QUANTITATIVE DETERMINATION OF ACTIVE BOWMAN-BIRK ISOINHIBITORS, IBB1 AND IBBD2, IN COMMERCIAL SOYMILKS

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Abbreviations used

BAPNA, N-α-benzoyl-DL-arginine-*p*-nitroanilide; BBI, Bowman-Birk inhibitors; BBIC, Bowman-Birk inhibitor concentrate; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; CIA, chymotrypsin inhibitor activity; CIU, chymotrypsin inhibitor units; CRC, colorectal cancer; DMH, dimethylhydrazine;

GIT, gastrointestinal tract; IU, inhibitor units; K_i, inhibition constant; KTI, Kunitz trypsin inhibitor; SM, soymilk; TIA, trypsin inhibitor activity, TIU, trypsin inhibitor units.

Running title: Active Bowman-Birk inhibitors in soymilks

Abstract

Naturally-occurring serine protease inhibitors of the Bowman-Birk family exert their potential chemopreventive and/or therapeutic properties *via* protease inhibition. In this study, we have quantified the amounts of active BBI isoinhibitors, IBB1 and IBBD2, in six commercial soymilks. By using cation exchange chromatography, the BBI isoinhibitors were isolated and their specific trypsin inhibitory activity was used to estimate their amounts in soymilk samples. IBB1 and IBBD2 concentrations ranged from 0.44 to 5.20 and 0.27 to 4.60 mg/100 ml of soymilk, respectively; total BBI, considered as the sum of both isoinhibitors, ranged from 0.60 to 9.07 mg/100 ml of soymilk. These data show that physiologically relevant amounts of active BBI are present in commercial soymilk and may exert potential health-promoting effects.

1. Introduction

In humans, aberrant functioning of certain serine proteases underlies pathological and physiological disorders. The therapeutic value of protease inhibitors, both natural and synthetic, as modulators of such proteolytic activities in disease is well-recognized (Turk, 2006; Drag & Salvensen, 2010; Deu, Verdoes, & Bogyo, 2012). Within this framework, there is a growing interest in naturally-occurring serine protease inhibitors of the Bowman-Birk family due to their potential chemopreventive and/or therapeutic properties which can impact on several human diseases, including cancer, neurodegenerative disorders and inflammatory processes (Clemente, Marín-Manzano, Arques & Domoney, 2013). Bowman-Birk inhibitors (BBIs) from soybean (*Glycine max*) are the most extensively studied members of this protein family. Soybean BBI and homologous proteins have been demonstrated to be effective at preventing or suppressing radiation- and chemical carcinogen-induced transformation, in a wide variety of *in vitro* assays and, carcinogenesis and inflammatory disorders in *in vivo* model systems (Kennedy, 1998; Clemente & Domoney, 2006; Carli et al., 2012; Magee, Owusu-Apenten, McCann, Gill & Rowland, 2012; Safavi & Rostami, 2012;). Experimental human trials utilizing BBI concentrate (BBIC), a protein extract of soybean enriched in BBI, have been completed in patients with oral leukoplakia, benign prostatic hyperplasia and ulcerative colitis. The strength of BBI doses in such intervention studies, measured in chymotrypsin inhibitory units (CIU), ranges from 25 to 800 CIU/d for a total of 6 months of BBIC treatment (Kennedy, 1998). The results of phase I clinical trials carried out with nineteen male patients with benign prostatic hyperplasia have shown that BBIC reduced prostate-specific antigen levels and prostate volume (Malkowicz et al., 2001). In the case of patients with ulcerative colitis, intake of BBIC was associated with a clinical response and induction of remission, as assessed by the Sutherland Disease Activity Index (an index that consists of four major criteria as follows: stool frequency, rectal bleeding, mucosal appearance, and physician rating of disease activity) (Lichtenstein, Deren, Katz, Lewis, & Kennedy, 2010); on the contrary, no clinical effects of BBIC in patients with oral leukoplakia were observed (Armstrong et al., 2013). Although the anti-nutritional effects of BBI

cannot be ignored, these intervention studies revealed that BBIC, orally administrated to human volunteers, was well-tolerated and no apparent toxicity or adverse side effects were elicited after long-term treatment.

In soybean, two major classes of protease inhibitors, Kunitz (KTI) and BBI, accounts for about 6% of the total seed protein (Brandon & Friedman, 2002). KTI is a 21 kDa protein with a single reactive site that binds trypsin. Soybean BBIs are proteins with molecular masses in the range of 6-9 kDa and comprise two distinct binding loops, responsible for the inhibition of two enzyme molecules, which may be the same or distinct types of enzymes (Birk, 1985). Two BBI isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their inhibitory domains, are predominant in soybean cultivars; IBB1 inhibits both trypsin and chymotrypsin whereas IBBD2 inhibits trypsin only (Clemente, Moreno, Marín-Manzano, Jiménez, & Domoney, 2010).

In order to quantify BBI in soy foods, enzymatic and immunological assays have been developed; however, no comprehensive information on the concentration of BBI in soy foods is currently available. The occurrence of BBI in soy foods (soymilk, soy infant formula, tofu, bean curd, soybean cake, and fermented soy products, among others) present in the US market is noteworthy, where BBI may be present in different amounts (Hernandez-Ledesma, Hsieh & de Lumen, 2009). The soy varieties used, the products themselves and the technological processes used in their preparations all contribute to variation in BBI concentration (Xiao, Wood, Robertson, & Gilani, 2012). In a recent study, BBI concentrations of twelve soymilk samples, ranging from 7.2 to 55.9 mg BBI/100 mL of soymilk, were reported (Hernandez-Ledesma et al., 2009). Such amounts seem to be physiologically relevant in order to exert anticancer effects in humans (Kennedy, 1998); nevertheless, these data are based on immunoreactive forms of BBI that could be functionally inactive. The emerging evidence suggests that soybean BBI exert their preventive and therapeutic properties via protease inhibition (Clemente, Sonnante, & Domoney, 2011). Thus, treatment of soybean BBI with reducing and alkylating agents, which substantially reduces inhibitory activity

against serine proteases, renders these dietary proteins unable to inhibit cell proliferation of colon cancer cells (Clemente et al., 2010). More recently, the anti-proliferative effect of rTI1B, a major pea BBI isoinhibitor expressed heterogously in *Pichia pastoris*, compared with those observed using a related inactive mutant, was evaluated (Clemente, Marín-Manzano, Arques, & Domoney, 2012). The proliferation of HT29 colon cancer cells was significantly affected by rTI1B in a dose-dependent manner, whereas the inactive mutant did not show any significant effect on colon cancer cell growth. These findings suggest that serine proteases should be considered as important targets in investigating the potential chemopreventive role of BBI during the early stages of colorectal carcinogenesis.

Although active BBI seems to be necessary to exert their reported anti-carcinogenic and anti-inflammatory properties, quantitative data regarding their presence in commercial soymilks has not been previously reported. Consequently, the aim of this study was to develop a suitable methodology that combines separation of protease isoinhibitors by liquid chromatography and further enzymatic determination of trypsin and chymotrypsin inhibitory activity in order to quantify the amounts of active BBI isoinhibitors, IBB1 and IBBD2, present in commercial soymilks that could exert potential health benefits to consumers.

2. Materials and methods

2.1 Materials

BBI (T9777) and KTI (T2327) from soybean, trypsin (type III) and α-chymotrypsin (type VII) from bovine pancreas, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma (Alcobendas, Spain). All other chemicals were of analytical grade.

2.2 Isolation of soybean protease inhibitors

A mixture of soybean BBI and KTI was prepared by dissolving 1 mg of each in 6 ml of 50 mM sodium acetate buffer, pH 4.4. The mixture was fractionated on a MonoS 5/50 GL cation exchange column (GE Healthcare, Uppsala, Sweden), connected to an AKTA FPLC system (GE Healthcare), using a linear gradient of 0-0.16 M NaCl in 50 mM sodium acetate buffer, pH 4.4, at a flow rate of 1 mL/min. The elution was monitored at 280 nm and 0.5 mL fractions were collected. Trypsin inhibitory activity (TIA) measurements of eluted samples were carried out in flat-bottom microtitre plates by using BAPNA as specific substrate; the assay products were measured at 405 nm, as previously described (Clemente, Marin-Manzano, Jimenez & Rubio, 2008). Chymotrypsin inhibitory activity (CIA) evaluation of eluted samples was carried out by using BTEE as specific substrate, as described below (see section 2.5).

2.3 Preparation of soymilk extracts

Six commercial soymilks (SM-1 to SM-6) were purchased from local stores in Granada, Spain. Four samples (500 mL each) from the same lot/brand were individually freeze-dried and stored at -20 °C. Freeze-dried soymilk (500 mg) were added to 10 mL of 50 mM sodium acetate buffer, pH 4.4, and stirred for 1 h at room temperature. The extracts were centrifuged at 3,500*g* for 15 min and the supernatants were dialysed extensively against 50 mM sodium acetate buffer, pH 4.4, at 4 °C. The soymilk preparations were fractionated on a MonoS 5/50 GL cation exchange column and monitored by TIA and CIA (see sections 2.2 and 2.5, respectively). The trypsin inhibitory profile of soymilks was used to define the chromatographic elution of their major protease inhibitors.

2.4 Mass peptide fingerprinting

Isolated soybean protease inhibitors (10 µg) were dissolved in NuPAGE lithium dodecyl sulphate sample buffer (Invitrogen, Paisley, UK) and separated by electrophoresis on Novex 12% Bis–Tris pre-cast gels using 2-N-morpholine-ethane sulphonic acid (NuPAGE MES, Invitrogen) as running buffer. Immediately before use, samples were reduced with dithiothreitol (DTT) and NuPAGE antioxidant added to the upper buffer chamber to prevent re-oxidation of reduced proteins during electrophoresis. Bands were excised from Colloidal Blue (Invitrogen)-stained gels and subjected to in-gel trypsin digestion. Peptide fragments from digested proteins were desalted and concentrated using C-18 ZipTip columns (Millipore, Madrid, Spain) and then, loaded directly onto the matrix-assisted laser desorption/ionization (MALDI) plate, using a-cyano-4-hydroxycinnamic acid as the matrix for MALDI-mass spectrometry (MS) analysis. MS spectra were obtained automatically in a 4700 Proteomics Analyzer (Applied Biosystems, Cheshire, UK) operating in reflectron mode with delayed extraction. Peptide mass data were used for protein identification against the MS protein sequence database (www.matrixscience.com).

2.5 Measurement of protease inhibitory activities

The major BBI isoinhibitors, IBB1 and IBBD2, and Kunitz inhibitor were assessed for TIA and CIA. TIA was measured using a modified small-scale quantitative assay with BAPNA as specific substrate, and using 50 mM Tris, pH 7.5 as enzyme assay buffer. One trypsin inhibitor unit (TIU) was defined as that which gives a reduction in absorbance at 410 nm of 0.01, relative to trypsin control reactions, in 10 min in a defined assay volume of 10 mL (Domoney & Welham, 1992). CIA was measured using BTEE as specific substrate. One chymotrypsin inhibitor unit (CIU) was defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to chymotrypsin control reactions, in 5 min in a defined assay volume of 10 mL, as previously described (Clemente, MacKenzie, Jeenes & Domoney, 2004). Specific TIA and CIA of IBB1,

IBBD2 and KTI, expressed as inhibitor units (IU) per mg of protein, were calculated. Such values were used to estimate the amount of individual protease inhibitors present in commercial soymilks.

3. Results

3.1 Isolation and functional characterization of major soybean BBI isoinhibitors, IBB1 and IBBD2, and Kunitz inhibitor

As previously demonstrated by chromatographic, electrophoretic and mass peptide fingerprinting analyses, commercially available BBI consisted in a mixture of two major isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their inhibitory domains (Clemente et al., 2010). In the present study, a mixture of commercial BBI and KTI from soybean was fractionated by MonoS cation exchange chromatography. The elution pattern of the mixture of protease inhibitors, monitored by TIA and CIA measurements, is shown in **Fig. 1a**. Up to three major chromatographic peaks were resolved; at pH 4.4, peak 1 was not retained by the MonoS column whereas peaks 2 and 3 were bound and eluted in the range 0.05-0.08 M NaCl and 0.11-0.14 M NaCl, respectively. Regarding their protease inhibitory activities, peak 1 showed both TIA and CIA whereas peaks 2 and 3 demonstrated TIA only. The chromatographic fractions containing the three proteins were pooled individually and analysed by SDS-PAGE, and showed to correspond to the main electrophoretic bands present in the starting material (**Fig. 1b**). When alkylated, the purified peaks 1 and 2 showed proteins with apparent molecular masses in the range 10-12 kDa whereas peak 3 showed a single electrophoretic band of 21 kDa. Further studies by mass peptide fingerprinting were carried out in order to reveal the identity of the three protease inhibitors. In-gel tryptic digestion of excised electrophoretic bands was performed followed by separation of the peptides generated and MS based analysis. A search of peptide mass data against the MS protein sequence database enabled the unambiguous identification of the protease inhibitors. The purified

proteins, corresponding to the chromatographic peaks 1, 2 and 3 (see **Figure 1a**), were identified as Bowman-Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN), Bowman-Birk type proteinase inhibitor D-II (Swiss-Prot entry: IBBD2_SOYBN) and Kunitz inhibitor (Swiss-Prot entry: 1BA7_A), respectively, with sequence coverage ranging from 52 to 86 % (**Table 1**). An amino acid sequence comparison of IBBD2 and IBB1 proteins is shown in **Table 2**, where the peptide sequences that contributed to protein identification by MS are indicated. As described for other BBI proteins, IBB1 and IBBD2 contain 14 Cys residues in conserved positions (Clemente et al., 2011). Following the nomenclature of Schechter & Berger (1967), IBBD2 showed almost identical amino acid sequences within the inhibitory domains, except for positions P_2' and P_4'; in both inhibitory domains, the P_1 position is occupied by Arg, conferring specificity for inhibition of trypsin-like proteases. In the case of IBB1, variation at several positions within the two inhibitory domains was observed; the presence of Lys or Leu in position P_1 confers specific inhibition of trypsin- or chymotrypsin-like proteases, respectively. In agreement with the identity of the P_1 residues of their inhibitory domains, IBB1 inhibited both trypsin and chymotrypsin, whereas IBBD2 inhibited trypsin only (**Table 3**). IBB1 showed a high specific CIA (2917 ± 292 CIU per mg of protein), in contrast to IBBD2, where CIA was not detected. IBBD2 showed a higher specific TIA than IBB1 (4919 ± 101 and 3828 ± 209 TIU per mg of protein, respectively). When compared with the BBI isoinhibitors, KTI showed lower specific TIA (2147 ± 105 TIU per mg of protein), being its ability to inhibit chymotrypsin almost null (**Table 3**). These significant differences in specific inhibitory activities are likely to reflect the variation in the amino acid sequences of the inhibitory domains that play an essential role in determining specificity and potency (Clemente & Domoney, 2006).

3.2 Evaluation of protease inhibitors in soymilks

When monitored by TIA and CIA, the elution pattern of the six commercial soymilks was similar to that obtained for the mixture of protease inhibitors on cation exchange chromatography (**Figure 2**). The three chromatographic peaks obtained from the different soymilks were collected, being the protein identification of electrophoretic bands confirmed by mass peptide fingerprinting (not shown). The specific TIA was used to estimate the content of individual protease inhibitors (IBB1, IBBD2 and KTI) in commercial soymilks. IBB1 and IBBD2 concentrations ranged from 0.44 to 5.20 and 0.27 to 4.60 mg/100 ml of soymilk, respectively; total BBI, considered as the sum of both isoinhibitors, ranged from 0.59 to 9.18 mg/100 ml of soymilk. In the case of KTI, its concentrations ranged from 1.82 to 5.50 mg/100 ml of soymilk (**Table 4**).

4. Discussion

BBIs appear to exert a protective effect against inflammatory disorders and cancer development; such beneficial effect has been specifically attributed to their intrinsic ability to inhibit serine proteases (Safavi & Rostami, 2012; Clemente et al., 2013). Inactive BBI forms render these dietary proteins unable to inhibit cell proliferation of colon cancer cells (Clemente et al., 2010; 2012). These findings reveal the need to evaluate the amounts of active BBI present in soy foods that could potentially exert a beneficial effect to consumers. To quantify BBI in soy foods, enzymatic and immunological methods using polyclonal or monoclonal antibodies have been developed (Brandon, Bates & Friedman, 2004). By using western blotting analysis, Hernandez-Ledesma et al. (2009) reported BBI concentrations ranging from 7.2 to 55.9 mg/100 ml of soymilk, showing most of the tested soymilks values higher than 23 mg/100 ml of soymilk. Although immunoassays offer the specificity and sensitivity necessary to recognize BBI in complex samples, they are unable to distinguish among active or inactive forms. In addition, unusual patterns of temperature-dependent binding displayed by monoclonal antibodies towards soybean BBI have

256 been reported (Brandon, Bates, & Friedman, 1989). Indeed, the lack of commercially available
257 antibodies against BBI makes difficult to measure this protein quantitatively in soybean products
258 that claim their reported health benefits (Losso, 2008). In the case of enzymatic inhibition, only
259 functional BBI proteins with the ability to form complexes with digestive proteases, trypsin and
260 chymotrypsin, can be evaluated (Clemente, Jiménez, Marín-Manzano & Rubio, 2008). Although
261 enzymatic methods offer useful information about the overall protease inhibitory activity in
262 complex samples, it gives no indication about which type of protease inhibitor is responsible of
263 such activity (DiPietro & Liener, 1989). To distinguish the inhibitory activities among major
264 soybean protease inhibitors, BBI and KTI, chromatographic fractionation of commercial soymilks
265 prior to enzymatic inhibition measurements is strictly necessary. In view of that, we have isolated
266 KTI and two major BBI isoinhibitors, IBB1 and IBBD2, from six commercial soymilks and
267 quantified the corresponding amounts taken into account their specific TIA (**Figure 2**). The
268 concentrations of active BBI, considered as the sum of IBB1 and IBBD2, ranged from 0.59 to 9.18
269 mg/100 ml of soymilk whereas KTI ranged from 1.82 to 5.50 mg/100 ml of soymilk. The reported
270 data reflects a significant variation on protease inhibitor concentrations among soymilks. The soy
271 varieties used as well as the processing conditions might be responsible for the variability found in
272 protease inhibitory activity among commercial soymilks. Given that beneficial effects of soybean
273 BBI in humans seem to be dose-dependent, qualitative and quantitative differences on protease
274 inhibitory activities among soymilks might be physiologically relevant.

275 The amounts of protease inhibitors reported in this study points out their significant resistance
276 to processing conditions during soymilk preparation. Heat treatment, a basic step of soymilk
277 preparation, may reduce TIA content at some extent. In a recent study, Xiao et al. (2012) reported a
278 TIA reduction of 44.4 % in soymilk when compared to that contained in whole soybean;
279 unfortunately, no data regarding the survival rates of KTI and BBI were available. Whereas KTI is
280 considered a heat-labile inhibitor, the ability of BBI to inhibit serine proteases seem not to be
281 significantly diminished (Rouhana, Adler-Nissen, Cogan, & Frokiaer, 1996) except when prolonged

heat treatment at high temperature is applied (Rayas-Duarte, Bergeron, & Nielsen, 1992; Osman, Reid, & Weber, 2002). The rigid structure provided by the seven intra-molecular disulphide bridges that maintain the structural and functional features of the binding sites by adding covalent attachment to the inhibitor core are responsible of such high stability (Trivedi, Laurence, & Siahann, 2009; Bateman & James, 2011; Kumar & Gouda, 2013). It has been demonstrated that BBI from chickpea seeds can resist both acidic conditions and the action of digestive enzymes, and transit through the stomach and small intestine of pigs, generally held as a suitable model for human digestive physiology (Clemente et al., 2008). The presence of active BBI (at least 5-8 % of the total ingested BBI) at the terminal ileum revealed the resistance of a significant proportion of these proteins to the extreme conditions of the gastrointestinal tract *in vivo*. Chromatographic, electrophoretic and enzymatic data obtained from ileal samples suggested that most of the BBI activity is derived from a protein core containing the two binding domains, and resistant to proteolysis. *In vitro* incubation studies of soybean BBI with mixed fecal samples of pigs showed that BBI remained active and their intrinsic ability to inhibit serine proteases was not significantly affected by the enzymatic or metabolic activity of fecal microbiota (Marin-Manzano, Ruiz, Jimenez, Rubio, & Clemente, 2009).

Purified BBI and BBIC has demonstrated to exert a protective and/or suppressive effect in dimethylhydrazine (DMH)-treated animals when used at concentrations as low as 10 mg/100 g diet, reducing the incidence and frequency of colon tumours in mice (St. Clair, Billings, Carew, Keller-McGandy, Newberne, & Kennedy, 1990) and rats (Kennedy, Billings, Wan, & Newberne, 2002). Such amount would be equivalent to that present in a single serving of SM-2 and SM-4 and suggest that a single cup of soymilk could have some protective effect against cancer development if the results from animal studies are extrapolated to humans. Autoclaved BBIC, in which serine protease inhibitory activity was abolished, did not show any significant suppressive effect on colon tumour development in rodents, suggesting that the intrinsic ability of BBI to inhibit serine proteases may be required for their anti-cancer properties (Kennedy et al., 2002). Recent studies have

demonstrated a significant concentration- and time-dependent decrease in the growth of HT29 human colon adenocarcinoma cells when treated with a mixture of IBB1 and IBBD2 (Clemente et al., 2010). These proteins have been shown to exert strong anti-proliferative effects of colon cancer cells at concentration as low as 20 µM and IC_{50} values in the range 40-50 µM; in contrast, the growth of non-malignant colonic fibroblastic CCD18-Co cells was unaffected. Interestingly, chemically inactivated soybean BBI did not demonstrate any significant effect of the proliferation of colon cancer cells suggesting that BBI exert their anti-proliferative properties via protease inhibition. In a recent study, a comparative study with rTI1B, a major pea BBI isoinhibitor expressed heterologously in *Pichia pastoris*, and a related synthetic mutant derivative lacking trypsin and chymotrypsin inhibitory activity was carried out (Clemente et al., 2002). Whereas the proliferation of HT29 colon cancer cells was inhibited significantly by rTI1B in a dose-dependent manner, the inactive mutant did not show any significant effect on colon cancer cell growth. These results support the relevance of quantify active BBI in soy-foods.

The anti-carcinogenic properties of soybean BBI have been linked to the chymotrypsin inhibitory domain, leading to the hypothesis that chymotrypsin-like proteases are potential targets of BBI in anti-cancer effects (Kennedy et al., 2002). Yavelow, Collins, Birk, Troll, & Kennedy (1985) reported that an enzymatically modified soybean BBI having only chymotrypsin inhibitory activity was still fully effective as an inhibitor of radiation-induced transformation *in vitro*, whereas the BBI form which inhibits trypsin-like proteases only was ineffective. In contrast, it has been demonstrated recently that IBBD2, with ability to inhibit trypsin only, exerts anti-proliferative effects on colon cancer cells (Clemente et al., 2012). This is the first indication of the involvement of the trypsin inhibitory domain of BBI on health benefits and reveals that both trypsin- and chymotrypsin-like proteases involved in carcinogenic processes should be considered as potential targets of BBI. Due that both therapeutic targets and the action mechanism of soybean isoinhibitors, IBB1 and IBBD2 remain unknown, it is difficult to recognize the relevance of differences in terms of qualitative and quantitative inhibitory capacities among soymilks. Finally, recent studies suggest

that BBI may play an important role in the protection of other bioactive compounds present in soymilk against degradation or gut proteolysis. An example of such is lunasin, a 43-amino acid peptide with demonstrated chemopreventive action in both culture and animal models (Hernandez-Ledesma et al., 2009; Hsieh, Hernandez-Ledesma, Jeong, Park, & de Lumen, 2010).

In summary, this paper reports for first time the amounts of active protease inhibitors, distinguishing between KTI and two major BBI isoinhibitors, present in commercial soymilks. The results obtained in this study suggest that soymilk might be considered as a rich source of active BBI to exert potential health benefits. Research is needed to investigate the bioavailability of active BBI present in soymilks and to study their contribution in chronic disease prevention in healthy subjects.

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Figure Captions

Figure 1. A) Elution profile of a mixture of commercial Bowman-Birk isoinhibitors, IBB1 and IBBD2, and Kunitz inhibitor (KTI) from soybean on a MonoS 5/50 GL cation exchange column. Absorbance (mAU) at 280 nm of the chromatographic elution and the linear gradient of NaCl (0-0.16 M) are shown (solid and dotted lines, respectively). Using BAPNA and BTEE as specific substrates, the trypsin (▲) and chymotrypsin (Δ) inhibitory activities, measured on every fraction, are shown. B**)** SDS-PAGE under denaturing and reducing conditions of the mixture (lane b) and the chromatographic peaks 1 (lane c), 2 (lane d) and 3 (lane e) that contain purified protease inhibitors. Molecular weight markers are shown in lane a.

Figure 2. Elution profile of six commercial soymilks on a MonoS 5/50 GL cation exchange column. Absorbance (mAU) at 280 nm of the chromatographic elution and the linear gradient of NaCl (0-0.16 M) are shown (solid and dotted lines, respectively). Using BAPNA and BTEE as specific substrates, the trypsin (▲) and chymotrypsin (Δ) inhibitory activities, measured on every fraction, are shown.

Table 1. Identification of major Bowman-Birk isoinhibitors, IBB1 and IBBD2, and Kunitz inhibitor (KTI) separated by cation-exchange chromatography

Chromatographic ID	Protein name	NCBI accession number	Entry name	Sequence coverage (%)	Matched peptides	Protein score[1]
1	Bowman–Birk proteinase inhibitor	GI:157830209	1BBI_A	52	3	123
2	Bowman-Birk proteinase inhibitor type D-II	GI:350045	IBBDII	86	5	228
3	Trypsin Inhibitor A (Kunitz type)	GI:3318877	1BA7_A	72	16	775

Database searching was performed using the MASCOT database (http://www.matrixscience.com).

Protein scores greater than 57 were significant ($P<0.05$).

Table 2. Amino acid sequence alignment of IBB1 and IBBD2

	1----------10----------20----------30----------40----------50----------60----------70----------
IBB1_SOYBN (GI:157830209)	- - - -- - - - - *DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACK*SCICA**LS**YPAQCFCVDITDFCYEPCKPSEDDKEN
IBBD2_SOYBN (GI:350045)	*SDQSSSYDDDEYSKPCCDLCMCTRSMPPQCSCEDIRLNSCHSDCKSCMCTRSQPGQCRCLDTNDFCYKPCK*SRDD-------
	1----------10----------20----------30----------40----------50----------60----------70----------

Accession numbers are from MASCOT database (http://www.matrixscience.com). Amino acid sequences of inhibitory domains are underlined. **P_1-$P_{1'}$** are the reactive peptide bond sites, in bold text. Either K or R at position P_1 determines specificity for trypsin, whereas L determines specificity against chymotrypsin. The peptides that contributed to protein identification are indicated in italics.

Table 3. Specific inhibitory activity for trypsin (T) and chymotrysin (C) of Bowman-Birk isoinhibitors, IBB1 and IBBD2, and Kunitz inhibitor (KTI)

Protease inhibitor	Amino acid sequence of inhibitory domains		Specific inhibitory activity (IU/mg protein)	
	Domain 1	Domain 2	TIA	CIA
IBB1	CT**KS**NPPQC	CA**LS**YPAQC	3,828 ± 209	2,917 ± 292
IBBD2	CT**RS**MPPQC	CT**RS**QPGQC	4,819 ± 101	ND
KTI	SPY**RI**R		2,147 ± 105	78 ± 5

Specific activities values represent means ± SD from at least three independent determinations. **P_1-P_1**· are the reactive peptide bond sites, in bold text. Either K or R at position P_1 determines specificity for trypsin, whereas L determines specificity against chymotrypsin. ND, not detected.

529

530 **Table 4.** Protease inhibitory activity and quantitative determination of Bowman-Birk isoinhibitor, IBB1 and IBBD2, and Kunitz inhibitor (KTI) in six commercial soymilks

	Total TIA	Total CIA	IBB1 (mg)	IBBD2 (mg)	Total BBI[1] (mg)	KTI (mg)
SM-1	6,853 ± 1,727	2,857 ± 382	0.44 ± 0.08	0.27 ± 0.05	0.71 ± 0.14	1.82 ± 0.58
SM-2	50,857 ± 4,895	15,356 ± 1,308	4.63 ± 0.62	4.44 ± 0.71	9.07 ± 1.18	5.50 ± 0.39
SM-3	11,858 ± 1,630	4,913 ± 305	1.11 ± 0.15	0.67 ± 0.15	1.77 ± 0.23	1.98 ± 0.20
SM-4	43,295 ± 6,012	14,368 ± 2,888	5.20 ± 0.82	3.54 ± 0.46	8.74 ± 1.25	2.96 ± 0.17
SM-5	8,495 ± 1,364	2,835 ± 171	0.49 ± 0.03	0.11 ± 0.03	0.60 ± 0.05	2.84 ± 0.48
SM-6	12,839 ± 1,408	3,198 ± 251	0.80 ± 0.11	0.33 ± 0.04	1.12 ± 0.11	3.74 ± 0.44

531

532 Data are mean ± SD per 100 ml of soymilk from at least three independent determinations. Quantitative data of an individual protease inhibitor was calculated taking into account their corresponding specific inhibitory activities for trypsin (see Table 2).
[1]Total BBI is the sum of IBB1 and IBBD2.