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Purification and Quantification of Kunitz Trypsin Inhibitor in Soybean Using Two-Dimensional Liquid Chromatography

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Abstract Kunitz trypsin inhibitor (KTI) is one of the major antinutritional factors in soybean and results in inhibition of digestion of dietary protein. In this study, we developed a novel strategy to purify and quantify KTI from soybean using two-dimensional liquid chromatography. Lipids from ground soybean were removed using hexane after which the ground soybean was extracted with protein extraction buffer. The crude extract was first purified by weak anion exchange chromatography, and then the fraction containing KTI was further separated by size exclusion chromatography. The fraction containing KTI was collected and analyzed by SDS-PAGE and electrospray ionization mass spectrometry. Results indicated that purified KTI has a molecular mass of 20 kDa and a purity of ~98% with inhibitory activity of 2425 TIU/mg protein. This assay was used for the quantification of KTI in soybean samples. The assay showed concentrations with a range between 7.81 and 500.00 μg/mL and a limit of detection of 0.12 mg/g. The recoveries of KTI in spiked soybean samples were between 82.19% and 86.65%, and intra- and interday precisions (% CV) were less than 7.35% and 8.42%. The developed method was used to analyze soybean samples from different sources and soybean products derived from different processing techniques, which demonstrated that the developed procedure provided an accurate and sensitive tool for separation and quantification of intact KTI in soybean.

Keywords Soybean · Kunitz trypsin inhibitor · Two-dimensional liquid chromatography · Purification · Quantification

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Introduction

Soybean, with about 40% crude protein, is one of the major vegetative protein sources used in the food and feed industries. Soybean meal provides ~70% of protein meal utilized in animal feed mixtures for poultry and swine. However, soybean meal contains a number of antinutritional factors, which can interfere with the digestion and absorption of nutrients, disturb normal metabolism and cause adverse physiological responses, and result in hypersensitivity and decrease the production performance of animals (Guo et al. 2007; Sun et al. 2008a, b). Trypsin inhibitors (TI) are important antinutritional factors in soybean seeds, which can lead to decrease of growth performance in poultry and swine. The antinutritive mechanism is manifested in decreased protein digestion, inducing the enlargement of the pancreas (hypertrophy or hyperplasia) and through damaging the pancreas secretive function, which results in decreased nitrogen utilization and increased endogenous loss of nitrogen (Friedman and Brandon 2001; Pacheco et al. 2014). Palliyeguru et al. reported that higher levels (>6.21 mg/g) of TI in nonroasted dietary soybean not only affected growth performance of broilers but also increased incidence of subclinical necrotic enteritis in the flock (Palliyeguru et al. 2011).

Two types of trypsin inhibitors are found in soybean: the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor

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(BBI). KTI is a strong inhibitor of trypsin with a molecular mass around 21 kDa, while BBI is much smaller (7–8 kDa) and inhibits both trypsin and chymotrypsin (Dipietro and Liener 1989). KTI is found in much higher concentration than BBI (4:1; *w*/*w*), and it has 170 to 200 amino acid residues and two intrachain disulfide bonds. KTI forms a specific three-dimensional structure and binds strongly to trypsin, instantly forming an irreversible compound, blocking its active site and halting digestion of certain proteins (Onesti et al. 1991). Long-term consumption of diets with high KTI activity may produce unwanted effects in humans and livestock. Thus, ensuring the lowest possible KTI activity in soybean meal is a valuable tool to improve the health and welfare of animals and reduce the financial losses from lower quality soybean meal.

KTI is heat-labile; its activity can be reduced or eliminated by thermal treatment (Chen et al. 2014). Recently, other technologies, such as fermentation or expansion, have been applied to reduce the amount of active KTI in soybean (Feng et al. 2007; Romarheim et al. 2005). However, there is a need to provide accurate and sensitive methods for identification and quantification of KTI in soybean seeds and soybean products to determine potential hazardous effects and to evaluate the efficacy of the different processing methods. Previously, methods for measuring the TI activity in soybean products involved detecting urease activity of the samples, since there is a positive correlation between urease activity and TI activity. However, these methods only indirectly estimate the TI level and there is significant variability in estimates of urease activity among the different methods for detecting urease activity (Araba and Dale 1990). Moreover, the methods have not been evaluated for heat-treated soybean meal. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a popular technique to detect and characterize proteins (Hsieh and Chen 2007). However, the sizing accuracy of SDS-PAGE depends on the protein characteristics, including amino acid sequence, isoelectric point, structure, and presence of certain side chains or prosthetic groups. Consequently, some proteins do not migrate according to their molecular weight (Goetz et al. 2004). Thus, this method can be inaccurate and variable and is most useful as a qualitative method. Recently, enzyme-linked immunosorbent assays (ELISA) with polyclonal antibodies (Pabs) have been used to detect KTI (Pedersen et al. 2008; Zhou et al. 2015). Although immunoassays generally are highly sensitive and provide a high throughput, they suffer from limitations such as antibody availability, high variability, and a limited range of specificity (Julka et al. 2012). So they are most useful as a screening and semiquantitative method. The other detection methods included microfluidic or lab-on-a-chip technology. Aleksandra et al. reported a lab-on-a-chip method to estimate KTI in soybean varieties, but the method requires specialized equipment (Torbica et al. 2010).

Thus, reported methodologies for estimation of KTI in soybean are of limited usefulness in quantification of KTI concentrations in soybean. There is a need for analytical methodology for precise quantification methods for detecting KTI in soybean and soybean products. Liquid chromatography is an excellent quantitative analysis method for protein quantification. Samir et al. reported quantification of Gly m 4 protein, a major soybean allergen, by two-dimensional liquid chromatography (Julka et al. 2012), which demonstrated the possibility of applying liquid chromatography (LC) to the analysis of soybean-related proteins. The objective of the present study was to develop a quantitative assay for the determination of KTI in soybeans using two-dimensional LC. Considering the complex protein profile in soybean, this would require the development of a novel strategy using offline two- dimensional chromatographic separations to increase the resolving power and separate KTI from co-eluting interferents. The method was evaluated for assay selectivity, linearity, sensitivity, and recovery. To the best of our knowledge, this is the first time that an offline two-dimensional liquid chromatography-based assay was used to accurately quantify KTI in soybean samples. The method was used to isolate and purify endogenous KTI from soybean seeds. The study will facilitate further investigation of antinutritional mechanisms of KTI, breeding of lower KTI content soybean lines, and development of soybean processing techniques to reduce soybean allergenicity.

Experimental Section

Reagents and Chemicals

KTI standard from soybean (Cat no. T2327, Sigma) was obtained from Sigma Company (St. Louis, MO) and used as received without further purification. BCA™ Protein Assay Kit was obtained from Pierce (Rockford, IL). PageRuler Prestained Protein Ladder was purchased from Thermo (Rockford, IL). Trypsin (Cat no. T0458, Amresco) was purchased from Amresco Company (Solon, OH). Benzoyl-dl-arginine-p-nitroanilide hydrochloride (BAPNA) was purchased from Sigma Company (St. Louis, MO). All other chemicals were of analytic grade. Each liter of Bis-Tris-HCl start buffer contained 4.18 g Bis-Tris and was adjusted to pH 6.4. Each liter of Bis-Tris-HCl elution buffer contained 4.18 g Bis-Tris and 58.44 g NaCl and was adjusted to pH 6.4. Each liter of phosphate buffer saline contained 5.96 g NaH_2PO_4 and 1.25 g $Na_2HPO_4{\cdot}2H_2O$ and was adjusted to pH 7.5. Each liter of Tris-HCl buffer contained 6.05 g Tris and 4.99 g $CaCl_2{\cdot}6H_2O$ and was adjusted to pH 8.2. All water used was Milli-Q water (>18.2 MΩ).

Soybean seeds were provided by the Institute of Crop Science Chinese Academy of Agricultural Sciences (Beijing,

China). Soybean products came from the Ministry of Agriculture Feed Safety and Bio-availability Evaluation Center (Beijing, China).

Instruments and Apparatus

Anion exchange chromatography was performed on an AKTA™ Pure system coupled with a UV detector (GE Healthcare Life Sciences, USA). Size exclusion chromatography separation was performed on an Agilent 1200 HPLC system coupled with a diode array detector (Agilent Technologies, Fremont, CA). An SDS-PAGE electrophoresis slot was purchased from Bio-Rad Laboratories (Herefordshire, England). In the assay for trypsin inhibitory activity, the residual enzyme activity was measured by monitoring absorbance at 410 nm on a Beckman Coulter DU-730 spectrophotometer. Mass spectrometry identification was performed on a NanoLC-Q-Orbitrap MS system (NanoLC, Waters NanoAcquity HPLC, Milford, MA, USA; MS, Thermo Fisher Q-Extractive, Waltham, MA, USA).

Sample Preparation and Extraction

Soybeans and soybean products were ground to fine powder to pass through a 60-mesh sieve. Lipids from 50 mg soybean samples based on fresh weight were extracted by mixing with hexane (soybean/hexane, 100 mg/3 mL) for 1 h at room temperature, followed by centrifuging at 12,000 rpm for 15 min to remove the supernatant fluid containing soybean oil. A vacuum rotatory evaporator (Thermo, USA) was used to remove traces of hexane in the soybean flour. After lipid extraction, the soybean flour was subsequently extracted with 1.5 mL protein extraction buffer (20 mM Bis-Tris-HCl, pH 6.4) with continuous agitation at 1200 rpm for 2 h at room temperature using a mixer, followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was filtered through a 0.2-μm filter and designated as crude extract.

Chromatographic Conditions of Offline Two-Dimensional Liquid Chromatography Assay

A 1-mL sample of crude extract was applied on weak anion exchange column of DEAE-Sepharose (90 μm particle size, 0.7 × 2.5 cm; GE Healthcare) using Fast Protein Liquid Chromatography system previously equilibrated with 10 column volumes of 20 mM pH 6.4 Bis-Tris-HCl. The mobile phase consisted of solutions A (20 mM pH 6.4 Bis-Tris-HCl) and B (20 mM pH 6.4 Bis-Tris-HCl, including 1 M NaCl). A stepped elution program was used: 15% solution B (initial) for 25 min, 15% –25% solution B (from 25 to 25.1 min), 25% solution B (from 25.1 to 30 min), 25%–100% solution B (from 30 to 30.1 min), and 100% solution B (from 30.1 to 35 min). A 5-min equilibration using 15% solution B was necessary before the next injection. The mobile phase was delivered at a flow rate of 1 mL/min at room temperature. The absorbance was monitored at 280 nm. The fraction containing KTI was pooled, filtered via 0.1 μm microporous film, and aliquoted for further purification and quantification.

The second chromatographic separation of KTI was achieved on a Waters XBridge® BEH SEC 200 Å column (3.5 μm particle size, 7.8 × 300 mm) using an HPLC system. The column temperature was room temperature. The mobile phase was 50 mM pH 7.5 phosphate buffer, which was delivered at a flow rate of 0.5 mL/min, and the injection volume was 20 μL. Detection with a diode array detector was carried out at 280 nm.

Polyacrylamide Gel Electrophoresis Analysis

SDS-PAGE and native-PAGE was performed to detect the protein profile of soybean samples (Oddepally et al. 2013). Protein samples (50 μg) were loaded onto 5%–12% polyacrylamide gel, and electrophoresis was performed in a vertical electrophoresis unit at 90 V constant voltage for 0.5 h and 120 V constant voltage for 1.5 h. The gel was stained with Coomassie Brilliant Blue R-250 (0.05%, *w*/*v*) in methanol-acetic acid-water (25:10:65, *v*/*v*/*v*) and destained in the same solution without dye. Trypsin inhibitor from Sigma was used as KTI marker. Gels were digitized with a UMAX Powerlock 2100XL scanner and quantitatively processed with Quantity One software.

Trypsin Inhibitory Assay

The inhibitory activity of KTI was determined as described by Cruz et al. (2013) with some modifications. Samples were diluted to reach an inhibiting capacity of 40%–60%; 2 mL of trypsin solution (200 mg/L trypsin in 0.001 M HCl buffer) was added to 2 mL sample solution and preincubated for 10 min at 37 °C. Five milliliters of 400 g/L α-*N*-benzoyl-dl-arginine-p-nitroanilidehydrochloride (BAPNA) solution in 50 mM pH 8.2 Tris-HCl containing 20 mM $CaCl_2$ was added, and the mixture was incubated at 37 °C for 10 min. The reaction was terminated by adding 1 mL of 30% acetic acid. The reaction mixture was centrifuged at 2.5 × 1000*g* and absorbance at 410 nm was measured. Trypsin inhibitor activity, expressed as trypsin inhibitor units/mg protein (TIU/mg protein), was calculated from the absorbance read at 410 nm against a reagent blank. One trypsin unit was defined as the increase by 0.01 absorbance unit at 410 nm of the reaction mixture.

Protein Identification Using NanoLC-MS

For the determination of molecular mass of purified KTI, purified KTI was diluted with 0.1% TFA and directly injected

into the ESI-MS. Raw data from the mass spectrometer were processed with Thermo Xcalibur 2.2. For the identification of amino acid sequences, 100 μg protein solution was digested with trypsin using FASP method (Maurer et al. 2013). Protein digest was diluted with 0.1% formic acid for nanoLC-MS analysis. Mobile phase A consisted of 0.1% formic acid in water (v/v) and mobile phase B consisted of 0.1% formic acid in acetonitrile (v/v). A gradient elution program is shown in Supplemental Table 1. Raw data from the mass spectrometer were preprocessed with Mascot Distiller 2.4 for peak picking. The resulted peak lists were searched against *Uniprot Glycine max* database using Mascot 2.4 search engine.

Validation Procedure

The validity of the method, including linearity, sensitivity, as well as recovery and accuracy, was evaluated in four replicates. Recoveries were determined by spiking samples with KTI standard solution at 4, 10, and 15 mg/g in soybean flour. The concentration of the standard solution was gradually diluted and analyzed in order to determine the limit of detection (LOD, $S/N = 3$). The amounts of KTI in the soybean samples were quantified using a calibration curve developed with the KTI standard solution.

Analysis of KTI in Soybean Seeds and Soybean Products

Ten soybean seeds harvested in 2016 from China with different origins and breeds and eight soybean products with four different processing methods, including fermented soybean meal, extruded soybean meal, extruded full-fat soybean, and dehulled soybean meal, were prepared in duplicate. The amounts of KTI were determined using the developed assay with calibration curve obtained from KTI standard.

Results and Discussion

Optimization of Sample Extraction

Soybean contains ~19% oil; the existence of fat in the sample may interfere with the detection of KTI; thus, soybean seeds were extracted using hexane. The result shows that, after lipid extraction, soybean samples had better accuracy and reproducibility compared with unextracted soybean. The existence of fat in the sample decreases solubility of proteins and increases nonspecific binding of the chromatographic column. Therefore, all the soybean samples were subjected to lipid extraction prior to protein extraction. Development of an efficient protein extraction procedure is the most important step for accurate determination of an analyte. A number of extraction methods were evaluated to obtain the best recovery of KTI. Conditions optimized included extraction solvent, ratio of seed and extract solution, and extraction time. Glycinin and β-conglycinin are the major components in soybean protein, which account for 65%–80% of the total protein. They also are the major interference for the separation of KTI. We found that 20 mM Bis-Tris-HCl buffer at pH 6.4 could significantly reduce solubility of the both glycinin and β-conglycinin, but had no influence on KTI from defatted soybean powder. With Bis-Tris-HCl buffer, the most reproducible and highest extraction efficiency for KTI was obtained, and this buffer was compatible with weak anion exchange column. So, Bis-Tris buffer was selected as the extraction buffer. The ratio between soybean sample and extraction buffer (1 g:10 mL, 1 g:30 mL, and 1 g:100 mL) and extraction time (0.5, 1, 2, and 3 h) were also tested. These results showed that 30 mL/g extract buffer and 2 h extract time were optimal due to the higher recovery and good repeatability (data not shown).

Method Development of the Two-Dimensional Chromatography

An offline two-dimensional liquid chromatographic assay was developed to purify and quantify KTI at the intact protein level. Figure 1 depicts the schematic for the analytical setup utilized for KTI purification and quantification. Soybean extract was separated on anion exchange column by stepped gradient elution. The fractions containing KTI were pooled and aliquoted into vials for further separation using size exclusion columns. Purity and molecular mass of the intact purified KTI were confirmed by SDS-PAGE and high-resolution ESI-MS, respectively.

When the crude extract solution was injected directly into the size exclusion column, high background interference was observed (Fig. 3a); thus, prepurification of the crude extract prior to size exclusion chromatography (SEC) was needed. The pH of the extract buffer was 6.4, higher than the isoelectric point of KTI ($pI = 4.5$); thus, in the crude extract, KTI carried a negative net charge and could bind to the anion exchanger. Therefore, an anion exchange column was chosen to purify KTI from the crude extract. Weak anion exchange and strong anion exchange resin were compared. The results showed that with strong anion exchange resin, a small interference peak co-eluted with the target KTI peak and the interference could not be separated from KTI by optimizing elution conditions (Fig. 2a). With weak anion exchange resin, the interference was eliminated (Fig. 2b). Thus, weak anion exchange column was used for the first dimension purification of KTI. Here, gradient elution was used to collect high content target protein with less elution volume and time. Three fractions (fraction 1, 17.5 to 25 min; fraction 2, 27.2 to 32 min; and fraction 3, 32.5 to 35 min) with absorbance higher than 5 mAU at 280 nm were collected for subsequent analyses (Fig. 2b). Specific KTI activities of the fractions were measured (Table 1). Fraction 2 showed the highest KTI

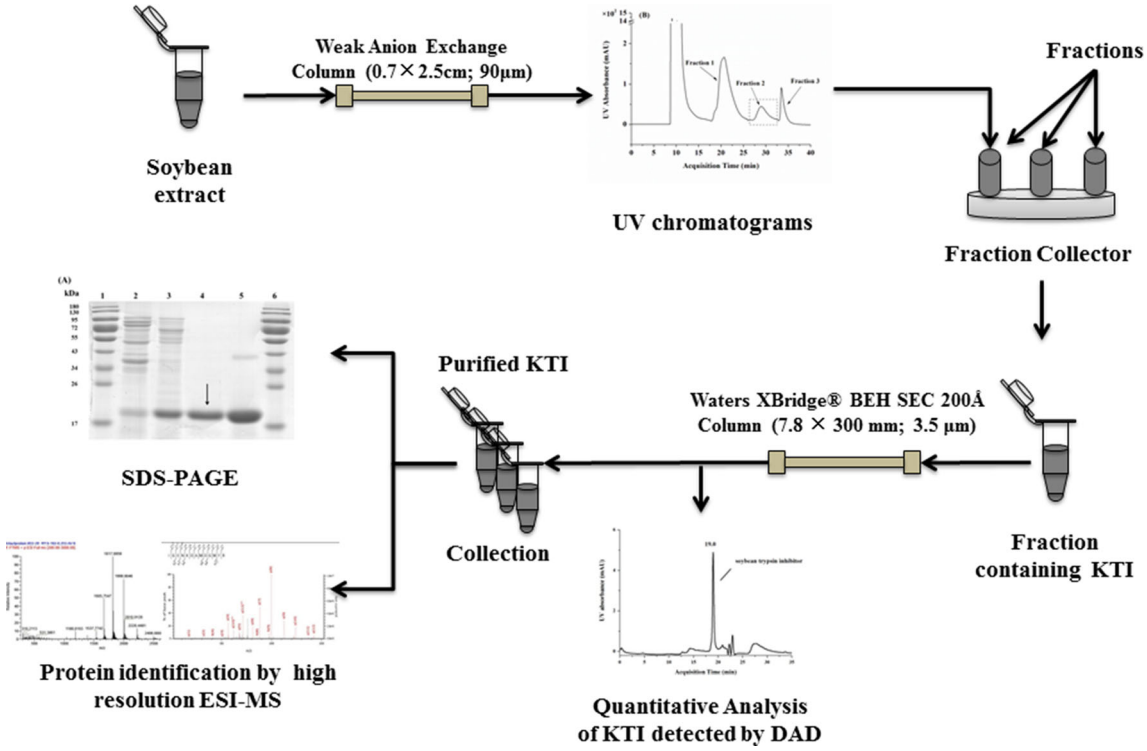

Fig. 1 The working principle of two-dimensional liquid chromatography for purification and quantification of KTI

concentration and the highest inhibitory activity of 1534 TIU/mg protein. Then fraction 2 was pooled and aliquoted into vials for further purification and quantification.

In the second dimension of separation and purification, chromatographic parameters, including choice of column, mobile phase composition, gradient condition, and flow rate, were tested to obtain the best separation of KTI. Reversed-phase (RP) chromatography column (Waters BEH C_4, 2.1 mm × 100 mm, 1.7 μm, 300 Å) was compared with SEC column (Waters XBridge® BEH SEC 200 Å column, 7.8 × 300 mm, 3.5 μm). The RP chromatography column separates proteins based on their hydrophobicity and is a rapid and efficient separation technique for intact protein analysis. Nonetheless, proteins with different sizes may show similar hydrophobicity and are therefore difficult to separate by RP-HPLC. In order to achieve better peak shape and minimize carryover, in RP separation, the column was often set at elevated temperature. This, however, raised questions on whether

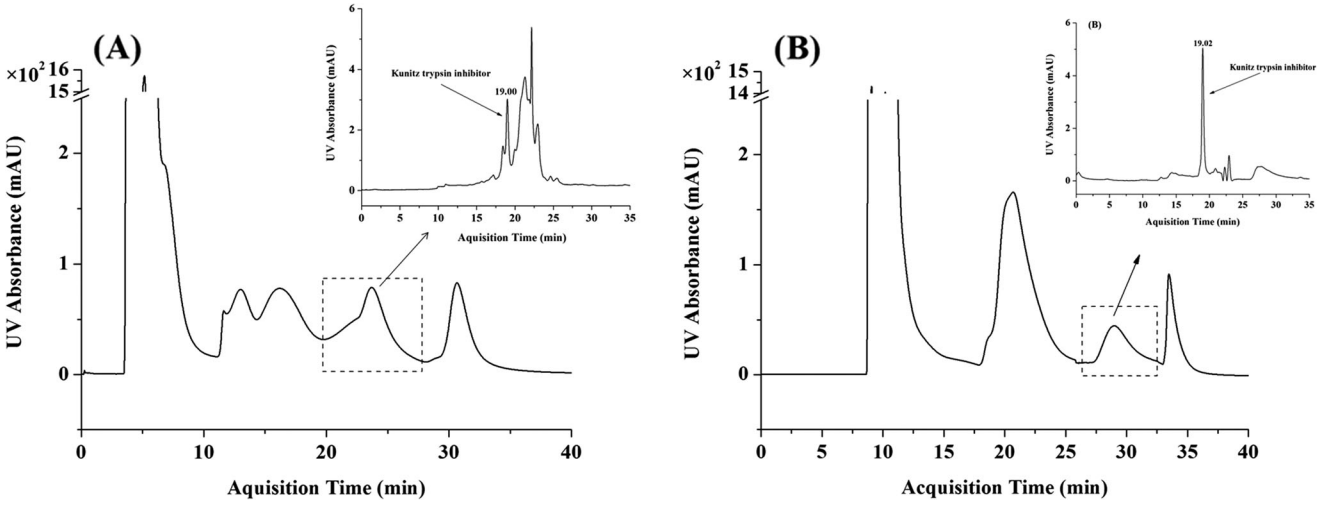

Fig. 2 The separation effect of soybean extract on two anion exchange columns using fast protein purification system. **a** UV monitoring chromatogram from strong anion exchange column (*inset*: the SEC chromatogram of the fraction containing KTI). **b** UV monitoring chromatogram from weak anion exchange column (*inset*: the SEC chromatogram of the fraction containing KTI). The *rectangle* indicates the fraction containing KTI

Table 1 Amount and activity of KTI from different analytical steps including various chromatographic fractions

Yields	Extract	Fraction 1[a]	Fraction 2[b]	Fraction 3[c]	Purified KTI	Standard
Total protein[d] (mg)	13.16	0.49	0.22	0.42	0.09	0.15
Specific activity[e] (TIU/mg)	290	410	1534	17	2425	2223

[a] Fraction 1 (retention time from 17.5 to 25 min) was collected from the weak anion exchange chromatography
[b] Fraction 2 (retention time from 27.2 to 32 min) was collected from the weak anion exchange chromatography
[c] Fraction 3 (retention time from 32.5 to 35 min) was collected from the weak anion exchange chromatography
[d] Expressed as [measured the protein concentration (mg/mL)] × the total volume of each fraction (mL)
[e] Expressed as [measured the total trypsin inhibitory units] / the total protein content of each fraction (mg)

high temperature and acetonitrile induces protein degradation and degeneration. The RP chromatogram of KTI standard sample (1 mg/mL) showed that there were several early eluting peaks followed by a prominent later eluting peak (Supplemental Fig. 1). The early peaks and latter peak were native and denatured forms of KTI in the organic phase, respectively (Reitsma and Yeung 1987). Thus, reversed-phase column could not be used for separation in the second dimension. SEC is one of the most commonly used chromatographic techniques for separating proteins. Generally, isocratic elution is used without the need of elevated column temperature. Mobile phase constitution, salt concentration, and pH would influence resolution of the separation. Here, we tested 50 mM pH 7.5 phosphate buffer and 50 mM pH 6.0 citrate buffer, and the results showed that 50 mM pH 7.5 phosphate buffer had better chromatographic separation of the soybean proteins. No remarkable difference was observed when using 50 or 250 mM phosphate buffer. The salt concentration was set at 50 mM to alleviate damages to the HPLC pump and plumbing. In SEC, the flow rate of the mobile phase greatly affects peak shape and helps resolve closely eluted compounds. After optimization, the flow rate of the mobile phase was set at 0.5 mL/min, and the mobile phase could improve the separation effect and provide better peak shapes and sufficient intensity. Finally, 50 mM pH 7.5 phosphate buffer was selected as the mobile solvent, and the mobile phase was delivered at a flow rate of 0.5 mL/min at room temperature. Under optimal conditions, a typical chromatogram of KTI standard is shown in Fig. 3c.

Characterization of Isolated KTI

Fraction 2 from the first dimension was separated by size exclusion chromatography (Fig. 3b) and the protein peak containing KTI was collected. The SEC-purified KTI was submitted to SDS-PAGE and a single protein band with a molecular mass of ~20 kDa was observed (Fig. 4a). Accurate molecular mass of purified KTI was confirmed by ESI-MS. The results showed a multiple charged $[M+10H]^{+10}$ ion at m/z 1998.805 Da, $[M+11H]^{+11}$ at m/z 1817.095 Da, and $[M+12H]^{+12}$ at m/z 1665.754 Da (Fig. 4c). Deconvolution of the mass spectrum revealed molecular weight of purified KTI at 19,964.956 Da which was consistent with the previous reports (Oddepally et al. 2013; Zhou et al. 2008). Meanwhile, the amino acid sequence of KTI was confirmed by nanoLC-high-resolution mass spectrometry. The MASCOT search results showed that the matched peptides of purified KTI covered 70% of the soybean Kunitz-type trypsin inhibitor (Supplemental Table 2 and Supplemental Fig. 2), and the MS/MS spectrum of one of the uniquely matched peptides is shown in Fig. 4d. Moreover, the accurate purity of the purified KTI was also analyzed by the size exclusion chromatography based on the normalized calculation of chromatographic peaks. The purity of the purified KTI was approximately 98% (Fig. 4b). The purified KTI contained trypsin inhibition with a specific activity of 2425 TIU/mg protein which was slightly higher than that of the KTI standard (Table 1).

Method Validation

KTI standard solutions at different levels showed satisfactory linearity within the concentration range of 7.81–500.00 μg/mL and correlation coefficients were better than 0.999 (Supplemental Fig. 3). The LOD, which was defined as the concentration at 3 times the chromatographic peak signal intensity of noise, was found to be 0.12 mg/g of KTI in fresh soybean samples. The limit of quantitation (LOQ), defined as the concentration at 10 times the signal intensity of noise, was 0.41 mg/g in fresh soybean sample. To evaluate the precision and accuracy of the method, the recoveries of KTI spiked in soybean flour were determined using four replicates at different concentrations. Using standard calibration, the average recoveries of KTI in spiked soybean samples were between 82.19% and 86.65% and coefficients of variations (CVs) were less than 7.35%. The interday CVs were less than 8.42% (Table 2). Thus, the chromatographic assay developed here can be used to accurately determine KTI concentration in soybean samples.

Analysis of KTI in Soybean Seeds and Soybean Products

Ten soybean seed samples of different origins and varieties were collected and tested with the developed method in three

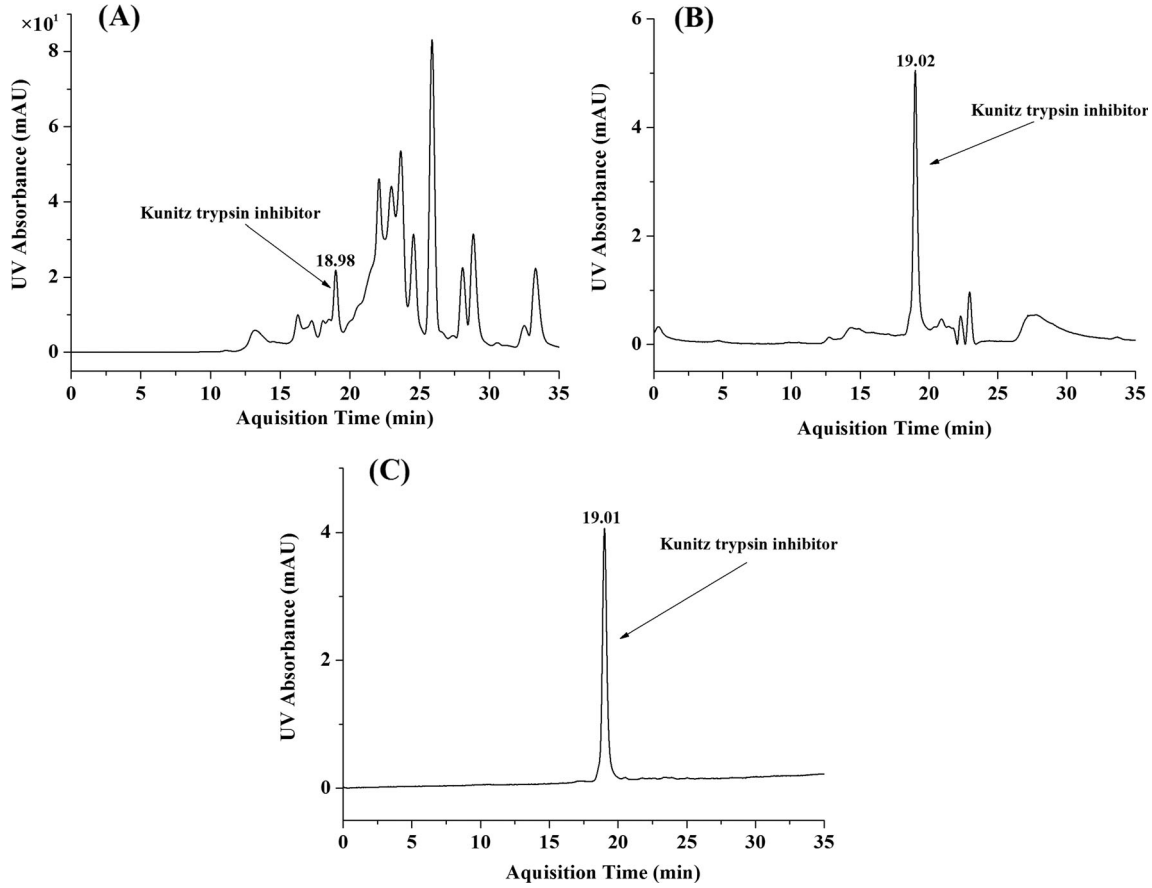

Fig. 3 The chromatograms of HPLC-DAD: **a** crude extract of soybean seed; **b** sample of soybean seed after purification with weak anion exchange column; **c** KTI standard sample with a concentration of 100 μg/mL

replications. The results showed that the concentration of KTI in soybean seeds ranged from 6.13 to 8.08 mg/g (Table 3), which was consistent with previous reports (Hong et al. 2004; Huisman et al. 1990). The results also showed that origins of

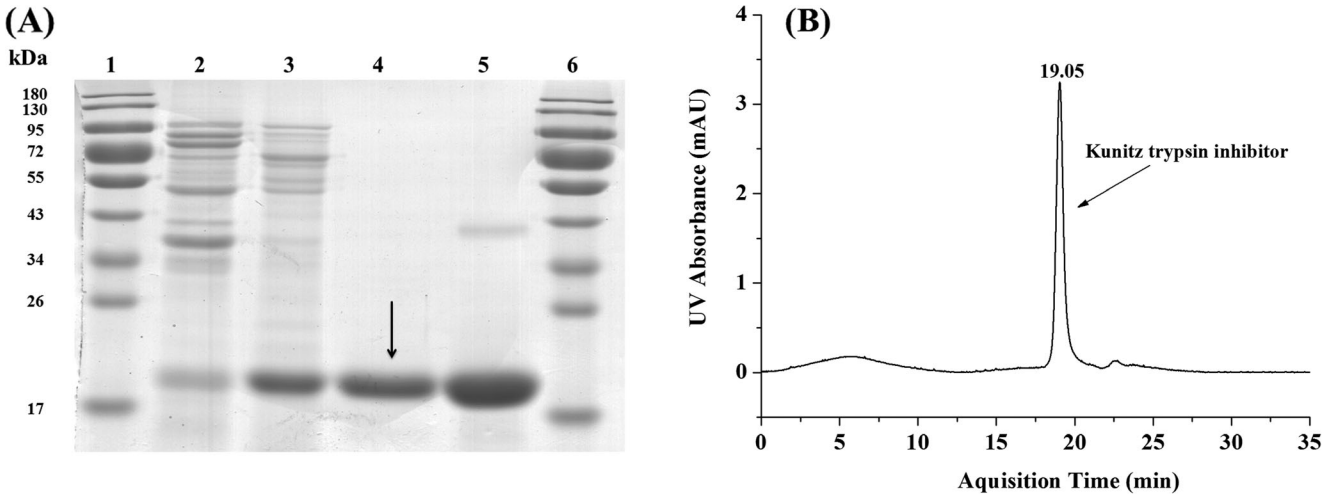

Fig. 4 Characterization of purified KTI from soybean seeds. **a** SDS-PAGE analysis of purified protein. *Lanes 1* and *6*, molecular weight standards; *lane 2*, soybean seed extract; *lane 3*, KTI isolated from weak anion exchange column; *lane 4*, KTI elution separated on a size exclusion chromatography; *lane 5*, the KTI standard sample from Sigma. The *arrow* indicates the KTI band. **b** A blank subtracted HPLC chromatogram of intact purified KTI sample at 280 nm. **c** ESI mass spectrum of purified KTI (*inset*: deconvoluted mass spectrum of KTI). **d** MS/MS spectrum of a doubly charged ion at *m/z* 769.8552 of peptide K.IGENKDAMDGWFR.L from the soybean Kunitz-type trypsin inhibitor A

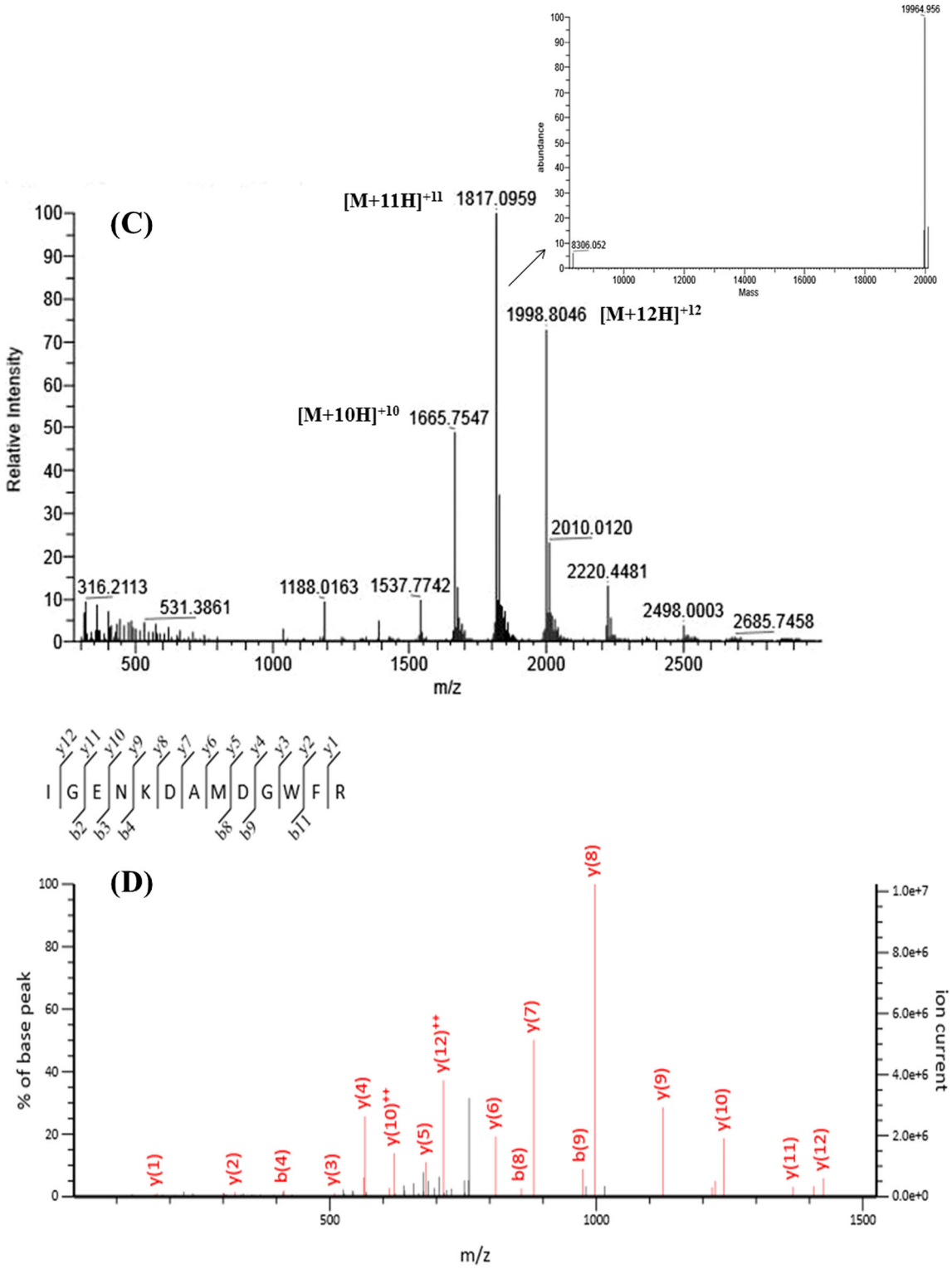

Fig. 4 (continued)

the soybean do not have significant effects on KTI concentration in the samples.

Various processing methods have been developed to reduce KTI activity. Here, with the developed chromatographic assay, we evaluated the effectiveness of different processing methods in removing KTI from soy products. Four soybean products, namely fermented soybean meal, extruded soybean meal, extruded full-fat soybean, and dehulled soybean meal

Table 2 Recoveries of KTI spiked in defatted soybean ($n = 4$)

Spiked level (mg/g)	Measured concentration (mg/g)	Mean recovery (%)	Intraday precision CV (%)	Interday precision CV (%)
4	3.47	86.65	7.35	8.42
10	8.22	82.19	6.41	6.68
15	12.33	82.23	0.90	3.59

were collected and tested (Table 3). The results showed that KTI in fermented soybean meal and extruded soybean had the lowest concentrations of KTI, even below LOQ in these samples. Previous research has shown that microbial fermentation could degrade more than 84% of TI into small-size peptide or amino acids from soybean meals (Hong et al. 2004). Since KTI is heat-labile (Perez-Maldonado et al. 2003), the high temperature and high pressure applied in extrusion process could effectively destroy KTI (Anuonye et al. 2007; Romarheim et al. 2005). Small amounts of KTI could still be detected in extruded full-fat soybean samples. One reason could be that the process conditions of extruded full-fat soybean are not as harsh as those of other processing methods to degrade KTI (Zarkadas and Wiseman 2005). Interestingly, KTI content in dehulled soybean meal was much lower than that in soybean seeds. The difference could be due to the mild heat treatment at temperature of 60–88 °C and mechanical crushing during the breakage and peeling of the soybean seeds which might destroy part of KTI. These results are qualitatively consistent with previous literature reports (Grieshop et al. 2003) and indicate that there are several effective processing techniques to remove KTI from soybean products, especially in fermented soybean and extruded soybean meal where KTI is nearly completely removed. Therefore, fermented soybean meals and extruded soybean meal can be used as a specialized feed ingredient, especially for young animal diets as a replacement for expensive animal-origin protein ingredients. Jansman et al. demonstrated that diets with a TI concentration of 0.7 mg/g and less did not reduce the growth of pigs (Jansman et al. 1998).

Crude protein extract from the above samples was also tested with native-PAGE (Supplemental Fig. 4). For fermented soybean meal and extruded soybean, native-PAGE showed no band around 20 kDa, which was consistent with HPLC results. For samples with higher KTI concentration detected by HPLC, such as soybean seeds, native-PAGE also showed bands. For extruded full-fat soybean and dehulled soybean meal, native-PAGE showed almost no band, while HPLC can still detect a trace amount of KTI in these samples. So, compared with native-PAGE, the two-dimensional chromatographic method developed here has better sensitivity and accuracy for trace amounts of KTI.

Conclusion

A two-dimensional liquid chromatography assay was successfully developed for purification and quantification of intact

Table 3 Concentrations of KTI in actual soybean samples from soybean seeds and soybean products

No.	Name	KTI concentration (mg/g)	No.	Name	KTI concentration (mg/g)
1	Southeast spring-summer-autumn soybean seeds	6.87	11	Fermented soybean meal	<0.41
2		6.62	12	Fermented soybean meal	<0.41
3		7.40	13	Extruded full-fat soybean	1.09
4	Huanghuai summer soybean seeds	6.48	14	Extruded full-fat soybean	0.57
5		6.45	15	Dehulled soybean meal	0.90
6		6.99	16	Dehulled soybean meal	<0.41
7	North spring soybean seeds	7.60	17	Extruded soybean meal	<0.41
8		6.13	18	Extruded soybean meal	<0.41
9		8.08			
10		6.74			

KTI from soybean samples. The assay showed an acceptable sensitivity toward KTI with a LOD at 0.12 mg/g and accuracy with recoveries of 82.19%–86.65%. The assay also showed good resolution with other soybean proteins even in complex samples such as soybean meals. The assay was successfully applied to detect levels of KTI in soybean seeds and soybean products with different processing techniques. Data from these real soybean samples show that the place of origin has very limited influence on KTI content of the soybean samples. Effective processing can remove most of the KTI from soybean products. This research provides a solid foundation for the evaluation of soybean, soybean products, and soybean processing techniques. Moreover, there are over 20 identified antinutritional proteins in soybean, so there exists considerable opportunity and challenge to develop efficient methods to eradicate these immunodominant allergens while maintaining nutritional value, as well as develop sensitive and accurate detection methods. The two-dimensional liquid chromatography concept developed here with its high sensitivity and accuracy is a very promising technique for the detection and quantification of other macromolecular antinutritional factors in soybean meals.

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Compliance with Ethical Standards

Conflict of Interest Tianjiao Zhou declares that he has no conflict of interest. Shuaijuan Han declares that he has no conflict of interest. Zhen Li declares that he has no conflict of interest. Pingli He declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

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