THE SUPEROXIDE DISMUTASE ACTIVITY OF VARIOUS PHOTOSYNTHETIC ORGANISMS MEASURED BY A NEW AND RAPID ASSAY TECHNIQUE

Landis E. A. HENRY, Barry HALLIWELL* and David O. HALL
*Department of Plant Sciences and *Biochemistry, King's College, 68 Half Moon Lane, London SE24 9JF, England*

Received 25 May 1976

1. Introduction

The superoxide radical O_2^- is a highly toxic species which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes which catalyse the breakdown of O_2^- according to the equation below and which appear to play an important protective role in vivo [1–4].

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Reactions dependent on the presence of O_2^- are inhibited by addition of superoxide dismutase [1] and this observation has led to the development of a number of assays for measuring the activity of this enzyme. The assays usually consist of (i) a system which generates O_2^-, e.g. mixtures of xanthine and xanthine oxidase [5], NADH and phenazine methosulphate [6] or an illuminated mixture of riboflavin and methionine [7] together with (ii) a compound which reacts with O_2^- such as cytochrome *c* [5], nitro-blue tetrazolium [7], adrenaline [8], pyrogallol [9] or hydroxylamine [10]. The ability of an added cell extract to inhibit the latter reactions is taken as a measure of its euperoxide dismutase activity. However, inhibition could also be attributed to prevention of the formation of O_2^- in these complex reaction mixtures (e.g. [11]). Further, it is very difficult to study the effects of metabolites on superoxide dismutase activity, because of the possibility of interference with different stages of these assay systems [1].

In the present paper a rapid and simple assay for superoxide dismutase is described, which utilizes the ability of these enzymes to inhibit reduction of nitro-blue tetrazolium by $K^+O_2^-$ added directly as a solution in dimethylsulphoxide [12,13].

2. Materials and methods

2.1. *Materials*

All reagents were of the highest quality commercially available. $K^+O_2^-$ was from ICN Pharmaceuticals Inc., New York, USA. Spinach (*Spinacia oleracea*) leaves were obtained from New Covent Garden, London, UK, *Chara fragilis* from the University of London Botanical Supply Unit, Egham, Surrey, UK, *Nitella transluscen* from Dr J. W. Lund, Freshwater Biological Association, Ambleside, Cumbria, UK, *Spirulina platensis* from Mlle. G. Clement, Institut Francais de Petrole, Paris, France, *Spirulina maxima* from Ing. H. Durand-Chastel SOSA Texcoco, Sullivan, Mexico, and *Scenedesmus obliquus* from Dr C. J. Soeder, University of Dortmund, West Germany.

2.2. *Preparation of plant extracts*

Unless otherwise stated, all procedures were carried out at 0–4°C. Spinach leaf extracts were obtained by homogenising 50 g of washed laminar tissue in 0.2 M Tris-HCl buffer, pH 7.5 (200 ml) for 3 × 5 sec periods in an Ato-Mix (MSE Ltd., Crawley, Sussex, UK). The homogenate was filtered through 2 layers of muslin before use.

Samples of fresh *Chara* and *Nitella* were washed in distilled water, contaminating material removed, then ground with 1.0 M potassium phosphate buffer, pH 7.8, in a mortar and pestle with the aid of acid-

washed sand. Cells of *Spirulina* and *Scenedesmus* were disrupted by sonication in 1.0 M potassium phosphate buffer, pH 7.8, using a Soniprobe (Type 1130 A, Dawe Instruments Ltd., London, UK) for five periods of 30 sec with intermediate cooling intervals of 60 sec. The homogenates were centrifuged at 3000 *g* for 15 min. Solid $(NH_4)_2\ SO_4$ was added to the supernatant to give 40% saturation, the pH being kept at 7.8 by addition of NH_3. The mixture was allowed to stand for 1 h and then centrifuged at 11 000 *g* for 30 min. Solid $(NH_4)_2\ SO_4$ was added to the supernatant to give 80% saturation. After standing for 2 h the mixture was centrifuged at 11 500 *g* for 30 min. The pellet was resuspended in the minimum volume of 5 mM potassium phosphate buffer, pH 7.8, and dialysed against this buffer for 48 h. The samples were then concentrated in an Amicon ultrafiltration cell incorporating a UM 20E membrane (cut-off mol. wt. 20 000), followed by further concentration involving vacumn dialysis in a Sartorius collodion membrane-filter apparatus with a SM 13 200 membrane (Sartorius-Membranfilter, 34 Göttingen, West Germany).

2.3. *Purified enzymes*

Erythrocuprein was prepared by the method of McCord and Fridovich [5]. Purified superoxide dismutases from *Spirulina platensis* and *Rhodopseudomonas spheroides* was a gift from J. Lumsden and was prepared as described by Lumsden et al. [14].

2.4. *Enzyme assays*

Assays of superoxide dismutase by the xanthine–xanthine oxidase or by the riboflavin–methionine methods were carried out by published procedures [5,7]. In the present assay technique, solid $K^+O_2^-$ was allowed to stand in contact with dry dimethylsulphoxide for at least 24 h. The solution was filtered immediately before use. 200 µl of filtrate was added to 2.8 ml of an aqueous solution containing nitroblue tetrazolium, (56 µM), EDTA 10 µM and potassium phosphate buffer pH 7.8 (10 mM). A blue colour developed almost instantaneously as formazan was produced from nitro-blue tetrazolium. The absorbance of each solution was read at 560 nm against a control in which pure dimethylsulphoxide had been added to the above reaction mixture.

Protein was measured by the Folin method [15] and chlorophyll by the method of Arnon [16].

3. Results

Fig.1 shows the effect of the three superoxide dismutases; bovine erythrocuprein, which contains copper and zinc (cyanide sensitive), an iron-containing enzyme from *Spirulina* and a manganoenzyme from *Rhodopseudomonas* (both cyanide insensitive) on the reduction of nitro-blue tetrazolium by $K^+O_2^-$ at pH 7.8. Inhibition of formazan production was directly proportional to the amount of enzyme added until 65–75% inhibition had been achieved. In contrast the following were without effect 7×10^4 units of highly purified catalase (see [17]), bovine serum albumin (100 µg) or superoxide dismutases treated with 20% trichloroacetate or heated at 100°C

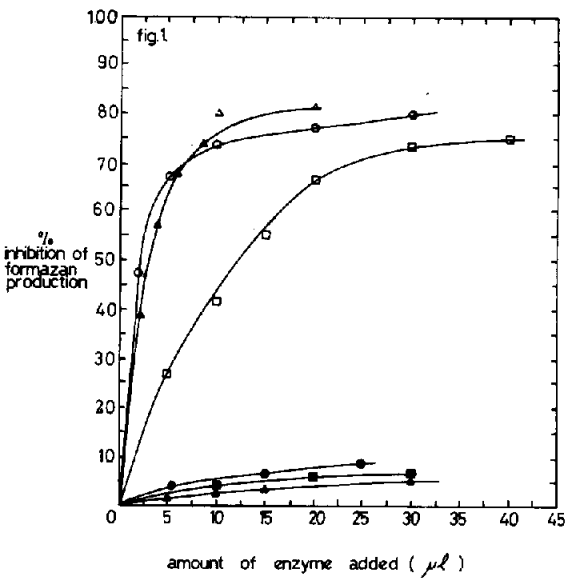

Fig.1. Effect of three purified superoxide dismutases on percentage formazan production. Assays were carried out at 20°C in a total volume of 3.0 ml, buffered at pH 7.8, by 0.01 M potassium phosphate buffer containing 10 µM EDTA. Enzymes and potassium superoxide in dry dimethylsulphoxide was added as described in the text and the absorbance of each solution was read at 560 nm in a 10 mm path length glass cuvette. Open symbols correspond to native enzymes and closed symbols to denatured enzymes. (△) *Spirulina platensis.* (○) *Rhodopseudomonas spheroides.* (□) *Bovine erythrocuprein.*

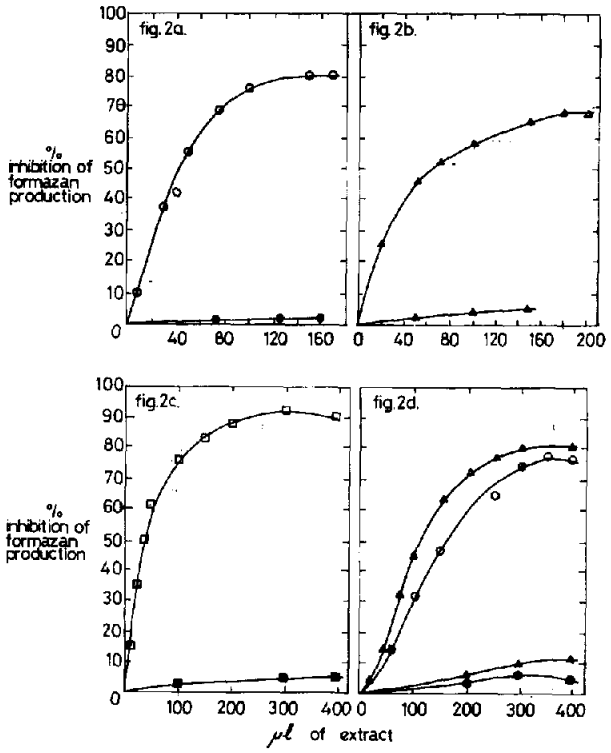

Fig. 2. Effect of partially purified superoxide dismutases on percentage inhibition of formazan production. Assays were carried out at 20°C in a total volume of 3.0 ml, buffered at pH 7.8, by 0.03 M potassium phosphate buffer containing 30 μM EDTA. Extracts and potassium superoxide in dry dimethylsulphoxide was added as described in the text and the absorbance of each solution was read at 560 nm in a 10 mm path length glass cuvette. Open symbols correspond to native extracts and closed symbols to denatured extracts (a) *Spinacia* extracts; (b) *Scenedesmus* extracts; (c) *Spirulina* extracts; (d) (△) *Chara* and (○) *Nitella* extracts.

for 30 min. Inclusion of KCN (100 μM) in the reaction mixtures abolished the inhibitory action of erythrocuprein, but did not affect inhibition by the iron or manganese-containing enzymes, in agreement with previous results [5,14]. Dimethylsulphoxide at the concentration (6.7%, v/v) present in the final reaction mixture had no effect on the activity of purified superoxide dismutases, as measured by the xanthine–xanthine oxidase assay [7].

In the $K^+O_2^-$ assay 5.850 μg of bovine erythrocuprein were required to give 50% inhibition of formazan production. However, only 0.135 μg of this enzyme was needed to reach 50% inhibition of the reduction of nitro-blue tetrazolium by a xanthine–xanthine oxidase system at pH 7.8 [11] and 0.180 μg gave 50% inhibition in the riboflavin–methionine assay [7]. It is clear that the $K^+O_2^-$ assay is less sensitive than the others.

The $K^+O_2^-$ assay was used to measure the superoxide dismutase activity of extracts from *Spinacia* leaves and from cells of *Chara, Nitella, Spirulina* and *Scenedesmus* (fig.2). In each case, inhibition was proportional to the amount of extract added until a maximum was achieved. Inhibition was abolished by heating the extracts at 100°C for 30 min, or by trichloroacetate treatment. Addition of KCN (1 mM) abolished the inhibition by *Spinacia* extracts but did

Table 1
Assay of extracts of photosynthetic organisms for superoxide dismutase by three different techniques

Organism used	*SOD activity present as μg erythrocuprein that would be required per ml of extract to give the same degree of inhibition.*		
	$K^+O_2^-$ assay	Xanthine–xanthine oxidase assay	Riboflavin–methionine assay
Spinacia	2.565	0.855	–
Chara	35.70	13.0	27.4
Nitella	21.50	7.20	16.20

Xanthine–xanthine oxidase assay was carried out as described by Halliwell [11]; riboflavin–methionine assay as described by Beauchamp and Fridovich [1]; $K^+O_2^-$ assay as described in the text.

not decrease inhibition by any of the algal extracts (fig.2). This confirms previous reports [18–20] that the superoxide dismutase activity of *Spinacia* leaves is mainly, if not totally, due to a copper–zinc enzyme. When purified erythrocyprein was mixed with *Spinacia* leaf extracts, the $K^+O_2^-$ assay was found to measure accurately the total enzyme activity present.

The calibration curves in fig.1 were used to calculate the amount of superoxide dismutase in extracts of *Spinacia, Chara* and *Nitella*; the results are presented in table 1, in terms of the amount of erythrocuprein that would be required per ml of extract to give the same degree of inhibition. The extracts were also assayed by the xanthine–xanthine oxidase [11] and riboflavin–methionine [7] methods and similar calculations were made. Table 1 shows that the amounts of enzyme measured in the extracts by these three assays are quite similar, but that the xanthine–xanthine oxidase assay gives consistently lower results than the others. The reason for this is not yet known, but the complexity of the reactions taking place during this assay [21,22] should be noted.

4. Discussion

The work presented in this paper has utilised the reduction of nitro-blue tetrazolium by $K^+O_2^-$ dissolved in dimethylsulphoxide (a reaction first reported by Valentine and Curtis [12]) to develop a new assay for superoxide dismutase. We have shown that it can be applied to crude tissue extracts as well as to purified enzymes. The advantages of this new assay are two-fold: firstly, direct addition of $K^+O_2^-$ overcomes the problem of including complex O_2^- generating systems in the reaction mixtures; secondly, this assay is very rapid, since the colour development is complete within a few seconds of adding the $K^+O_2^-$. One potential disadvantage of the assay is its lower sensitivity but we have not found this to be a practical problem because most tissue extracts are rich in dismutase activity and require considerable dilution before assaying by the other techniques used in this paper. Additionally the sensitivity of the $K^+O_2^-$ assay can be increased 10 × raising the pH of the reaction mixture to 10.2. Further, nitro-blue tetrazolium could easily be replaced by any other compounds capable of reacting with O_2^-, such as cytochrome *c*, adrenaline or hydroxylamine.

Acknowledgements

The authors wish to thank Mr John Carpenter for his assistance in matters concerning algal ecology and classification and Dr A. Slabas for his helpful discussions.

References

[1] Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147–159.
[2] Halliwell, B. (1974) New Phytol. 73, 1075–1086.
[3] Hewitt, J. and Morris, J. G. (1975) FEBS Lett. 50, 315–318.
[4] Lumsden, J. and Hall, D. O. (1975) Nature 257, 670–672.
[5] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[6] Nishikimi, M., Appaji Rao, N. and Yagi, K. (1972) Biochem. Biophys. Res. Commun. 46, 849–854.
[7] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[8] Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
[9] Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474.
[10] Elstner, E. F. and Heupel, A. (1976) Anal. Biochem. 70, 616–620.
[11] Halliwell, B. (1975) FEBS Lett. 56, 34–38.
[12] Valentine, J. S. and Curtis, A. B. (1975) J. Amer. Chem. Soc. 97, 224–226.
[13] Younes, M. and Weser, U. (1976) FEBS Lett. 61, 209–212.
[14] Lumsden, J., Cammack, R. and Hall, D. O. (1976) Biochim. Biophys. Acta, in the press.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[16] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[17] Halliwell, B. (1973) Biochem. J. 135, 379–381.
[18] Asada, K., Urano, M, and Takashi, M. (1973) Eur. J. Biochem. 36, 257–266.
[19] Allen, J. F. and Hall, D. O. (1973) Biochem. Biophys. Res. Commun. 52.
[20] Elstner, E. F. (1975) Planta 123, 145–154.
[21] Hodgson, E. K. and Fridovich, I. (1976) Arch. Biochem. Biophys. 172, 202–205.
[22] Hodgson, E. K. and Fridovich, I. (1976) Biochim. Biophys. Acta 430, 182–188.