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Co-purification of a heat-stable antioxidant with the superoxide dismutase activity from dried peas

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A heat-stable antioxidant component was observed to co-purify with the superoxide dismutase (SOD) activity from dried peas. The heat-stable component co-purified by size (gel filtration on Sephadex G-75) and by charge (flat-bed isoelectric focusing (IEF) and Rotofor IEF) coincidently with the SOD activity. Throughout the purification procedure the SOD fractions all exhibited heat-stable antioxidant activity. The autoxidation of linoleic acid in the presence of the pea SOD fraction was delayed for more than four weeks. Purified pea SOD may provide a novel protein-bound, thermostable natural oxidant.

INTRODUCTION

Lipid oxidation is a major cause of quality changes in foods, both in those with a minor fat content and in those where most of the food is fat. Processing increases the susceptibility of substances such as fats to oxidative deterioration, and the use of antioxidants in processed foods is nowadays commonplace; without them the shelf-life of many foods would be unsatisfactory. The use of synthetic antioxidants such as butylated hydroxytoluene (BHT) is widespread in the food industry. However, since 1985, 'naturalness' has been heavily promoted by the food manufacturers and retailers. This, and the concerns of the consumer, have promoted much research into the area of natural antioxidants. The use of the enzyme superoxide dismutase (SOD) (EC 1.15.1.1) as a natural food antioxidant was first discussed in a patent application by Michelson & Monod (1975).

Commercially available bovine SOD has been shown to act as an antioxidant in a fatty acid model autoxidation system by prolonging the initiation phase of autoxidation (Nice & Robinson, 1992). SOD is known to catalyse the dismutation of the superoxide radical ($O_2^{\cdot-}$) (reaction (1)):

$$O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2 \qquad (1)$$

$O_2^{\cdot-}$ is one of the reactants in the metal-catalysed Haber–Weiss reaction (reaction (2)), which results in the formation of the hydroxyl radical ($OH^{\cdot}$), considered to be ultimately responsible for the initiation of oxidation in the absence of additional pro-oxidants (Miller & Aust, 1989). We have proposed (Nice & Robinson, 1992) that SOD removes $O_2^{\cdot-}$ and prevents it from taking part in the metal-catalysed Haber-Weiss production of $OH^{\cdot}$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Metal}} OH^- + OH^{\cdot} + O_2 \qquad (2)$$

If the $O_2^{\cdot-}$ scavenging and antioxidant property of SOD is to be exploited commercially, an inexpensive form of relatively stable enzyme must be obtained. The present study set out to assess the antioxidant activity of partially purified SOD from dried peas, a relatively cheap home-produced crop. The antioxidant activity was determined throughout the purification procedures to determine the minimum purity required for the SOD fraction to delay the onset of fatty acid oxidation significantly. The thermostability of the preparations was also examined.

MATERIALS AND METHODS

Nitroblue tetrazolium (NBT), diethylenetriaminepentaacetic acid (DETAPAC), riboflavin, Triton X100, linoleic acid (99%) and Tween 20 were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Bovine superoxide dismutase was obtained from BCL (Lewes, East Sussex, UK). Ampholine carrier ampholytes for IEF and Ultrodex gel were obtained from Pharmacia–LKB Ltd (Milton Keynes, UK). All other chemicals were analytical reagent grade.

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Extraction of SOD

Split dried green peas were purchased from the Leeds Market. The dried peas were ground in a coffee mill for two intervals of 10 s and then extracted in sodium phosphate buffer (50 mM, pH 7·8) in the ratio 1 g : 5 ml at 4°C for 2 h. The homogenate was filtered through two layers of muslin and then centrifuged.

Ammonium sulphate precipitation

Ammonium sulphate was added to the centrifuged extract to give 45% saturation at 4°C. Precipitated protein was removed by centrifugation (17 500 *g* for 20 min) and further amounts of ammonium sulphate were added to the supernatant to give 75% salt saturation. After equilibration the precipitate was collected by centrifugation and dissolved in a two-fold volume of sodium phosphate buffer (50 mM, pH 7·8) and held at −18°C.

Enzyme assay

Solutions were measured for SOD activity by the nitroblue tetrazolium/riboflavin assay (Beauchamp & Fridovich, 1971).

The photosensitive assay reagent contained 0·05% (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED), $5{\cdot}6 \times 10^{-5}$ M nitroblue tetrazolium (NBT), $5{\cdot}85 \times 10^{-7}$ M riboflavin, 0·01% (wtv) Triton X100 in sodium phosphate buffer containing 1 mM diethylenetriaminepentaacetic acid (DETAPAC).

The photosensitive assay solution was dispensed into a 4 ml standard disposable plastic cuvette, containing the sample or standard to be measured (0–60 μl), to give a total volume of 3 ml. The sample cuvettes were placed in a 24-slot cuvette rack interspaced with a corresponding control containing only assay solution, and then the rack was placed in a foil-lined lightbox (64 cm × 25 cm) and illuminated for 6 min by two 20 W fluorescent strips placed 8 cm apart.

The absorbance at 560 nm was measured before and after the 6 min illumination period. The results were expressed in μg equivalents of pure bovine SOD, by first constructing a standard calibration curve by including varying amounts of commercial bovine SOD in the assay mixture. The assay was performed in triplicate for each standard or sample to be measured. The calibration curve was linearised by the method of Asada *et al.* (1974).

Protein assay was carried out by the method of Bradford (1976).

Purification by gel filtration

A 5 ml aliquot of the extract obtained from ammonium sulphate fractionation was applied to a Sephadex G-75 column (16 mm i.d. × 580 mm). The column was equilibrated and eluted with sodium phosphate buffer (50 mM, pH 7·8) at a flow rate of 18 ml h^{-1}. Fractions (4 ml) were collected and the SOD activity and protein content were measured.

Purification by flat-bed isoelectric focusing

The fractions from the Sephadex G-75 column containing SOD activity were pooled and concentrated to 2 ml by ultrafiltration using an Amicon PM10 membrane. The concentrate was dialysed against a 100-fold volume of glycine (1% aq.).

The dialysed solution of partially purified SOD was incorporated into a slurry containing Ultrodex gel (4%, w/v), and Ampholine carrier ampholytes, pH 4·0–6·0 (5%, v/v). In a shallow plastic tray, 100 mm × 240 mm × 10 mm deep, the gel slurry was dried under a stream of air until 38% of the water had been removed. Electrode strips moistened with sodium hydroxide (1 M) for the cathode, and ortho-phosphoric acid (1 M) for the anode, were placed at either end of the gel bed. These were supported by further electrode strips soaked in Ampholine solution (5%, w/v). Focusing was carried out at high power, using the running conditions of 16 W with the limits of 16 mV and 2000 V for 6 h at 4°C.

After completion of focusing a metal grid was placed in the gel bed, dividing the gel into 30 segments parallel to the electrode strips. The pH of each gel segment was determined using a surface-pH electrode, before the proteins were eluted from the separate gel segments with approximately 4 ml sodium phosphate buffer (50 mM pH 7·8). The eluted fractions were measured for SOD activity and protein content.

Purification by Rotofor isoelectric focusing

Ammonium sulphate extract was dialysed overnight against deionised water. Portions (2·5 ml) of Ampholine ampholytes (pH 3–10) were mixed with a 10 ml aliquot of dialysed extract and 37·5 ml of deionised water. The sample was loaded into the chamber and focused for 5 h at a constant power of 12 W as directed in the BioRad Rotofor operation manual. The fractions containing SOD activity were pooled and re-run in the Rotofor for 4 h at 12 W, and then after collection were re-run for a further 3 h at 12 W.

Autoxidation model system—assessment of antioxidant activity

A solution of linoleic acid (2·5 mM) was prepared by emulsification with Tween 20 (0·5%) in sodium phosphate buffer (50 mM, pH 7·8). Aliquots (20 ml) were placed in clean, disposable plastic bottles and the test sample was added. The bottles were incubated open to the air, in the dark. The oxidation of linoleic acid was followed by measuring the $OD_{234\ nm}$ of a 100 μl sample of the incubation solution diluted to 2 ml with distilled water. A corresponding control was performed in the absence of test sample.

RESULTS AND DISCUSSION

The antioxidant activity has been measured for pea extracts fractionated by both size (gel filtration) and charge (isoelectric focusing) (IEF). Surprisingly, for each of the two very different fractionation techniques, the results show that while potent antioxidant activity is associated with pea SOD, there is also a thermostable component of the sample which was able to inhibit the autoxidation of linoleic acid.

The elution profile for gel filtration as measured at 280 nm, is shown in Fig. 1. SOD activity was mainly found in fractions 15–18 inclusive, which also showed strong antioxidant activity. Autoxidation of linoleic acid was delayed for more than four weeks in the presence of a 150 μl aliquot of combined fractions 15–18 inclusive. In Fig. 2 the effect is shown for the first three weeks. This antioxidant activity was thermostable, as it was not affected by heat treatment at 100°C for 30 min. Therefore the measured antioxidant activity was not entirely due to the enzymic activity of SOD alone, but could be due to the presence of a heat-stable antioxidant component co-purified by size with the SOD protein.

Fractions 10, 25 and 30, which contained no SOD activity, were also tested in the model autoxidation system. As shown in Fig. 2, fraction 25 possessed virtually no antioxidant activity, whereas in the presence of aliquots (150 μl) of either fractions 10 or 30 an atypically shaped autoxidation curve was observed. Instead of the linear propagation phase observed with the control (Fig. 2), the propagation phase, in the presence of fractions 10 or 30, was non-linear with just over half of the maximum absorbance at 234 nm achieved compared with the control.

Measurements of the protein content of the fractions showed that fractions 10 and 30 contained very different concentrations of protein (9500 and 65 μg ml^{-1}, respectively) but produced similar autoxidation curves. Fraction 25 contained more protein (205 μg ml^{-1}) than fraction 30, but was virtually devoid of antioxidant activity. These results show that antioxidant activity against linoleic acid was not a property of all the pea proteins, but was mainly associated with the SOD-rich fraction and possibly fractions 10 and 30.

The shapes of the autoxidation curves (Fig. 2) in the presence of both fractions 10 and 30 indicate that these fractions contained substances able to decompose lipid peroxy radicals as these propagate the linear phase of autoxidation. As this phenomenon was observed with both the high-molecular-weight fraction (fraction 10) and the low-molecular-weight fraction (fraction 30), we conclude that antioxidant activity is associated with components of different molecular weight. In the case of fraction 10, low-molecular-weight antioxidants may be bound to the higher-molecular-weight proteins.

In view of these unexpected findings, and especially that of co-purification by molecular size of pea SOD with thermostable antioxidant activity, the pea extract was fractionated by isoelectric focusing as an alternative purification technique. After flat-bed preparative IEF, the SOD activity was mainly found in fraction 6, which also possessed strong antioxidant activity. Again both the heated and unheated sample (200 μl) inhibited the initiation phases of the reactions sufficiently to delay the onset of autoxidation by more than four weeks; the inhibition for the first two-week period is shown in Fig. 3. Similar results were obtained with the SOD fractions collected after isoelectric focusing using the preparative IEF Rotofor. In this case both the heated and unheated SOD samples were able to delay autoxidation by more than three weeks (Fig. 4). For control purposes samples (200 μl) of ampholines (0·125%, v/v), tested for antioxidant activity, delayed the autoxidation of linoleic acid for only 2–3 days, after which autoxidation proceeded as for the control.

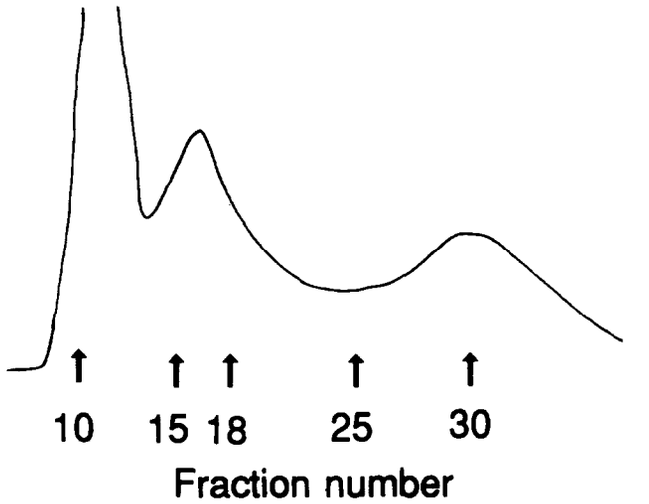

Fig. 1. Gel filtration of the ammonium sulphate extract. The precipitate obtained at 75% saturated ammonium sulphate was dissolved in phosphate buffer (50 mM pH 7·8) prior to gel filtration on Sephadex G-75. Measurements were made for protein at 280 nm.

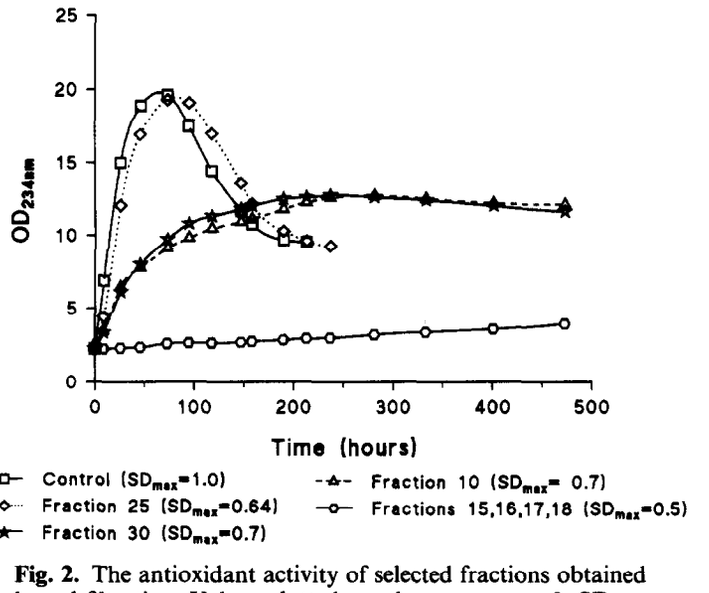

Fig. 2. The antioxidant activity of selected fractions obtained by gel filtration. Values plotted are the means, n = 9. SD_{max}, maximum standard deviation. Fractions 10, 25 and 30 exhibited no SOD activity. Fractions 15, 16, 17 and 18 exhibited strong SOD activity.

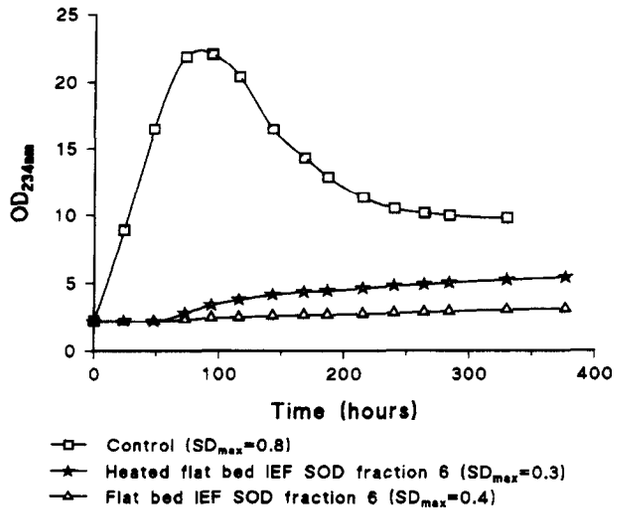

Fig. 3. The antioxidant activity of the SOD fraction from flat-bed preparative IEF. Values plotted are the means, $n = 9$. SD_{max}, maximum standard deviation.

Our results suggest that either the heat-stable antioxidant component has a similar molecular weight and charge to pea SOD or that it was complexed to the SOD protein. However, it is extremely unlikely that a protein of virtually identical molecular size to SOD would also have a pI within 0·5 of that of SOD as determined by IEF.

A wide variety of phenolic substances have been reported to be present in peas, e.g. kaempferol and quercetin (both flavonoids), ferulic acid (a phenylpropanoid), and vanillic acid (a phenolic acid) (Mendez & Lojo, 1971). The antioxidant activity of phenolic compounds is primarily due to their ability to act as hydrogen donors to break the oxidation radical chain reaction. In addition some phenolic compounds also possess $OH^{\cdot}$ scavenging ability (Chimi *et al.*, 1991; Husain *et al.*, 1987), $^1O_2$ quenching ability (Sorata *et al.*, 1984) and iron-chelating ability (Laughton *et al.*, 1989), which could inhibit some of the autoxidation reactions. Phenolic compounds are in general heat-stable and continue to be active antioxidants even after boiling for 30 min. They have been reported to bind strongly to proteins by hydrogen bonding (Haslam, 1974), by hydrophobic interactions (Hoff *et al.*, 1980) and indirectly by the formation of covalent bonds between the protein and the oxidation product of the phenolics (Mason, 1955; Loomis & Battaile, 1966). It is therefore proposed that the heat-stable antioxidant component associated with the pea SOD activity contains at least some phenolics. While we have previously shown that bovine SOD acts as an inhibitor of autoxidation of linoleic acid in a model system, the pea extract containing pea SOD seems also to contain the additional proposed bound phenolic compounds conferring thermostable antioxidant activity. Further investigations are in hand to characterise the thermostable antioxidant component.

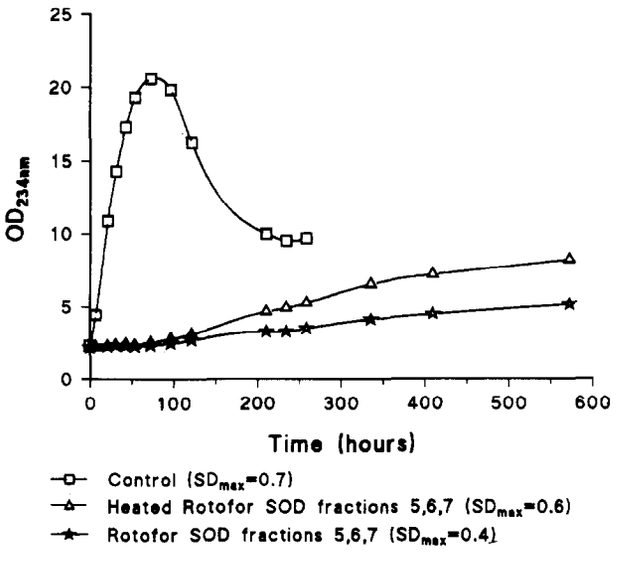

Fig. 4. The antioxidant activity of the SOD fractions from Rotofor preparative IEF. Values plotted are the means, $n = 9$. SD_{max} maximum standard deviation.

Depending on the specificity of binding to SOD protein, co-purification with the SOD activity may provide a means to obtain a novel, highly active and heat-stable natural protein-bound antioxidant.

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REFERENCES

Asada, K., Takahashi, M. & Nagate, M. (1974). Assay and inhibitors of spinach superoxide dismutase. *Agric. Biol. Chem.*, **38**, 471–3.

Beauchamp, C. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analyt. Biochem.*, **44**, 276–87.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analyt. Biochem.*, **72**, 248–54.

Chimi, H., Cillard, J., Cillard, P. Rahmani M. (1991). Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. *J. Am. Oil Chem. Soc.*, **68**, 307–12.

Haslam, E. (1974). Polyphenol–protein interactions. *Biochem. J.*, **139**, 285–8.

Hoff, J. E., Oh, H. I., Armstrong, G. S. & Haff, L. A. (1980). Hydrophobic interactions in tannin–protein complexes. *J. Agric. Food Chem.*, **28**, 394–8.

Husain, S. F., Cillard, J. & Cillard, P. (1987). Hydroxyl radical scavenging activity of flavonoids. *Phytochem.*, **26**, 2489–91.

Laughton, M. J., Halliwell, B., Evans, P. J. & Hoult, J. B. S. (1989). Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem. Pharmacol.*, **38**, 2859–65.

Loomis, W. D. & Battaile, J. (1966). Plant phenolic compounds and the isolation of plant enzymes. *Phytochem.*, **5**, 423–38.

Mason, H. S. (1955). Reactions between quinones and proteins. *Nature (London)* **175**, 771–2.

Mendez, J. & Lojo, M. I. (1971). Phenolic and indole constituents of edible peas. *J. Food Sci.*, **36**, 871–2.

Michelson, A. M. & Monod, J. (1975). Superoxide dismutases and their applications as oxidation inhibitors. US Patent 3 920 521.

Miller, D. M. & Aust, S. D. (1989). Studies of ascorbic-dependent iron-catalysed lipid peroxidation. *Arch. Biochem. Biophys.*, **217**, 113–19.

Nice, D. J. & Robinson, D. S. (1992). Inhibition of lipid autoxidation by bovine superoxide dismutase. *Food Chem.*, **45**, 99–103.

Sorata, Y, Takahama, U. & Kimura, M. (1984). Protective effect of quercetin and rutin on photosensitised lysis of human erythrocytes in the presence of hematoporphyrin. *Biochim. Biophys. Acta*, **799**, 313–17.