-glucopyranoside	280
1% Triton-X-100	480
2.5% Triton-X-100	480
5% Triton X-100	800

Activity was measured in the supernatant obtained after treating the pellet with the extractants for 2 h with stirring. Each value represents mean of 4 determination ± S.E.M.

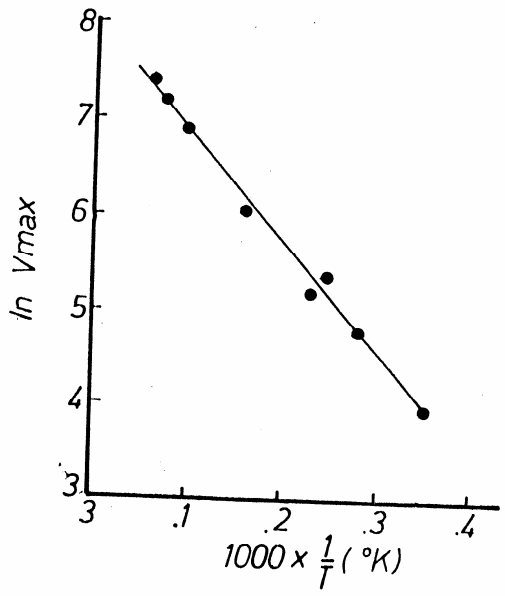

Fig. 1: Arrhenius relationship of ln V_{max} of sulphite oxidase activity as a function of absolute temperature (in reciprocal form). Enzyme activity was expressed in nkat mg^{-1} chlorophyll.

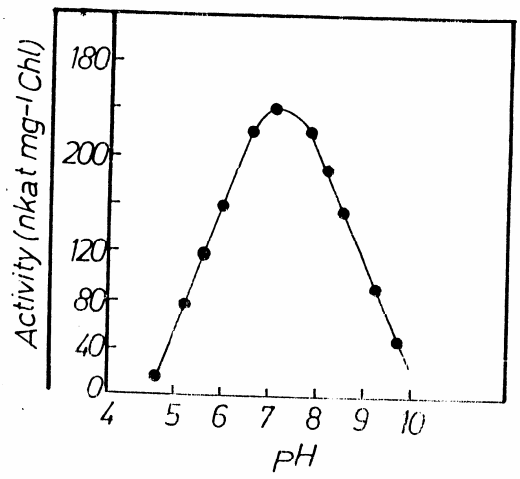

Fig. 2: pH dependence of sulphite oxidase activity. Buffers employed were: 90 mM citrate-phosphate, pH 4 to 7 and 100 mM Tris-HCl from pH 7 to 9.5. Each point represents the mean of 4 determinations ± S.E.M.

Table 4: Effect of various concentrations of radical scavengers on sulphite oxidation by yam thylakoid dispersed in Triton X-100.

Carbohydrate concentration (mM)	Relative sulphite oxidase activity		
	Mannitol	Mannose	Fructose
0	100	100	100
10	105	100	98
20	105	98	99
40	104	98	100

Activity of the enzyme was assayed using ferricyanide reduction method with thylakoid concentration of 0.08g chlorophyll per litre. Activity obtained in the absence of effectors was 8000 nkat mg^{-1} chlorophyll.

Effect of pH on Sulphite oxidase activity

The effect of pH on sulphite oxidase activity was investigated between pH range of 4 and 9.5, and the results are shown in Fig. 2. The optimum pH obtained from the pH profile is 7.6, while the pK values are 6.0 and 9.0. Since the difference between the observed pK values is greater than 3, the observed pK and Vmax are close to the true values (24). The pK of imidazol group in protein varies from 5 to 7 and that of sulphydryl group of cysteins varies from 8 to 11 (22). These pK values of 6.0 and 9.0 could refer to histidine and cystein respectively. However, it has to be remembered that it is difficult to assign an experimental pK value to the reactive group of amino acid in protein (15).

Inhibitors of enzyme activity

It has been suggested (12) that in plants sulphite oxidation could be initiated by (i) superoxide anion formed on the reduction side of electron transport system in chloroplasts, (ii) by free radical e.g. OH^- or (iii) by H_2O_2. In order to check that sulphite oxidation observed was not due to free radical, O^{-2} and H_2O_2 enzyme activity was assayed in the presence of various concentration of mannitol, fructose and mannose, which were known to be potent free radical scavengers (5, 13). Enzyme activity was also assayed in the presence of catalase or superoxide dismutase which catalyses the dismutation of O^{-2} to H_2O_2 (26). No inhibition was observed in the presence of the radical scavengers. Catalase has no effect on the activity of yam sulphite oxidase. The effect of all the inhibitors tested indicates that the observed sulphite oxidation was not induced through electron transport system in the chloroplasts. It was reported that spinach superoxide dismutase is a potent inhibitor of sulphite oxidase (13) and it was therefore proposed that superoxide anion formed by univalent reduction of oxygen by illuminated chloroplasts is the initiator of sulphite oxidation. However, Miszalski and Ziegler (25) observed in their *in vitro* study that super-oxide dismutase did not change sulphite oxidation due to free radical producing system. They concluded therefore that superoxide anion was not inhibitor of the aerobic oxidation of sulphite. In this study, it was observed that bovine erythrocyte superoxide dismutase inhibited the activity of yam thylakoid sulphite oxidase at high concentration (Table 2). The non-inhibition of sulphite oxidase by catalase has been reported (18) and these observations confirm that H_2O_2 is not a reactant in sulphite oxidase catalysed reaction in the chloroplasts (14, 25). The results of this study confirm the presence of an enzyme system containing sulphite oxidase activity in yam thylakoid membrane.

It has been reported that sulphite oxidase was inhibited by sulphate when cytochrome c was used instead of ferricyanide and that the inhibition was competitive in *Thiobacillus thioporus* enzyme. It was also observed that lack of sulphate inhibition of *T. novellus* enzyme was due to the use of ferricyanide as

electron acceptor (27). In this investigation, sulphate has no effect on the yam sulphite oxidase either in phosphate buffer or Tris-HCl buffer.

Strong phosphate inhibition has been reported in Thiobacilli (18, 27) and it was said that this inhibition was as a result of structural similarity between phosphate and sulphate (end product). In the case of hepatic sulphite oxidase, it was reported (18) that the reduction of cytochrome c by sulphite oxidase was sensitive to sulphate and phosphate inhibition but these compounds had no effect on the reduction of oxygen. In contrast, to sulphite oxidase from the above bacteria, yam sulphite oxidase is better coupled with ferricyanide than with cytochrome c.

Results of this study shows that yam sulphite oxidase differs from hepatic sulphite oxidase but similar to spinach sulphite oxidase (15) and sulphite oxidase from bacteria.

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