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Heat Stability of Superoxide Dismutase in Cabbage

J. L. Walker, K. M. McLellan & David S. Robinson

Procter Department of Food Science, University of Leeds, Leeds LS2 9JT, Great Britain

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ABSTRACT

*The occurrence of superoxide dismutase activity (SOD) in extracts of cabbage (*Brassica oleracea *var.* capitata*) was detected by use of the nitroblue tetrazolium assay method for the measurement of superoxide anion (O_2^-). The SOD activity found in aqueous extracts of cabbage was destroyed at temperatures greater than 45°C, whereas, for commercial preparations of bovine SOD, temperatures in excess of 65°C were required for deactivation of the enzyme. Heat inactivation energies have been calculated. Tests for sensitivity to cyanide have indicated that a substantial proportion of the cabbage SOD activity is due to the CuZn type of enzyme. As shown by isoelectric focusing, three SOD isoenzymes were present in the aqueous extracts of cabbage.*

INTRODUCTION

Superoxide dismutase (SOD) is an oxidoreductase enzyme (EC 1.15.1.1) catalysing the disproportionation of superoxide anion radicals in living cells to molecular oxygen and hydrogen peroxide.

$$2O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

The superoxide radical is itself relatively unstable and in aqueous solution undergoes a spontaneous dismutation reaction to form molecular oxygen and hydrogen peroxide. At neutral pH, however, the radical exists mainly as O_2^-, and the dismutation reaction is relatively slow due to electrostatic

repulsion of the negatively charged radicals. At lower pH values the radical exists as the weak acid $HO_2^{\cdot}$, and the rate of dismutation increases very substantially, the equilibrium lying favourably in the direction of dismutation.

$$2HO_2^{\cdot} \rightleftharpoons H_2O_2 + O_2$$

The enzyme SOD accelerates the reaction at physiological pH, and a very fast rate of reaction has been reported for the catalysis of dismutation by SOD ($K = 1{\cdot}6 \times 10^9\,mol^{-1}\ s^{-1}$) (Rotilio *et al.*, 1972). The mechanism involves a metal ion prosthetic group: copper, manganese or iron. In eukaryotic cells the form of SOD present in the cytosol contains copper and zinc ions (the CuZn form), while the sOD in the mitochondria contains manganese ions (the Mn form).

The enzyme-catalysed dismutation by CuZn-SOD is believed to proceed by subsequent reduction and oxidation of a copper ion acting as an electron carrier.

$$SOD\text{-}Cu^{2+} + O_2^{\cdot-} \rightleftharpoons SOD\text{-}Cu^{+} - O_2$$

$$SOD\text{-}Cu^{+} - O_2 + O_2^{\cdot-} + 2H^{+} \rightleftharpoons SODCu^{2+} + H_2O_2 + O_2$$

Bovine CuZn-SOD is claimed to be an unusually stable enzyme, retaining most of its activity after treatment with 10M urea, 4·8M guanidium chloride or 4% sodium dodecyl sulphate (Forman & Fridovich, 1973). Upon heating, the holoenzyme is said to suffer no perceptible loss of activity after 20 min at 70°C (McCord & Fridovich, 1969*b*; Forman & Fridovich, 1973; Hicks *et al.*, 1979). This thermal stability, which is dramatically reduced by the removal of the Cu and Zn ions, is fully restored by their replacement (Forman & Fridovich, 1973). Thermal inactivation of the purified bovine CuZn enzyme at temperatures greater than 70°C has been suggested to be irreversible (Forman & Fridovich, 1973) and a first order process (McCord & Fridovich, 1969*b*), with an activation energy of 72·4 kcal mol^{-1} (Forman & Fridovich, 1973). The CuZn-SOD in bovine milk can withstand pasteurisation at 63°C (Korycka-Dahl *et al.*, 1979), but at temperatures higher than 70°C the degree of enzyme inactivation increases rapidly with temperature. Raw milk undergoing HTST pasteurisation at 80°C loses approximately half its SOD activity, while the purified enzyme from bovine milk was found to be less stable to heat (Hicks *et al.*, 1979).

Little information is available on the form of SOD present in vegetables, its heat stability and the fate of the enzyme during thermal processing of vegetables. In crude extracts of corn and oats the SOD activity has been found to be more resistant to boiling than the peroxidase activity (Giannopolitis & Ries, 1977). Furthermore, the SOD activity in the crude

corn extract was found to be more heat stable than the SOD partially purified from corn.

In the current investigation, the sensitivity of SOD activity in cabbage extracts to heating over a range of temperatures is determined. Isoelectric focusing is used in conjunction with heat treatment to demonstrate the differential thermal stability of the SOD isoenzymes.

MATERIALS

Bovine erythrocyte superoxide dismutase was purchased from B.C.L., Lewes, Sussex, Great Britain. Nitroblue tetrazolium was obtained from Sigma, Poole, Dorset, Great Britain, and *o*-dianisidine from Koch-Light Laboratories, Colnbrook, Great Britain. Ampholine carrier ampholytes were obtained from LKB Instruments, S. Croydon, Great Britain.

All other chemicals were from BDH Chemicals, Poole, Dorset, Great Britain, in the Analar grade where available. White cabbage (*Brassica oleracea*, var. *capitata*) was purchased from a local supermarket.

METHODS

Superoxide dismutase extraction

The extracting buffer was 0·05M sodium phosphate at pH 7·8. Soluble solids were extracted from white cabbage by homogenising one part cabbage with two parts (w/v) ice-cold extracting buffer. The homogenate was filtered through a double layer of muslin, then centrifuged at 17 500 *g*, 4°C, for 25 min. The supernatant was filtered through a Whatman No. 1 paper, then frozen at −18°C until required, when it was dialysed against extracting buffer containing 0·1 mM EDTA.

Superoxide dismutase assay

Samples were assayed for SOD activity by the photochemical method, in which nitroblue tetrazolium (NBT) was reduced to a blue formazan by superoxide anions (Beauchamp & Fridovich, 1971). The method was modified by replacing methionine with tetramethylethylenediamine (TEMED) in the superoxide generating system. The reaction mixture contained riboflavin ($2{\cdot}34 \times 10^{-6}$ M), TEMED ($4{\cdot}3 \times 10^{-3}$ M), NBT $1{\cdot}12 \times 10^{-4}$ M), sodium phosphate (0·05 M) and EDTA (1×10^{-4} M) at pH 7·8 and room temperature. 100 μl of sample were added to the reaction

mixture in a cuvette to bring the final volume to 3 ml. Cuvettes were illuminated for 6 min in a foil lined box by a 30 W fluorescent light strip. The absorbance at 560 nm was measured before and after the period of illumination, and ΔA_{560}/6 min was calculated. The rate of increase in absorbance was linear for the first 10 min of reaction. Control measurements were made by replacing the sample with 100 μl buffer.

Heat treatment

For the heat treatments 0·2-ml aliquots were pipetted into test tubes previously equilibrated in a waterbath at the appropriate temperature. After the required period of heating the test tubes were quickly placed on ice and 0·6 ml of ice cold buffer added to each. 100 μl of each mixture was assayed for SOD activity.

Isoelectric focusing

Flat bed isoelectric focusing was carried out on an LKB Ultrophor Electrofocusing Unit using 5% polyacrylamide gels (LKB Technical Publication). Power was supplied by an LKB 2197 Power Supply Unit and the plate was cooled to 10°C using the LKB 2209 Multitemp temperature control unit. The gels were prepared by mixing 29% w/v aqueous acrylamide solution (2·8 ml), 0·9% w/v aqueous bisacrylamide solution (2·8 ml), Ampholine carrier ampholytes, pH range 4–6·5 (1·2 ml), TEMED (8 μl) and distilled water (9·6 ml). The gels were polymerised with ammonium persulphate, 0·4 ml of 2% w/v aqueous solution and allowed to set for 90 min. Orthophosphoric acid (1 M) and sodium hydroxide (1 M) were used for the anode and cathode solutions, respectively. Samples were applied to the gel by means of absorbant paper strips placed 35 mm from the cathode. Isoelectric focusing was carried out for 120 min with maximum settings of 20 W, 20 mA and 1600 V. For visualising SOD activity, the staining method of Beauchamp & Fridovich (1971) was slightly modified by halving the NBT concentration. The gels were illuminated for up to 40 min, during which time they became dark blue except for the colourless zones which marked the position of the SOD enzymes.

Gels were stained for peroxidase activity using *o*-dianisidine as described by Gkinis & Fennema (1978).

Cyanide inhibition of superoxide dismutase

For testing for inhibition by cyanide, polyacrylamide disc gel electrophoresis was carried out by the method of Davis (1964). Staining reagents for cyanide inhibition experiments contained 2 mM NaCN.

RESULTS AND DISCUSSION

Most of the work already carried out on the thermal stability of SOD has only been with purified bovine SOD (McCord & Fridovich, 1969*b*; Forman & Fridovich, 1973; Hicks *et al.*, 1979 and Korycka-Dahl *et al.*, 1979). For this reason a commercially available, purified sample of bovine SOD was included in the present investigation for comparative purposes.

Enzyme assay and quantification

The nitroblue tetrazolium (NBT) assay was chosen over the more widely used cytochrome c assay (McCord & Fridovich, 1969*a*), as we have found xanthine oxidase inhibitors and cytochrome c oxidants to be present in the crude cabbage extract in quantities which interfered with the cytochrome c assay. However, crude extracts of cabbage were found to be free of substances capable of either oxidising the blue formazan, reducing NBT or generating O_2^- in quantities sufficient to interfere with the NBT assay. In the presence of fluorescent light and TEMED the riboflavin is photoreduced and superoxide anion radicals are generated. These radicals reduce NBT to a blue formazan at a rate which is linear for the period of the assay. In the presence of SOD the superoxide anion radicals undergo enzyme-catalysed dismutation in preference to reducing NBT and hence the rate of production of formazan is reduced by SOD. By the inclusion of

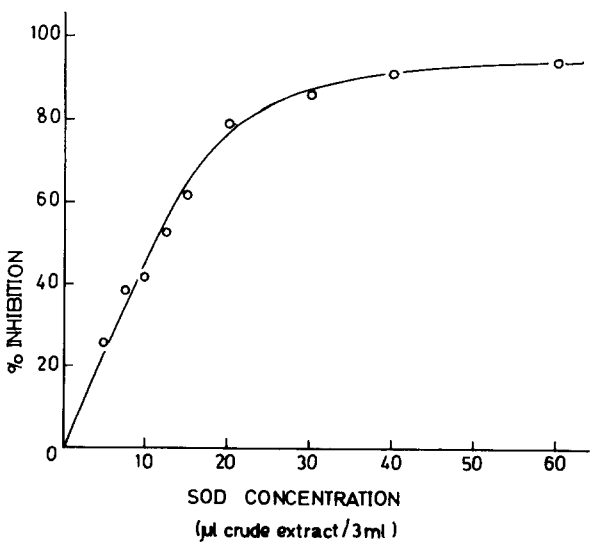

Fig. 1. Effect of crude extract of cabbage on the production of blue formazan from NBT reduced by O_2^-.

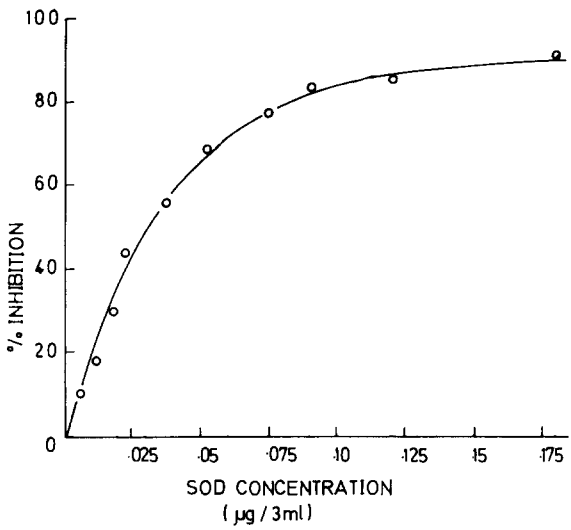

Fig. 2. Effect of purified bovine SOD on the production of blue formazan from NBT reduced by $O_2^{\cdot-}$.

2×10^{-5} M cyanide in an assay, which is sufficient to inhibit peroxidase but does not affect SODs (Beauchamp & Fridovich, 1971), it was found that the measured SOD activity was not sensitive to the presence of 2×10^{-5} M cyanide. It was therefore concluded that SOD measurements made of the extract were not affected by the presence of peroxidase.

Percentage inhibition of formazan production for both the crude cabbage extract and the purified bovine SOD was approximately linear with the concentrations of either cabbage extracts or purified bovine SOD up to 50% inhibition. Beyond 50% inhibition by SOD, the relationship between apparent enzymic activity and concentration was markedly non-linear, as seen in Figs 1 and 2, and in this respect was similar to results reported by other workers (McCord & Fridovich, 1969; Sawada & Yamazaki, 1973; Baker, 1976). These calibration graphs (Figs 1 and 2) were used to quantify the amounts of residual SOD activity still present in samples assayed after heat treatment, which were then expressed as a percentage of the amount of enzyme present before heat treatment, to give the values for 'percentage activity remaining' (Figs 3, 4 and 5).

Thermal stability

As can be seen in Fig. 3, 2 minutes' heating at 70°C dstroys virtually all of the SOD activity in the cabbage extract. At 60°C, there appears to be a biphasic relationship between enzyme inactivation and heating time. The

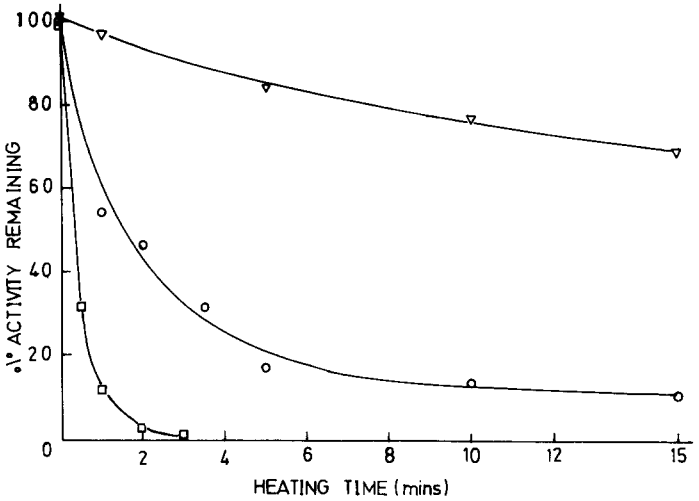

Fig. 3. Heat inactivation of crude cabbage extract SOD. ▽—▽ Heating at 50°C. ○—○ Heating at 60°C. □—□ Heating at 70°C.

biphasic form of the heat inactivation curve is less obvious in the case of the purified bovine enzyme (Fig. 4), which is slowly deactivated at 70°C and 75°C. Various sources (McCord & Fridovich, 1969*b*; Forman & Fridovich, 1973; Hicks *et al.*, 1979) have quoted bovine SOD as being stable at 70°C for periods of up to 30 min. Here we have used small quantities of enzyme solution which quickly attained the desired temperature and found that 60% of the bovine enzyme was inactivated after 30 min at 70°C.

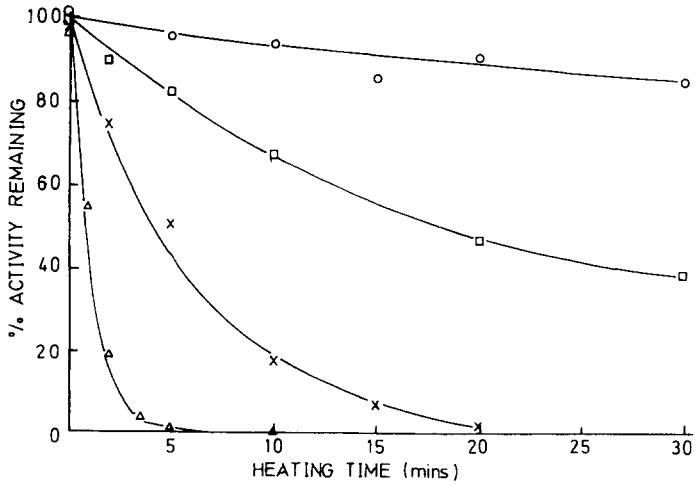

Fig. 4. Heat inactivation of purified bovine SOD. ○—○ Heating at 60°C. □—□ Heating at 70°C. × — × Heating at 75°C. △—△ Heating at 80°C.

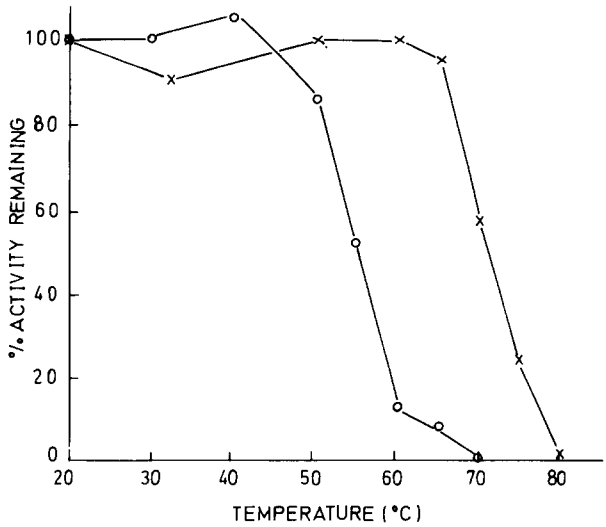

Fig. 5. Effect of temperature on crude cabbage extract SOD and purified bovine SOD. ○—○ Cabbage extract heated for 10 min. × — × Purified bovine SOD heated for 10 min.

Both the bovine and cabbage forms of SOD were heated for 10 min at various temperatures to obtain temperature profiles (Fig. 5). Purified bovine SOD was stable up to 65°C, but the cabbage extract SOD activity underwent deactivation at temperatures greater than 45°C. No residual enzymic activity was found after heating to 80°C and 70°C for the bovine and cabbage extract SODs, respectively.

Comparative stabilities of the different types of SOD at different temperatures are shown in Table 1.

The activation energies (Table 2) for the heat inactivation of the enzymes, calculated from the results in Figs 3 and 4 using Arrhenius plots of $1/T$ versus ln (rate constant), confirmed that the cabbage SOD enzymes were more heat-labile than the purified bovine SOD.

TABLE 1
Half life of SOD at Various Temperatures ($t_{1/2}$)

Temperature (°C)	*Cabbage extract* (*min*)	*Purified bovine SOD* (*min*)
50	>30	
60	1·5	>30
70	0·25	18·5
75	—	4·5
80	—	1·0

TABLE 2
Activation Energy for the Thermal Inactivation of SOD

Energy	E_a ($kJ\ mol^{-1}$)
Cabbage SOD	215
Bovine SOD	250

Isoelectric focusing

Superoxide dismutases from various sources (Beauchamp & Fridovich, 1971; Weisinger & Fridovich, 1973; Giannopolitis & Ries, 1977) have been detected using gel electrophoresis coupled with an enzyme-specific staining technique (Beauchamp & Fridovich, 1971). However, isoelectric focusing has the advantage of concentrating the protein zones, thus allowing for greater resolution of isoenzymes and the detection of smaller amounts of enzyme. The technique also confirms that high molecular weight compounds with isoelectric points, proteins, for example, are responsible for catalysis of the dismutation reaction.

Isoelectric focusing (Fig. 6) revealed the presence of three SOD isoenzymes in the crude cabbage extract and three in the purified bovine SOD.

Heat treatment of the bovine SOD to destroy 50% of the enzymic activity, as measured by assay, followed by isoelectric focusing, resulted in the loss of activity at the position of isoenzyme 1 (Fig. 6(ii) and (v)). Heating to destroy 90% of the SOD activity resulted in a further loss at the position of isoenzyme 3 (Fig. 6(iii)).

No SOD activity was seen after isoelectric focusing of bovine or cabbage samples that had been heated to destroy all activity detectable by assay (Fig. 6(iv) and (x)).

Heating of the cabbage extract to destroy 50% of the SOD activity, followed by isoelectric focusing, resulted in the loss of activity at the position of isoenzyme 3, and a reduction in the other bands of SOD activity (Fig. 6(vii)). Isoelectric focusing of unheated cabbage extract (Fig. 6(vi)), shows bands 1 and 2 to be of similar size and intensity; however, isoenzyme 2 is more thermostable and retains some activity after heat treatment to destroy 90% of the SOD activity (Fig. 6(viii) and (ix)).

Inhibition by cyanide

CuZn-SOD can be distinguished from the Mn-SOD and the Fe-SOD by the inhibition of only CuZn-SOD in the presence of millimolar levels of

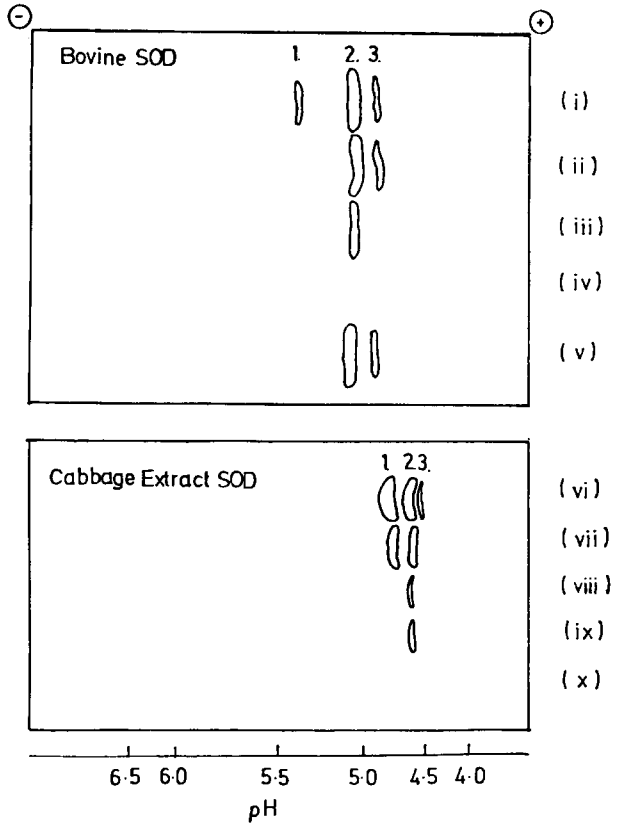

Fig. 6. Isoelectric focusing of superoxide dismutases. Gels were stained dark blue with the exception of colourless zones indicating SOD activity. (i) Purified bovine SOD (0·5 μg sample) unheated. (ii) Purified bovine SOD (0·5 μg sample) heated 1 min at 80°C. (iii) Purified bovine SOD (0·5 μg sample) heated 3 min at 80°C. (iv) Purified bovine SOD (0·5 μg sample) heated 5 min at 80°C. (v) Purified bovine SOD (0·5 μg sample) heated 20 min at 70°C. (vi) Cabbage extract (40 μl sample) unheated. (vii) Cabbage extract (40 μl sample) heated 1 min at 60°C. (viii) Cabbage extract (40 μl sample) heated 15 min at 60°C. (ix) Cabbage extract (40 μl sample) heated 1 min at 70°C. (x) Cabbage extract (40 μl sample) heated 5 min at 70°C.

cyanide (Weisinger & Fridovich, 1973). Electrophoresis of the cabbage extract on polyacrylamide gels and subsequent staining for SOD revealed two main bands of SOD activity, both of which were inhibited by the inclusion of 2 mM NaCN in the staining reagents (Fig. 7). Addition of 1 mM NaCN to the NBT assay of crude cabbage extract resulted in a 99% loss of activity which indicated that the detectable SOD activity in the cabbage extract was due to the CuZn-SOD type of enzyme.

Isoelectric focusing gels, and disc electrophoresis gels of the cabbage extract tested for peroxidase activity, showed that observed bands of SOD activity were not caused by peroxidatic oxidation of the blue formazan. It

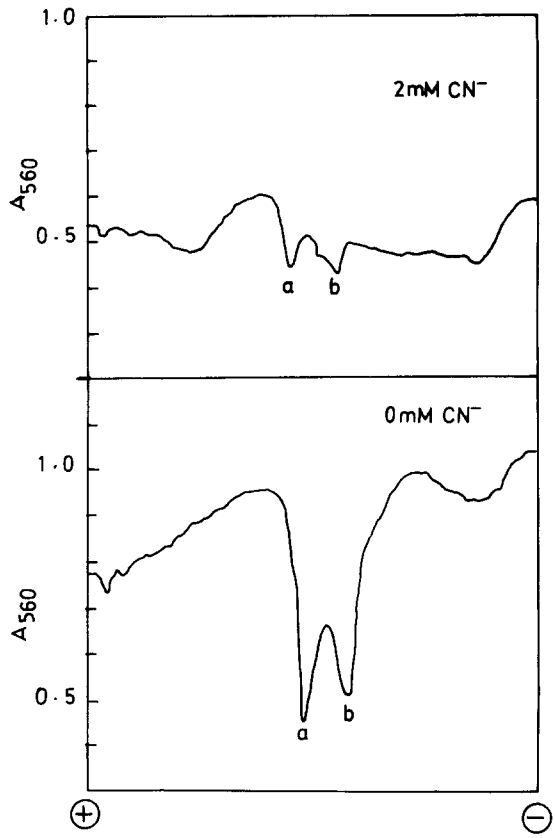

Fig. 7. Densitometer scans of electrophoresis gels showing the inhibition by cyanide of two bands (a and b) of cabbage superoxide dismutase activity. 40 μl of cabbage extract was applied to the gels.

was found that SOD and peroxidase bands did not coincide on either type of gel; therefore, peroxidases were not mimicking SOD activity on the gels stained for SOD.

On heating the crude extract of cabbage for 5 min at 70°C, 100% of the original SOD activity is destroyed, which therefore establishes the enzymic nature of the measured SOD activity in the crude extract. Different isoenzyme patterns of SOD activity resulted from heat treatments that involved different time/temperature combinations calculated to give the same degree of enzyme inhibition. This may arise because some of the cabbage SOD isoenzymes are more susceptible to lower temperatures for longer times. However, as the method of detecting SOD activity by isoelectric focusing is not quantitative, the differential thermal stability of the various isoenzymes cannot be determined without separation of the isoenzymes.

CuZn-SODs are widely reported to be inhibited by millimolar levels of cyanide (Rotilio *et al.*, 1972; Weisinger & Fridovich, 1973) and this was found to be the case with cabbage SOD enzymes separated by electrophoresis (Fig. 7). However, when the same extract was separated by isoelectric focusing, 20 mM cyanide in the staining reagents had no effect on the SOD enzymes, and the enzymic activity patterns were identical to those stained in the absence of cyanide. It was considered that the lack of effect of cyanide in the isoelectric focusing gels was probably due to the use of ampholytes in the gels, which seemed to prevent the cyanide ion from combining with the active site of the CuZn-SOD enzymes.

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