Food Chemistry 114 (2009) 1405–1412

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Purification and characterisation of a novel antioxidant protein molecule from *Phyllanthus niruri*

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ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form 26 September 2008
Accepted 10 November 2008

Keywords:
Tertiary butyl hydroperoxide
Hepatocyte
Oxidative stress
Phyllanthus niruri
Antioxidant activity
35 kDa protein molecule of *P. niruri* (PNP)
Purification
Amino acid sequence
Functional characterisation

ABSTRACT

The herb *Phyllanthus niruri* is known to possess antioxidant activity, but the nature of the responsible active principle(s) is not well defined. The present study reports the purification and characterisation of a 35 kDa antioxidant protein molecule from this herb using a bioassay in which oxidative stress was introduced in hepatocytes with the help of a suitable free radical inducer, tertiary butyl hydroperoxide (TBHP), and the prevention of that stress was monitored using the protein fraction(s) obtained at each step of the purification. Partial amino acid sequence of this protein revealed its unique structural features. The purified protein possesses potent radical-scavenging activity, enhances intracellular antioxidant power and protects the TBHP-induced alterations of the cytoprotective and antioxidant molecules, as well as other parameters used in this particular study. Combining, data suggest that the antioxidant activity of *Phyllanthus niruri* is at least partly due to this unique protein molecule.

1. Introduction

Since ancient times, medicinal plants, in parts or as a whole, are being used as human consumables, as well as therapeutic agents, because of their easy availability, low cost and minimal side effects. Some well-known medicinal plants of this type are *Silybum marianum* (Farghali, Kamenikova, Hynie, & Kmonickova, 2000), *Terminalia arjuna* (Karthikeyan, Sarala Bai, Gauthaman, Sathish, & Niranjali Devaraj, 2003; Sinha, Manna, & Sil, 2006), *Andrographis paniculata* (Singha, Roy, & Dey, 2007) *and Cajanus indicus* (Ghosh & Sil, 2007; Sinha, Manna, & Sil, 2007). Active ingredients such as alkaloids (Racková, Májeková, Kost'álová, & Stefek, 2004), flavonoids (Pietta, 2000) and phenolics (Santos-Gomes, Seabra, Andrade, & Fernandes-Ferreira, 2003) were purified and characterised from these plants.

Phyllanthus niruri (*P. niruri*) is also an important medicinal plant of this type, widely used in the preparation of various ayurvedic formulations (Barros et al., 2006; Bhattacharjee & Sil, 2007; Venkateswaran, Millman, & Blumberg, 1987). Phyllanthin (Harish & Shivanandappa, 2006) and corilagin (Cheng, Lin, & Hsu, 1995) are the well-characterised bioactive compounds isolated from the organic extracts of this herb. We have shown earlier that the aqueous extract of *P. niruri* possesses reasonable antioxidant activity (Chatterjee & Sil, 2006; Sarkar, Sarkar, Bhattacharjee, Chatterjee, & Sil, 2005). In addition, the aqueous extract has been shown to have better antioxidant activity when administered intraperitoneally (Chatterjee & Sil, 2006). Heat and protease treatments partially destroyed the antioxidant activity of the aqueous extract of the herb. However, almost complete loss of protein isolate-induced antioxidant activity was observed under the same treatment conditions (Chatterjee, Sarkar, & Sil, 2006). The protein isolate could also affect various oxidative stress-related parameters induced by

Abbreviations: ALT, alanine aminotranferase; CID, collisionally induced dissociation; DCF-DA, dichlorofluorescein diacetate; DEAE, diethyl amino ethyl; DMEM, Dulbecco's modified eagle's medium; DTNB, 5,5' dithiobis-2-nitrobenzoic acid; FRAP, ferric reducing/antioxidant power; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione s-transferase; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MDA, malonaldehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PNP, 35 kDa *P. niruri* protein; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulphate–polyacryl amide gel electrophoresis; SOD, super oxide dismutase; TBHP, tertiary butyl hydroperoxide; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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doi:10.1016/j.foodchem.2008.11.022

a number of toxins and drugs (Bhattacharjee & Sil, 2006, 2007; Sarkar & Sil, 2007). Combining all, the data indicate that the protein of this herb might be responsible, at least partly, for its antioxidant activity.

The aim of the present study was, therefore, to identify and characterise the responsible antioxidant protein molecule(s) from this medicinal plant. We used a bioassay in which tertiary butyl hydroperoxide (TBHP) was used to induce oxidative stress in murine hepatocytes, and that oxidative impairment (ROS generation) was protected by the addition of the protein fractions. The homogeneous preparation of the active principle was achieved by homogenisation and several chromatographic procedures. Its characterisation was performed by confirming its homogeneous preparation by HPLC, PAGE and SDS–PAGE, determining its molecular weight by SDS–PAGE, determining the effect of heat treatment and enzymatic digestion on its bioactivity, and finally by determining its amino acid sequence using MALDI-TOF as well as LC–MS analyses and comparing that with other proteins present in the database. In addition, dose- and time-dependent experiments have been carried out to evaluate the optimum antioxidant effect of the active principle. Its cytoprotective activity was determined by measuring the cell viability and ALT leakage in hepatocytes. The antioxidant activity of the protein was further confirmed by determining its effects on cellular lipid peroxidation, GSH contents and catalase activity.

2. Materials and methods

2.1. Animals

Male swiss albino mice, of body weight 20 ± 2 g, were acclimatised in the laboratory for a fortnight before starting the experiments. They were fed *ad libitum* with food and water. The mice were fasted for 16–18 h before performing any experiment.

2.2. Chemicals

TBHP, DCF–DA, Collagenase IV and Dulbecco's modified eagle's medium (DMEM) were obtained from Sigma chemical co., USA. An ALT measurement kit was purchased from span diagnostic, India. All the chemicals were of reagent grade with the highest laboratory purity.

2.3. Purification of the protein

The leaves from *P. niruri* were homogenised in 50 mM phosphate buffer, pH 7.4, and the soup (after centrifugation at 15,000*g*) was brought to 60% ammonium sulphate saturation. The pellet was reconstituted and dialysed against 50 mM phosphate buffer, passed through DEAE–cellulose column and eluted using a linear gradient of 0–1 M NaCl in the same phosphate buffer. Two major peaks were observed. The protein fractions from the first peak (showed maximum biological activity) were collected, concentrated and dialysed in 50 mM phosphate buffer. The active material of the peak-I fractions was subjected to gel filtration chromatography and re-chromatography using a gel filtration column (BIOSEP-SEC-S200, 600 × 7.8 mm) attached to the HPLC. Biological activity of each fraction was checked, and the material of the active peak was subjected to re-chromatography under identical conditions.

2.4. Test of homogeneity

The homogeneity and the molecular weight of the protein were checked according to the method of Laemmli (1970). SDS–PAGE was performed with 12% resolving and 5% stacking gels. A set of molecular weight marker proteins (25–225 kDa) was run in the gel to determine the molecular weight of the protein.

2.5. Effect of temperature on the biological activity of the protein

To check the effect of temperature on the protein, the protein was heated at 90 °C for 5 min, cooled and then applied to hepatocytes (10^6cells/ml) at a dose of 10 μg/ml for 20 min before TBHP administration. The incubation was continued for 2 h. A control was kept where the biologically active protein was administered at the same dose.

2.6. Effect of protease on the biological activity of the protein

For this experiment, 35 kDa protein molecules of *P. niruri* (PNP) and trypsin were incubated at 37 °C for 1 h and then experimental hepatocytes were treated with the mixture for 20 min before TBHP intoxication. The effect was compared with that of the biologically active protein.

2.7. Determination of the amino acid sequence of the protein

For the protein digestion, the bands were cut from the gel as closely as possible, with either a scalpel or a punch, and washed/destained in 50% ethanol and 5% acetic acid. The gel pieces were then reduced with DTT and alkylated with iodoacetamide before digestion with trypsin overnight. The peptide fragments were extracted from the polyacrylamide gel and evaporated to 30 μl for LC–MS analysis. The LC–MS system was a ThermoFisher LTQ ion trap mass spectrometer system. The HPLC column was a self-packed 8 cm × 75 μm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. - Ten microliter volumes of the extract were injected using an Eksigent splitless nanoflow LC system, and the peptides were eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of 250 nl/min introduced into the source of the mass spectrometer. The microelectrospray ion source was operated at 2.5 kV. The digest was analyzed, using the data-dependent multitask capability of the instrument acquiring full scan mass spectra, to determine the molecular weights of the peptides and product ion spectra and to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 3500 collisionally induced dissociation (CID) spectra of ions, ranging in abundance over several orders of magnitude. The data were analysed by using all CID spectra collected in the experiment to search the NCBI databases with the search programme mascot. Each identification was verified by manual inspection of several matching spectra. The interpretation process was aided by additional searches using the programme Sequest as needed.

2.8. Hepatocyte isolation

Hepatocytes were isolated from mice liver according to the method of Sarkar and Sil (2006) with some modifications. The livers were isolated under aseptic conditions and placed in phosphate-buffered saline. The livers were irrigated in buffer A (10 mM HEPES, 3 mM KCl, 130 mM NaCl, 1 mM $NaH_2PO_4{\cdot}H_2O$ and 10 mM glucose, pH 7.4) and then incubated with buffer B (5 mM $CaCl_2$, 0.03% collagenase, type IV) for about 45 min at 37 °C. The liver was then passed through a wide bore syringe and 80 μm decron mesh, respectively. The dissociated cells were centrifuged at 500*g*, and the pellet was suspended in Dulbecco's minimal essential medium (DMEM) containing 10% foetal calf serum and 5 μg/ml of insulin, 5 μM hydrocortisone, 100 U/ml of penicillin

and 100 μg/ml of streptomycin. The suspension was adjusted to obtain $\sim 1 \times 10^6$ cells/ml.

2.9. Determination of cell viability

MTT is converted to an insoluble formazan by the cleavage of its tetrazolium ring by active mitochondrial dehydrogenase in living cells (Edmondson, Armstrong, & Martinez, 1988). To determine cell viability, cells were preincubated with the protein (10 μg/ml) for 20 min. Following aspiration and washing with PBS, cells were exposed to 250 μM TBHP for 2 h. The resulting cells were rinsed and incubated with 0.5% FBS–DMEM containing 0.1 vol of MTT (5 mg/ml) for 4 h. At the end of the incubation period, the medium was removed and the converted dye was solubilised with DMSO (0.3 ml). Absorbances were measured at 570 nm, and cell viability was expressed as a percentage of the corresponding control.

2.10. Cell leakage

After the appropriate experimental procedure, as described earlier, hepatocyte suspensions were centrifuged at 60*g*. The leakage of the enzyme ALT (secreted outside the cells) was determined from the supernatant using a kit and following the manufacturer's protocol (Reitman & Frankel, 1957).

2.11. Assay of antioxidant power of hepatocytes: Ferric reducing/antioxidant power (FRAP) assay

The antioxidant power of hepatocytes was determined using FRAP assay, following the method of Benzie and Strain (1999). Briefly, 50 μl of the hepatocytes (normal as well as experimental cells) suspension was added to 1.5 ml of freshly prepared and pre-warmed (37 °C) FRAP reagent (300 mM acetate buffer, pH = 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1) and incubated at 37 °C for 10 min. The absorbance of the sample was read against reagent blank (1.5 ml FRAP reagent + 50 μl distilled water) at 593 nm.

2.12. Determination of reactive oxygen species

The intracellular ROS production in hepatocytes was measured by using 2,7-dichlorofluorescein diacetate (DCF–DA) as a probe, following the method of LeBel and Bondy (1990) as modified by Kim, McCarter, and Yu (1996). After treatment with the protein (appropriate concentration as needed for different assays) and TBHP (250 μM), the cells (hepatocytes) were incubated with 20 μM DCF–DA from the stock solution of 10 mM DCF–DA in ethanol at 37 °C for 10 min. The culture medium was removed, and the cells were washed with PBS and 2 ml of PBS was added to each well. The fluorescence intensity of the cells was measured with excitation at 488 nm and emission at 525 nm. The untreated groups were used as control. The results were expressed as the percentage of fluorescence intensity with respect to control.

2.13. Measurement of lipid peroxidation

The assay of lipid peroxidation was performed by a colorimetric reaction, using thiobarbituric acid (TBA), following the method of Esterbauer and Cheeseman (1990). Hepatocytes containing about 1 mg of protein were mixed with TCA–TBA mixture and heated at 100 °C for 30 min. The flocculent precipitate was removed by centrifugation, and the absorbance of thiobarbituric acid-reactive substance (TBARS) formed was measured at 532 nm. TBARS concentrations of the samples were calculated using the extinction coefficient of MDA, i.e. 1.56×10^{-5} $mmol^{-1}$ cm^{-1}.

2.14. Determination of cellular GSH

GSH concentration in control and experimental hepatocytes was determined by the method of Tietze (1969). Hepatocytes were deproteinated with trichloroacetic acid (TCA) by centrifugation, and GSH released in the supernatant was derivatized with 5,5′ dithiobis-2-nitrobenzoic acid (DTNB). The development of colour was measured at 412 nm.

2.15. Determination of catalase activity

Catalase activity in hepatocytes (normal and experimental) was measured by the method of Bonaventura, Schroeder, and Fang (1972). For the assay, the sonicated hepatocytes containing 5 μg total protein were mixed separately with 700 μl 5 mM hydrogen peroxide and incubated at 25 °C. The disappearance of peroxide was observed at 240 nm for 15 min. One unit of catalase activity is that which reduces 1 μmole of hydrogen peroxide per minute.

2.16. Protein estimation

Protein concentrations of all the experimental samples were estimated according to the method of Bradford (1976). Bovine serum albumin (BSA) was taken as a standard protein.

2.17. Statistical analysis

For the statistical analysis, we used the Student's *t*-test and analysis of variance when appropriate. Results were expressed as means ± S.D.

3. Results

3.1. Purification of the protein

Fig. 1a represents the chromatogram of the protein samples obtained after 60% ammonium sulphate saturation and subjected to a DEAE-ion-exchange column. Two distinct protein peaks appeared in this separation. The material of the first one showed prominent biological activity and was further subjected to gel filtration chromatography using a gel filtration column attached to the HPLC. Fig. 1b shows the gel filtration chromatogram. Several peaks were obtained and the major one eluted at 20.785 min and showed potent biological activity. It was re-chromatographed under the same conditions. A sharp symmetrical peak with potent biological activity was obtained, indicating that a homogeneous preparation of the antioxidant protein molecule from *P. niruri* had been accomplished (Fig. 1c). The summary of purification of PNP is shown in Table 1. The protein was purified 240-fold with a recovery of 1.1%.

3.2. Characterisation of the protein

3.2.1. Molecular weight

Fig. 1d represents the SDS–PAGE pattern of the purified protein (PNP) obtained at 20.589 min by gel filtration re-chromatography using HPLC. Appearance of a single sharp protein band in the region of 35 kDa proved that the molecular mass of the protein was 35 kDa. This result shows that the purified protein from *P. niruri* was homogeneous and consisted of a single polypeptide chain.

3.2.2. Effect of temperature and protease digestion

The preheated PNP (PPNP) treatment could not practically alter the ROS production in hepatocytes induced by TBHP. Trypsin digested protein (TPNP) treatment also had very little effect on the

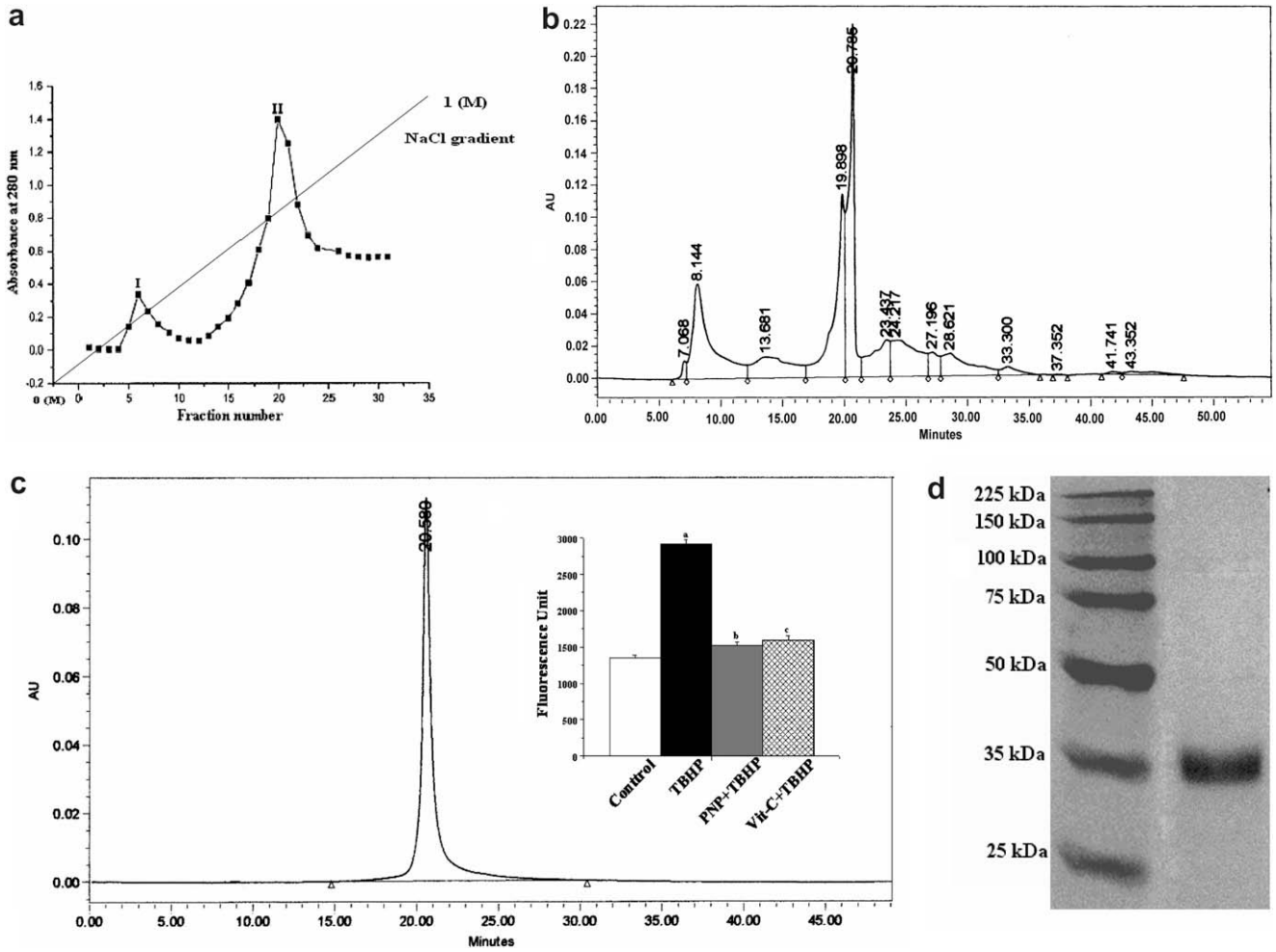

Fig. 1. Purification of the antioxidant protein molecule from *P. niruri*. (a) ion-exchange chromatogram of the protein isolate eluted from a DEAE–Cellulose column. The column was eluted using a linear gradient of NaCl (0–1 M) in 50 mM phosphate buffer, pH 7.4, at a flow rate of 1 ml/min. UV absorbance of the elutants from the column was measured at 280 nm. Each peak material was used to study the antioxidant activity, (b) gel filtration chromatogram of the peak-I materials collected from DEAE–Cellulose ion-exchange chromatography. The chromatography has been carried out by using a gel filtration column [BIOSEP-SEC-S200, 600 × 7.8 mm] attached to HPLC. The column was eluted using 50 mM phosphate buffer, pH 7.4, at a flow rate of 1 ml/min. Each peak material was used to study the antioxidant activity, (c) re-chromatogram of the materials of the active peak eluted at 20.786 min from the previous gel filtration column attached to HPLC. Inside bar graph represents the ROS generation in hepatocytes of different experimental sets expressed in fluorescence units. Control: ROS generation in normal hepatocytes, TBHP: ROS generation in TBHP-intoxicated hepatocytes, TBHP + Protein: ROS generation in hepatocytes treated with PNP before TBHP administration. Each value represents mean ± S.D. (Pa < 0.01; Pb < 0.01; Pc < 0.01) and (d) SDS–Polyacrylamide gel electrophoresis pattern of the protein obtained after gel filtration re-chromatography.

free radical production in hepatocytes induced by TBHP administration. Experimental results suggest that both the heat treatment and enzymatic digestion resulted in loss of biological activity of PNP (Fig. 2).

3.2.3. Amino acid sequence of the protein

Several peptide fragments of PNP generated by trypsin cleavage were subjected to MALDI-TOF as well as LC–MS analyses. Although a number of peptides were obtained through these combined procedures, only four, of nominal mass 2128 Da, 2392 Da, 2533 Da and 2719 Da, were prominent. These peptides were subjected to MS–MS analysis for sequencing. The amino acid sequence of (a) the 2128 Da peptide is: - - - KTDQDXFTNXPAXVDR; (b) the 2392 Da peptide is - - - SDNPDPNXDPTYAATXR ... (c) the 2533 Da peptide is: FSNXXXDDTDLVALSEHTFVQR, and finally (d) the 2988 Da peptide is GKFSNXXXDDTDLVALSEHTFVQR (Table 2). When the amino acid sequences of the peptides were compared with the sequences present in the NCBI non-redundant database, none of these was found to be matched with any peptide sequences in the NCBI non-redundant or refseq databases.

Table 1
Summary of purification of the antioxidant protein from *P. niruri*.

Purification steps	Total protein (μg)	Amount of protein required for biological activity (μg)	Fold of purification	Yield (%)
Crude extract	4,500,000	2400	1	100.0
60% Ammonium sulphate saturation	1,200,000	800	3	80.0
DEAE–cellulose ion-exchange chromatography	200,000	250	9.6	43.0
Gel filtration on HPLC	5000	20	120	13.3
Re-chromato-graphy on HPLC	2000	10	240	1.1

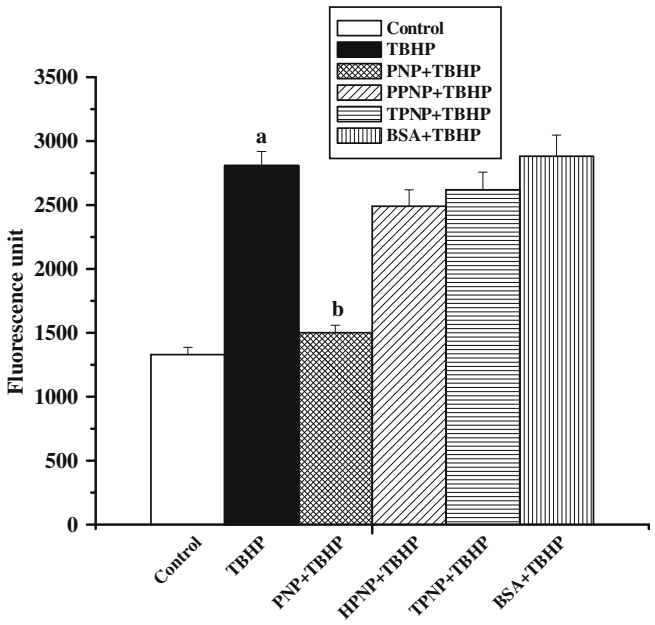

Fig. 2. Effects of heat and protease treatments on the biological activity of PNP (10 μg/ml) against TBHP-induced ROS generation expressed in fluorescence units. Control: ROS generation in normal hepatocytes, TBHP: ROS generation in TBHP-intoxicated hepatocytes, PNP + TBHP: ROS generation in the bioactive PNP-treated hepatocytes before TBHP administration, PPNP + TBHP: ROS generation in the hepatocytes treated with the preheated (at 90 °C for 5 min) PNP before TBHP administration, TPNP + TBHP: ROS generation in hepatocytes treated with the trypsin-digested (at 90 °C for 5 min) PNP before TBHP administration. BSA + TBHP: ROS generation in BSA-treated hepatocytes before TBHP administration. Each value represents mean ± SD (n = 6). (Pa < 0.01; Pb < 0.01).

3.3. Effect of PNP on cell viability

Table 3 shows that 250 μM TBHP treatment for 2 h caused a loss of about 50% of the cell viability. When hepatocytes were treated with PNP 20 min prior to the addition of TBHP, a significant increase in cell viability was observed. About 97% cell viability was restored at a protein concentration of 10 μg/ml for 2 h incubation. When Vit-C was used instead of PNP, the cell viability was restored almost completely.

3.4. Time- and dose-dependent prevention of ROS generation by PNP

Fig. 3 shows the time- and dose-dependent prevention of TBHP-induced ROS generation by PNP expressed in fluorescence units. Incubation of hepatocytes with TBHP tremendously enhanced the ROS production. PNP could prevent that in a time- and dose-dependent manner. As evidenced from the figure, treatment of hepatocytes with 10 μg PNP, prior to TBHP (250 μM) administration, almost reduced the ROS production to normal levels.

3.5. Cytoprotective action of PNP

Table 3 demonstrates the cytoprotective effect of PNP (expressed as ALT leakage) from normal and experimental hepatocytes. ALT leakage was elevated to a very high level (more than double) with respect to normal hepatocytes upon TBHP treatment, indicating severe cellular damage. PNP treatment before TBHP intoxication significantly inhibited the damage, as evidenced from the less ALT leakage. Vit-C administration also showed a similar result.

Table 2
Amino acid sequence of the peptides obtained by the tryptic digestion of the 35 Da protein purified from *P. niruri*.

	(M + H$^+$)	Z[a]	Sequence[b]
1	935	2	CXTFQQR (C = cyclized carbamidomethylated C)
2	953	2	CXTFQQR (C = carbamidomethylated C)
3	967	2	CXTFQQR (C = acrylamide modified C)
4	1104	2	(209 Da) CXTFQQR
5	1332	2	PNXDPTYAATXR
6	1751	2,3	- - TAXPGPFDTFTQNR
7	1794	2,3	- - - AXPGPFDTFTQNR
8	2128	2,3	- - - KTDQDXFTNXPAXVDR
9	2392	2,3	- - - SDNPDPNXDPTYAATXR (Neutral loss of 146 Da from precursor and sequence specific ions)
10	2533	2,3	FSNXXXDDTDLVALSEHTFVQR
11	2719	3,4	GKFSNXXXDDTDLVALSEHTFVQR
12	3215	2,3	(- - -GF - - -)[c]

[a] Charge state.
[b] X = I or L, which cannot be distinguished by low energy CIC.
[c] Not sure of sequence direction.

Table 3
Effect of the pretreatment of PNP (10 μg/ml) on TBHP-induced alterations of cell viability; intracellular ferric reducing/antioxidant power (FRAP); membrane leakage enzyme ALT; the thiol-based antioxidant, GSH and antioxidant enzyme catalase.

Name of the parameters	Control	TBHP	PNP + TBHP	Vit-C + TBHP
Cell viability (% over control)	100.00 ± 4.80	53.12 ± 2.93[a]	95.62 ± 5.10[b]	93.16 ± 4.03[c]
FRAP levels (% over control)	100 ± 4.72	62.6 ± 3.25[a]	89.1 ± 4.20[b]	91.1 ± 4.12[c]
ALT (IU/l)	40.1 ± 1.80	86.3 ± 3.65[a]	45.1 ± 2.19[b]	47.4 ± 2.12[c]
MDA (nmol/mg protein)	3.92 ± 0.18	6.63 ± 0.30[a]	4.88 ± 0.22[b]	4.49 ± 0.23[c]
GSH (nmol/mg protein)	25.5 ± 1.34	10.0 + 0.59[a]	23.8 + 1.22[b]	22.6 + 1.13[c]
CAT (μmol/min/mg protein)	412.1 ± 20.8	260 ± 16.1[a]	383 ± 16.8[b]	398 ± 17.1[c]

p < 0.01.
[a] Values differ significantly from normal control.
[b] Values differ significantly from toxin control.
[c] Values differ significantly from toxin control.

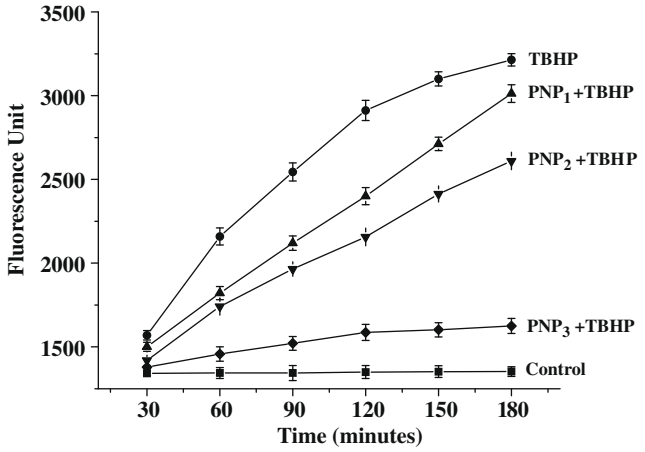

Fig. 3. Time- and dose-dependent prevention of ROS generation by PNP induced by TBHP expressed in fluorescence units. Control: Time-dependent fluorescence values when normal hepatocytes were incubated in DMEM for up to 180 min. TBHP: Time-dependent fluorescence values when hepatocytes were incubated with TBHP (250 μM) in DMEM up to 180 min. PNP_1 + TBHP, PNP_2 + TBHP and PNP_3 + TBHP: Time-dependent fluorescence values when hepatocytes were incubated with 2.5 μg, 5.0 μg and 10.0 μg PNP for 20 min prior to TBHP (250 μM) administration in DMEM and the incubation continued up to 180 min.

3.6. Effect on intracellular antioxidant power

As evidenced from Table 3, TBHP administration decreases the antioxidant power of the hepatocytes by approximately 40%. PNP treatment before TBHP administration almost restores the antioxidant power.

3.7. Effect on GSH and MDA levels

Table 3 shows the levels of GSH and MDA in normal (as well as in experimental) sets of hepatocytes. TBHP administration caused a significant reduction in GSH level although great enhancement of MDA level was detected under the same conditions. Pretreatment of hepatocytes with PNP could protect the alterations of these prooxidant–antioxidant-related parameters and keep their values almost normal.

3.8. Effect on antioxidant enzyme, catalase

Table 3 shows the activity of the intracellular antioxidant enzyme, catalase, in normal and experimental sets of hepatocytes. TBHP administration significantly decreased the catalase activity. Treatment with PNP, prior to the toxin administration, prevented this change and maintained the activity of this particular enzyme at nearly that of normal hepatocytes.

4. Discussion

Oxidative stress is defined as the disturbance between the prooxidant and antioxidant balance in favour of the increased level of the former in organs, tissues and cells. This in vivo pathophysiological situation arises as a result of either the increased production of reactive oxygen species (ROS) or the decreased level of the antioxidant defence. Under normal physiological conditions, about 1–4% of the cellular oxygen consumed in mitochondria, is converted into superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). These free radicals, in turn, react with the cellular macromolecules and cause peroxidative tissue damage (Dalle-Donne, Giustarini, Colombo, Rossi, & Milzani, 2003). To counter this oxidative stress, the natural antioxidative defence mechanism of the body operates to detoxify or scavenge the ROS. However, if the rate of production of ROS overcomes the rate of their consumption, the body needs the supplementation of antioxidants from outside, to cope with the excess ROS. It is, therefore, sensible to elucidate the detailed protective mechanisms of these medicinal (human-consumable) plants in organ pathophysiology and to look for the biologically active compounds possessing intrinsic antioxidant activity therein.

The crude aqueous fraction of *P. niruri* possesses antioxidant activity, and intraperitoneal administration of the same amount of extract showed better action than did oral administration. We, therefore, conducted the present study for the purification of a protein molecule possessing antioxidant activity from the aqueous extract. Primary murine hepatocytes were used for the bioassay throughout the purification procedure.

With the help of a well-defined bioassay, and following four steps of purification, we have isolated and purified the protein (240-fold) to homogeneity. Heat and enzymatic treatments destroyed the biological activity of the protein. SDS–PAGE demonstrated a single symmetrical band in the region of 35 kDa, suggesting that the protein is composed of a single polypeptide chain with an apparent molecular mass of 35 kDa. Its partial amino acid sequences were obtained from four prominent peptide fragments by MALDI-TOF as well as LC–MS analyses. Several different forms of the CXTFQQR peptide were identified (Table 2), including the carbamidomethylated, acrylamide and cyclised carbamidomethylated C forms. The peptide with a mass of 1104 Da has an additional mass of 209 Da that is either a modification of the C residue or additional N-terminal sequence or both. The peptide with a mass of 1332 Da has the sequence PNXDPTYAATXR and corresponds to the C-terminal portion of the larger peptide. . . . .SDNPDPNXDPTYAATXR (2392 Da). The 1332 Da peptide is a non-tryptic peptide in which cleavage occurs at the D–P bond. The full length peptide has a molecular weight of 2392 Da, and the N-terminal portion of this peptide could not be determined. This peptide undergoes a prominent neutral loss of 146 Da from both the precursor ion and sequence specific ions, suggesting that this peptide is modified and this modification occurs on the unknown N-terminal portion of the sequence. At this stage, the antioxidant protein of the herb could be considered as unique, as none of the sequences of tryptic peptides showed similarities to those present in the database.

The role of protein molecules in drug- and toxin-induced oxidative stress is not clearly defined. In fact, very little information is available in the literature in this regard. A few articles, published very recently (Lee, Oh, Ko, Lim, & Lim, 2006; Oh & Lim, 2006; Sivapriya & Srinivas, 2007; Tsoi, Ng, & Fong, 2005), have revealed that some protein molecules from various plant sources possess activities like our protein, although these reports do not describe any mechanism of protective action or structural features of the active principles. We have also isolated and partially characterised another protein molecule of this type from the herb *Cajanus indicus* (Sarkar, Ghosh, Kinter, Mazumder, & Sil, 2006). However, this molecule does not have any primary structural similarity to this novel protein (PNP) isolated from *P. niruri,* despite functionally sharing many similar activities.

Oxidative stress inducers (e.g. TBHP) could cause a significant increase in the rate of formation of ROS, such as superoxide anion radical (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). These could also cause enhanced lipid peroxidation and oxidation of proteins as well as of DNA. Intracellular defence systems consisting of antioxidant enzymes SOD, catalase, GST, GR and GPx, along with integral antioxidant molecules, GSH and its metabolites, e.g. oxidised glutathione (GSSG), on the other hand, inhibit that stress. The antioxidant enzyme SOD quenches O_2^- by converting it into O_2 and H_2O_2 (Fridovich, 1972), and catalase converts H_2O_2 into H_2O and O_2 with the help of another enzyme, GST (Jones & Suggett,

1968). GSH interacts with residual free radicals escaping decomposition by the antioxidant enzymes and is consequently converted into its metabolite GSSG.

Our experimental findings show that TBHP administration increased the lipid peroxidation and decreased the level of GSH. This phenomenon leads to depletion of the activities of antioxidant enzymes, e.g. catalase, in hepatocytes. Protein pretreatment, however, could prevent all these alterations. In addition, we found that TBHP induced a significant reduction in cellular antioxidant power (as indicated by lower FRAP value than that of the normal control). Treatment with the protein increased the same by enhancing the FRAP value compared to the toxin control. Recently, different laboratories (Bruck et al., 2002; Lu, Huang, Yang, & Tsukamoto, 1999; Schreck, Albermann, & Baeuerle, 1992) have reported that antioxidative agents could suppress the activation of NF-κB, a ubiquitous transcription factor, and these are activated in pathophysiological conditions, e.g. ROS generation. Since the 35 kDa protein could reduce ROS generation and enhance intracellular antioxidant power, it is likely that it would also reduce the NF-κB activation by scavenging the ROS or by engulfing the ROS-producing substrate (TBHP) during its protective action. Future studies on the effect of the protein on NF-κB activation will address this issue and may help to elucidate its mechanism of antioxidant action. Besides antioxidant activity, this protein molecule shows cytoprotective activity by preventing the cell membrane damage induced by TBHP.

In conclusion, the 35 kDa protein molecule isolated from the herb *P. niruri* shows antioxidant as well as cytoprotective activities in hepatocytes, although the mechanism of its protective action is not clearly known. Further studies are, therefore, necessary to fully characterise this active principle, both structurally and functionally, to arrive at a clear picture of its role as an antioxidant molecule. These are currently in progress.

Acknowledgements

The work has been supported by the Council of Scientific and Industrial Research, Government of India (a Grant-in-Aid to PCS, Scheme Number: 01(1788)/02/EMR-II). MKS acknowledges the receipt of a CSIR (partly) fellowship, as well as the fellowship from the Bose Institute. BM is supported by Grant HL 079164 from the National Institute of Health, USA. The authors are grateful to the Director of the Bose Institute for providing the laboratory facilities and Mr. Prasanta Pal for excellent technical assistance for the study.

References

Barros, M. E., Lima, R., Mercuri, L. P., Matos, J. R., Schor, N., & Boim, M. A. (2006). Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis. *Urology Research, 34*, 351–357.

Benzie, I. F. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymolozy, 299*, 15–27.

Bhattacharjee, R., & Sil, P. C. (2006). The protein fraction of *Phyllanthus niruri* plays a protective role against acetaminophen-induced hepatic disorder via its antioxidant properties. *Phytotherapy Research, 20*, 595–601.

Bhattacharjee, R., & Sil, P. C. (2007). Protein isolates from the herb, *Phyllanthus niruri* L. (Euphorbiaceae), plays hepatoprotective role against carbon tetrachloride-induced liver damage via its antioxidant properties. *Food and Chemical Toxicology, 45*, 817–826.

Bonaventura, J., Schroeder, W. A., & Fang, S. (1972). Human erythrocyte catalase: An improved method of isolation and a revaluation of reported properties. *Archives of Biochemistry & Biophysics, 150*, 606–617.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Bruck, R., Aeed, H., Schey, R., Matas, Z., Reifen, R., Zaiqer, G., et al. (2002). Pyrrolidine dithiocarbamate protects against thioacetamide-induced fulminant hepatic failure in rats. *Journal of Hepatology, 36*, 370–377.

Chatterjee, M., Sarkar, K., & Sil, P. C. (2006). Herbal (*Phyllanthus niruri*) protein isolate protects liver from nimesulide-induced oxidative stress. *Pathophysiology, 13*, 95–102.

Chatterjee, M., & Sil, P. C. (2006). Hepatoprotective effect of aqueous extract of *Phyllanthus niruri* on nimesulide-induced oxidative stress in vivo. *Indian Journal of Biochemistry and Biophysics, 43*, 299–305.

Cheng, J. T., Lin, T. C., & Hsu, F. L. (1995). Antihypertensive effect of corilagin in the rat. *Canadian Journal of Physiology and Pharmacology, 73*, 1425–1429.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R., & Milzani, A. (2003). Protein carbonylation in human diseases. *Trends in Molecular Medicine, 9*, 169–176.

Edmondson, J. M., Armstrong, S. L., & Martinez, A. O. (1988). A rapid and simple MTT based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *Methods in Cell Science, 11*, 15–17.

Esterbauer, H., & Cheeseman, K. H. (1990). Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods in Enzymolozy, 186*, 407–421.

Farghali, H., Kamenikova, L., Hynie, S., & Kmonickova, E. (2000). Silymarin effects on intracellular calcium and cytotoxicity: A study in perfused rat hepatocytes after oxidative stress injury. *Pharmacological Research, 41*, 231–237.

Fridovich, I. (1972). Superoxide radical and superoxide dismutase. *Accounts of Chemical Research, 5*, 321–323.

Ghosh, A., & Sil, P. C. (2007). Antioxidative effect of a protein from *Cajanus indicus* L against acetaminophen-induced hepato-nephro toxicity. *Journal of Biochemistry and Molecular Biology, 40*, 1039–1049.

Harish, R., & Shivanandappa, T. (2006). Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri. Food Chemistry, 95*, 180–185.

Jones, P., & Suggett, A. (1968). The catalase–hydrogen peroxide system. A theoretical appraisal of the mechanism of catalase action. *Biochemical Journal, 110*, 621–629.

Karthikeyan, K., Sarala Bai, B. R., Gauthaman, K., Sathish, K. S., & Niranjali Devaraj, S. (2003). Cardioprotective effect of the alcoholic extract of *Terminalia arjuna* bark in an in vivo model of myocardial ischemic reperfusion injury. *Life Sciences, 73*, 2727–2739.

Kim, J. D., McCarter, R. J. M., & Yu, B. P. (1996). Influence of age, exercise and dietary restriction on oxidative stress in rats. *Aging Clinical and Experimental Research, 8*, 123–129.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

LeBel, C. P., & Bondy, S. C. (1990). Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. *Neurochemistry International, 17*, 435–440.

Lee, S. J., Oh, P. S., Ko, J. H., Lim, K., & Lim, K. T. (2006). Protective effect of glycoprotein isolated from Ulmus davidiana Nakai on carbon tetrachloride-induced mouse liver injury. *Journal of Pharmacy and Pharmacology, 58*, 143–152.

Lu, S. C., Huang, Z. Z., Yang, H., & Tsukamoto, H. (1999). Effect of thioacetamide on the hepatic expression of gamma-glutamylcysteine synthetase subunits in the rat. *Toxicology and Applied Pharmacology, 159*, 161–168.

Oh, P. S., & Lim, K. T. (2006). Plant originated glycoprotein has anti-oxidative and anti-inflammatory effects on dextran sulfate sodium-induced colitis in mouse. *Journal of Biomedical Science, 13*, 549–560.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*, 1035–1042.

Racková, L., Májeková, M., Kost'álová, D., & Stefek, M. (2004). Antiradical and antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects. *Bioorganic and Medicinal Chemistry, 12*, 4709–4715.

Reitman, S., & Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *American Journal of Clinical Pathology, 28*, 56–63.

Santos-Gomes, P. C., Seabra, R. M., Andrade, P. B., & Fernandes-Ferreira, M. (2003). Determination of phenolic antioxidant compounds produced by calli and cell suspensions of sage (*Salvia officinalis* L.). *Journal of Plant Physiology, 160*, 1025–1032.

Sarkar, K., Ghosh, A., Kinter, M., Mazumder, B., & Sil, P. C. (2006). Purification and characterisation of a 43 kD hepatoprotective protein from the herb *Cajanus indicus* L. *Protein Journal, 25*, 411–421.

Sarkar, M. K., Sarkar, K., Bhattacharjee, R., Chatterjee, M., & Sil, P. C. (2005). Curative role of the aqueous extract of the herb, *Phyllanthus niruri,* against nimesulide-induced oxidative stress in murine liver. *Biomedical Research, 16*, 171–176.

Sarkar, K., & Sil, P. C. (2006). A 43 kD protein from the herb *Cajanus indicus* L. Protects thioacetamide-induced cytotoxicity in hepatocytes. *Toxicology in Vitro, 20*, 634–640.

Sarkar, M. K., & Sil, P. C. (2007). Hepatocytes are protected by herb *Phyllanthus niruri* protein isolate against thioacetamide toxicity. *Pathophysiology, 14*, 113–120.

Schreck, R., Albermann, K., & Baeuerle, P. A. (1992). Nuclear factor kappa B: An oxidative stress-responsive transcription factor of eukaryotic cells. *Free Radical Research Communication, 17*, 221–237.

Singha, P. K., Roy, S., & Dey, S. (2007). Protective activity of andrographolide and arabinogalactan proteins from *Andrographis paniculata* Nees. Against ethanol-induced toxicity in mice. *Journal of Ethnopharmacology, 111*, 13–21.

Sinha, M., Manna, P., & Sil, P. C. (2006). Aqueous extract of the bark of *Terminalia arjuna* plays protective role against sodium fluoride induced hepatic and renal oxidative stress. *Journal of Natural Medicine, 61*, 251–260.

Sinha, M., Manna, P., & Sil, P. C. (2007). A 43 kD protein from the herb *Cajanus indicus* L., protects against fluoride induced oxidative stress in mice erythrocytes. *Pathophysiology, 14*, 47–54.

Sivapriya, M., & Srinivas, L. (2007). Isolation and purification of a novel antioxidant protein from the water extract of Sundakai (*Solanum torvum*) seeds. *Food Chemistry, 104*, 510–517.

Tietze, F. (1969). Enzymic method for quantitative determination of nanogram amount of total and oxidizes glutathione: Applications to mammalian blood and other tissues. *Analytical Biochemistry, 27*, 502–522.

Tsoi, A. Y., Ng, T. B., & Fong, W. P. (2005). Antioxidative effect of a chymotrypsin inhibitor from *Momordica cochinchinesis* (Cucurbitaceae) seeds in primary rat hepatocyte culture. *Journal of Peptide Science, 11*, 665–668.

Venkateswaran, P. S., Millman, I., & Blumberg, B. S. (1987). Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: In vitro and in vivo studies. *Proceedings of National Academy of Sciences USA, 84*, 274–278.