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Isolation and purification of a novel antioxidant protein from the water extract of Sundakai (*Solanum torvum*) seeds

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Abstract

Reactive oxygen species (ROS) at physiological concentrations may be required for normal cell function. Excessive production of ROS can be detrimental to cells, because ROS can cause oxidative damage to lipids, proteins, and DNA. Herein, we describe the isolation and purification of a novel antioxidant protein the water extract of dried, powdered Sundakai (*Solanum torvum* [Solanaceae]) seeds. Sundakai belongs to the Solanaceae family, a small shrub, which is distributed widely in India, Malaya, China, Phillipines and tropical America. Fifty percent of ammonium sulphate-precipitated crude water extract was fractionated on a Sephadex G100 column, which yielded two peaks, PI and PII. Peaks PI and PII inhibited lipid peroxidation up to 40% and 89%, respectively in linolenic acid micelles. Rechromatographing of peak PII on Sephadex G100 yielded a single peak, indicating the homogeneity of the purified protein. SDS–PAGE analysis indicated the molecular weight of the purified protein to be ~28 kDa. The purified protein, at 0.8 μM, inhibited deoxyribose degradation induced by generation of hydroxyl radicals by 90% and scavenged DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals by 76%. The reducing power and chelating power of the purified protein, at 0.8 μM, were found to be 72% and 85%, respectively. The protein, at 0.8 μM, also offered significant protection to calf thymus DNA damage induced by H_2O_2 (1 mM). Therefore, the present study demonstrates, for the first time, a novel protein from the water extract of Sundakai seeds as an excellent antioxidant.

Keywords: Sundakai seeds; Water extract; Lipid peroxidation; DPPH radicals; Antioxidant

1. Introduction

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Porter, Caldwell, & Mills, 1995), are constantly generated in aerobic organisms during normal respiration. Although ROS at physiological concentrations may be required for normal cell function, excessive production of ROS can be detrimental to cells, because ROS can cause oxidative damage to lipids, proteins, and DNA. Polyunsaturated fatty acids (PUFA), which are found predominantly in cellular membranes, are especially vulnerable to attack by ROS, because of the high concentration of allylic hydrogens in their structure. The resulting lipid hydroperoxides can affect membrane fluidity and the function of membrane proteins. In addition, lipid hydroperoxides can undergo iron-mediated, one-electron reduction and oxygenation to form epoxyallylic peroxyl radicals, which trigger a chain reaction of free radical-mediated lipid peroxidation (Girrotti, 1998). The end-products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl nonenal and malondialdehyde, many of which are highly toxic to cells (Yu & Yang, 1996). In addition, reactive aldehydes, generated by lipid peroxidation, can attack other cellular targets, such as proteins and DNA, thereby propagating the initial damage in cellular membranes to other macromolecules. Because lipid hydroperoxides, formed in membranes, are important components of ROS generation in vivo, their detoxification appears to be critical for the survival of an organism in oxidative stress (Dargel, 1992). Therefore antioxidants play a

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vital role in inhibition of lipid peroxidation or by protecting against cellular damage by free radicals.

Synthetic compounds, such as butylated hydroxyanisole (BHA), propyl gallate and *tert*-butyl hydroquinone are widely used as antioxidants. However, they are toxic at higher doses. Therefore much attention has been paid to identification of effective natural antioxidants (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995; Van Poppel & Van den Berg, 1997).

Many scientists have suggested that dietary antioxidants, such as ascorbate, α-tocopherol and carotenoids, from fruits and vegetables could help to protect the cells from damage caused by oxidative stress and to fortify the defence system against degenerative diseases (Ames, 1983; Frei, Forte, Ames, & Cross, 1991).

Earlier studies in our laboratory have shown that dietary components, such as turmeric (*Curcuma longa*) and vegetables extracts, effectively modulate ROS-induced lipid peroxidation and DNA damage (Shalini & Srinivas, 1987; Srinivas & Shalini, 1991; Sujatha & Srinivas, 1995).

Solanum torvum (Sundakai) is a small shrub of the Solanaceae family, distributed widely in India, Malaya, China, Phillipines and tropical America. Its edible fruits are used as a vegetable. A decoction of fruits is given for cough ailments and is considered useful in cases of liver and spleen enlargement (Siemonsma & Piluek, 1994). The plant is sedative and diuretic and the leaves are used as a haemostatic. The ripened fruits are used in the preparation of tonic and haemopoietic agents and also for the treatment for pain (Kala, 2005).

Phytochemical screening of methanolic extract of sun-dried Sundakai fruits gave positive tests for alkaloids, flavonoids, saponins, tannins and glycosides (Chah, Muko, & Oboegbulem, 2000). Previous studies have shown that no significant changes were observed on supplementation of dry Sundakai powder w.r.t. glucose, lipid profile, glycated proteins, total amino acids and uronic acid levels in NIDDM patients (Iyer, Mehta, Mani, & Mani, 1992). Antiviral isoflavonoid sulfate and steroidal glycosides were also isolated from the fruits of *S. torvum* (Arthan et al., 2002). In this regard, the present investigation was focussed on isolation and purification of a novel antioxidant protein from the water extract of dried, powdered Sundakai seeds.

2. Materials and methods

2.1. Plant material

Authentic Sundakai (*S. torvum*) fruits were collected from reputable farms. The fruits were dried at room temperature in shade for 30 days. The seeds and fruit coat were separated after the fruits were dried. The seed was powdered and sieved through 100 mesh and used for the study.

2.2. Preparation of the crude extract

Ten grams of the powdered seed material of Sundakai (*S. torvum*) were suspended in 100 ml of double-distilled water, vortexed thoroughly and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was collected and filtered through Whatman no. 1 filter paper. The filtrate was lyophilized. The lyophilized filtrate was designated as crude extract of Sundakai seed and was used for the isolation of a novel antioxidant protein.

2.3. Purification of the crude extract

The crude extract was subjected to 50% ammonium sulphate fractionation. The fractionated extract was dialyzed against double-distilled water for 48 h with four changes at intervals of 12 h. The dialysate was lyophilized and fractionated on a Sephadex G100 column (V_o 54 ml, V_t 164 ml, flow rate 1.5 ml/5 min) using water as eluent. The fractions, which inhibited lipid peroxidation by up to 80% were pooled, lyophilized and rechromatographed on Sephadex G100 column (V_o 29 ml, V_t 86 ml, flow rate 1.5 ml/5 min) using water as eluent. The fractions, which inhibited lipid peroxidation by up to 80% were pooled, lyophilized and used for further studies. The purified protein was analysed by SDS–PAGE (Laemmli, 1970) with 12% separating gel and 5% stacking gel.

2.4. Estimations

The total protein content of the crude extract was determined by Bradford's method (Bradford, 1976); the total sugar was estimated by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), sulphydryl group was estimated by the Ellman (1959) method. Total phenolic content was determined by the Folin–Ciocalteau reagent (Kujala, Loponen, Klika, & Pihlaja, 2000); ascorbic acid and chlorophyll contents were estimated according to the method described by Sadasivam and Manickam (1997a, 1997b); α-tocopherol was estimated using the Emmerie–Engel reaction (Rosenberg, 1992). The ninhydrin test was done according to the method described by Sadasivam and Manickam (1997c).

2.5. Determination of antioxidant activity using linolenic acid micelles

Lipid peroxidation induced by the Fe^{2+}–ascorbate system in linolenic acid micelles was estimated by the method of Shimazaki, Ueta, Mowri, and Inoue (1984) and Dahle, Hill, and Holman (1962). The reaction mixture contained linolenic acid micelles in TBS (10 mM, pH 7.4), $FeSO_4$:ascorbate (10:100 μmol) and increasing concentrations (0.2–0.8 μmol) of the protein in a final reaction volume of 0.5 ml. The reaction mixture was incubated at 37 °C for 1 h. The reaction mixture was treated with 1% TBA and incubated in a hot water bath for 15 min. This assay was carried out in triplicates. The colour developed was measured spectrophotometrically at 535 nm.

2.6. Hydroxyl radical-scavenging assay

The hydroxyl radical-scavenging assay was done according to the method of Halliwell, Gutteridge, and Aruoma (1987). The reaction mixture contained 2.8 mM 2-deoxyribose, 100 μM $FeCl_3$ and ascorbate, 104 μM EDTA and 1 mM H_2O_2 and increasing concentrations (0.2–0.8 μM) of the protein. The volume was made up to 1.0 ml with potassium phosphate buffer (20 mM, pH 7.4). The mixture was incubated at 37 °C for 1 h. The colour developed by adding 1% TBA was measured spectrophotometrically at 535 nm. This assay was done in triplicates.

2.7. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay

The assay was performed according to the method of Schimada, Fujikawa, Yahara, and Nakamura (1992). Reactions were performed in 1.25 ml of methanol containing (0.5 mM) freshly prepared DPPH, and increasing concentrations (0.2–0.8 μM) of the protein. The reaction mixture was incubated at 37 °C for 30 min and the absorbance was measured spectrophotometrically at 517 nm. This assay was carried out in triplicate.

2.8. Reducing power

Increasing concentrations (0.2–0.8 μM) of the protein were mixed with 200 μM ferric chloride and 400 μM potassium ferricyanide. To this, 10% trichloroacetic acid was added. The reaction mixture was made up to 1 ml with distilled water and incubated at room temperature for 10 min. The absorbance was measured spectrophotometrically at 700 nm (Oyaizu, 1986). This assay was done in triplicate.

2.9. Ferrous ion chelating effect

Reaction mixture contained 200 μM ferrous chloride and 400 μM ferric cyanide and increasing concentrations (0.2–0.8 μM) of the protein. The reaction volume was made up to 1.0 ml with water and incubated at 37 °C for 10 min. The absorbance was read spectrophotometrically at 700 nm (Dinis, Madeira, & Almeida, 1994). This assay was done in triplicate.

2.10. DNA protectant activity

The reaction mixture contained calf thymus DNA (1 mg/ml PBS, pH 7.4), 1 mM H_2O_2 and 0.8 μmol of the protein in 100 μl of TBE (10 mM Tris–boric acid–EDTA, pH 7.4). The mixture was incubated at 37 °C for 30 min and the total volume was loaded onto 0.8% agarose gel dissolved in 0.5× TBE buffer, pH 7.4, with 1 mg/ml of ethidium bromide. The bands were visualized in a UV transilluminator.

2.11. Statistical analysis

Comparisons between control and treated groups were performed with a Student's *t*-test (Snedecor & Cochran, 1976) and a *p*-value of less than 0.05 was considered significant.

3. Results and discussion

There is a great interest in the use of antioxidants that intercept ROS to ameliorate oxidative stress-induced diseases. Many plant materials, such as vegetables, fruits, spices and herbs, are established sources of natural antioxidants. Green tea and Ginkgo biloba extracts are rich in antioxidants, such as vitamin C, flavonoids and polyphenols (Myerscough, 1998) and such plant-derived compounds and extracts are inexpensive and have no toxicity. In the present investigation, an attempt was made to isolate, purify and study the antioxidant activity of the protein from the water extract of Sundakai seeds.

3.1. Purification of the crude extract

Fifty percent of ammonium sulphate precipitation and fractionation, through Sephadex G100, of water extract of Sundakai seed yielded two peaks. The peaks were designated as PI and PII (Fig. 1). The fractions of peak PI inhibited lipid peroxidation only by 40%. The fractions of peak PII inhibited lipid peroxidation by upto 90%. The fractions of peak PII were pooled separately and rechromatographing through a Sephadex G100 column yielded a single peak (Fig. 2), indicating homogeneity of the purified protein. A single band was obtained on 12% SDS–PAGE, indicating

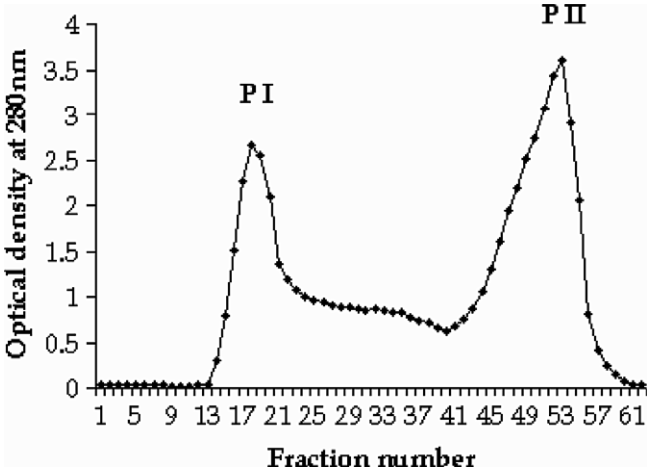

Fig. 1. Fractionation of crude water extract of Sundakai seed through Sephadex G 100 column. Crude protein (1.7 mg), 50% ammonium sulphate-fractionated, dialyzed (2500MWCO) against water, concentrated, fractionated on Sephadex G 100 column (V_o 54 ml, V_t 164 ml, flow rate 1.5 ml/5 min), eluted with water; fractions monitored at 280 nm. Antioxidant activity was determined for each fraction of two peaks in terms of lipid peroxidation in linolenic acid micelles and peak II was designated as the active peak.

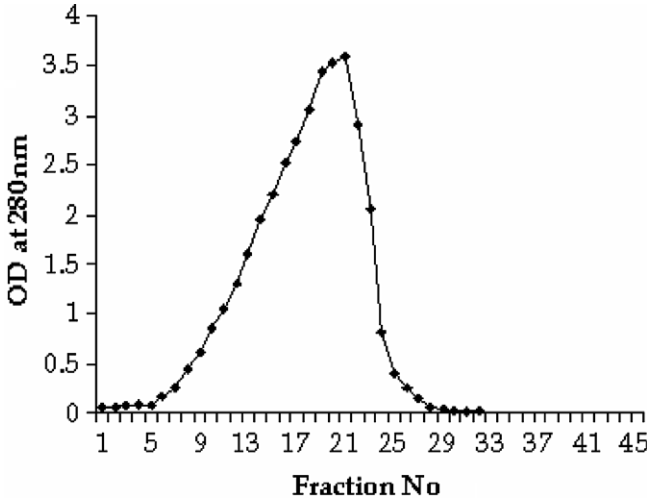

Fig. 2. Rechromatographing of peak PII of Sundakai seed protein on Sephadex G 100. Peak PII fractions of the Sundakai seed protein were pooled, lyophilized and 1.0 mg of protein was rechromatographed on Sephadex G 100 (V_o 29 ml, V_t 86 ml, flow rate 1.5 ml/5 min), eluted with water; fractions monitored at 280 nm.

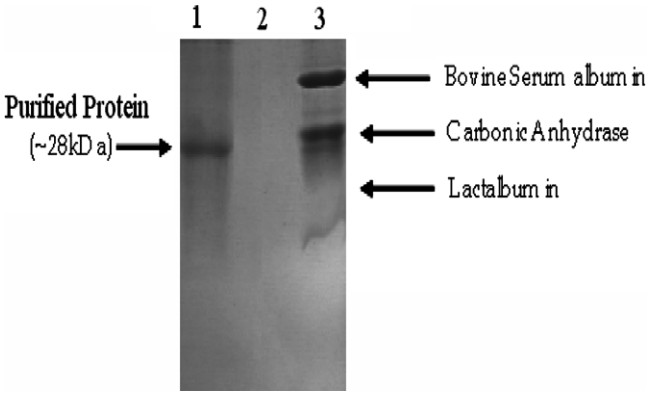

Fig. 3. SDS–PAGE of the purified protein of Sundakai seed. Lane 1: Purified protein (25 μg), Lane 2: Blank, Lane 3: Protein molecular weight markers (10 μg each). Purified protein was obtained from Sephadex G 100 column (25 μg), Protein molecular weight markers (10 μg each), in 2× sample buffer containing SDS, were loaded onto SDS gel containing 4% acrylamide in stacking gel and 12% acrylamide in separating gel. Electrophoresis was carried out with running buffer (25 mM Tris, 190 mM glycine, 10% SDS) at 50 V for stacking gel and 100 V for separating gel. Bands were stained using Coomassie brilliant blue.

purity of the protein, and the molecular weight of the purified protein was found to be ~28 kDa (Fig. 3).

3.2. Estimations

The proximate analysis of the crude Sundakai seed extract showed that the seed extract was rich in protein. It also had significant amounts of ascorbic acid, α-tocopherol and total sugar. The protein content of the crude seed extract was found to be 3.7% (dry weight). The total sugar was found to be 0.454% (dry weight). The polyphenols was twofold less than sugars, i.e. 0.235% (dry weight). The ascorbic acid and α-tocopherol were found to be 2.18% and 0.647% (dry weight), respectively. The test for the presence of chlorophyll proved negative. The test for sulphydryl group also proved negative. The test for ninhydrin proved positive (Table 1). Literature evidence indicates that plant phenols, and many sugars (glucose and mannitol) possess hydroxyl radical-scavenging activity (Byers & Perry, 1992; Guo, Cao, Sofic, & Prior, 1997) and significant increase of total antioxidant capacity occurs upon supplementation with vitamin C and phenolics of green and black tea (Serafini, Ghiselli, & Ferro-Luzzi, 1996).

Table 1
Proximate analysis of the crude water extract of Sundakai seed

Analysis	Seed (g % dry weight)
Protein	3.7 ± 0.08
Sugars	0.454 ± 0.06
Polyphenols	0.235 ± 0.01
Ascorbic acid	2.18 ± 0.01
Alpha tocopherol	0.647 ± 0.009
Ninhydrin test	Positive
Chlorophyll	Negative
Sulphydryl groups	Negative

Protein, sugars, polyphenols, ascorbic acid, α-tocopherol, chlorophyll, sulphydryl groups and ninhydrin test were done as described in Section 2 and values are expressed as grammes present per 100 g of Sundakai dry powder.
Values are means ± SD of six experiments.

3.3. Determination of antioxidant activity using linolenic acid as model system

When free radicals attack membranes, which are high in lipids, they form lipid peroxides. Hence, we initially used an iron-dependent system (ferrous sulphate:ascorbate) for induced lipid peroxidation to investigate the antioxidant activities of the purified protein using linolenic acid micelles as lipid phase model systems. Increasing concentrations of the purified protein were tested for antioxidant activity in the linolenic acid medium. The protein exhibited a dose-dependent inhibition of $FeSO_4$ and ascorbate-induced lipid peroxidation. A maximum concentration of 0.8 μmol of the protein significantly inhibited the lipid peroxidation to a maximum of 89% (Fig. 4). The antioxidant activity of the protein was compared with standard antioxidants, such as BHA, curcumin and α-tocopherol. Each of these antioxidants inhibited lipid peroxidation by 78%, 87% and 92% at 400 μM concentration, respectively. These results suggest that the magnitude of the antioxidant potential of purified protein is high at low concentration compared to known standard antioxidants. Similar studies have reported that vegetables extracts could modulate lipid peroxidation (Sujatha & Srinivas, 1995).

3.4. Hydroxyl radical-scavenging assay

Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to

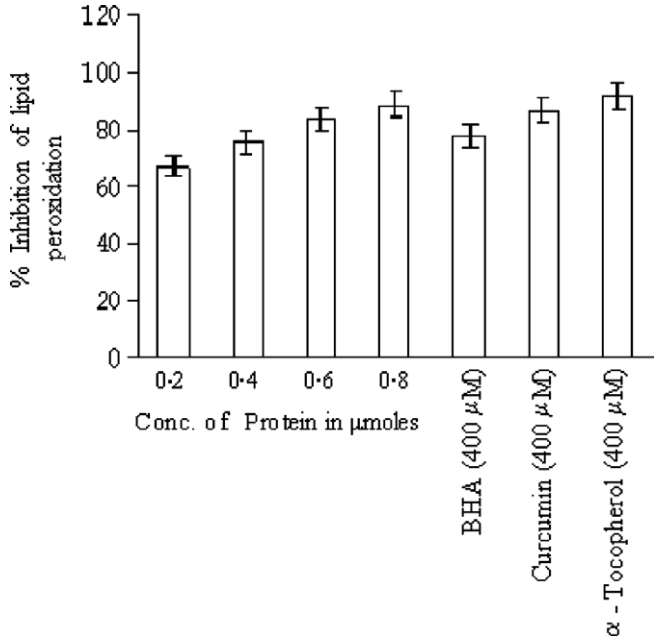

Fig. 4. Dose-dependent inhibition of lipid peroxidation by the purified protein by TBARS method. Linolenic acid (6 μM) + ascorbic acid (100 μmol) + ferrous sulphate (10 μmol) ± various concentrations of purified protein (0.2–0.8 μmol) in 500 μl of TBS (10 mM Tris, pH 7.4, 0.15 M saline), incubating at 37 °C for 60 min. Lipid peroxidation was measured by TBARS. Values are means ± SD of triplicates.

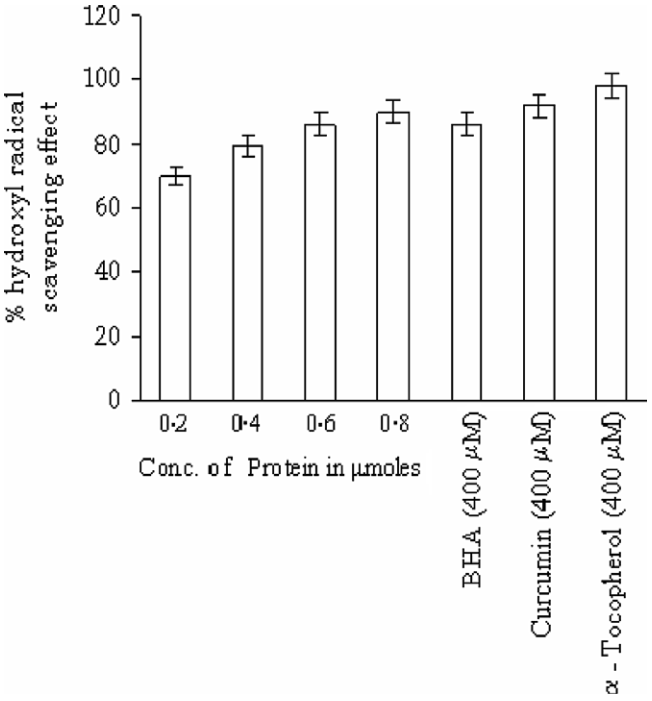

Fig. 5. Dose-dependent scavenging effect of hydroxyl radicals by the purified protein by deoxyribose method. Deoxy-D-ribose (2.8 mM) + ferric chloride (100 μM) + ascorbic acid (100 μM) + H_2O_2 (1 mM) + EDTA (104 μM) ± various concentrations of purified protein (0.2–0.8 μmoles) in 1.0 ml PB (0.02 M phosphate buffer, pH 7.4), incubating at 37 °C for 60 min. Hydroxyl radical-scavenging effect was estimated by deoxyribose method. Values are means ± SD of triplicates.

initiate cell damage *in vivo* (Rollet Labelle et al., 1998). Further, we studied the effect of the protein on hydroxyl radicals generated by Fe^{3+} ions, as measured by determining the degree of deoxyribose degradation, an indicator of TBA–MDA adduct formation. As shown in Fig. 5, the purified protein exhibited a dose-dependent inhibition of the hydroxyl radical scavenging activity. A maximum of 90% inhibition was obtained at 0.8 μmol of the protein. The inhibition was statistically significant when compared to that without any inhibitor. The scavenging potential was compared with known antioxidants, such as BHA, curcumin and α-tocopherol. Each of these antioxidants inhibited lipid peroxidation by 86%, 92% and 98% at 400 μM concentration, respectively. Among the various concentrations tested, the protein showed maximum hydroxyl radical-scavenging activity at 0.8 μmoles, which was a much lower concentration than those of standard antioxidants, such as BHA, curcumin and α-tocopherol.

3.5. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay

The indirect evidence for scavenging activity of the protein on Fe^{3+}-dependent hydroxyl radical generation was further confirmed using a direct approach with DPPH radicals, a stable radical used to evaluate the antioxidant activity of plant and microbial extracts (Chang et al., 2001; Hu & Kitts, 2000). As shown in Fig. 6, the protein showed a dose-dependent DPPH radical-scavenging activity, and

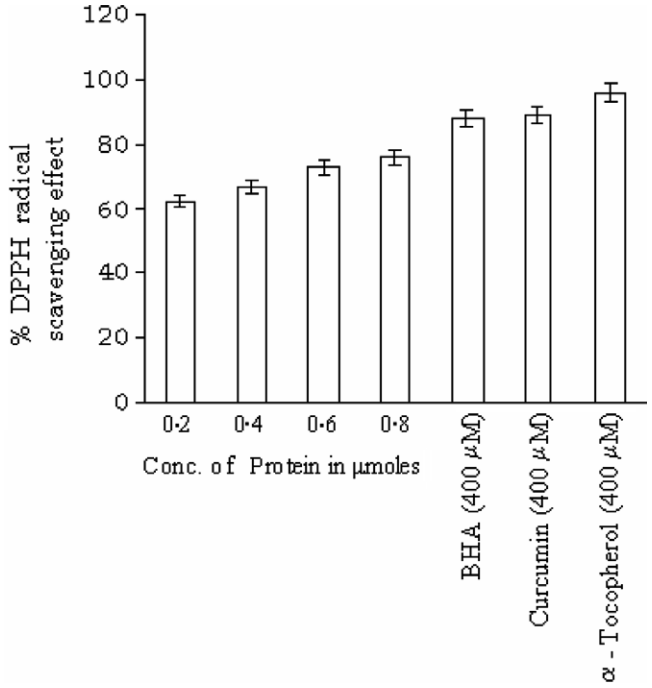

Fig. 6. Dose-dependent scavenging of DPPH radicals by the purified protein. DPPH (0.5 mM) + increasing concentrations (0.2–0.8 μM) of the protein. Mixture was incubated at 37 °C for 30 min and the absorbance was measured spectrophotometrically at 517 nm. This assay was carried out in triplicate and the values are means ± SD.

0.8 μmol of the protein showed maximum inhibition. DPPH radical-scavenging activity of the purified protein was found to be 76% at 0.8 μmol. The protein exhibited a statistically significant DPPH radical-scavenging activity when compared to the group without any inhibitor. Standard antioxidants, such as BHA, curcumin and α-tocopherol, showed 88%, 89% and 96% scavenging of DPPH radicals, respectively, at 400 μM concentration.

3.6. Reducing and chelating power

The extracts of spices and herbs may well act as electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions and it has been shown that the antioxidant effect exponentially increases as a function of the development of the reducing power (Tanaka, Kuie, Nagashima, & Taguchi, 1988). As shown in Fig. 7, the protein exhibited a maximum reducing power in a dose-dependent manner. The protein (at 0.8 μmol) exhibited a maximum reducing power (72%), which was statistically significant. EDTA at 375 μM showed 94% reducing power. Similar studies have reported that antioxidant activities of natural antioxidants are involved in termination of free radical reactions and exhibit reducing power (Wang, Yen, Ling, & Wu, 2003). The reducing power of the antioxidants might be due to their hydrogen-donating ability (Schimada et al., 1992).

It is evident that the strong reductive power of antioxidants may also affect ions, especially Fe^{2+} and Cu^{2+}. Iron is an essential mineral for normal physiology, but excess can result in cellular injury. If they undergo the Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals and thereby contribute oxidative stress (Hippeli & Elstner, 1999). The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, and carbohydrates and lead to cellular impairment. Since ferrous ions are the most effective pro-oxidants in food systems, the good chelating effect would be beneficial and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress-induced diseases. When iron ion is chelated, it may lose pro-oxidant properties.

Hence, we tested the chelation of Fe^{2+} by the purified protein in a competition assay with potassium ferricyanide. Interestingly, as seen in Fig. 7, the antioxidant factors of the protein were found to be capable of binding Fe^{2+} ions. The iron-chelating effect of the protein was comparable to that of EDTA. The protein, at 0.8 μM, exhibited a maximum chelating effect (85.6%), which was statistically significant. EDTA, at 375 μM, showed 95% chelating effect. Similar studies have reported that extracts of mushrooms exhibit chelating effect of ferrous ions and reducing power (Mau, Lin, & Song, 2002).

3.7. DNA protectant activity

Oxidative DNA damage has been implicated in various degenerative diseases (Halliwell & Gutteridge, 1981; Jenner, 1991). The protectant effect of the purified protein on hydrogen peroxide-mediated DNA damage was analyzed in agarose gel. As shown in Fig. 8, when calf thymus DNA was treated with hydrogen peroxide for 30 min, extensive DNA damage was observed in Lane 2, where the DNA was treated with 1 mM H_2O_2. The purified

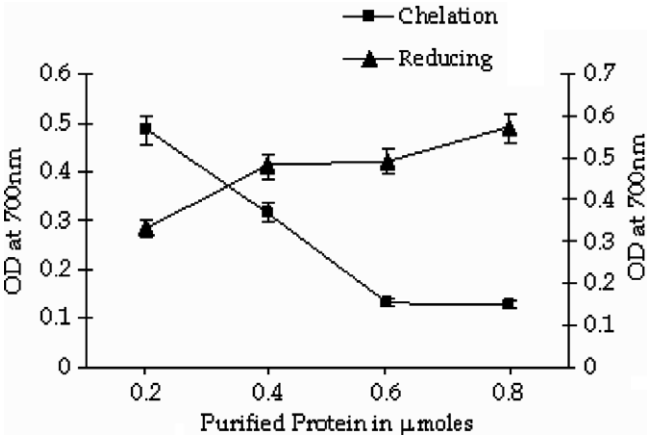

Fig. 7. Chelating and reducing activities of the purified protein. Chelating and reducing effects of the purified protein were estimated. The absorbance of the reaction mixture was read at 700 nm. Lower absorbance indicated stronger chelating effect and increased absorbance indicated increased reducing power. The assay was done in triplicate and the values are expressed as means ± SD.

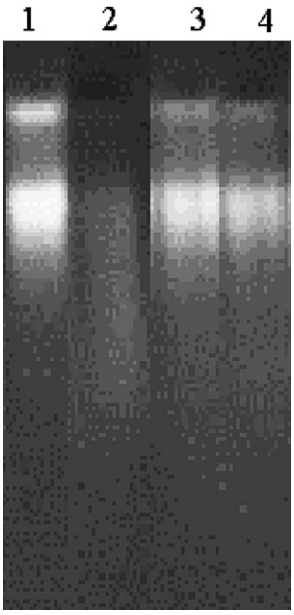

Fig. 8. DNA protectant activity of the purified protein against H_2O_2-induced calf thymus DNA damage. Lane 1: Calf thymus DNA (10 μg), Lane 2: As 1 + H_2O_2 (1 mM), Lane 3: As 2 + purified protein (0.8 μM), Lane 4: As 2 + BHA (400 μM) Calf thymus DNA (10 μg) + H_2O_2 (1 mM) ± purified protein (0.8 μM), BHA (400 μM) in 100 μl TBE (10 mM Tris–boric acid–EDTA, pH 7.4), incubated at 37 °C for 30 min. Electrophoresis was carried out at 80 V. Bands were visualized in a UV transilluminator.

protein, at 0.8 μM, protected against the DNA damage induced by H_2O_2 (Lane 3), whereas BHA also offered protection to H_2O_2-induced DNA damage (Lane 4).

4. Conclusion

The novel protein from the water extract of Sundakai seed was proved to be an effective antioxidant, even at low dose, when compared to well known standard synthetic antioxidants. This study warrants further understanding of the mechanism of the antioxidant activity exhibited by the purified Sundakai seed protein.

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