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Characterisation of a heat-stable antioxidant co-purified with the superoxide dismutase activity from dried peas

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Heat-stable antioxidant activity co-purified with the pea superoxide dismutase (SOD) activity is believed to be due to bound phenolic compounds. Digestion with trypsin showed that the native SOD protein structure was not required for the expression of the thermostable antioxidant activity. Part of the bound phenolic compounds can be removed from the SOD protein by alkaline hydrolysis. The dissociated phenolic fraction was able to inhibit the autoxidation of linoleic acid by more than one week. Incubation of the phenolic fraction with bovine SOD (BSOD) and serum albumin (BSA) resulted in the transfer of thermostable antioxidant activity to the SOD protein but not to serum albumin.

The results indicate that pea SOD can act as a carrier for certain pea phenolic compounds and thus facilitate a protein-bound thermostable antioxidant.

INTRODUCTION

A heat-stable antioxidant component has been observed to co-purify with the superoxide dismutase (SOD) activity isolated from dried peas (Nice & Robinson, 1993). It was reported that the antioxidant component was co-purified by salt precipitation, by gel filtration and by isoelectric focusing with the pea SOD activity. We proposed that the strong antioxidant activity observed was due to the presence of phenolic compounds bound to the SOD protein.

A wide variety of phenolic compounds has been reported to be present in peas, e.g. kaempferol, quercetin, ferulic acid, vanillic acid (Mendez & Lojo, 1971). In general, phenolic compounds possess antioxidant activity which is predominantly due to their ability to act as hydrogen donors to break the oxidative free radical chain reactions. Some phenolic compounds also possess hydroxyl radical ($\dot{O}H$) scavenging ability (Husain *et al.*, 1987; Chimi *et al.*, 1991; singlet oxygen ($^1O_2$) quenching ability (Sorata *et al.*, 1984) and iron-chelating ability (Laughton *et al.*, 1989), each of which may inhibit some of the oxidation reactions.

Phenolic compounds constitute a distinctive group of higher plant metabolites. Their uniqueness lies not only in their phenolic character and the range of molecular weights they possess, but also in their ability to complex strongly with proteins, certain types of polysaccharides and carbohydrates, nucleic acids and alkaloids.

Phenolic compounds associate with proteins principally by means of hydrogen bonding and hydrophobic interactions (Hoff *et al.*, 1980). At high pH there is the possibility of the formation of ionic bonds with the basic amino acid residues. Covalent bonds may also be formed between the oxidation product of the phenolic compounds and proteins. The highly reactive quinones which result from enzymic and non-enzymic oxidation readily polymerise and condense with reactive groups of proteins (Mason, 1955; Loomis & Battaile, 1966).

The present study was designed to assess the role of the suspected phenolic compounds bound to pea SOD protein (Nice & Robinson, 1993). Hydrolysis with sodium hydroxide was used to partially remove the suspected phenolic compounds from pea SOD protein in order to determine their antioxidant activity and examine their ability to re-associate with either pea or bovine superoxide dismutases.

MATERIALS AND METHODS

Nitroblue tetrazolium (NBT), diethylenetriamine-pentacetic acid (DETAPAC), riboflavin, Triton X100, trypsin, 2,6 dichloroquinone, Folin–Ciocalteau solution, vanillin, linoleic acid (99%) and Tween 20 were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Bovine superoxide dismutase was obtained from BCL

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(Lewes, East Sussex). All other chemicals were analytical reagent grade.

Extraction and the partial purification of SOD from dried peas in the presence of polyvinyl pyrrolidone (PVP)

Split dried green peas were purchased from Leeds Market. The dried peas (100 g) were ground in a coffee mill for two intervals of 10 s then extracted in sodium phosphate buffer (50 mM, pH 7·8) containing either 1% or 10% (w/v) polyvinyl pyrrolidone (PVP) in the ratio 1 g:5 ml for 2 h. The homogenate was filtered through two layers of muslin then centrifuged.

Ammonium sulphate was added to the centrifuged extract to give 45% saturation at 4°C. Precipitated protein was removed by centrifugation and further amounts of ammonium sulphate 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).

A 5-ml aliquot of ammonium sulphate extract 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}. Then 4-ml fractions were collected and the SOD activity and protein content measured. The fractions containing the greatest SOD activity were collected and stored at −18°C.

Enzyme assay

Solutions were measured for SOD activity by the light-induced nitroblue tetrazolium/riboflavin assay (Beauchamp & Fridovich, 1971), as described by Nice and Robinson (1993).

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

Partial removal of the heat-stable antioxidant

Partially purified SOD protein was prepared by gel filtration as above, except that PVP was omitted from the extraction buffer to enhance suspected binding of the phenolic compounds to the SOD protein. Following gel filtration, the 4-ml fractions containing the maximum SOD activity were pooled and concentrated to 3 ml using an Amicon Centriprep Concentrator with a molecular weight cut-off of 10 000. The concentrate was treated for 15 min at ambient temperature with 1 M sodium hydroxide (NaOH) to give a final concentration of 0·1 M NaOH. The solution was then acidified to pH 6·0 with acetic acid and the resulting precipitate removed by centrifugation for 5 min. The supernatant was separated in an Amicon Centriprep Concentrator into a low molecular weight fraction (the filtrate) and a high molecular weight fraction (the retentate).

This procedure was repeated using a final concentration of 0·5 M NaOH for 30 min. The stronger hydrolysis conditions were used in an attempt to remove a greater proportion of the bound phenolic compounds.

Identification of the heat-stable antioxidant

Aliquots (60 μl) of the filtrate from the hydrolysed G-75 SOD fractions were spotted onto a TLC cellulose plate. The spots were sprayed with one of the following chromogenic spray reagents:

- Folin–Ciocalteu (50% (v/v) in water);
- Folin–Ciocalteu (50% (v/v) in water) followed by ammonia fuming;
- 1% (w/v) aqueous ferric chloride/1% (w/v) aqueous potassium ferricyanide;
- 2% (w/v) alcoholic 2,6-dichloroquinone chlorimide (Gibbs reagent) followed by a 20% (w/v) sodium carbonate overspray;
- 10% (w/v) vanillin in concentrated HCl (the vanillin was dissolved in a small quantity of methanol before adding the HCl);
- diazotized *p*-nitroaniline, prepared by dissolving 0·5 g *p*-nitroaniline in 100 ml hot N HCl then cooling in ice before mixing with a solution of sodium nitrite (27 g $litre^{-1}$). Spraying with diazotized *p*-nitroaniline was followed by a 20% (w/v) sodium carbonate overspray.

An identical plate was prepared but before spraying, thin-layer chromatography was carried out with *n*-butanol: acetic acid: ether: water (9:6:3:1) for 4 h to separate the phenolic components.

Digestion of the BSOD protein by trypsin

A 2-ml aliquot of the pooled G-75 SOD fractions prepared in the absence of PVP, was incubated with trypsin at ambient temperature for 2 and 4 days to give a final trypsin concentration of 1 mg ml^{-1}. Following incubation, the samples were tested for antioxidant activity in the autoxidation model system.

Association of the heat-stable antioxidant to BSOD or BSA

A 0·5-ml aliquot of the filtrate from the hydrolysed G-75 peak SOD fractions prepared in the absence of PVP, was incubated with 0·5 ml of either BSOD or BSA (5 mg ml^{-1}) for 1 h.

The samples were applied in turn to a Biogel P-4 column (16 mm i.d. × 300 mm) equilibrated and eluted with distilled water at a flow rate of 18 ml h^{-1}. The first eluted peak containing the protein was tested for antioxidant activity in the autoxidation model system.

Autoxidation system — assessment of antioxidant activity

The autoxidation of an emulsified solution of linoleic acid (2·5 mM) was followed by measuring the absorbtion at 234 nm (Nice & Robinson, 1993).

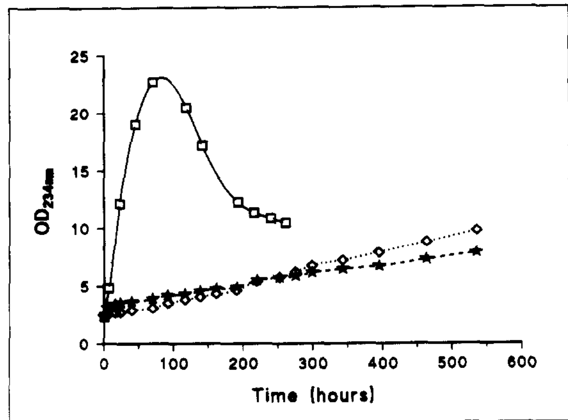

Fig. 1. The antioxidant activity of the gel filtration SOD fractions following extraction in the presence of 10% polyvinyl pyrrolidone. Values plotted are the means, n = 9. SD_{max} = maximum standard deviation.

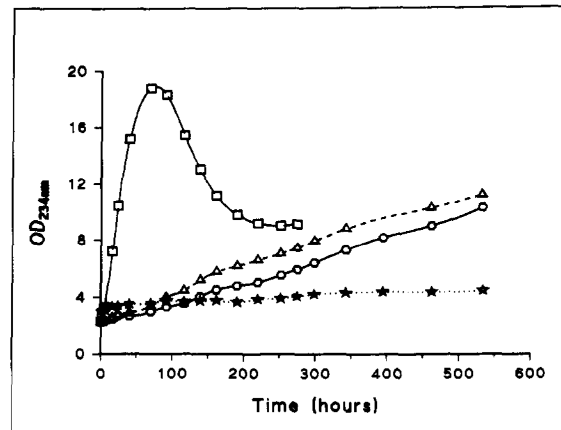

Fig. 3. The antioxidant activity of the filtrate and the retentate following partial hydrolysis of the gel filtration SOD fractions with 0·5 M NaOH for 30 min. Values plotted are the means, n = 9. SD_{max} = maximum standard deviation. The filtrate contains substances with <10 000 molecular weight and the retentate contains substances with >10 000 molecular weight.

RESULTS AND DISCUSSION

The present study was designed to characterise suspected phenolic compounds bound to pea SOD. Hydrolysis with sodium hydroxide was used to partially remove the phenolic compounds from the SOD in order to first test their antioxidant activity and secondly examine their ability to reassociate with proteins and especially bovine superoxide dismutase.

The pooled SOD fractions obtained by gel filtration prepared in the presence of either 1% or 10% PVP showed strong antioxidant activity when added to the linoleic acid autoxidation model system. Heat-inactivation of the samples by boiling in a sealed test-tube for 30 min did not abolish the antioxidant activity as observed for the 10% PVP G-75 fractions in Fig. 1. Although these results may indicate that protein-bound phenolic compounds may not be responsible for the thermostable antioxidant activity, it is known that PVP is not completely effective in preventing protein–phenolic interactions.

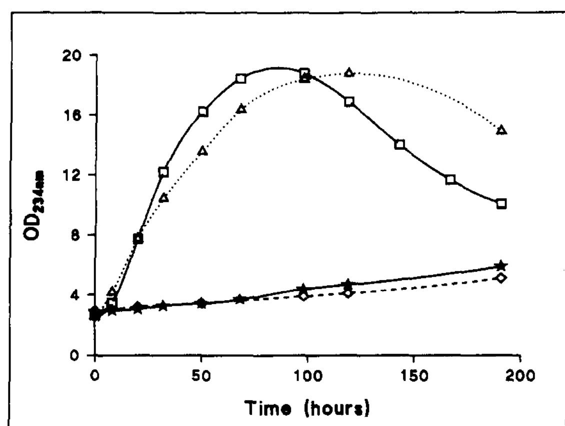

Fig. 2. The antioxidant activity of the 4-day trypsin-treated gel filtration SOD fractions. Values plotted are the means, n = 9. SD_{max} = maximum standard deviation.

The native protein structure of the pea SOD was not required for antioxidant activity as limited proteolysis by trypsin did not diminish the antioxidant activity. As shown in Fig. 2, heated trypsin-treated pea SOD still inhibited the oxidative reaction where heated trypsin itself was inactive.

Autoxidation tests after hydrolysis with 0·5 M NaOH and size separation by ultrafiltration using a PM10 membrane showed that both the filtrate (containing substances with <10 000 molecular weight) and retentate (containing substances with >10 000 molecular weight) possessed strong antioxidant activity (Fig. 3). Thus low molecular weight substances released by NaOH and the retained higher molecular weight protein components were each able to inhibit autoxidation in the test system. As the alkaline hydrolysis was only carried out at room temperature and is a crude method for removal of bound phenolic compounds, or indeed other protein-bound conjugates, then it is not surprising that some of the antioxidant activity remained attached to the protein.

Qualitative analysis of the filtrate has indicated the presence of phenolic compounds. After thin-layer chromatography of the filtrate, all the chromogenic sprays used for the detection of phenolic compounds yielded positive results, including the reaction with acidified vanillin, which produces a red-violet colour and is almost specific for phenolic compounds containing the phloroglucinol or resorcinol-type oxygenation pattern where the substituents are hydroxy or alkoxy groups (Geiger, 1985). In the solvent systems used, the reactive compounds had a very low mobility (Fig. 4) and the streaking observed indicated the possible presence of bound sugars (Fry, 1983).

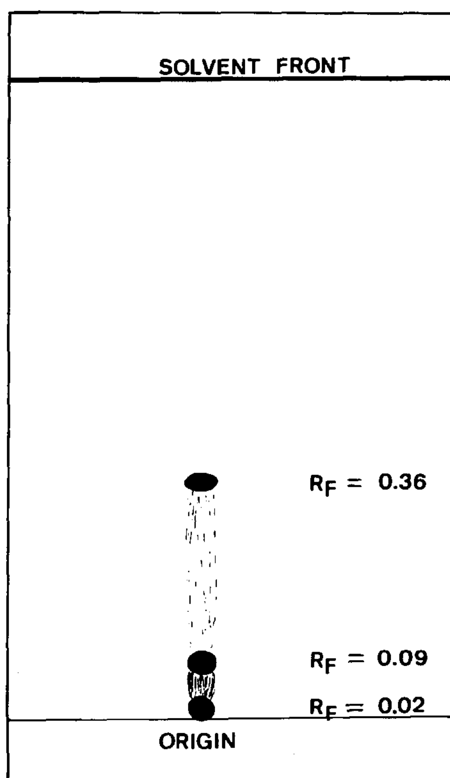

Fig. 4. The TLC pattern of the filtrate from the 0·5 M NaOH-treated gel filtration SOD fractions. TLC was performed in *n*-butanol : acetic acid : ether : water (9 : 6 : 3 : 1) for 4 h. R_F = mobility relative to the solvent front.

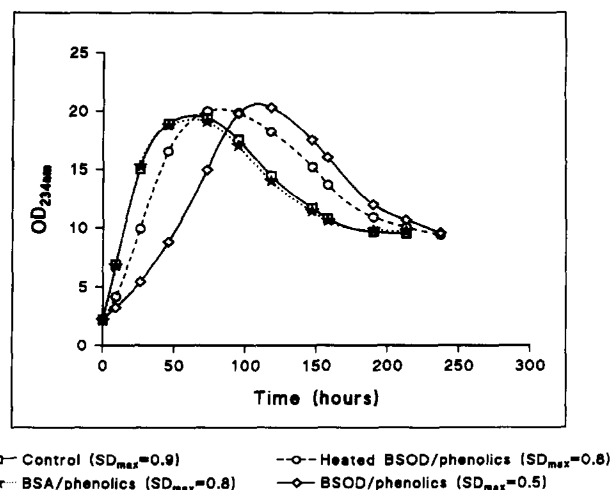

Fig. 5. The antioxidant activity of BSOD and BSA following incubation with the removed pea phenolics. Values plotted are the means, n = 9. SD_{max} = maximum standard deviation. BSA/phenolics and BSOD/phenolics are the BSA and BSOD proteins respectively, following incubation with the removed pea phenolics.

The above results suggest that phenolic compounds bind to pea SOD either prior to or during the isolation of the enzyme. Therefore, in these circumstances we considered that it may be possible to recombine the phenolic compounds present in the alkaline filtrate, obtained by NaOH treatment, with the SOD protein and possibly even other proteins. Indeed tests carried out with bovine SOD have shown that BSOD, previously exposed to the phenolic filtrate, and eluted in the high molecular weight fraction from a Biogel P-4 column, possessed antioxidant activity (Fig. 5). The BSOD/phenolic complex was able to delay the autoxidation of linoleic acid by 1–2 days and heat-inactivation diminished this effect only slightly (Fig. 5). The heat-sensitive antioxidant activity can be attributed to the SOD activity, as BSOD has been reported to delay the autoxidation of linoleic acid (Nice & Robinson, 1992) with no antioxidant activity remaining after heating (Nice, 1992), whereas the heat-stable antioxidant activity observed in Fig. 4 is thought to be due to the presence of bound phenolic compounds attached to the BSOD protein during the incubation in the presence of the previously dialysed alkaline pea SOD filtrate. For a bovine serum albumen control, antioxidant activity was not observed in the respective fraction eluted from the Biogel P-4 column (Fig. 5).

Other work has more generally indicated that the interaction of phenolic compounds with proteins may be a relatively non-specific surface phenomenon. Hagerman and Butler (1981) showed that the protein structure can play a role in determining its susceptibility to phenolic complex formation. They showed that proline-rich and conformationally mobile proteins may be preferentially precipitated by phenolic compounds in the presence of other proteins. The molecular size and conformational flexibility and mobility of the phenolic compounds is also reported to be important (McManus *et al.*, 1985). BSA can be thought of as an extremely flexible protein and might be expected to readily bind phenolic compounds. Its flexibility arises from both its loop–link–loop structure and the absence of disulphide bonds linking distant parts of the peptide chain (Peters, 1985). BSA has the ability to bind a wide variety of biological materials, from metal ions to fatty acid molecules. Most of the associations involve hydrophobic interactions and the flexibility allows it to adapt to the shape of the ligand (Peters, 1985). It might therefore be predicted that BSA would readily bind phenolic compounds due to its apparent flexibility and readiness to form hydrogen bonds.

McManus *et al.* (1985) reported that BSA was able to bind various phenolic compounds and was maximal at around pH 4·0 where the N (normal) → F (fast) transition of BSA occurs (Peters, 1985). This change corresponds to a partial opening of the BSA molecule, causing exposure of nearly one-half of the carboxyl groups and hence presumably to an increase in the number of possible binding sites.

A possible explanation for the observed lack of conferred antioxidant activity is that the binding sites on BSA were occupied more readily by other substances in the phenolic sample (e.g. metal ions), or that binding of the phenolic compounds to BSA occurs in such a way as to prevent the expression of their antioxidant activity. Alternatively, the phenolic compounds removed from pea SOD may have a particular affinity for the SOD protein and bind strongly to other sources of

SOD. Nevertheless, the binding of the low molecular weight dialysable fraction containing phenolic compounds, to either bovine or pea SOD to form a thermostable protein-bound antioxidant, may still be due to a specific affinity of superoxide dismutases for the isolated phenolic compounds.

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