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# A Simple and Rapid Method to Isolate Low Molecular Weight Proteinase Inhibitors from Soybean

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**ABSTRACT : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the 60% isopropanol extract of soybean (*Glycine max* [L.] Merr.) seed revealed two abundant proteins with molecular masses of 19 and 10 kDa. Amino acid analysis revealed that the isopropanol-extractable protein fraction was rich in cysteine. Two-dimensional gel electrophoretic analysis indicated that the 19 kDa and 10 kDa proteins had pI of 4.2 and 4.0 respectively. Peptide mass fingerprints of trypsin digests of the two proteins obtained using matrix-assisted, laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy revealed the 19 kDa protein was Kunitz trypsin inhibitor and the 10 kDa protein was Bowman-Birk proteinase inhibitor. When resolved under non-denaturing conditions, the isopropanol-extracted proteins inhibited trypsin and chymotrypsin activity. Results presented in this study demonstrate that isopropanol extraction of soybean seed could be used as a simple and rapid method to obtain a protein fraction enriched in Kunitz trypsin and Bowman-Birk proteinase inhibitors. Since proteinase inhibitors are rich in sulfur amino acids and are putative anticarcinogens, this rapid and inexpensive isolation procedure could facilitate efforts in nutrition and cancer research.**

***Keywords*:** Bowman-Birk proteinase inhibitor, isopropanol-soluble proteins, storage proteins, trypsin inhibitor.

The classical work of Osborne (1924) initiated efforts to identify and characterize seed storage proteins. These proteins have traditionally been placed into one of four categories based upon solubility. Albumins are soluble in water, globulins in salt solutions, while prolamins dissolve in alcohol solutions, and glutelins in dilute acid or alkali. Prolamins are the most prevalent storage protein in cereal grains, while leguminous plants synthesize globulins as protein reserves (Nielsen, 1996). Among the cultivated crops, soybean (*Glycine max* [L.] Merr.) assimilates the highest percentage of protein per mass of seed (Nielsen, 1996; Krishnan, 2000)

Salt-soluble globulins, glycinins (11S), and β-conglycinins (7S) account for 70% of the total seed protein in soybean (Thanh & Shibasaki, 1976; Hu & Esen, 1981). Since leguminous globulins are deficient in sulfur-containing amino acids with respect to monogastric nutritional requirements, efforts to increase the methionine and cysteine content of these proteins are ongoing. Although mutational breeding to increase sulfur amino acid content has previously met with limited success, a recent study demonstrated that this approach might be a viable option (Imsande, 2001). Smartt *et al.* (1975) proposed improving the nutritional quality of legume proteins by increasing the concentration of endogenous proteins that are rich in specific amino acids. This proposal necessitates the identification of seed proteins that have a high content of methionine and cysteine. Hu & Esen (1981, 1982) carried out a detailed investigation to identify and characterize soybean seed proteins rich in sulfur amino acids using one and two-dimensional electrophoresis. They separated the soybean seed proteins into water-, sodium chloride-, isopropanol-, glacial acetic acid-, sodium hydroxide-, and sodium dodecyl sulfate (SDS)-soluble fractions and determined the relative contribution of each to the total seed protein. Although relatively poor in sulfur amino acids, the water-, salt-, and acetic acid-soluble fractions cumulatively represented 91% of the total seed proteins. The isopropanol-soluble fraction, which represents less than 1% of the total seed protein, was not considered further. In this study, we report the characterization of the isopropanol-soluble fraction and demonstrate its significant contribution to the overall sulfur amino acid content of soybean seed proteins. In addition, we demonstrate that isopropanol extraction of soybean seed flour can be used as a simple and rapid method to isolate low-molecular-weight proteinase inhibitors.

## MATERIALS AND METHODS

### Extraction of soybean proteins

Pioneer Brand 4362 soybean seeds were ground to a fine powder using a mortar and pestle. Isopropanol-soluble proteins were obtained by adding 1 ml of the solvent to 100 mg of seed powder in a 2-ml eppendorf tube. The slurry was placed in a 30 °C shaker for 1 h. Samples were clarified by

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centrifugation at 15,800 g for 5 min. A 500 µl aliquot of the supernatant was mixed with three volumes of ice-cold acetone and stored overnight at -20 °C. Precipitated proteins were recovered by centrifugation as above The protein pellet was air-dried and resuspended in 200 µl of SDS-sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 5% β-mercaptoethanol) and boiled for 5 min. A 10 µL aliquot was loaded onto the polyacrylamide gel for electrophoretic analysis.

### Polyacrylamide gel electrophoresis (SDS-PAGE)

Soybean seed proteins were resolved by SDS-PAGE on a $10 \times 8 \times 0.75$ cm gel comprised of 4% stacking and 13.5% resolving gel in a discontinuous buffer system (Laemmli, 1970). Electrophoresis was conducted at 20 mA constant current at room temperature. After completion of electrophoresis, the resolved proteins were detected by Coomassie brilliant blue staining.

### Amino acid analysis

Amino acid analyses were performed at the University of Missouri Experiment Station Chemical Laboratories. Samples for amino acid analysis were degassed and subjected to hydrolysis for 16 h at 155 °C with 6N HCL under nitrogen atmosphere in furnaced $13 \times 199$ mm glass tubes equipped with PTFE-faced, rubber-lined screw caps. Separation of the amino acids was performed on a Beckman 6300 Amino Acid Analyzer equipped with a high performance cation-exchange-resin column. Analyses of variance on amino acid levels were conducted with PROC GLM in SAS (1996). Multiple range tests between the treatments were made invoking Fisher's protected least significant difference test option.

### Two-dimensional gel electrophoresis

Isopropanol-extracted soybean seed proteins were resuspended in a minimum volume of isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 50 mM dithiothreitol, 2 mM tributylphosphene, 2% CHAPS, 2% Triton X-100) by gentle vortexing for 1h. Insoluble material was removed by centrifugation at 12000 g for 10 min. The supernatant was decanted and the protein concentration determined by Bradford assay using IEF buffer as the blank. One mg protein was loaded on a 13 cm, immobilized pH gradient (pH 3-10) strip (Amersham Biosciences, Piscataway, NJ) by active rehydration at 50 V for 12 h. Isoelectric focusing was performed in a step-wise gradient as follows: 500 V for 1.5 h, 1000 V for 2 h, and 8000 V for 3 h. Current was limited to 50 microA per strip. Reduction, alkylation, and SDS-PAGE-equilibration steps immediately followed IEF. Strips were equilibrated in SDS-PAGE equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% glycerol, 5% SDS) plus 2% DTT for 15 min, followed by 15 min incubation in SDS-PAGE equilibration buffer plus 2.5% iodoacetamide. The pH gradient strips were then layered on top of a 13% acrylamide gel and sealed with agarose sealing buffer (60 mM Tris-Cl pH 6.8, 60 mM SDS, 100 mM DTT, 0.05% bromophenol blue, 0.5% agarose). Using the SE600 system (Amersham), SDS-PAGE was conducted as described (Laemmli, 1970). After overnight immersion in colloidal Coomassie blue (20% ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, 0.008 % Coomassie brilliant blue G-250), the gels were destained in water and imaged using a UMAX Powerlook 2100XL flatbed scanner.

### Sample preparation for MALDI and peptide mass fingerprinting

Gel plugs corresponding to 19- and 10-kDa molecular weights were excised from the Coomassie blue stained gels and destained in 200 µL of 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate. Digestion with sequencing-grade trypsin at 37 °C for 16 h assured complete proteolytic cleavage. Peptides were extracted from the gel plugs with 50 µL of 60% (v/v) acetonitrile, 1% (v/v) formic acid, and concentrated to 5-15 µL by centrifugal vacuum evaporation. Analyses of trypsin-digested protein samples were carried out on an Applied Biosystems Voyager-DE Pro Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometer (MALDI-TOF MS). The MALDI-TOF MS was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized/desorbed with a 337-nm laser and spectra acquired at 20 kV accelerating potential with optimized parameters. Spectra were automatically processed for baseline correction, noise removal, and peak deisotoping prior to submission to a local copy of version 3.2.1 of the MS Fit program of Protein Prospector (http.//prospector.ucsf.edu). Search criteria required the match of at least four peptides with a mass error of less than 150 ppm for a tentative protein assignment

### Gel electrophoretic analysis of trypsin and chymotrypsin inhibitor activity

Proteinase inhibitor activity was detected according to Pichare & Kachole (1994) with slight modifications. Isopropanol-extracted proteins dissolved in sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol (v/v)) were resolved on a non-denaturing PAGE gel. After electrophoresis, the gels

were washed for 10 min in 50 ml of 100 mM Tris-HCl (pH 7.8). Subsequently, the gels were incubated for 10 min with either 0.1 mg/ml trypsin or 0.1 mg/ml chymotrypsin prepared in 100 mM Tris-HCl (pH 7.8). After brief rinsing in 100 mM Tris-HCl (pH 7.8), gels were placed on exposed undeveloped X-ray film and incubated at 37 °C. Appearance of the proteinase inhibitor bands was observed visually. The X-ray film was rinsed in distilled water to remove the hydrolyzed gelatin. Unhydrolyzed regions of the gel represented zones of proteinase inhibitor activity.

## RESULTS

### Analysis of isopropanol-extractable soybean seed proteins

Hu & Esen (1981, 1982) separated soybean proteins into six fractions using sequential extraction with different solvents. They determined that the water-soluble fraction contained about 75% of the total seed protein, while isopropanol solubilized only 1% of the total protein. To characterize the isopropanol-soluble fractions, we sequentially extracted the soybean seed powder with increasing concentrations of the alcohol. Soybean seeds extracted with a 60% isopropanol solution and fractionated by SDS-PAGE revealed several low molecular weight proteins (Fig. 1). Most prominent were the19-kDa and 6-kDa proteins. Between these two polypeptides were proteins in the molecular weight range of 10 to 16 kDa that exhibited a diffuse migration pattern (Fig. 1) These diffuse proteins were not visible in the 70% isopropanol extract, but the 19 and 6 kDa proteins were still observed (Fig. 1) Inclusion of the reducing agent, β-mercaptoethanol, to the solvent did not result in the extraction of additional proteins but did increase the yield (data not shown). Amino acid analysis revealed that the 60%-isopropanol extract contained four-fold higher cysteine when compared to the total protein (Table 1). The methionine content of the isopropanol extract was not significantly different from that of the total seed protein (Table 1). The total sulfur amino acid (TSAA; cysteine + methionine) concentration of 60%-isopropanol extract was 2 8 times higher than that of the total protein fraction (Table 1).

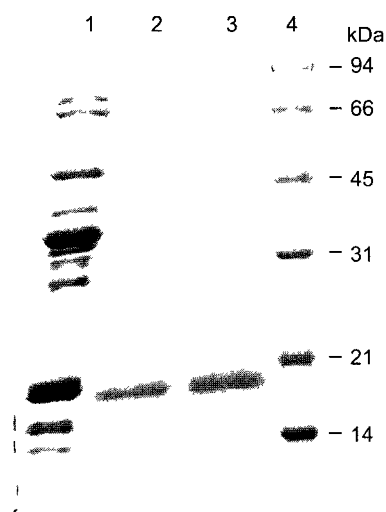


**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of soybean seed proteins Total protein (Lane 1), 60% isopropanol (Lane 2), and 70% isopropanol (Lane 3) extracted soybean seed proteins were resolved on a 13% SDS-PAGE gel and the proteins visualized by staining the gel with Coomassie Brilliant Blue. Sizes of molecular weight markers (Lane 4) in kDa are also shown

**Table 1.** Sulfur amino acid composition of prolamin and total protein of soybean

| Amino acid | Total protein | Prolamin |
|---|---|---|
| Cysteine | 1.81%a | 7 69%b |
| Methionine | 1.44%a | 1 38%a |

Data are the means of three different preparations. Means showing the same letter within each row are not significantly different at $P\leq$ 0 05

### Isopropanol-soluble fraction reveals proteinase inhibitor activity

Soybean seeds contain several low molecular weight proteins that are rich in cysteine. Prominent among them are the Bowman-Birk proteinase and Kunitz trypsin inhibitors. These inhibitory proteins have been estimated to contain nearly half the cysteine content of soybean seed (Nielsen, 1996). Since we determined the isopropanol-soluble proteins contained a significant quantity of cysteine, the possibility that this fraction was enriched in proteinase inhibitors was examined. When the isopropanol-soluble proteins were incu-

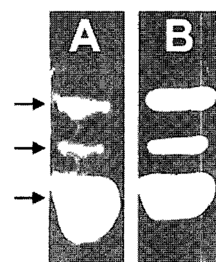


**Fig. 2.** Gel electrophoretic analysis of protease inhibitor activity Isopropanol-extracted proteins were resolved in a native polyacrylamide gel and incubated with either chymotrypsin (Lane A) or trypsin (Lane B) Proteinase inhibitor activity was detected by exposing the gel was to X-ray film Unhydrolyzed regions represent zones of proteinase inhibitor activity

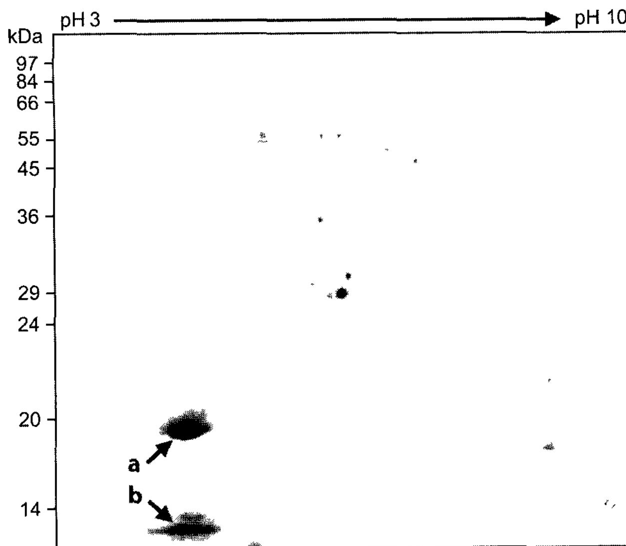


**Fig. 3.** 2-D gel of 60% isopropanol-extracted proteins from soybean seeds Proteins were separated in the first dimension on an IPG strip pH 3-10 and in the second dimension on a 13% acrylamide SDS-gel The gel was stained with colloidal Coomassie blue The two prominent spots used for peptide mass fingerprint are indicated.

bated with either trypsin or chymotrypsin and exposed to X-ray film, proteinase inhibitor activity was detected (Fig. 2). These results confirmed that the isopropanol-soluble fraction contains both Bowman-Birk proteinase and Kunitz trypsin inhibitors.

### Kunitz trypsin inhibitor and Bowman-Birk proteinase inhibitor are the abundant proteins in the isopropanol-soluble fraction

Isopropanol-extracted soybean proteins, when resolved by two-dimensional gel electrophoresis and visualized with Coomassie-blue, revealed by two prominent spots (Fig. 3). The molecular weights of these two spots based upon relative mobility were 19,807 and 10,892 Daltons with calculated isoelectric points of 4.2 and 4 0, respectively. After excision from the gel, the proteins were subjected to trypsin digestion. High-quality mass spectra were obtained for peptide components of the two segregated proteins utilizing MALDI-TOF MS. A search of the protein database using PEPTIDENT SOFTWARE identified the 19-kDa protein as soybean trypsin inhibitor and the 10-kDa protein as the Bowman-Birk protease inhibitor (Figs. 4 and 5). These two spots represented the majority of the protein in the isopropanol-extractable fraction. We also performed Western blot analysis using antibodies raised against purified soybean trypsin inhibitor. The antibodies reacted against the 19-kDa protein confirming that it was a Kunitz trypsin inhibitor (data not shown).

## DISCUSSION

Traditional salt extraction of soybean seed yields a myriad of proteins, which are classified as 11S, 8S, 7S, or 2S as determined by sucrose-density sedimentation (Nielsen, 1996) Because 11S and 7S, the major storage proteins of soybean, contain marginal amounts of sulfur amino acids, a significant portion of the sulfur amino acid content of the soybean seed can be attributed to the sulfur-rich 8S and 2S proteins (Nielsen, 1996). In addition, a few low-abundance proteins, which are rich in methionine and cysteine, also contribute to the sulfur amino acid content of soybean (George & de Lumen, 1991; Revilleza *et al.,* 1996). Results presented in this paper reveal that the 60%-isopropanol extract of soybean contains proteins rich in cysteine A preponderance of this fraction was comprised of proteinase inhibitors. However, the isopropanol-soluble proteins represent less than 1% of the total seed proteins (Hu & Esen, 1981, 1982). Thus, increasing the concentration of this cysteine-rich protein fraction should result in an increase in the overall sulfur amino acid content of soybean.

Genetic engineering approaches have been used to elevate the sulfur amino acid content of soybean and other legumes (Muntz *et al.,* 1998). Attempts have been made to introduce heterologous sulfur rich proteins into soybean. Methionine-rich proteins have been identified in Brazil nut [*Bertholletia excelsa* H B.K.] (Altenbach *et al.,* 1987), sunflower [*Helianthus annuus* L.] (Kortt *et al.,* 1991), rice [*Oryza sativa* L.] (Masumura *et al.,* 1989), and corn [*Zea mays* L.] (Kirihara *et al.,* 1988). Expression of Brazil nut 2S albumin (BNA) resulted in elevating the methionine content of soybeans (Townsend & Thomas, 1994). Since the BNA albumin was an allergen, no commercial cultivars were subsequently released. Introduction of methionine-rich zeins have resulted only in marginal increases in the sulfur amino acid content of transgenic soybeans (Dinkins *et al.,* 2001; Kim & Krishnan, 2004). Even though genetic engineering provides a means to increase the protein quality of soybean, several obstacles remain to be surmounted to achieve a significant increase in sulfur amino acid content. From our studies, it is clear that the isopropanol-soluble fraction contributes the majority of sulfur amino acids to the soybean seed protein profile. Screening the USDA soybean germplasm for lines that preferentially accumulate isopropanol-soluble protein can be used as method to identify soybean cultivars containing high levels of cysteine. This selection process would facilitate improvement of seed storage protein quality.

Several studies have demonstrated that diets containing soybean protein contribute to human health beyond the nutritional aspects (Messina *et al.,* 1994, Friedman & Brandon, 2001; Barnes & Messina, 2002). Among soybean seed

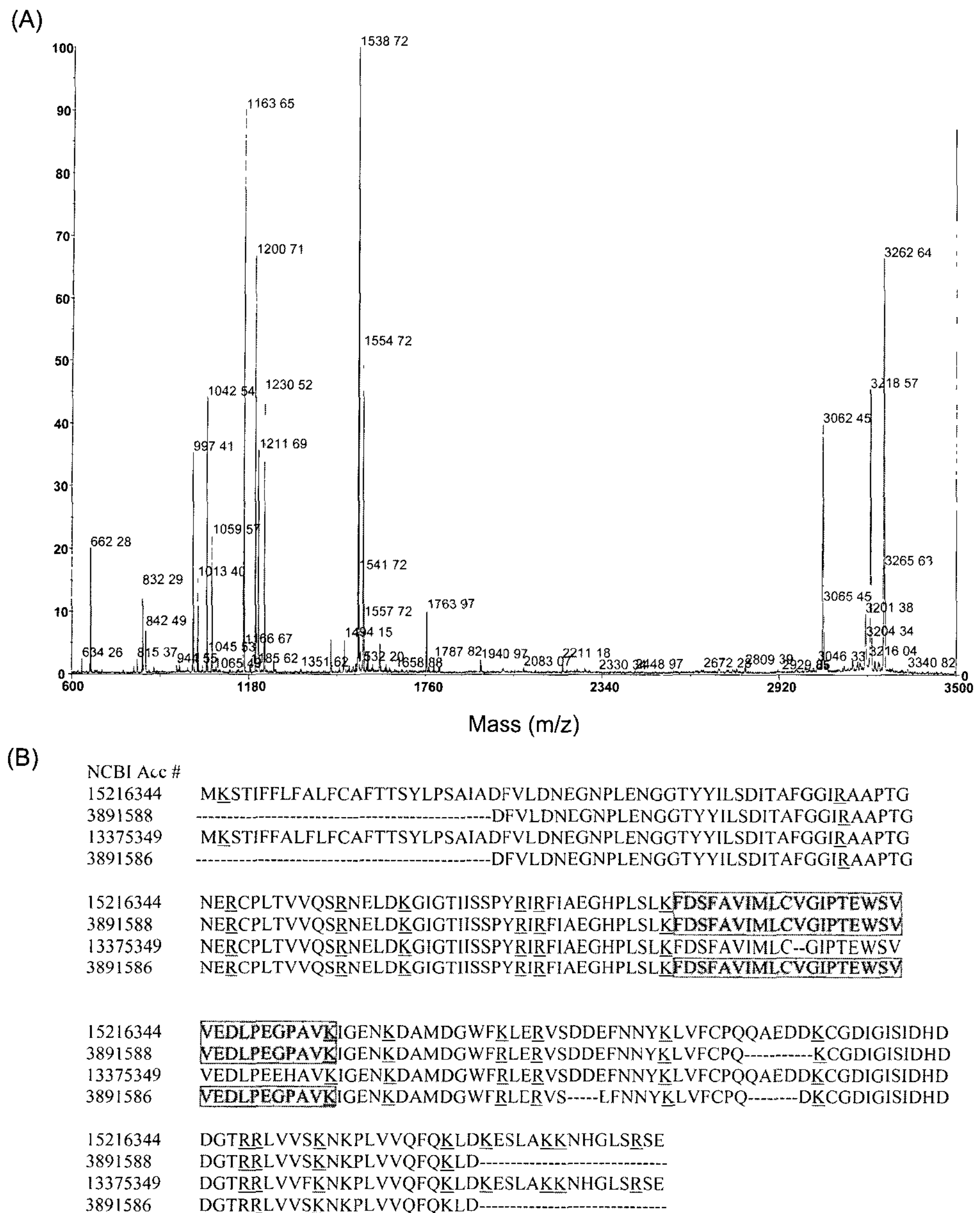


**Fig. 4.** Peptide mass fingerprint of spot 'a' from Fig 3 obtained with matrix-assisted, laser desorption/ionization-time of flight mass spectroscopy (Panel A) The peptides were identified using protein databases as described in Materials and Methods The peptide fragments matching Kuntz trypsin inhibitors protein sequences found in National Center for Biotechnology Information protein database (ACC# 15216344, 3891588, 13375349, and 3891586) are highlighted (Panel B)

proteins, Bowman-Birk protease inhibitor has received particular attention because of its potential to prevent malignant transformation (Yavelow *et al.*, 1985) and carcinogenesis *in vitro* (Kennedy *et al.*, 1996). Based on these observations, it was suggested that BBI could serve as a human cancer chemopreventive agent (Kennedy, 1998). These low molecular weight proteinase inhibitors are traditionally purified by ammonium-sulfate precipitation, gel filtration, column chromatography, or HPLC (Birk *et al.*, 1963, Hwang *et al.*, 1977; Deshimaru *et al.*, 2002). Each of these procedures is time consuming and results in limited amounts of purified material. Therefore establishing a simple protocol for the isolation and purification of these proteins would be beneficial. The results presented in this study show that direct extraction of soybean seed with 60% isopropanol resulted in a fraction that was enriched in the low molecular weight proteinase inhibitors. Previously, it was shown that a soybean seed extract containing the Bowman-Birk protease inhibitor and other proteins was as affective as the purified protease inhibitor fraction as a cancer preventative agent

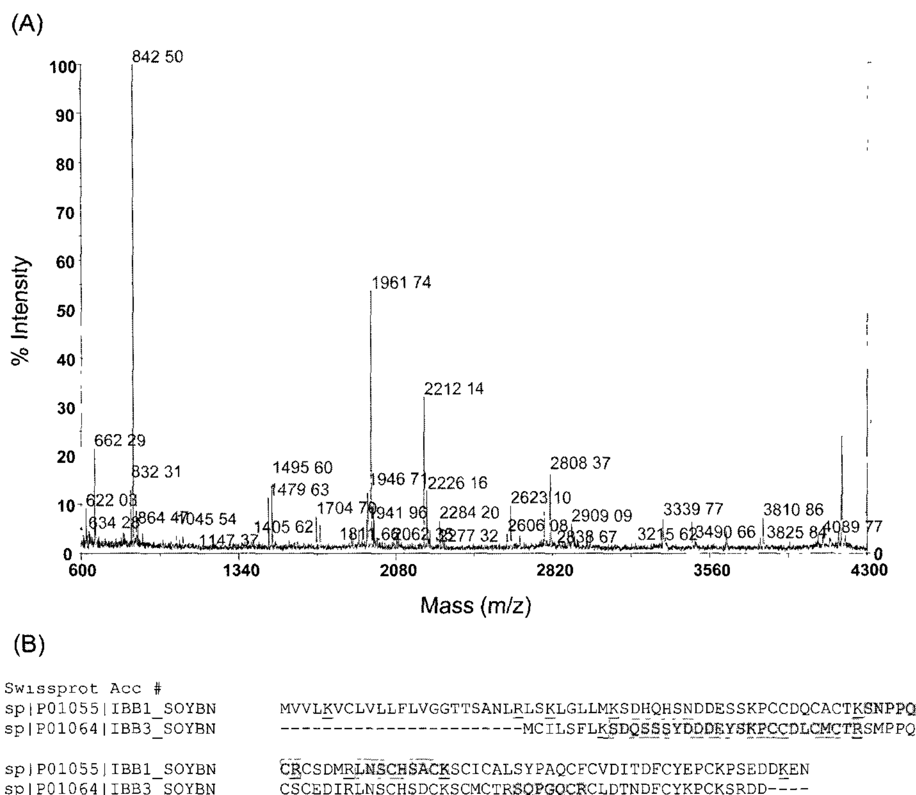


**Fig. 5.** Peptide mass fingerprint of spot 'b' from Fig 3 obtained with matrix-assisted, laser desorption/ionization-time of flight mass spectroscopy (Panel A) The peptides were identified using protein databases as described in Materials and Methods The peptide fragments matching Bowman-Birk proteinase inhibitors found in Swiss-Prot database (ACC# P01055 and P01064) are highlighted (Panel B)

(Kennedy *et al.,* 1993). Because the isopropanol extraction procedure is very simple and results in large amounts of the Bowman-Birk protease inhibitor-enriched fraction, it could be the method of choice for epidemiological and laboratory studies dealing with carcinogenesis.

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