Article

Purification of lectin and Kunitz trypsin inhibitor from soya seeds

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Abstract

The search for potent and selective therapeutic agents is progressing by the study of natural compounds in plants. Plant-derived macromolecules are considered emerging therapeutic agents and an alternative to synthetic and small molecule drugs. Where it has long been known that plants possess medicinal properties, the compounds responsible for their action are in many cases still unknown: often only whole crude plant extracts or fractionated extracts are tested for the ability to inhibit common pathogens. Here, we present a fast protein liquid chromatography method for the separation of crude plant proteins. Kunitz trypsin inhibitor (KTI; 24.2 kDa) and lectin (31 kDa) were purified from *Glycine max* by liquid extraction followed by ion exchange column chromatography. The need for serial chromatographic separation steps has been eliminated by introducing more complex elution profiles hence reducing cost, time and improving recovery. The identity of KTI-A and lectin was confirmed by MALDI-TOF/TOF Mass Spectrometry. Cell proliferation assays using B16F1 melanoma cells revealed that both KTI and the monomeric lectin retained some antiproliferative activity. This method could be useful for rapid and cost-effective purification of bioactive compounds from plant material.

Introduction

Epidemiologic studies have demonstrated that consumption of soya products or food may prevent the progression of type 2 diabetes mellitus (1), could reduce cholesterol and the risk of cardiovascular disease (2) or possibly decrease the risk of some cancers (3–7). Many proteins found in soya seeds are active ingredients and could be used for therapeutic purposes (8, 9). Examples of soya bean-derived bioactive proteins include lectins (10), β-conglycinin (11), lunasin (12, 13) and trypsin inhibitors such as Bowman–Birk (14) and Kunitz trypsin inhibitors (KTIs) (15, 16) (Table I). KTIs (~20 kDa) have been extracted from different seeds (17). Recent studies investigating *in vitro* bioactivity suggested that soya bean-derived KTI possess the ability to prevent the proliferation of cancer cells including liver, breast, nasopharyngeal and ovarian cancer cells (15, 16). Due to the ability of protease inhibitors to interfere with physiological activities of not only prokaryotic but also eukaryotic organisms, they are explored for various possible applications in the biotechnological, pharmaceutical, agriculture and food industries. Lectins are a group of proteins that are capable of binding to glycoproteins, glycolipids and polysaccharides without chemical modification (18). Preclinical studies have suggested plant lectins including soya bean lectins can inhibit cancer cells proliferation (19–21).

Due to the therapeutic applications of KTI and lectin, and with new functionalities still emerging, a rapid extraction and purification method to obtain pure lectin or KTI from soya bean or other natural sources is desirable. In addition, KTI is an important anti-nutritional factor in soya bean seeds and there is a need for more straightforward analytical methods to obtain functional protein for quantification and characterization. Preparative column chromatography is a popular separation method due to its high reproducibility, the ability to yield large quantities of pure protein. It has been used for the purification of plant extracts including KTI and lectins and generally involves multiple steps. Typically, anionic and cationic exchange column chromatography is used in combination with size exclusion

Table I. Examples of bioactive proteins extracted from soya bean

Proteins	Activity	References
β-Conglycinin[a]	Reduce cholesterol, anticancer	(11, 22)
Glycinin[a]	Reduce cholesterol, antimicrobial	(23, 24)
Lectin[a]	Anticancer	(19, 21)
Lunasin[a]	Anticancer	(12, 13, 19)
44-kD Soya bean toxin[a]	Antifungal	(25, 26)
Peroxisomal proteins[b]	Numerous, including fatty acid β-oxidation, metabolite transport, photorespiratory glycolate metabolism and stress response	(27)
Soya bean peribacteroid membrane proteins[c]	Protein translocation, folding, maturation or degradation in symbiosomes	(28)
Glysojanin[a]	Antifungal	(29)
β-Glucan-binding protein[a]	Pathogen recognition and activation of defense	(30)
Neutral PR-5 protein (GmOLPb)[d]	Protect the plant from stress imposed by high salt	(31)
GmPep914 (DHPRGGNY) and GmPep890 (DLPRGGNY) peptides[d]	Plant defense	(32)
Allergen P34[a]	Human allergen	(33)
Bowman–Birk inhibitor[a]	Anticancer	(14, 34)
Kunitz trypsin inhibitor[a]	Anticancer and HIV-1 reverse transcriptase inhibitory activities	(15, 16)

Protein source:[a] seeds;[b] cotyledons;[c] roots;[d] leaves

chromatography or gel filtration. Most elution profiles in the ionic chromatographic separation comprise a linear or stepwise gradient of NaCl (16, 18, 20, 21).

Eluting proteins with a stepwise gradient could reduce the numbers of chromatography steps leading to a faster and cost-effective purification process. Here, we describe a fast preparative method for the isolation and purification of lectin and KTI from the crude extract of soya bean (*Glycine max*) using anionic and cationic exchange column chromatography. To prove that the purified proteins remained biological active, their effect on the metabolic activity of B16F1 melanoma cells was determined.

Materials and methods

Materials

Soya bean (*G. max* (L.) Merr.) seeds were sourced from Sema Foods Ltd, UK. Chromatographic columns (Bio scale™ Macro-prep DEAE cartridge (weak anionic), Bio scale™ Macro-prep High S cartridge (strong cationic), 5 mL) were purchased from Bio-Rad Laboratories Ltd, UK. RPMI 1640, penicillin/streptomycin/glutamine (PSG), FBS and dialysis cassettes (Slide-A-Lyzer™, 2 kDa) were purchased from life technologies (Thermo Scientific, UK). Mouse melanoma (B16F1) cells were from the European Collection of Cell Cultures. All other chemicals and reagents were of analytical grade, purchased from Sigma Aldrich Company Ltd, UK.

Preparation of soya bean extract

Air dried mature soya seeds (100 g) were ground in liquid nitrogen with pestle and mortar to a fine powder, defatted with diethyl ether (soya powder to diethyl ether ratio of 1:6 (w/v)) under overnight continuous stirring and then air dried. Crude proteins were extracted using buffer X (25 mM Tris-HCl, 10 mM PMSF, 1 mM EDTA, pH 7.5). Samples (20 g of powder into 100 mL buffer X) were stirred for 4 h at 4°C and centrifuged (21,000 *g*) for 30 min at 4°C. The supernatant was collected and the extraction process repeated twice. The supernatants were pooled to obtain a crude extract from which proteins were precipitated using ammonium sulfate (30–95% saturation for 1 h at 4°C). After centrifugation (21,000 *g*) of the mixture for 20 min at 4°C, the new supernatant was discarded; the residue was dissolved in buffer A (20 mL, 25 mM Tris-HCl, pH 8.1) and dialyzed against dH_2O (MWCO 3 kDa) for 24 h at 4°C. The total protein content was determined by Bradford protein assay using BSA (5–100 µg/mL) as a standard (35).

Chromatographic isolation and purification of soya bean proteins

Optimization of separation conditions involved changing the flow rate (1–3 mL/min) and the gradient elution profile. In the optimized method, dialyzed crude extract (4 mL) was loaded onto a weak anionic exchange column (Macro-prep DEAE) equilibrated with buffer A (25 mM Tris-HCl, pH 8.1). The adsorbed fractions were eluted using a stepwise saline gradient at a flow rate of 2.5 mL/min [0–45 min: 100% buffer A; 46–52 min: 0–7% buffer B (25 mM Tris-HCl, 0.75 M NaCl, pH 8.1); 53–60 min: 7–10% buffer B; 61–71 min: 10–12% buffer B; 72–80 min: 12–20% buffer B; 81–90 min: 20–25% buffer B; 91–95 min: 25–30% buffer B; 96–105 min: 30–35% buffer B; 106–115 min: 35–40% buffer B; 116–123 min: 40–50% buffer B; 124–131 min: 50–60% buffer B; 132–139 min: 60–80% buffer B; 140–147 min: 80–100% buffer B; 148–158 min: 100% buffer B]. Proteins elution were recorded at 280 nm and fractions collected every 30 s. Ten different peaks (noted I–X) were identified. After dialysis against water and freeze drying, the fractions corresponding to Peaks II and III, and Peaks VI and VII were pooled separately (A1 and A2, respectively) and further purified on a strong cationic exchange column (Macro-prep High S) equilibrated with buffer C (20 mM ammonium acetate buffer, pH 4.5). The adsorbed fractions were eluted as previously described using buffer D (20 mM ammonium acetate buffer, 0.5 M NaCl, pH 4.5 [0–20 min: 100% buffer C; 21–25 min: 0–10% buffer D; 26–34 min: 10–15% buffer D; 35–42 min: 15–25% buffer D; 43–50 min: 25–40% buffer D; 51–58 min: 40–60% buffer D; 59–66 min: 60–80% buffer D; 67–74 min: 80–100% buffer D; 75–85 min: 100% buffer D]. To determine the degree of reproducibility, the anionic exchange column chromatography was repeated three times using ammonium sulfate precipitated (40–95%) fractions of the crude extract from three different extractions.

Gel electrophoresis

Proteins in crude extracts and in eluted fractions were visualized by SDS-PAGE (4% stacking gel, 12% resolving gel). Collected fractions were dialyzed against dH_2O, lyophilized and reconstituted in HEPES buffer (10 mM, pH 7.4). After electrophoresis (2 h, 100 V), the gel was stained with Coomassie blue (R 250, BioRad) solution (200 mL, 1–2 h) and de-stained (methanol:water:acetic acid; 3:6:1) until the protein bands were clearly visible. The molecular weight of the proteins were estimated by comparison with the protein molecular mass markers (i.e., albumin (Bovine serum, 66 kDa), ovalbumin (Egg, 45 kDa), carbonic anhydrase (Bovine erythrocytes, 29 kDa), trypsin inhibitor (Soya bean, 20 kDa), α-lactalbumin (bovine milk, 14.2 kDa) and cytochrome C (Equine heart, 12.4)) (Sigma Aldrich).

Identification of purified proteins by MALDI-TOF/TOF

In-gel digestion

Proteins of interest (20, 30 and 55 kDa) were cut out using a sterile scalpel from the SDS-PAGE gel under sterile conditions and each protein band was transferred to a sterile Eppendorf tube. After washing the gel bands in HPLC grade water (500 μL), dithiothreitol (DTT) (10 mM, 30 μL) in ammonium bicarbonate (100 mM) was added and then the gel pieces were incubated at 56°C for 30 min. After removing DTT, iodoacetamide (IAA) (55 mM, 30 μL) in ammonium bicarbonate (100 mM) was added and incubated for 45 min in the dark and at room temperature (r.t.). After decanting IAA, the gel pieces were incubated at 37°C for 30 min in 500 μL of ammonium bicarbonate (100 mM)/MeCN (50:50). The washing procedure was repeated twice and the gels were dried in MeCN (100 μL) for 15 min at r.t. trypsin (20 μg/vial, Sigma Aldrich) was reconstituted in 100 μL of HCl (1 mM) and 900 μL of ammonium bicarbonate (40 mM) in 9% MeCN. Trypsin solution (30 μL) was added to the dried gel pieces, which were left on ice for 30 min, 40 μL of ammonium bicarbonate (50 mM, pH 8.0) was then added to it and then incubated overnight at 37°C. The supernatant was transferred to a methanol washed Eppendorf tube and the gel pieces were incubated in extraction buffer (0.1% formic acid in 50% MeCN solution) for 15 min at r.t. Supernatants were combined and concentrated by evaporation to <20 μL for mass spectrometric analysis.

MALDI-TOF/TOF

Aliquots of the in-gel trypsin digested purified proteins were desalted by zip (C_{18}) tip. The desalted tryptic peptides were eluted in 0.1% TFA in 50% MeCN (10 μL) and added to a saturated solution of CCA (10 μL). About 2 μL of the mixture was loaded onto the MALDI plate and air dried. Mass spectrometry experiment was performed on a Bruker Ultraflex III MALDI-TOF/TOF (Bruker, USA) MS with the positive reflection mode. Ions were generated by a nitrogen laser emitting at 337 nm. Data were acquired over the mass range 50–2,500 Da. LIFT method in flex control was used to acquire mass spectra. Spectrum processing and annotation were performed in flex analysis using Sophisticated Numerical Annotation Procedure (SNAP) peak picking algorithm. The peptide mass lists obtained by MALDI-TOF/TOF were exported (MASCOT generic format) for MASCOT searching (Matrix Science, London, UK). Searches were done against preferred taxonomy against the Uni Prot sequence database (fixed modification: carbamidomethyl (C) and variable modification: deamidated (NQ), oxidation (M)).

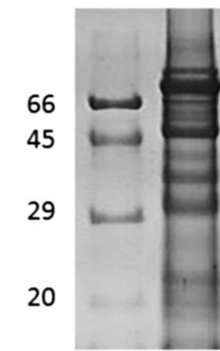

Figure 1. SDS-PAGE analysis of soya bean crude extract following precipitation in ammonium sulfate. M: molecular weight markers—trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and albumin (66 kDa).

Biological activity

B16F1 melanoma cells were cultured at 37°C in a 5% CO_2 atmosphere and using RPMI supplemented with 10% FBS and 1 × PSG. The viability of the cells was evaluated using an MTT assay. About 100 μL of B16F1 cells were seeded into a 96-well plate (10^4 cells/well). After 24 h, 20 μL of Kunitz inhibitor A (0–480 μg/mL) or lectin (0–35 μg/mL) in HEPES buffer (10 mM, pH 7.4) was added to the cells which were incubated for a further 24 h. About 20 μL of MTT reagent (5 mg/mL) was added to each well and the cells were further incubated at 37°C for 2 h. The supernatant was removed and 100 μL of DMSO was added. The plates were left at 37°C for 30 min and the absorbance was measured at 540 nm. The viability of the cells was determined relative to untreated control.

Results

Following grounding of soya bean (*G. max* (L.) Merr.) and liquid–liquid extraction, the total protein content in the crude extract was estimated to be 0.21 mg of protein per gram of soya seeds. The analysis of the crude extract by SDS-PAGE revealed the presence of ~15 different protein bands (Figure 1).

The extract was initially fractionated using a weak anionic exchange column (Macro-prep DEAE). Following optimization, crude samples were loaded onto the DEAE column equilibrated with 25 mM Tris-HCl at pH 8.1. Unadsorbed proteins were eluted with the buffer and adsorbed fractions were obtained using a combination of stepwise and linear gradients (0–0.75 M NaCl in 25 mM Tris-HCl, pH 8.1) (Figure 2A). The SDS-PAGE analysis of the resulting fractions indicated that the proteins were partially separated (Figure 2B). Proteins with high molecular weight (>30 kDa) were mainly eluted in the earlier fractions (Peaks I–IV) at low salt concentration (0.05–0.18 M NaCl) whereas lower molecular weight proteins (<25 kDa) were essentially eluted a higher salt concentration (0.19–0.60 M NaCl).

Peaks II and III (60–86 min) corresponding to proteins eluting at a salt concentration ranging from 0.075 to 0.15 M, displayed two intense bands (~30 and ~55 kDa), whereas Peaks VI and VII (105–124 min) corresponding to proteins eluting from 0.22 to 0.30 M NaCl displayed one intense band (~20 kDa) (Figure 2B).

Fraction A1 and Fraction A2, corresponding to the dialyzed pooled fractions from Peaks II and III, and Peaks VI and VII, respectively, were further purified by cationic exchange chromatography

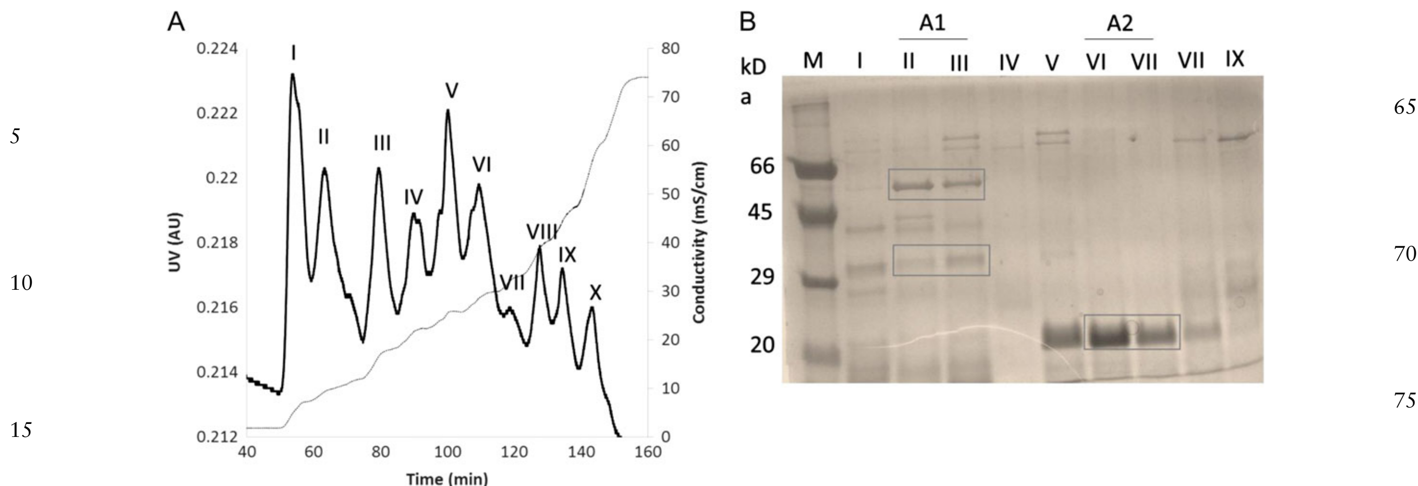

Figure 2. (A) Purification of the soya bean crude extract by anionic exchange chromatography The extract was loaded onto a weak anion exchange column (Macro-prep DEAE (40 mm × 12.6 mm)) equilibrated with 25 mM Tris-HCl, pH 8.1. The adsorbed proteins were eluted using stepwise and linear NaCl gradients (0–0.75 M NaCl in 25 mM Tris-HCl, pH 8.1) at a flow rate of 2.5 mL/min; fractions were collected every 30 s. The conductivity (—) indicates the NaCl gradient (B) SDS-PAGE analysis of the collected fractions (Peaks I–IX); M: molecular weight markers. Fractions II and III, and Fractions VI and VII were pooled together (A1 and A2), dialyzed and further purified by cationic exchange chromatography.

(Figure 3A). The Macro-prep High S column (strong cationic exchange) was equilibrated with an ammonium acetate buffer (20 mM, pH 4.5) which was also used to elute the unadsorbed proteins. As previously, the adsorbed proteins were obtained using a combination of stepwise and linear NaCl gradients (0 0.5 M NaCl in 20 mM ammonium acetate buffer, pH 4.5). For Fraction A1, two peaks were observed at high salt concentration (>0.2 M). Peak B was at ~60 min with a NaCl concentration between 0.20 and 0.25 M and Peak C was at ~70 min with a NaCl concentration between 0.25 and 0.30 M. For Fraction A2, the elution of the bound proteins (~60 min) was observed at even higher ionic strength with a salt concentration between 0.30 and 0.40 M. The SDS-PAGE analysis of the collected fractions (i.e., B, C and D) (Figure 3B) correlated with previous results (Figure 2B) and confirmed the estimation of the proteins molecular weight (Peak B: protein 1, ~55 kDa; Peak C: protein 2, ~30 kDa; Peak D: protein 3, ~20 kDa). The purification method was found to be highly reproducible, yielding a similar elution profile for different crude extracts (interday precision (based on retention time of Peaks II, III, VI and VII) between 0.49–0.91%CV, $n = 3$).

The identity of the proteins was determined by MALDI-TOF/TOF. The proteins (protein 1: ~55 kDa, protein 2: ~30 kDa and protein 3: ~20 kDa) were digested within the gels using trypsin. The resulting peptidic fragments were analyzed by MALDI-TOF-TOF and identified using the Uni Prot sequence database (MASCOT). Protein 1 was identified as a *G. max* β-amylase with a protein score of 776, protein 2 was identified as *G. max* lectin with a protein score of 481 and protein 3 was identified as *G. max* KTI-A with a protein score of 414 (Table II).

To determine if the proteins recovered after our purification process retained some activity, the effect of KTI (protein 3) and lectin (protein 2) on the viability of B16F1 melanoma cells was investigated using an MTT assay. The cells viability was determined relative to untreated control cells. Figure 4 shows that after an incubation period of 24 h, the activity of both proteins was dose dependent over the range of concentration used (0–100 μg/mL). The purified KTI had an IC_{50} value of ~160 μg/mL (~6.6 μM) whereas the purified lectin displayed minor inhibition with ~30% cell growth inhibition at a concentration of 35 μg/mL (~1.67 μM).

Discussion

Generally, lectin and KTI-A extracted from plant are purified using a combination of ion exchange, affinity and size exclusion column chromatography (16, 18, 21, 36), Supplementary data Table S1. For example, a melibiose-binding lectin from Chinese black soya beans was purified using a four steps column chromatography method using a strong anionic exchange column (Q Sepharose) with a stepwise elution profile (0.2, 0.5 and 1 M NaCl) which was followed by a strong cationic exchange column (SP Sepharose) also eluted with a stepwise profile (0.2 and 1 M NaCl) (21). These were then followed by another strong anionic exchange column (Mono Q) eluted with two consecutive NaCl linear gradients (0–0.3 M and 0.3–1 M) and finally a size exclusion column (Superdex 75 HR). Another study used a similar four steps procedure for the purification of KTI derived from Korean black soya beans (16). A three steps method developed with weak anionic and cationic columns (DEAE Sepharose and CM Sepharose, respectively) in combination with a size exclusion column (Sephacryl S-200) was used to purify lectin from *Typhonium divaricatum* (L.) (18). In both case, a NaCl linear gradient (0–0.5 M) was used to elute the bound proteins from the ionic columns. Lectin from *Sophora alopecuroides* seeds was also successfully purified using the similar columns and NaCl gradient (0.1–0.5 M) followed by size exclusion chromatography (Shephadex 75) (36). Here, we report a method using exclusively ion exchange columns resulting in pure proteins after a two-step chromatographic separation of crude extract. The elution profiles were optimized by changing the ionic strength of the mobile phase rather than its pH. Indeed, Dia *et al.* (37) have reported that optimization of protein separation by varying the pH of the mobile phase may not be an effective method as proteins in the mixture can display a similar binding profile. The pH of the buffer (25 mM Tris-HCl) was therefore fixed at pH 8.1, while its ionic strength was increased by a stepwise gradient, which was held in an isocratic mode for various times. Stepwise elution profiles tend to increase the separation time but they minimize the number of chromatographic steps required and improved the resolution of the peaks (17). Simply, by optimizing the elution profile, the use of multiple step separations are

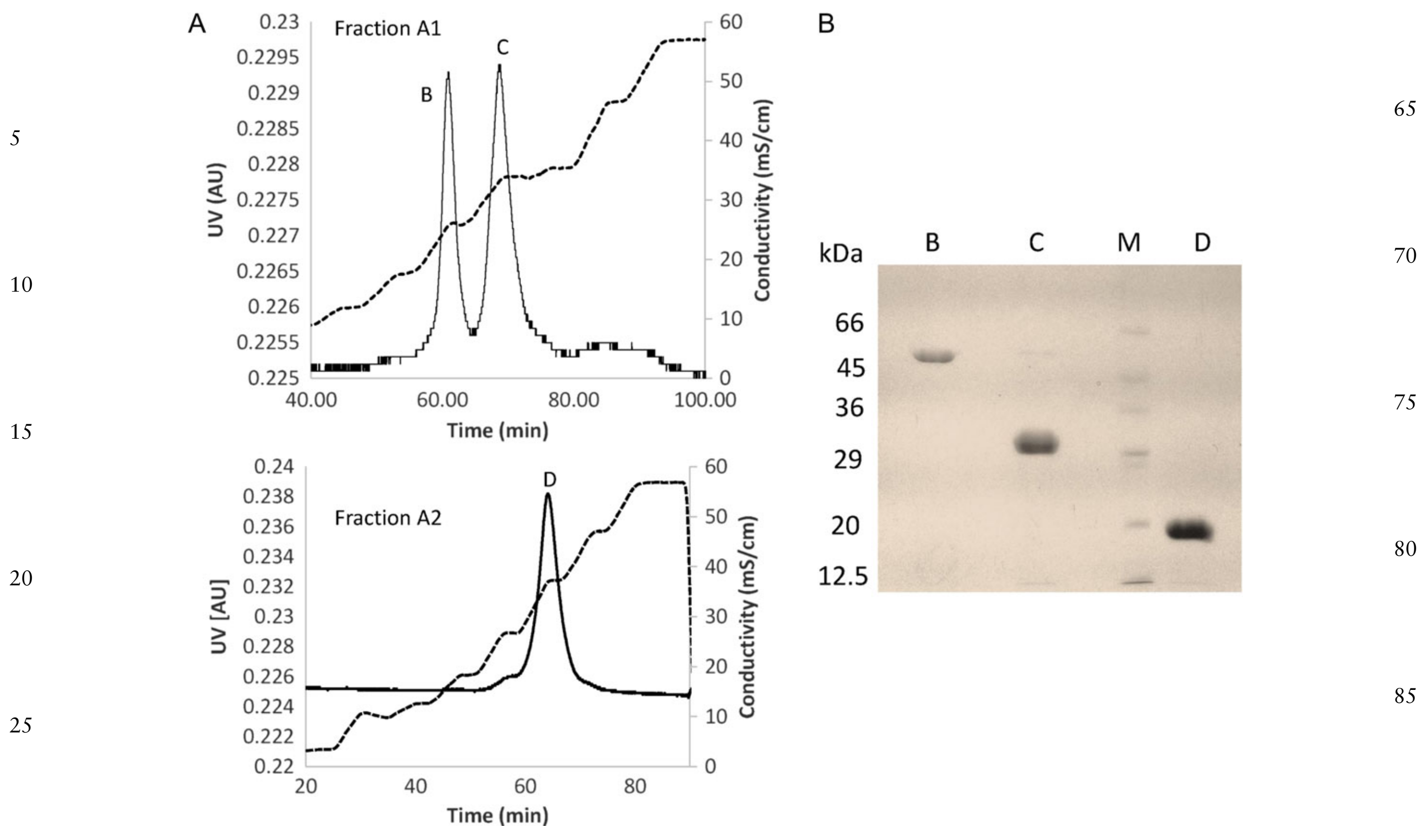

Figure 3. (A) Purification of Fractions A1 and A2 by cationic exchange chromatography. Freeze dried Fraction A1 and Fraction A2 were loaded onto a strong cationic exchange column (Macro-prep High S (40 mm × 12.6 mm)) equilibrated with 20 mM ammonium acetate buffer, pH 4.5. After elution of unadsorbed proteins, the adsorbed samples were eluted using a stepwise and linear saline gradient (0–0.5 M NaCl in 20 mM ammonium acetate buffer, pH 4.5) at a flow rate of 2.5 mL/min, fractions were collected every 30 s. The conductivity (—) indicates the NaCl gradient. (B) SDS-PAGE analysis of the collected fractions for Peaks B and C and Peak D; M: Molecular weight markers—cytochrome C (12.4 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and albumin (66 kDa).

Table II. Mass spectrometry protein identification (Mascot server, Matrix Science) results

	Protein 1	Protein 2	Protein 3
Amino acid sequence coverage (%)	15	25	18
Protein score at significance level (<0.05)	776	481	414
Nominal mass (kDa)	56.4	30.9	24.2
Match sequences	6	5	5
Match protein	β-Amylase	Lectin	Kunitz trypsin inhibitor A
Access number	13229323905428193	13229323905428192	13229323905428191
PI	5.40	5.65	4.99

minimized resulting in an overall shorter and more cost-effective way of obtaining pure proteins. Drifting of the baseline has been reported when using sodium sulfate or sodium acetate as ionic strength modifier (38). To avoid such issue, sodium chloride (NaCl) was used. The flow rate of the eluent could also have an impact on the separation time and/or on the peak resolution (39). During the separation, the flow was fixed at 2.5 mL/min as this value showed the best peak resolution compared to 1, 1.5, 2 and 3 mL/min.

Preclinical studies have demonstrated that both trypsin inhibitors and lectins possess some antitumor activity (40–42). Using different cell lines (i.e., liver and breast cancer cells), Lin *et al.* reported IC_{50} values ranging from 40 to 70 μM for KTI extracted from Chinese black soybean (17) and trypsin inhibitors from other plants, such as seeds of *Bauhinia variegata*, have also displayed comparable activities (IC_{50} ~54 μM) towards nasopharyngeal carcinoma cells (43). Similar studies have demonstrated the potent bioactivity of lectins from different sources (18, 21). These results demonstrates that the developed chromatographic method, using a combination of stepwise and linear gradients and two ion exchange columns, allowed us to reduce the number of chromatographic steps during the purification process (16, 17, 44) and that the purified proteins seemed to retain higher biological activity (KTI: IC_{50} ~6.6 μM towards B16F10 cells).

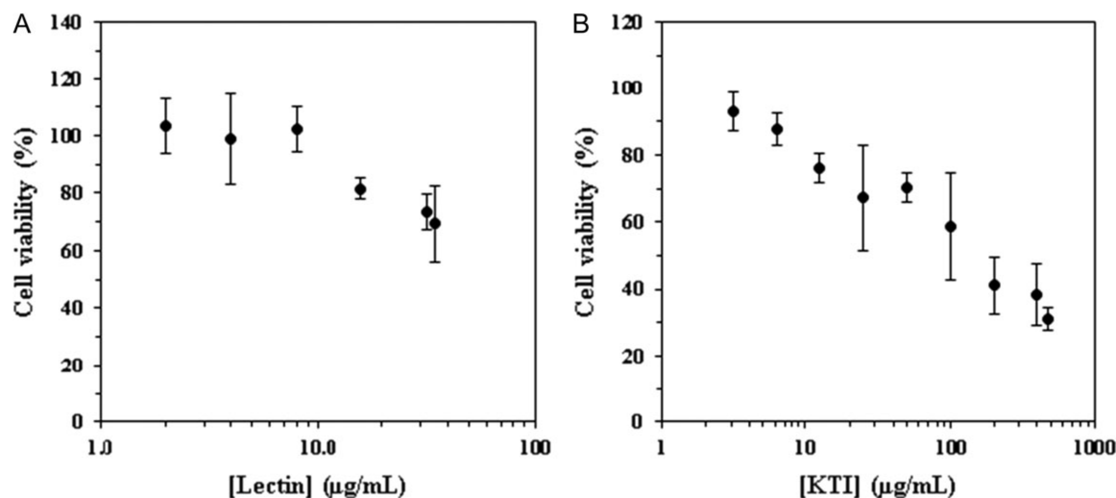

Figure 4. Viability of B16F1 cells in presence of proteins extracted from soya bean. Melanoma cells (10^4 cells/well) were incubated with different concentration of (A) Lectin or (B) Kunitz trypsin inhibitor A. Viability of the cells, relative to untreated samples, was estimated at 24 h using an MTT assay. Data represent Mean ± SD, $n = 3$.

Conclusion

A novel ion exchange column chromatography approach for the purification of soya-derived lectin (~30 kDa) and KTI-A (~20 kDa) resulting in the recovery of functional proteins has been developed. The proteins were purified by anionic and cationic exchange column chromatography. The use of small volume column bed, low pressure generating device and two steps column chromatography make the method more cost-effective and less time-consuming. Cell viability study against B16F1 melanoma cell lines demonstrates the antiproliferative activity of KTI-A and lectin. A complex elution profile has enabled the purification of proteins with minimized column chromatography. Soya is, therefore, an excellent source of bioactive trypsin inhibitor and lectin and could be used for obtaining these molecules in accord with its demand in medicine and other industries. This method will also be applied in order to purify lectin and KTI from other plant material as part of the search for novel, potent inhibitors and other active biomolecules as lectin. Further investigations should focus on the efficacy of the purified bioactive proteins.

Supplementary Data

Supplementary material is available.

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