

A RAPID PROCEDURE FOR THE PREPARATION OF LIGHT HARVESTING CHLOROPHYLL *a/b* PROTEIN COMPLEX

An assessment of its manganese content

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Received 20 March 1979

1. Introduction

Following our recent study of the distribution of superoxide dismutase activity in green leaves [1], which showed that the majority of the total leaf enzyme was located in the chloroplasts, we became interested in thylakoid bound forms of superoxide dismutase activity. Although 20–70% of the total Cu–Zn SOD of the chloroplast stroma has been reported to be bound to the thylakoids [2,3] we find that all the SOD can be removed from the intact membranes by hypotonic washing. The possibility that some of the internal manganese of the thylakoid could possess SOD activity has been considered in the past [4] and the presence of a cyanide-insensitive SOD activity on sub-thylakoid particles prepared by detergent extraction has been described [5,6]. These particles were reported to have a low chlorophyll *a/b* ratio and this suggested to us that they might consist largely of light-harvesting chlorophyll *a/b* protein complex. The preparation of the particles by the original method proved arduous and it was modified to obtain a higher yield and remove contamination. The resultant preparation yielded LHC of a high purity. The LHC purified by this method and a standard method [7] was examined and found to contain manganese and SOD activity.

Abbreviations: SDS, sodium dodecyl sulphate; LHC, light-harvesting chlorophyll *a/b* protein complex; SOD, superoxide dismutase; MOPS, morpholinopropane sulphonic acid; ESR, electron spin resonance

2. Materials and methods

Spinach (*Spinacia oleracea*) leaves were obtained from New Covent Garden Market, London. Lettuce (*Lactuca sativa*) was bought locally. French beans (*Phaseolus vulgaris*) and barley (*Hordeum vulgare*) were grown on moistened vermiculite under optimum conditions. All reagents were of the highest quality commercially available. Nitro-blue tetrazolium and polypropylene glycol 2025 were products of BDH Chemicals Ltd (London). Xanthine oxidase was obtained from International Enzymes Ltd (Windsor).

SOD was assayed by the inhibition of the reduction of nitro-blue tetrazolium by superoxide produced by the xanthine/xanthine oxidase generating system at pH 7.8 as in [8]. Triton X-100 interferes with the assay for SOD and erroneous results can be obtained if the detergent is not largely removed. Removal of Triton X-100 is accomplished with Bio-Beads SM2 (Bio-Rad UK Ltd) using methods in [9]. The freshly prepared complex is initially dialysed against the beads in 10 mM potassium phosphate buffer (pH 7.8) for 24 h and the larger micelles are filtered off through a column of the beads until no more detergent can be removed. Protein was measured by a modification of the Folin method [10] and chlorophyll by the method in [11]. Gel electrophoresis in the presence of SDS was performed essentially as in [12] in collaboration with Gunilla Høyer-Hanson of the Carlsberg Laboratories, Copenhagen. The manganese content of the complex was measured by atomic absorption after digestion with concentrated nitric acid. ESR measure-

ments were carried out on a Varian E4 EPR spectrometer.

2.1. Preparation of the complex

Chloroplasts were prepared as in [1]. The grinding medium was 20 mM MOPS (pH 7.5) containing 1 mM EDTA, 0.3 M mannitol, 0.2% BSA, 0.05% cysteine and 0.6% insoluble polyvinylpyrrolidone. Chloroplasts were pelleted with centrifugation at $2500 \times g$ for 5 min. The chloroplast pellet was redispersed by pipette in 10 vol. 50 mM potassium phosphate buffer (pH 7.8). After standing for 5 min on ice to ensure complete rupture of the chloroplasts the membranes were pelleted at $5000 \times g$ for 5 min. The washing procedure was repeated once more to remove the stromal SOD. The final thylakoid pellet was dispersed in a minimum volume of the buffer to 5 mg chlorophyll/ml. To this was added an equal volume of solution of 10% (w/v) Triton X-100, 0.1 M sucrose, 0.1 M potassium phosphate buffer pH 7.8 to give a chlorophyll : Triton X-100 ratio of 50 mg/g detergent. The mixture was stirred on ice for 30 min and then centrifuged at $5000 \times g$ for 5 min to remove debris. The supernatant was thoroughly mixed with 1.5 vol. cold polypropylene glycol 2025 and layered over 30% (w/v) sucrose in 50 mM potassium phosphate buffer (pH 7.8) in a centrifuge tube. This was centrifuged at $40\,000 \times g$ for 1.5 h after which time a dark green pellet was obtained beneath the clear sucrose layer. The layers above the pellet were poured off and the pellet washed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.25 M sucrose and pelleted at $10\,000 \times g$ for 10 min. The resultant pellet consists of LHC with few impurities.

LHC was also prepared by the method in [7] for comparison in collaboration with Birger Lindberg Møller of the Carlsberg Laboratories, Copenhagen.

3. Results

The purification procedure described can be completed in 4 h with a 10% yield ($10.6\% \pm 1.8\%$, 5 determinations) on a total chlorophyll basis. This procedure was used successfully on all the species described. The following results are for spinach but comparable results were obtained for other species. The room temperature absorption spectrum of the

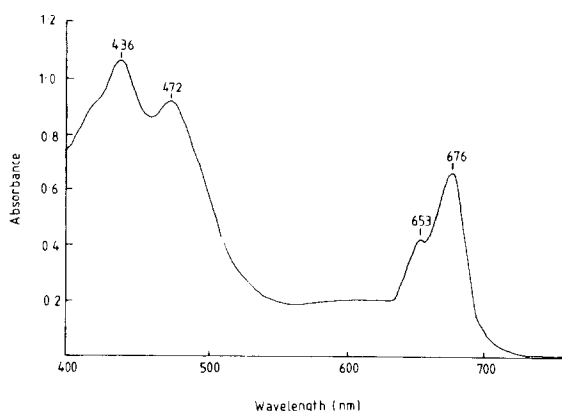


Fig.1. Absorption spectrum of the 1.5 h washed $40\,000 \times g$ LHC pellet dispersed in 0.25 M sucrose 50 mM potassium phosphate buffer (pH 7.8).

purified material (fig.1) shows A_{\max} at 436, 472, 653 and 676 nm and the chlorophyll *a/b* ratio was calculated to be 1.12 ± 0.1 (5 determinations). The protein/chlorophyll ratio was 5.14 ± 0.96 (5 determinations). When subjected to flat bed gel electrophoresis in the presence of SDS the purified complex revealed three major polypeptide bands (fig.2) with mol. wt 29 000, 26 000 and 23 000 as determined by co-electrophoresis with protein standards. The purified complex readily formed aggregates in the presence of cations which resembled stacked thylakoid membranes (fig.3) under high magnification. The purified complex showed no electron transport capabilities using any of the standard electron donors or acceptors for either photosystem and oxidised minus reduced spectra confirmed the absence of major electron-transport components. We therefore conclude that the preparation yields LHC as in [14,15].

The complex purified by both the method described and also by the method in [7] was examined by ESR at room temperature, liquid nitrogen and liquid helium temperatures. The liquid nitrogen and liquid helium spectra were featureless and no copper or iron signals were detectable. The room temperature spectra of the freshly prepared complex showed only a chlorophyll free-radical signal (fig.4). After treatment with concentrated HCl, heating or freezing and thawing a Mn^{2+} spectrum was evident. This spectrum was also produced by ageing or washing with 0.8 M



Fig.2. SDS-polyacrylamide flat bed gels stained with Coomassie brilliant blue showing: (A) washed barley thylakoids; (B) LHC prepared from barley by the method described; (C) LHC from barley prepared by the method in [7]; (D) LHC prepared from spinach by the method described; (E) washed spinach thylakoids.

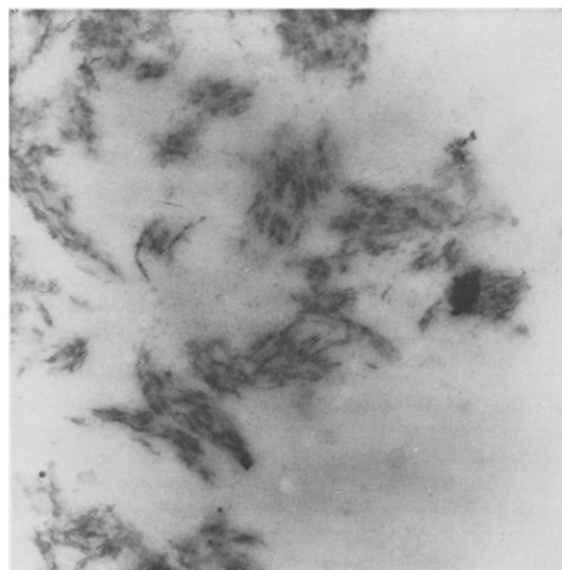


Fig.3. Electron micrograph of the 1.5 h washed $40\,000 \times g$ LHC pellet, aggregated by the addition of 10 mM $MgCl_2$ and fixed in 1% glutaraldehyde. It was then post-fixed in aqueous 2% OsO_4 /h and dehydrated in a graded ethanol series and embedded in Fluka durcupan ACM. Magnification $12\,000 \times$.

Tris-HCl (pH 8.0). Atomic absorption measurements of the total manganese routinely gave 50–70 ng Mn/g protein. The complex prepared by either method showed a cyanide-insensitive SOD activity after the removal of detergent (fig.5). This activity was decreased or completely inhibited by heating at $60^\circ C$ for 5 min, Tris treatment or incubation with 3.0 mM EDTA.

4. Discussion

The LHC produced by the method described and by the method in [7] was consistently found to contain manganese. It is known that redistribution of some of the thylakoid manganese takes place upon detergent treatment, and thus the manganese of the complex could either be truly bound or nonspecifically adsorbed. However, the complex retains its manganese after repeated washing and dialysis and it is therefore not loosely attached. The SOD-like activity described here can be equated with that reported for sub-thylakoid particles [5]. The activity appears to be due

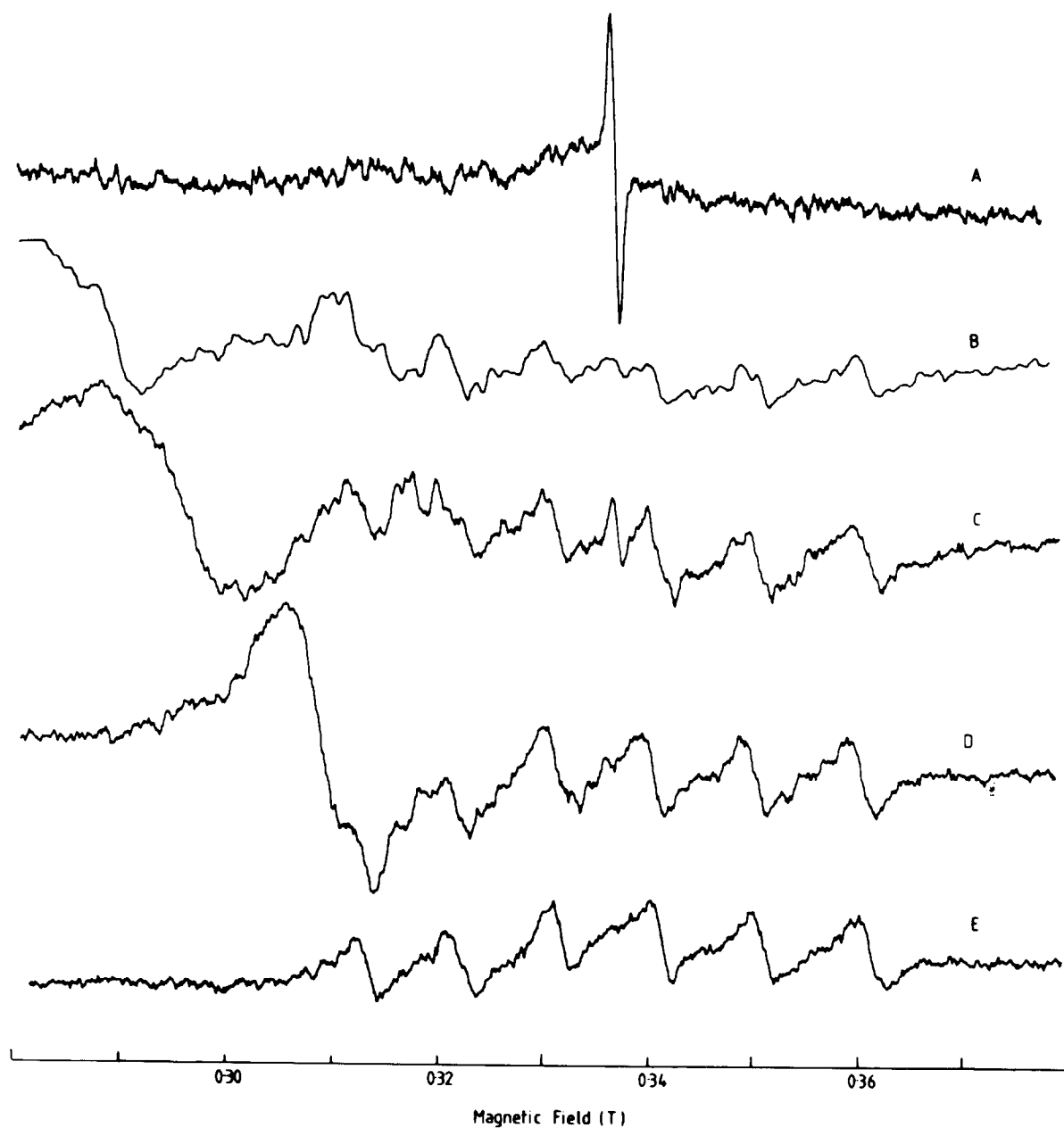


Fig.4. Room temperature ESR spectra of: (A) freshly prepared LHC; (B) the complex aged at 0°C for 1 week; (C) LHC frozen and thawed twice; (D) after 30 min in conc. HCl; (E) 1 μ M MnCl₂. Measurements were made in a flat cell at field set 3300 G, scan range 1000 G, time constant 0.3 s. Scan time 4 min, mod. ampl. 10 G, gain 2000, power 100 mW. Chlorophyll conc. 3 mg/ml.

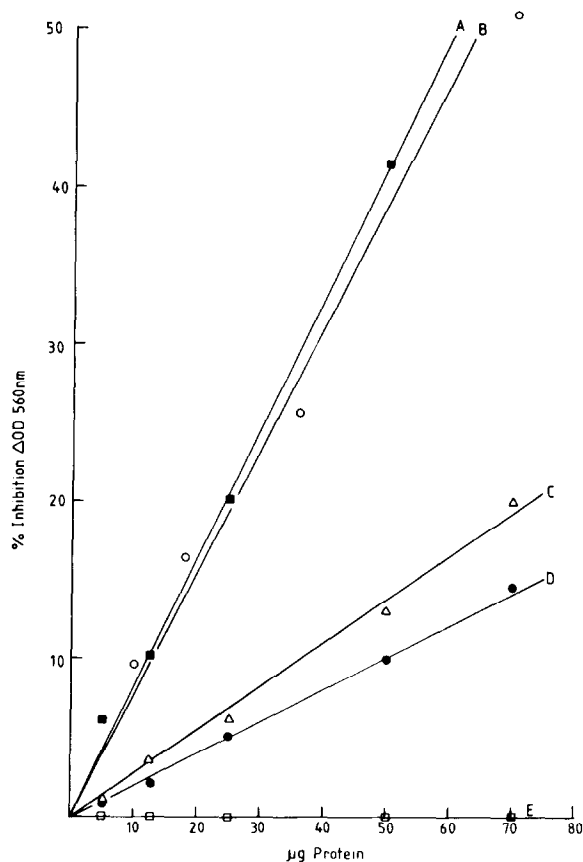


Fig.5. SOD activity of: (A) LHC prepared by the method described; (B) LHC purified by the method in [7]; (C) sample (A) in the presence of 1 mM EDTA; (D) sample (A) after heating at 60°C for 50 min; (E) sample (A) washed once with 0.8 M Tris-HCl buffer (pH 8.0) or in the presence of 3.0 mM EDTA. Similar results were obtained for sample (B) treated as in (C), (D) and (E).

to the presence of manganese as the other components of the electron-transport chain, e.g., plastocyanin, which might cause SOD-like activity have been removed during the preparation procedure. Cyanide-insensitive SOD could not be detected [16] in digitonin-fractionated membranes. This can be explained by the solubilisation capacity of digitonin. Unlike Triton X-100, digitonin fails to remove the permeability barrier between the SOD of the LHC and the components of the SOD assay. The activity is not due to Mn^{2+} which is known to react with superoxide [17] as the particles which have the SOD activity do not

show a Mn^{2+} ESR spectrum. Treatments which produce the Mn^{2+} signal severely inhibit the SOD activity. It therefore appears that it is a higher valency state manganese which promotes the dismutation of superoxide.

Acknowledgements

The generous assistance and collaboration of Birger Lindberg Møller and Gunilla Høyer-Hansen in elucidating the polypeptide composition of the detergent preparations and in the production of the LHC by the method in [7] is gratefully acknowledged, as is the assistance of Mr M. D. Ryan in measuring the ESR spectra. This work was supported by the Science Research Council.

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