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Characterization of anticancer, DNase and antifungal activity of pumpkin 2S albumin

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ABSTRACT

The plant 2S albumins exhibit a spectrum of biotechnologically exploitable functions. Among them, pumpkin 2S albumin has been shown to possess RNase and cell-free translational inhibitory activities. The present study investigated the anticancer, DNase and antifungal activities of pumpkin 2S albumin. The protein exhibited a strong anticancer activity toward breast cancer (MCF-7), ovarian teratocarcinoma (PA-1), prostate cancer (PC-3 and DU-145) and hepatocellular carcinoma (HepG2) cell lines. Acridine orange staining and DNA fragmentation studies indicated that cytotoxic effect of pumpkin 2S albumin is mediated through induction of apoptosis. Pumpkin 2S albumin showed DNase activity against both supercoiled and linear DNA and exerted antifungal activity against *Fusarium oxysporum*. Secondary structure analysis by CD showed that protein is highly stable up to 90 °C and retains its alpha helical structure. These results demonstrated that pumpkin 2S albumin is a multifunctional protein with host of potential biotechnology applications.

1. Introduction

The plant 2S albumins constitute an important class of low-molecular-weight multifunctional seed storage proteins. The 2S albumins are typical 1:1 disulfide linked heterodimers made of approximately 4 kDa small and 10 kDa large subunits [1,2]. Most 2S albumins possess eight conserved cysteines forming four disulfide bridges, two interchain and two intrachain in large subunit [3,4]. Apart from conserved cysteines, the amino acid sequence homology is relatively low within and among plant species. They exhibit a compact and stable structure made of five α-helices [5]. The members of this family have been bestowed with one or more functions which may include translational inhibitory, antimicrobial and serine proteinase inhibitory activities [5–9]. The pumpkin 2S albumin, apart from having translational-inhibitory activity, has been shown to possess RNA hydrolytic activity not reported for any other member of this family [10–12]. There is no report of anticancer activity for 2S proteins except for lunasin peptide derived from soybean 2S albumin [13].

The pumpkin (*Cucurbita* sp.), with its most parts edible, is one of the most widely used vegetable. The pumpkin seeds are rich source of minerals and protein and have been shown to have therapeutic potential [14]. The post-translational processing, purification and characterization of a 2S albumin from pumpkin seeds has been reported earlier [10,11]. The present study extends the characterization of pumpkin 2S albumin for its anticancer, DNase and antimicrobial activities. Also, secondary structure content and stability of pumpkin 2S albumin has been analyzed. These novel properties of pumpkin 2S albumin will add to its potential applications in biotechnology.

2. Material and methods

2.1. Materials

The seeds of *Cucurbita maxima* were obtained locally. DEAE-Sepharose, CM-Sepharose, Cibacron Blue 3GA, SDS–PAGE reagents and BSA were procured from Sigma–Aldrich (St. Louis, MO, USA), Human estrogen receptor positive, MCF-7 (adenocarcinoma) breast cancer cells; human androgen receptor negative prostate cancer cell lines DU-145 and PC-3; human ovarian cancer cells PA-1; and human liver cancer cells HepG2; were all obtained from National Center for Cell Science (NCCS) Pune, India. All cell culture reagents were from GIBCO (Invitrogen, CA, USA). MTT (3-(4,5-dimethyl-2-thiazolyl)2,5diphenyl-2H-tetrazoliumbromide), cell culture grade dimethyl sulfoxide (DMSO), agarose and all analytical grade chemicals were from HiMedia (Mumbai, India).

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2.2. Purification of protein

The seed extract, obtained after grounding and soaking of pumpkin seeds overnight in 50 mM of Tris–HCl, was filtered and centrifuged for 45 min at 18,000 rpm. The cleared supernatant was applied onto a pre-equilibrated DEAE-Sepharose column (1.5 × 10 cm, Bio-Rad). The flow through of DEAE column containing protein of interest was applied on to a CM-Sepharose column (1.5 × 8 cm, Bio-Rad) pre-equilibrated with 50 mM Tris–HCl buffer, pH 7.4. The bound proteins on CM column were eluted with a step gradient of NaCl in same buffer (50, 100, 300 and 500 mM). The desalted protein was concentrated using a 3 kDa cut off Centricon. The protein of interest was chromatographed on a Cibacron Blue 3GA affinity column (1.5 × 5 cm, Bio-Rad) in Tris–HCl, pH 7.4. The protein was eluted in same buffer containing 500 mM NaCl after extensive washing. The eluted protein was gel-filtered on a Superdex 75, 10/300 GL (GE healthcare) pre-equilibrated with 50 mM Tris–HCl buffer, pH 7.4. The purity of the protein was monitored on a 12% SDS–PAGE under both reducing and non-reducing conditions. To determine relative molecular weight of the protein, SDS–PAGE was performed along with molecular weight standards [15]. Protein concentration was determined by microbiuret method [16].

2.3. Activity on cell lines

2.3.1. Cell culture

MCF-7, PC-3, DU-145 and PA-1 cancer cells were maintained in Dulbecco's modified Eagle's media (DMEM) while HepG2 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (heat inactivated) (both from Invitrogen, Carlsbad, CA, USA) and 1% antibiotic (100 U/ml of penicillin and 100 μg/ml streptomycin) (Himedia, Mumbai, India) mix at 37 °C in humidified atmosphere in a CO_2 incubator.

2.3.2. Cytotoxicity assays

Different human cell lines, breast cancer cell line MCF-7, ovarian teratocarcinoma cell line PA-1, prostate cancer cell line PC-3 and DU-145 and liver hepatocellular carcinoma (HepG2) were taken as target for protein. MTT assay was carried out as described previously [17]. In brief, 5×10^3 cells in 200 μl of medium were seeded in 96-well plates (Griener, Germany). Different concentration of protein: 1, 5, 10, 20and 30 μM in buffer (Tris–Hcl, 20 mM, pH-7.4) were added to the monolayer. The buffer without protein was used as vehicle control. The cultures were assayed after 24 h by adding 20 μl of 5 mg/ml MTT followed by incubating at 37 °C for 4 h. The MTT-containing medium was then aspirated and 200 μl of DMSO (Himedia, Mumbai, India) was added to dissolve the formazone crystals. The optical density was measured at 570 nm using ELISA plate reader (Fluostar optima, BMG Labtech, Germany).

The percentage inhibition was calculated as

$$= 100 - [(\text{Mean OD of treated cell} \times 100)/\text{mean OD of vehicle treated cells(negative control)}]$$

2.3.3. Acridine orange staining

In order to check the plasma-membrane permeability, nuclear morphology and the chromatin condensation, the MCF-7 cells were stained with Acridine orange (AO)/Ethidium bromide (EB) dye mixture (100 μg/ml AO and 100 μg/ml EB) according to the protocol described earlier [18]. In brief, 0.5×10^6 cells were seeded for the assay in a 6-well plate and incubated with concentrations of 5, 10 and 20 μM of protein for 24 h and then washed properly with PBS (phosphate buffered saline). Then 500 μl AO/EB dye mixture dissolved in PBS was added to the plate and observed under fluorescent microscope (Zeiss, Axiovert 25, Germany).

2.3.4. DNA fragmentation assay

The DNA cleavage pattern due to the cytotoxicity of the compound was analyzed by agarose gel electrophoresis according to the protocol described earlier [19]. Briefly, 3×10^6 cells were exposed to protein at concentration 10 μM for 24 h. Thereafter cells were centrifuged, washed with PBS and the pellet was lysed with 400 μl of hypotonic buffer solution (10 mM Tris pH 7.5, 1 mM EDTA, 0.2% Triton X-100) for 15 min at room temperature and then centrifuged for 15 min at 12,000 rpm. Then 350 μl of the supernatant was again lysed in 106 μl of the second lysis buffer containing 150 mM NaCl, 10 mM Tris–HCl pH 8.0, 40 mM EDTA, 1% SDS, 0.2 mg/ml proteinase K (at final concentration) for 4 h at 37 °C. The DNA was then extracted with phenol/chloroform/isoamyl alcohol (25:25:1) and the pellet obtained was washed with ethanol and resuspended for RNase digestion in 15 μl of 10 mM Tris, 1 mM EDTA, pH 8.5, and 50 μg/ml RNase for 1 h at 37 °C. The DNA was analyzed by electrophoresis at 50 V/cm in a 2% agarose gel.

2.3.5. Statistical analysis

Data are expressed as mean ± SEM and statistically evaluated using one way ANOVA followed by Bonferroni *post hoc* test using Graph Pad Prism 5.04 (Graph Pad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered to be statistically significant.

2.4. Assay for deoxyribonuclease activity

DNase activity of pumpkin 2S albumin was carried out on both closed circular plasmid DNA (pBR-322 plasmid DNA) and liner double stranded DNA (BL-21 genomic DNA and calf thymus DNA). The activity on circular DNA was carried out by incubating 100 ng of supercoiled plasmid with increasing concentrations of purified pumpkin 2S albumin (50, 100, 200, 300, 400 and 500 ng) in 50 mM Tris, pH 7.4 for 60 min at 37 °C in total volume of 10 μl. Effect of divalent and monovalent cations was examined by performing the experiment in presence of 5 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$ and 100 mM of Licl, NaCl and KCl. The effect of 1 mM DTT and EDTA was also examined. Only plasmid and plasmid with 500 ng of Bovine serum albumin was taken as control. For linear DNA, 100 ng of BL-21 genomic DNA was incubated with increasing concentrations of protein (10, 30, 50 and 100 ng) in 50 mM Tris, pH 7.4 for 60 min at 37 °C in total volume of 10 μl. To determine effect of temperature on protein's DNase activity on pBR-322 plasmid DNA, 100 ng of protein incubated at different temperature from 30 to 90 °C for 15 min. The reactions were terminated after incubation by adding 10 μl of Ficoll solution (30% Ficoll, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF). The samples were electrophoresed on a horizontal setup under non-denaturing conditions in standard TAE buffer in 1% agarose gel at 60 V. For in-gel assay, DNase activity on SDS–PAGE was performed according to the methods of Blank et al. [20]. A 12% polyacrylamide gel containing 0.1 mg/ml of Calf thymus DNA was used. Samples were electrophoresed without prior treatment of 2-mercaptoethanol or boiling. After electrophoresis, the gels were rinsed with two changes of 25% isopropanol in 50 mM Tris–HCl (pH 7.0), followed by three changes of reaction buffer. All rinses were for 30 min at room temperature in 250 ml of the respective buffer. Rinsing was followed by 10 h of incubation at 55C in reaction buffer. The incubated gels were then rinsed and stained with 0.5 μg/ml Ethidium bromide for 30 min, the dark band produced by DNase activity of protein was observed under ultraviolet light.

2.5. Circular dichorism studies

Circular dichroism analysis of protein was carried out on a Chirascan CD spectrometer (Applied Photophysics, UK) in Sodium phosphate buffer (20 mM, pH 7.4). To record Far-UV (190–260 nm) CD spectra, 0.2 mg/ml protein was used in quartz cell of 1 mm path length at 1 nm band width and 0.25 s per point. Sample cuvette has been attached to peltier system, incubated for 5 min and the conformational stability of protein at different temperature (30–90 °C) was examined by recording the spectra. Three data sets were recorded and then average CD spectra were used for analysis. The analyses of CD spectra were performed using online DichroWeb programme [21].

2.6. Antifungal activity

The antifungal activity of protein against the growth of the different fungal species such as *Fusarium oxysporum* (NCIM 1281), *Phanerochaete chrysosporium* (MTCC 767) and *Aspergillus flavus* (MTCC 1783) grown on PDA medium was tested. The growth inhibition was monitored by placing the 50 and 100 μg of protein on sterile paper disc at margin of the fungal mycelia. Sterilized 50 mM Tris–HCl buffer, pH 7.4 and cyclohexamide (50 μg) were used as negative and positive controls respectively. Fungal cultures were allowed to grow at 30 °C. The occurrence of the inhibition zone near the protein containing paper disc was used as an assessment of the growth inhibition.

3. Results and discussion

3.1. Purification of protein

The purification of pumpkin 2S albumin was accomplished using ion-exchange (anion- and cation-exchange), affinity and gel filtration chromatography. In anion exchange chromatography, most of the higher molecular weight proteins were adsorbed on to the DEAE column while protein of interest came in flow through. The flow through was applied on to a CM Sepharose column. The bound fractions were eluted using a step gradient of NaCl (100–500 mM). The fraction containing protein of interest got eluted at 300 mM NaCl. The protein was then desalted by dialysis and then loaded on to a Cibacron Blue 3GA affinity column. The column was washed thoroughly and adsorbed protein was eluted at 500 mM NaCl. The eluted fraction from affinity column was applied on to a size exclusion chromatography on Superdex S-75 column. The purity of the protein was determined by a single band on a non-reducing 12% SDS–PAGE (Supplementary Fig. S1). The SDS–PAGE analysis under both reducing conditions showed that protein is made of two disulfide-linked polypeptide chains of approximately 7 and 5 kDa and a total molecular weight of around 12.5 kDa.

3.2. Activity on cancer cell lines

3.2.1. Estimation of cytotoxicity

The cytotoxic effect of pumpkin 2S albumin toward selected cancer cell lines was estimated using MTT assay in a dose dependent manner (Fig. 1). The cells were incubated with pumpkin 2S albumin at various concentrations 1, 5, 10, 20, 30 and 40 μM. Cell viability of breast cancer (MCF-7), ovarian teratocarcinoma (PA-1), prostate cancer (PC-3 and DU-145) and hepatocellular carcinoma (HepG2) by treatment of 20 μM of protein was found to be 43.40%, 54.81%, 49.12%, 43.3% and 45.69%, respectively (Fig. 1). Among all the cell lines tested the protein was found to be more sensitive to MCF-7 cells where significant inhibition in cell viability was seen at 5 μM. A range of low-molecular-weight plant proteins including lunasin peptide derived from soybean 2S albumin have shown antitumor potential [22].

3.2.2. Pumpkin 2S albumin induced apoptosis and induction of DNA fragmentation

Acridine orange–Ethidium bromide staining and DNA fragmentation assay were employed to investigate the role of apoptosis in cytotoxicity of pumpkin 2S albumin. MCF-7 cells under the stress of early and late apoptosis can also be distinguished by the clear chromatin condensation and percentage uptake of AO: EB dye mixture. AO permeates all cells and makes the nucleus appear green while EB is taken up by the cells only when the cytoplasmic membrane integrity is lost as in late apoptosis or in necrosis staining the nucleus red (Fig. 2A). As shown in the Fig. 2A, the buffer (20 mM, pH 7.4) treated cells (control) had maximum number of viable cells showing Acridine orange staining with normal cell morphology. On the contrary, in case of cells treated with 10 and 30 μM of pumpkin 2S albumin there was increased number of EB stained cells as compared to vehicle treated cells indicating apoptotic cell death which was more prominent in a dose dependent manner.

To further confirm the above results, classical hallmark of apoptosis such as DNA fragmentation assay was carried out. As shown in Fig. 2B, the protein was almost equally potent and caused fragmentation of DNA in MCF-7 cells when treated at 10 and 30 μM concentrations respectively as compared to control cells. The results obtained from Acridine orange staining and DNA fragmentation assays thus indicate that the protein induced apoptosis in MCF-7 cells.

3.3. Deoxyribonuclease and antifungal activities

The pumpkin 2S albumin exhibited DNase activity on both closed circular and linear double stranded DNA. Pumpkin 2S albumin showed strong DNase activity on closed circular supercoiled DNA. At low concentration of protein (50 ng), 100 ng of DNA was transformed to nicked circular and linear DNA. With increasing concentrations of protein, the nicked and linear DNA was hydrolyzed completely (Fig. 3A). The presence of Mg^{2+} and Mn^{2+} enhanced the DNase activity of protein while $Zncl_2$ inhibited the activity. The presence of Ca^{2+} and Ni^{2+} marginally enhanced the activity (Fig. 3B). Among the monovalant cations, only LiCl showed activity almost similar to that of $NiCl_2$ whereas NaCl and KCl did inhibit the conversion of nicked DNA to liner. Pumpkin 2S albumin hydrolyzed 100 ng BL-21 genomic of linear DNA with complete hydrolysis at 100 ng of protein. The presence of EDTA inhibited the DNase activity on closed circular DNA (Fig. 3B). Effect on liner BL-21 genomic DNA shown that protein is very effective at 30 ng concentration while BSA confirms that there is no pseudo activity (Fig. 3C). Incubation of protein for 15 min up to 90 °C has little effect on activity demonstrating the stability of pumpkin 2S albumin (Supplementary Fig. S2a). In gel assay on polyacrylamide containing 0.1 mg/ml calf thymus DNA shows the clear dark band at position of 5 and 10 μg of protein on gel that shows the degradation of DNA at that position while in only loading dye position there is no degradation (Supplementary Fig. S2b). The results demonstrated that in addition to RNA hydrolytic activity reported earlier [12], pumpkin 2S albumin exhibited DNase activity indicating dual DNase/RNase nature. The results demonstrated the non-specificity of DNA hydrolytic activity. It is interesting to note that Mg^{2+} and Mn^{2+} enhance whereas Zn^{2+} inhibited the activity. Also, DNase activity was found to be sensitive to EDTA implying the effect of the presence of metal ion. Earlier report on pumpkin 2S albumin showed that the RNase activity was inhibited in the presence of metal ions particularly Zn^{2+} [12]. Among low-molecular-weight proteins, the dual DNase/RNase activity has been reported for 14 kDa PR4 protein from *Capsicum chinense* [23].

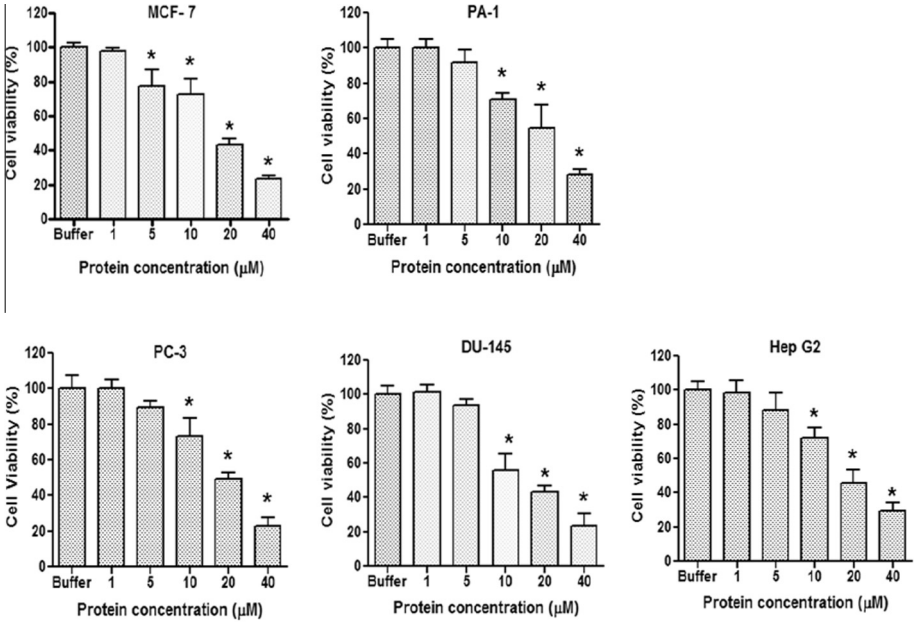

Fig. 1. Results of MTT assay on different cell lines. Histogram indicates the sum of cells analyzed in presence of buffer and different concentration of protein, representing total number of apoptotic cells. Results are the mean ± SEM of three independent experiments. *Represent statistically significant difference from control cells by multiple comparisons test at $p < 0.05$.

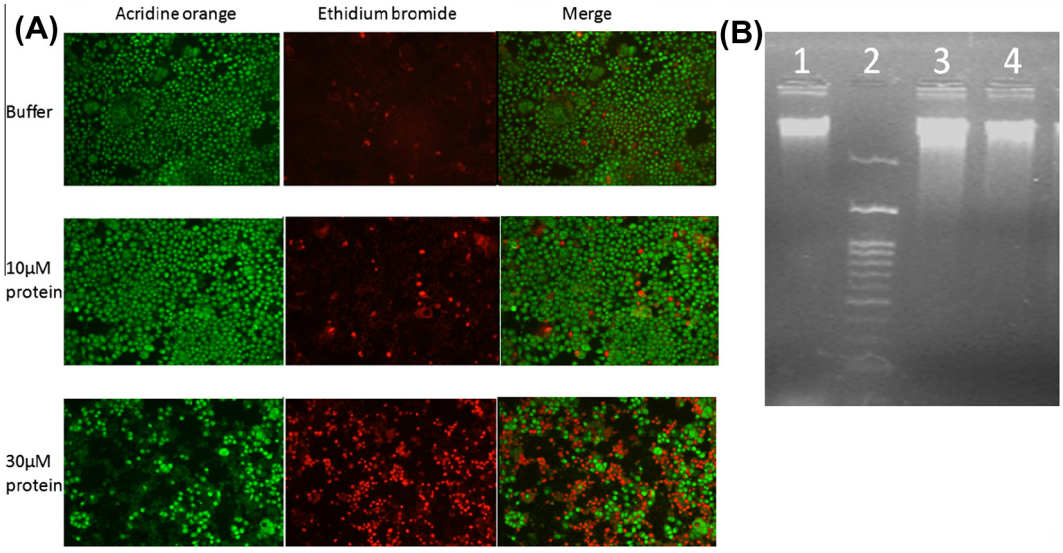

Fig. 2. (A) Evaluation of apoptosis by using Acridine orange/Ethidium bromide staining on MCF 7 cells treated with buffer, 10and 30 μM of protein for 24 h. The green staining indicates that the cell membrane integrity does not allow the entry of Ethidium bromide in the cytoplasm. Apoptotic cells are stained red due to the entrance of the Ethidium bromide. The nuclear staining was visualized at 200× magnification of fluorescent microscope. The experiment was performed in triplicate and a representative data is presented. (B) Induction of DNA fragmentation by protein in MCF-7 cells. Genomic DNA of normal cell (lane 1), and 10and 20 μM protein treated cells (lanes 3 and 4). Lane 4 shows the molecular weight marker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Pumpkin 2S albumin exerted antifungal activity against *F. oxysporum* as 50 and 100 μg of protein is making clear zone around periphery of fungal growth (Fig. 3D). The antifungal activity has been reported for 2S seed storage proteins [24,25].

3.4. Circular dichorism studies

Far-UV (190–260 nm) CD spectroscopy studies were performed for secondary structure analysis and conformational stability of protein at different temperature (Fig. 4A). CD spectra of native protein contained negative peaks at around 208 nm and 222 nm which clearly showed that it is a predominantly α-helical protein. The structural studies of 2S albumins from plants by NMR have demonstrated that they adopt a compact three-dimensional structure made of five α-helices [26]. There is very little change in helical structure of protein up to 90 °C. After heating at 90 °C for 15 min and then cooling at room temperature or at 30 °C, protein nearly retains its original state. The most 2S proteins are very stable owing to their compact structure stabilized by disulfide bonds. Napin proteins, belonging to 2S seed storage family, from some plants have been shown to have their helical structure intact at high temperature as 80–90 °C [27].

In conclusions, the present study demonstrated the cytotoxicity of pumpkin 2S albumin toward cancer cell lines which could add to

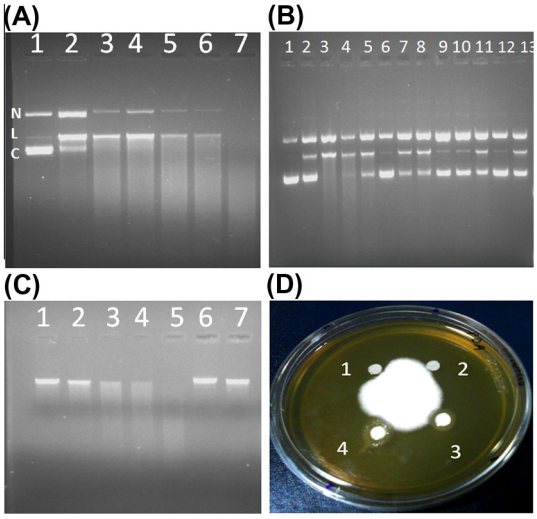

Fig. 3. DNase and antifungal activity of pumpkin 2S albumin. (A) Effect of protein concentration on DNase activity. Lanes 1–7 represent 100 ng of pBR322 closed circular double stranded DNA incubated with 0, 50, 100, 200, 300, 400 and 500 nM protein respectively examine for DNase activity on plasmid DNA. Closed circular, nicked and liner plasmid denoted by C, N and L, respectively. (B) Effect of metal ions on Dnase activity. L1, 100 ng of pBR322 plasmid in buffer only, L2, activity of 50 ng of protein only, L3–10, activity of protein in presence of $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $NiCl_2$, LiCl, NaCl, KCl, L11–12, effect of DTT and EDTA on activity. (C) DNase activity on linear DNA. BL-21 genomic DNA was incubated with buffer (lanes 1 and 7), BSA (lane 6) and 10, 30, 50 and 100 ng of protein (lanes 2–5, respectively). (D) Antifungal activity of protein against *Fusarium oxysporum*. Fungus has been grown on PDA agar plate. 1, positive control (50 μg of cyclohexamide); 2, negative control as buffer; 3 and 4 with 50 and 100 μg of protein.

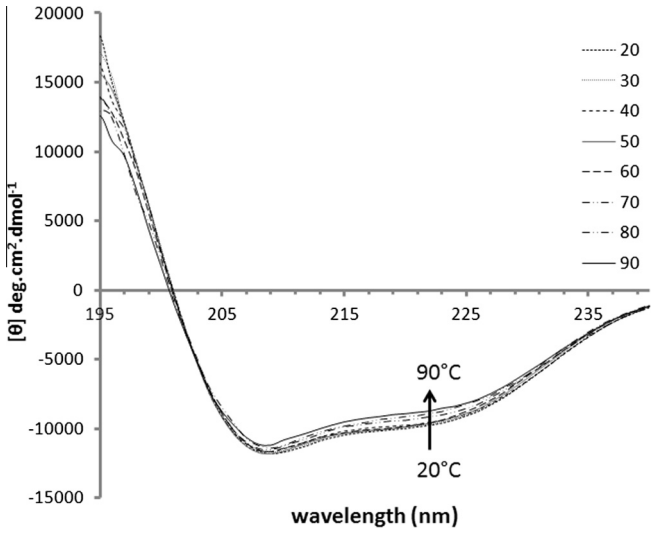

Fig. 4. Far-UV CD studies of pumpkin 2S albumin at increasing temperature (20 to 90 °C).

its biotechnological applications as potential antitumor agent. Pumpkin is one of the dietary sources in many countries. Also, it seems that full functional potential of many 2S albumin has not been investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.158.

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