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Subcellular Location of Sulphite Reductase in Plant Tissues

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Sulphite reductase (E.C. 1.8.1.2) is able to cause the reduction of inorganic sulphite to sulphide. This enzyme has been studied in a number of micro organisms (1, 2, 3, 4, 5). Although the enzyme from *Allium odorum* has been purified to homogeneity (6, 7) its characterisation is still incomplete. Recently Asada & Bandurski (8) and Asada et al. (9) have described some of the properties of a partially purified sulphite reductase from spinach and showed that the enzyme catalyses not only the reduction of sulphite but also that of nitrite and hydroxylamine. These findings threw doubt on the specificity of this enzyme, which in certain cases seems to be identical with enzymes involved in the reduction of nitrate to ammonia (6). Further doubts about the nature of sulphite reductase arise from the observation of Torii & Bandurski (5, 10) that free sulphite is not a true intermediate in the reduction of sulphate. Furthermore, it is not clear at present in which part of the plant, root, stem or leaves, sulphite is reduced. Since sulphite reductase appears to be a very electro-negative reductase, it is possible that the enzyme, as usually isolated, is part of an electron transport system. Such systems are usually associated with the particulate fraction of the cell. It therefore appeared important to determine in which subcellular fraction of the cell sulphite reductase of the higher plant is located and whether enzyme systems capable of reducing sulphite to sulphide occur in both photosynthetic and non-photosynthetic tissues.

Chloroplasts were isolated from spinach or pea leaves. The leaves were ground in 0.4 M sucrose, 0.05 tris, 0.01 M NaCl buffer, pH 7.8 (1–2 ml/g fr wt of leaves), the extract filtered through gauze and centrifuged at 300 g for 5 minutes and the residue discarded. The supernatant solution was then centrifuged at 300 g for 20 minutes, when very pure chloroplasts were required or at 2000 g for 10 minutes, when a more quantitative yield was needed. The residual supernatant was used as source of the soluble enzyme. The chloroplasts were washed in the same buffer and then reprecipitated.

Chloroplasts were also prepared by isolation in a non-aqueous medium according to Smillie (11) but using an initial density of the isolation medium containing *n*-hexane and carbon tetrachloride, of 1.32. The leaves were starved for 24 hours in the dark, at room temperature to reduce the starch content, frozen with liquid nitrogen, freezedried, ground in the non-aqueous medium and then fractionated. Extracts of the isolated chloroplasts, prepared by either the aqueous or non-aqueous techniques, were made by suspending them overnight in 0.05 M potassium phosphate buffer (pH 7.6) and then removing the fragmented chloroplasts by centrifugation. An active enzyme preparation was prepared from the supernatant solution by collecting the fraction precipitating between 35 to 65 % saturation with ammonium sulphate, resuspending it in 0.006 M potassium phosphate buffer (pH 7.6) and dialysing overnight against the same buffer. Almost all sulphite reductase activity present in the extract could be recovered in this fraction.

The soluble enzyme was prepared by fractionating the supernatant solution, after initial removal of the chloroplasts, with ammonium sulphate, the 35 to 65 % saturation fraction again being collected. Again virtually all activity was found in this fraction, and no activity could be detected in the fraction precipitating between 0 to 35 % saturation.

A particulate fraction which may be assumed to contain primarily mitochondria was prepared from pea or barley roots, oats, cauliflower, or sweet potatoes by grinding the tissue with the same buffer used for isolating chloroplasts. After filtration through gauze, the extracts were centrifuged for 10 minutes at 1000 g and the precipitate discarded. The supernatant solution was centrifuged for 20 minutes at 20,000 g. The particles were washed with the same buffer and reprecipitated. The precipitate was regarded as a mitochondrial fraction. The enzyme was extracted from the mitochondria by resuspending them in 0.05 M Tricine buffer, pH 7.2 (12) and sonicating the suspension for 90 seconds using a Raytheon Sonic oscillator, 10 K-c, at full output. Alternately the mitochondria were extracted with 0.05 M phosphate buffer pH 7.6 containing 1 % Triton X-100. Following sonication, the suspensions were centrifuged at 25,000 g for

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25 minutes and the supernatant solution used as a source of the enzyme. All isolation procedures were carried out in the cold at 2 to 6°. Sulphite reductase was assayed by a method developed by Asada (personal communication) and Asada et al. (8), based on the bleaching of reduced methyl viologen (MV.) in vacuo which was followed at 604 mμ. The reaction mixture contained the following components: 0.5 mM potassium sulphite, 0.05 M Tricine pH 7.2, 2 mM ATP, 2 mM EDTA, 0.27 mM reduced methyl viologen, and enzyme. The unit of activity is μmoles reduced methyl viologen oxidized/minute. Sulphide was measured by the method of Siegel (13).

Attempts to measure sulphide formation by isolated chloroplasts in the light, when sulphite was provided as substrate, were unsuccessful. Even added sulphide appeared to be bound by the tissue and only 15 % could be recovered. A light induced sulphite disappearance could be shown in isolated chloroplasts, but no sulphide formation could be demonstrated, so that no definite conclusion could be drawn from these experiments. Moreover, it was shown that boiled controls were not valid in such experiments, since boiling reduced the protein-sulphydryl groups detectable in the preparation by more than 50 %. This could induce a disappearance of sulphite non-enzymically, by reaction with the S-S groups in the protein.

In view of these difficulties the extraction procedures described above were adopted. In typical experiments 5 to 10 % of the total sulphite reductase activity was located in the chloroplasts, isolated in aqueous medium, and remainder in the soluble fraction. The formation of inorganic sulphide by such preparations could readily be demonstrated with a stoichiometry of 6 moles MV oxidised per mole of sulphide formed. Distribution between chloroplasts and soluble fraction was affected by the molarity of the aqueous isolation medium. Increasing the sucrose concentration increased recovery from the chloroplast fraction (table I).

Although the bulk of enzyme activity appeared to be present in the soluble fraction of the cell, the fact that part could be found in the chloroplasts and the increased retention in them, as the sucrose concentration of the isolation medium increased strongly suggest that this soluble activity originated in the chloroplasts. This has been shown to be true for a number of other chloroplast enzymes (11). When chloroplasts were isolated, using a non-aqueous technique, 88 % of the activity could be recovered from the chloroplast fraction (table II). These results indicate that the bulk of, if not all, sulphite reductase activity of green leaves is associated with the chloroplasts, provided that the leakage of soluble proteins is prevented. Both the distribution pattern and the higher activity/mg chlorophyll found in the chloroplast fraction prepared in non-aqueous medium support this point of view.

When the distribution pattern of enzymic activity is compared in a green tissue and a tissue lacking chlorophyll, pea shoots and pea roots, a striking difference is noted (table III). In shoot tissue, using an aqueous isolation technique, the

Table I. *Distribution of Sulphite Reductase Activity Between Chloroplast and Soluble Fractions of Spinach Leaves, when the Tissue was Ground in Aqueous Media, (Sucrose, Tris, NaCl Buffer), Containing Different Sucrose Concentrations and Activity Measured both in the Chloroplast Fraction and the Supernatant*

Sucrose concentration in isolation medium	% of Total activity recovered from chloroplasts	Unit of activity/mg chlorophyll in chloroplast fraction	Units of activity recovered/100 g fr wt of leaf tissue
			chloroplast and soluble
0.4 M	6.7	0.015	2.5
0.8 M	10.5	0.029	2.9
2.0 M	15.5	0.09	3.7

Table II. *Distribution of Sulphite Reductase Activity Between Soluble and Chloroplast Fractions of Freezedried Spinach Leaves, Prepared Using Non-aqueous Isolation Procedures*

Activity/mg chlorophyll of chloroplast fraction 0.224 units. (5 g freezedried spinach leaves were ground and chloroplasts prepared. Total activity recovered was 0.73 units/5g).

	Total chlorophyll, mg isolated in fraction	Total protein, mg isolated in fraction	Sulphite reductase activity (% of total) present in $(NH_4)_2SO_4$ fraction 35 to 65 % saturation
Chloroplast fraction	2.84	180	88
Non-chloroplast fraction	1.0	58	12

Table III. *Recovery of Sulphite Reductase Activity from Particulate and Soluble Fractions of Shoots and Roots of Ten Day Old, Light Germinated Peas*

Particulate fraction in both was precipitated at 12.000g for 20 minutes. The particulate fraction of shoots was extracted with buffer, while that of roots was extracted with buffer containing 1 % Triton X-100.

	Distribution of sulphite reductase activity	
	Roots	Shoots
Soluble	25 %	90 %
Particulate	75 %	10 %
Units of activity recovered/100 g tissue	1.07	10.7

bulk of the activity appears in the soluble fraction, being apparently leached out readily from the chloroplasts. In contrast, activity in the roots is found predominantly in the particulate fraction. Activity could only be extracted from it by treatment with detergent such as 1 % Triton X-100 or by sonication as described above. The presence of sulphite reductase activity could by this means be demonstrated in particulate fractions from cauliflower florets, sweet potatoes, (storage organ) and barley and pea roots. In all cases, sulphide formation as the end product of sulphite reduction was demonstrated.

The bulk of sulphite reductase activity is associated with the particulate fractions of the cell of both photosynthetic and non-photosynthetic tissues. Whether, in addition, a genuine soluble enzyme also exists could not be ascertained from these experiments. The level of enzymic activity is much greater in leaf than in root tissue (table III). The presence of an enzyme capable of reducing sulphite in both these tissues might be expected, since sulfate is assimilated in both.

The assignment of a subcellular location to an enzyme which is readily solubilised requires great caution. However, since sulphite reductase could be located in the particulate, probably mitochondrial fraction of non-photosynthetic tissue and since it could be shown to be present in chloroplasts, the conclusion that the apparently soluble enzyme originates from the chloroplasts seems justified. The fact that sulphite reductase occurs in the particulate fraction of the cell, that it is non-specific (2, 8, 9) and the strong electro-negativity of the enzyme suggest that the enzyme is in fact part of an electron transport system. The electron transport system present in the chloroplast and mitochondrial fractions differ and therefore the sulphite reductase demonstrated in them may also well be different. In both organelles probably relatively non-specific systems are present, which may have a more general role in the reductive processes of the cell.

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