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Isolation and Purification of Superoxide Dismutase Purified from Brussels Sprouts (*Brassica oleracea* L. var. *bullata* sub var. *gemmifera*)

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ABSTRACT

*The isoenzymes from Brussels sprouts (*Brassica oleracea *var.* gemmifera*), were characterised as CuZn–SOD by molecular weight, metal ion content and inhibition by cyanide.*

Isoelectric focusing (IEF) showed Brussels sprouts superoxide dismutase to consist of three isoenzymes. The two major isoenzymes, pI 4·5 and 4·7, A and B of Brussels sprouts SOD were separated and shown to possess different thermal inactivation properties. A subunit (approx. MW 16 000) for isoenzyme B has been detected, whereas for isoenzyme A two possible subunits (MW 16 000 and 19 000) may have been detected. Isoenzyme A was more stable to heat than isoenzyme B.

INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) is an oxidoreductase enzyme, but unusual in that the substrate is an unstable free radical, $O_2^{\cdot-}$. The reaction catalysed is as follows:

$$2O_2^{\cdot-} + 2H^+ \rightleftharpoons O_2 + H_2O_2$$

The biochemical role of the enzyme in animals, plants and micro-organisms is generally accepted to be protection of the living cell from oxygen toxicity caused by reactive oxygen species, which include superoxide

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radicals, hydrogen peroxide and the very reactive hydroxyl radical ($OH^{\cdot}$). Normally in living cells the H_2O_2 product of the dismutation reaction would be removed through the actions of catalase or peroxidase. However, for foods where the cellular structure breaks down during post-mortem and postharvest changes and particularly for comminuted foods and processed foods, there is less certainty that all these enzymes will remain under cellular control or retain their activity.

Previously we have reported (Walker *et al.*, 1987) SOD activity in crude extracts of cabbage (*Brassica oleracea* var. *capitata*). Inhibition by cyanide indicated the presence of the CuZn–SOD and analytical isoelectric focusing revealed the existence of three isoenzymes in the crude cabbage extracts. The SOD activity of the extract was relatively stable to heat, though less stable than a commercially purified bovine CuZn–SOD. In this communication we report the detection of separate SOD isoenzymes from Brussels sprouts (var. *gemmifera*) and isolation of the two major SOD isoenzymes.

MATERIALS AND METHODS

Extraction of SOD

Brussels sprouts were purchased from Leeds market.

Raw material was washed before homogenisation with chilled buffer (0·05M sodium phosphate, pH 7·8) in the ratio 1 g:2 ml. The homogenate was filtered through muslin, then centrifuged to remove cell debris. The supernatant was dialysed at 4°C against extraction buffer containing 0·1 mM EDTA.

Enzyme assay

Solutions were assayed for SOD by the riboflavin/nitroblue tetrazolium (NBT) assay (Beauchamp & Fridovich, 1971), modified as described previously (Walker *et al.*, 1987). One unit of SOD activity was defined as that contained in the amount of sample required in the assay to inhibit the production of formazan by 50%. As reported previously (Walker *et al.*, 1987) the inhibition of production was non-linear with enzyme concentration beyond 50% inhibition by SOD. A calibration curve was used, in order to quantify the amounts of SOD activity.

Protein assay was carried out by the method of Lowry (Layne, 1957).

Electrophoresis

Polyacrylamide disc gel electrophoresis (PAGE) was carried out by the method of Davis (1964) using Tris–glycine buffer at pH 8·3. SOD activity was

visualised on the gels by the riboflavin/NBT staining method of Beauchamp & Fridovich (1971). Protein subunits were observed and molecular weights calculated after SDS–PAGE on 10% polyacrylamide gels.

Analytical IEF was carried out in thin-layer (0·5 mm) polyacrylamide gels containing ampholytes (pH 4·0–6·5) as described previously (Walker *et al.*, 1987). Focused gels were stained for SOD activity as described above. Gels were stained for protein with Coomassie Blue R250 (0·1% w/v in 25% ethanol and 8% acetic acid) after immersion for 30 min in a fixing solution (sulphosalicylic acid, 3·5% w/v and trichloroacetic acid, 11·5% w/v). Destaining was carried out in ethanol (25% v/v) and acetic acid (8% v/v) for 15 min and finally overnight in several changes of destaining solution. Gels were stained for peroxidase activity by the *o*-dianisidine method described by McLellan & Robinson (1987).

Isolation of isoenzymes

Ammonium sulphate precipitation

Ammonium sulphate (243 g/litre) was added to the centrifuged and dialysed extract of Brussels sprouts to give 40% salt saturation. Precipitated protein was removed by centrifugation and further amounts of ammonium sulphate (390 g/litre) added to the supernatant to give 60% salt saturation. After equilibration the precipitate was collected by centrifugation, dissolved in a minimum volume of water and dialysed overnight at 4°C against a glycine solution (1% aq.) in preparation for preparative isoelectric focusing.

Preparative isoelectric focusing

The dialysed solution of partially purified SOD was incorporated into a slurry containing Ultrodex gel (from LKB), 4% w/v, and Ampholine carrier ampholytes, pH 4·0–6·5, 5% v/v. In a shallow plastic tray, 110 mm × 240 mm × 10 mm deep, the gel slurry was dried under a stream of warm air until the moisture content specified by the manufacturers was attained. Paper electrode strips moistened with *o*-phosphoric acid (1M) for the anode, and sodium hydroxide (1M) for the cathode, were placed at either end of the gel bed. These were supported by paper strips soaked in Ampholine solution (5% w/v). Focusing was carried out with maximum settings of 1170 V, 14 mA and 8 W for 24 h. The gel bed temperature was maintained at 10°C. After completion of focusing a metal grid was inserted into the gel bed, dividing the gel into 30 segments parallel to the electrode strips. Proteins were eluted from the separate gel segments with approximately 5 ml of phosphate buffer, 0·05M, pH 7·8, 0·1 mM EDTA. Eluents containing SOD activity of >900 units/ml were combined and subjected to gel filtration.

Separate Brussels sprouts SOD isoenzymes were obtained at the stage of preparative IEF. Individual gel segment eluents, shown by analytical IEF to contain a single isoenzyme, were separately subjected to gel filtration as described below.

Gel filtration
Solutions were applied to a column of Sephadex G-75, 16 mm i.d. × 600 mm, connected serially to a similarly packed column of the same dimensions. This configuration gave the most efficient separation. The columns were equilibrated and eluted with phosphate/EDTA buffer as above. Fractions containing SOD activity of >1000 units/ml were combined to provide a purified solution of SOD isoenzymes.

Enzyme characterisation

The metal ion content of the purified *Brassica* SOD enzymes in solution was determined by atomic absorption spectroscopy, using a Pye-Unicam SP90A atomic absorption spectrophotometer.

The molecular weight of the whole enzyme was determined by gel filtration on Sephadex G-75, using bovine albumin (MW 67 000), bovine β-lactoglobulin (MW 36 500), myoglobin (MW 17 800) and cytochrome c (MW 12 400) as molecular weight markers.

Cyanide inhibition

Solutions of purified SOD were assayed for enzyme activity by the riboflavin/NBT assay in the presence and absence of sodium cyanide (1 mM). PAGE gels of purified SOD were stained for enzyme activity in the presence and absence of sodium cyanide (2 mM).

Heat stability

Heat treatments were carried out in 0·05 M sodium phosphate buffer (pH 7·8) containing 0·1 mM EDTA, as described by Walker *et al.* (1987). The units of SOD activity present after heat treatment were calculated and expressed as a percentage of the units present in an unheated sample, to obtain the values for '% activity remaining'.

RESULTS

Analytical isoelectric focusing (IEF) of crude extracts of Brussels sprouts showed that the SOD activity was due to the presence of three isoenzymes in

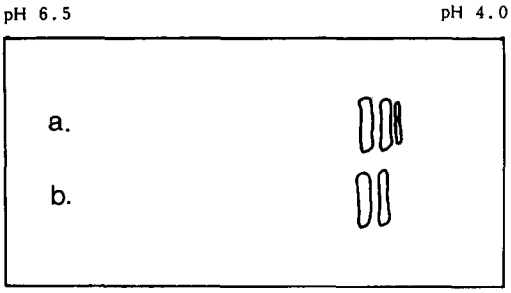

Fig. 1. Diagram of focused analytical IEF gels. (a) Brussels sprouts extract, 40 μl; (b) purified Brussels sprouts SOD solution, 10 μl. Areas of SOD activity appeared colourless against a purple-blue background.

Brassica species (Fig. 1). The isoenzyme distribution pattern is very similar to that previously reported for SOD in cabbage extracts. As shown in Fig. 1 it seems that the two most significant isoenzymes were present in approximately equal amounts and the third isoenzyme with a slightly lower isoelectric point was a minor component. As found previously for the cabbage extract (Walker *et al.*, 1987), staining for peroxidase activity of Brussels sprouts extract after isoelectric focusing showed that focused peroxidases and SOD isoenzymes did not coincide, and therefore the SOD activity observed was not due to the mimicking activity of peroxidases (Giannopolitis & Ries, 1977).

A summary of the results for the purification procedure for Brussels sprouts SOD isoenzymes is presented in Table 1, where the results are averages of the purification of several batches of material and are based on a starting material fresh weight of 500 g per batch.

Preparative IEF, in a granulated gel bed, resulted in the focusing of 95% of the recoverable SOD activity in the two major isoenzyme bands.

TABLE 1
Purification of Superoxide Dismutase from Brussels Sprouts

Purification	*SOD activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor (-fold)*
Crude extract (500 g)	285 000	2 190	130	100	1·0
Ammonium sulphate precipitate	156 800	580	270	55	2·1
Preparative IEF (pH 4·0–6·5)	74 680	33	2 263	26	17·4
Gel filtration (Sephadex G-75)	67 220	14	4 801	24	36·9

However, attempts to recover the minor third isoenzyme were not successful due to the inclusion of extraneous protein. The solutions containing SOD activity had negligible peroxidatic activity as measured by the *o*-dianisidine assay for peroxidase (McLellan & Robinson, 1981). The purified SOD enzyme solution consisted of two isoenzymes, with pI values of pH 4·5 and pH 4·7 (Fig. 1(b)).

After isoelectric focusing on the analytical gels the location of protein bands coincided with the location of SOD activity and only traces of protein not associated with SOD were observed for the preparations of purified Brussels sprouts SOD isoenzymes after PAGE electrophoresis. The isoenzymes were named A and B, with the isoenzyme having the lower, more acidic isoelectric point as isoenzyme A. After preparative isoelectric focusing, analytical IEF of the two separated isoenzymes showed that each purified isoenzyme was not contaminated with the other.

Enzyme characterisation

The UV spectrum of isoenzyme A was characteristic of a CuZn–SOD in lacking an absorbance peak at 280 nm, whereas isoenzyme B, typical of a protein, showed the characteristic spectrum of constituent aromatic amino acids. Similar differences have been reported for SOD isoenzymes isolated from pea leaves (Duke & Salin, 1983).

The inclusion of cyanide (1 mM) in the NBT assay resulted in the complete inhibition of the activity of the purified SOD activity indicating that enzymatic activity was due to CuZn–SOD enzymes. Mn–SOD and Fe–SOD are unaffected by this concentration of cyanide (Asada *et al.*, 1975). Inclusion of cyanide (2 mM) in the staining reagents gave complete inhibition of all enzymic activity on PAGE gels of purified Brussels sprouts isoenzymes. Analysis by atomic absorption spectroscopy showed the presence per mole of approximately 1·1 copper atom and approximately 1·3 zinc atom in the purified SOD. The molecular weight determined by gel filtration was approximately 31 000 for purified Brussels sprouts SOD. This value is consistent with those found (31 000–35 000) by other workers for CuZn–SOD from a variety of sources (Steinman, 1982).

PAGE gels of the separated isoenzyme, as described by Davis (1964) and stained for SOD activity, showed single bands of enzymic activity which corresponded to areas stained for protein with Coomassie Blue.

For the detection of protein subunits, SDS–PAGE of separated isoenzymes showed the isoenzyme A (pI 4·5) preparation to contain two proteins of equal staining intensity with molecular weights of approximately 16 000 and 19 000 plus two minor proteins of higher molecular weight (Fig. 2). The 16 000 component was probably a monomer of isoenzyme A

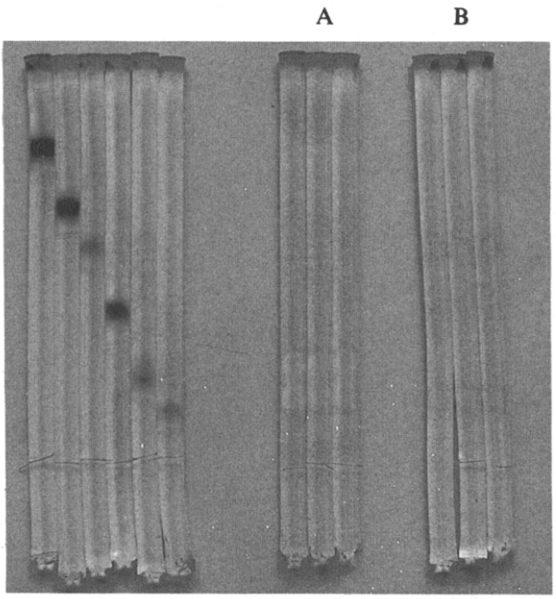

Fig. 2. Triplicate SDS–PAGE 10% gels. Solutions of the isoenzymes A and B were held at 37°C for 2 h prior to electrophoresis in phosphate buffer at pH 7·0 containing 0·035M SDS and 0·143M 2-mercaptoethanol, 6M urea. Protein was stained with 0·25% Coomassie Brilliant Blue R250 in 40% methanol and 7% acetic acid.

(MW 31 000). However, the presence of an apparent equivalent amount of a 19 000 component is more difficult to explain unless isoenzyme A contains two non-identical subunits. For isoenzyme B, SDS–PAGE showed the presence of one major component (approx. MW, 16 000) plus two minor proteins of higher molecular weight.

The thermal inactivation curves for the individually purified isoenzymes illustrated the differences in their thermal stability (Fig. 3). Isoenzyme B was completely denatured after 5 min at 60°C, whereas isoenzyme A had 50% of its original activity remaining after the same heat treatment. A trace of isoenzyme A activity (7%) remained after a further 25 min at this temperature (Fig. 3). The thermal inactivation curve at 60°C for the purified Brussels sprouts SOD isoenzymes obtained after gel filtration approximated to the average of the two heat inactivation curves for isolated isoenzymes A and B, indicating that the two isoenzymes may be present in approximately equal proportions in the purified SOD. The enzymatic activity was not found to regenerate after heat treatment.

DISCUSSION

Extracts of SOD activity purified from Brussels sprouts, like cabbage (Walker *et al.*, 1987), were found to consist of three isoenzymes. The SOD

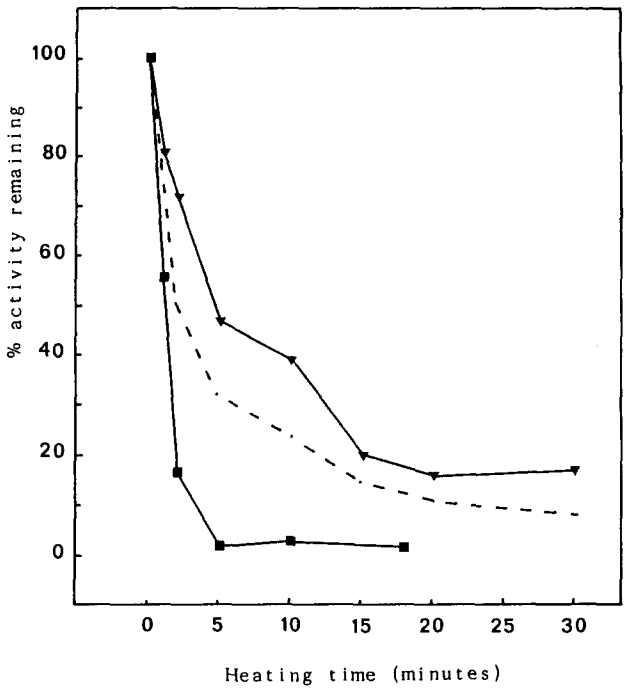

Fig. 3. Relative thermal stability of Brussels sprouts SOD isoenzymes. Aliquots (0·2 ml) were heated at 60°C, cooled and assayed in triplicate. Purified isoenzyme A solution (▼—▼) s.d. = 3·5%, purified isoenzyme B solution (■—■) s.d. = 2·9%, whole purified Brussels sprouts SOD (---●---●) s.d. = 4·1%.

enzymes isolated were characterised as typical of the Cu–Zn type as determined by molecular weight, metal ion composition, and sensitivity to inhibition by cyanide. The homogeneity of the separated SOD isoenzymes A and B was established by IEF and PAGE, which in each case showed the presence of a single band of SOD activity and corresponding protein bands on the gels after electrophoresis. Possible protein subunits of isoenzymes A and B were detected by SDS–PAGE (Fig. 2). For isoenzyme B the subunit molecular weight of 16 000 fits very well to the molecular weight estimated for the whole molecule which from other sources has generally been found to be composed of two identical monomers. For isoenzyme A, the position is less clear as a protein band with a molecular weight of 19 100 was also found as well as the 16 000 subunit on SDS–PAGE gels. The relative sizes of stained areas of both the 16 000 and 19 000 protein components were very similar and therefore it is presently assumed that these may be genuine subunits of isoenzyme A. It seems likely that both isoenzymes A and B from Brussels sprouts contain similar monomers; however, at present it has not been proved that the protein (MW 19 100) present in the isoenzyme B

preparations is a contaminating protein or fragment of the native dimer. There was no evidence that any of the isoenzymes found were of the Mn–SOD or Fe–SOD types. Isoenzymes of CuZn–SOD have been reported from several other sources: wheatgerm (Beauchamp & Fridovich, 1973), bovine blood (Civalleri *et al.*, 1982), mouse (Bloor *et al.*, 1983) and pea leaf (Duke & Salin, 1983).

The two major isoenzymes of Brussels sprouts SOD, when heated separately, showed remarkable differences in their thermostabilities. Isoenzyme A was more resistant to heat than isoenzyme B.

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