

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

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29 **Running title:** Active Bowman-Birk inhibitors in soymilks

30

31 **Abstract**

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

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52 **1. Introduction**

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

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

81 In soybean, two major classes of protease inhibitors, Kunitz (KTI) and BBI, accounts for about
82 6% of the total seed protein (Brandon & Friedman, 2002). KTI is a 21 kDa protein with a single
83 reactive site that binds trypsin. Soybean BBIs are proteins with molecular masses in the range of 6-
84 9 kDa and comprise two distinct binding loops, responsible for the inhibition of two enzyme
85 molecules, which may be the same or distinct types of enzymes (Birk, 1985). Two BBI
86 isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their
87 inhibitory domains, are predominant in soybean cultivars; IBB1 inhibits both trypsin and
88 chymotrypsin whereas IBBD2 inhibits trypsin only (Clemente, Moreno, Marín-Manzano, Jiménez,
89 & Domoney, 2010).

90 In order to quantify BBI in soy foods, enzymatic and immunological assays have been
91 developed; however, no comprehensive information on the concentration of BBI in soy foods is
92 currently available. The occurrence of BBI in soy foods (soymilk, soy infant formula, tofu, bean
93 curd, soybean cake, and fermented soy products, among others) present in the US market is
94 noteworthy, where BBI may be present in different amounts (Hernandez-Ledesma, Hsieh & de
95 Lumen, 2009). The soy varieties used, the products themselves and the technological processes used
96 in their preparations all contribute to variation in BBI concentration (Xiao, Wood, Robertson, &
97 Gilani, 2012). In a recent study, BBI concentrations of twelve soymilk samples, ranging from 7.2 to
98 55.9 mg BBI/100 mL of soymilk, were reported (Hernandez-Ledesma et al., 2009). Such amounts
99 seem to be physiologically relevant in order to exert anticancer effects in humans (Kennedy, 1998);
100 nevertheless, these data are based on immunoreactive forms of BBI that could be functionally
101 inactive. The emerging evidence suggests that soybean BBI exert their preventive and therapeutic
102 properties via protease inhibition (Clemente, Sonnante, & Domoney, 2011). Thus, treatment of
103 soybean BBI with reducing and alkylating agents, which substantially reduces inhibitory activity

104 against serine proteases, renders these dietary proteins unable to inhibit cell proliferation of colon
105 cancer cells (Clemente et al., 2010). More recently, the anti-proliferative effect of rTI1B, a major
106 pea BBI isoinhibitor expressed heterogously in *Pichia pastoris*, compared with those observed
107 using a related inactive mutant, was evaluated (Clemente, Marín-Manzano, Arques, & Domoney,
108 2012). The proliferation of HT29 colon cancer cells was significantly affected by rTI1B in a dose-
109 dependent manner, whereas the inactive mutant did not show any significant effect on colon cancer
110 cell growth. **These findings suggest that serine proteases should be considered as important targets**
111 **in investigating the potential chemopreventive role of BBI during the early stages of colorectal**
112 **carcinogenesis.**

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

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121 **2. Materials and methods**

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123 *2.1 Materials*

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

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130 *2.2 Isolation of soybean protease inhibitors*

131 A mixture of soybean BBI and KTI was prepared by dissolving 1 mg of each in 6 ml of 50 mM
132 sodium acetate buffer, pH 4.4. The mixture was fractionated on a MonoS 5/50 GL cation exchange
133 column (GE Healthcare, Uppsala, Sweden), connected to an AKTA FPLC system (GE Healthcare),
134 using a linear gradient of 0-0.16 M NaCl in 50 mM sodium acetate buffer, pH 4.4, at a flow rate of
135 1 mL/min. The elution was monitored at 280 nm and 0.5 mL fractions were collected. Trypsin
136 inhibitory activity (TIA) measurements of eluted samples were carried out in flat-bottom microtitre
137 plates by using BAPNA as specific substrate; the assay products were measured at 405 nm, as
138 previously described (Clemente, Marin-Manzano, Jimenez & Rubio, 2008). Chymotrypsin
139 inhibitory activity (CIA) evaluation of eluted samples was carried out by using BTEE as specific
140 substrate, as described below (see section 2.5).

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142 *2.3 Preparation of soymilk extracts*

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144 Six commercial soymilks (SM-1 to SM-6) were purchased from local stores in Granada, Spain.
145 Four samples (500 mL each) from the same lot/brand were individually freeze-dried and stored at -
146 20 °C. Freeze-dried soymilk (500 mg) were added to 10 mL of 50 mM sodium acetate buffer, pH
147 4.4, and stirred for 1 h at room temperature. The extracts were centrifuged at 3,500g for 15 min and
148 the supernatants were dialysed extensively against 50 mM sodium acetate buffer, pH 4.4, at 4 °C.
149 The soymilk preparations were fractionated on a MonoS 5/50 GL cation exchange column and
150 monitored by TIA and CIA (see sections 2.2 and 2.5, respectively). The trypsin inhibitory profile of
151 soymilks was used to define the chromatographic elution of their major protease inhibitors.

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153 *2.4 Mass peptide fingerprinting*

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

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169 *2.5 Measurement of protease inhibitory activities*

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171 The major BBI isoinhibitors, IBB1 and IBB2, and Kunitz inhibitor were assessed for TIA
172 and CIA. TIA was measured using a modified small-scale quantitative assay with BAPNA as
173 specific substrate, and using 50 mM Tris, pH 7.5 as enzyme assay buffer. One trypsin inhibitor unit
174 (TIU) was defined as that which gives a reduction in absorbance at 410 nm of 0.01, relative to
175 trypsin control reactions, in 10 min in a defined assay volume of 10 mL (Domoney & Welham,
176 1992). CIA was measured using BTEE as specific substrate. One chymotrypsin inhibitor unit (CIU)
177 was defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to
178 chymotrypsin control reactions, in 5 min in a defined assay volume of 10 mL, as previously
179 described (Clemente, MacKenzie, Jeenes & Domoney, 2004). Specific TIA and CIA of IBB1,

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

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184 **3. Results**

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186 *3.1 Isolation and functional characterization of major soybean BBI isoinhibitors, IBB1 and IBBD2,* 187 *and Kunitz inhibitor*

188 As previously demonstrated by chromatographic, electrophoretic and mass peptide
189 fingerprinting analyses, **commercially available** BBI consisted in a mixture of two major
190 isoinhibitors, IBB1 and IBBD2, showing considerable amino acid sequence divergence within their
191 inhibitory domains (Clemente et al., 2010). In the present study, a mixture of commercial BBI and
192 KTI from soybean was fractionated by MonoS cation exchange chromatography. The elution
193 pattern **of the mixture of** protease inhibitors, monitored by TIA and CIA measurements, is shown in
194 **Fig. 1a**. Up to three major chromatographic peaks were resolved; at pH 4.4, peak 1 was not retained
195 by the MonoS column whereas peaks 2 and 3 were bound and eluted in the range 0.05-0.08 M NaCl
196 and 0.11-0.14 M NaCl, respectively. Regarding their protease inhibitory activities, peak 1 showed
197 both TIA and CIA whereas peaks 2 and 3 demonstrated TIA only. The chromatographic fractions
198 containing the three proteins were pooled individually and analysed by SDS-PAGE, and showed to
199 correspond to the main electrophoretic bands present in the starting material (**Fig. 1b**). When
200 alkylated, the purified peaks 1 and 2 showed proteins with apparent molecular masses in the range
201 10-12 kDa whereas peak 3 showed a single electrophoretic band of 21 kDa. Further studies by mass
202 peptide fingerprinting were carried out in order to reveal the identity of the three protease inhibitors.
203 In-gel tryptic digestion of excised electrophoretic bands was performed followed by separation of
204 the peptides generated and MS based analysis. A search of peptide mass data against the MS protein
205 sequence database enabled the unambiguous identification of the protease inhibitors. The purified

206 proteins, corresponding to the chromatographic peaks 1, 2 and 3 (see **Figure 1a**), were identified as
207 Bowman-Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN), Bowman-Birk type
208 proteinase inhibitor D-II (Swiss-Prot entry: IBBD2_SOYBN) and Kunitz inhibitor (Swiss-Prot
209 entry: 1BA7_A), respectively, with sequence coverage ranging from 52 to 86 % (**Table 1**). An
210 amino acid sequence comparison of IBBD2 and IBB1 proteins is shown in **Table 2**, where the
211 peptide sequences that contributed to protein identification by MS are indicated. As described for
212 other BBI proteins, IBB1 and IBBD2 contain 14 Cys residues in conserved positions (Clemente et
213 al., 2011). Following the nomenclature of Schechter & Berger (1967), IBBD2 showed almost
214 identical amino acid sequences within the inhibitory domains, except for positions P₂' and P₄'; in
215 both inhibitory domains, the P₁ position is occupied by Arg, conferring specificity for inhibition of
216 trypsin-like proteases. In the case of IBB1, variation at several positions within the two inhibitory
217 domains was observed; the presence of Lys or Leu in position P₁ confers specific inhibition of
218 trypsin- or chymotrypsin-like proteases, respectively. In agreement with the identity of the P₁
219 residues of their inhibitory domains, IBB1 inhibited both trypsin and chymotrypsin, whereas
220 IBBD2 inhibited trypsin only (**Table 3**). IBB1 showed a high specific CIA (2917 ± 292 CIU per mg
221 of protein), in contrast to IBBD2, where CIA was not detected. IBBD2 showed a higher specific
222 TIA than IBB1 (4919 ± 101 and 3828 ± 209 TIU per mg of protein, respectively). When compared
223 with the BBI isoinhibitors, KTI showed lower specific TIA (2147 ± 105 TIU per mg of protein),
224 being its ability to inhibit chymotrypsin almost null (**Table 3**). These significant differences in
225 specific inhibitory activities are likely to reflect the variation in the amino acid sequences of the
226 inhibitory domains that play an essential role in determining specificity and potency (Clemente &
227 Domoney, 2006).

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229 *3.2 Evaluation of protease inhibitors in soymilks*

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231 When monitored by TIA and CIA, the elution pattern of the six commercial soymilks was
232 similar to that obtained for the mixture of protease inhibitors on cation exchange chromatography
233 (**Figure 2**). The three chromatographic peaks obtained from the different soymilks were collected,
234 being the protein identification of electrophoretic bands confirmed by mass peptide fingerprinting
235 (not shown). The specific TIA was used to estimate the content of individual protease inhibitors
236 (IBB1, IBBD2 and KTI) in commercial soymilks. IBB1 and IBBD2 concentrations ranged from
237 0.44 to 5.20 and 0.27 to 4.60 mg/100 ml of soymilk, respectively; total BBI, considered as the sum
238 of both isoinhibitors, ranged from 0.59 to 9.18 mg/100 ml of soymilk. In the case of KTI, its
239 concentrations ranged from 1.82 to 5.50 mg/100 ml of soymilk (**Table 4**).

240

241 **4. Discussion**

242

243 BBIs appear to exert a protective effect against inflammatory disorders and cancer
244 development; such beneficial effect has been specifically attributed to their intrinsic ability to
245 inhibit serine proteases (**Safavi & Rostami, 2012**; Clemente et al., 2013). Inactive BBI forms render
246 these dietary proteins unable to inhibit cell proliferation of colon cancer cells (Clemente et al.,
247 2010; 2012). These findings reveal the need to evaluate the amounts of active BBI present in soy
248 foods that could potentially exert a beneficial effect to consumers. To quantify BBI in soy foods,
249 enzymatic and immunological methods **using polyclonal or monoclonal antibodies** have been
250 developed (Brandon, Bates & Friedman, 2004). **By using western blotting analysis, Hernandez-**
251 **Ledesma et al. (2009) reported BBI concentrations ranging from 7.2 to 55.9 mg/100 ml of soymilk,**
252 **showing most of the tested soymilks values higher than 23 mg/100 ml of soymilk.** Although
253 immunoassays offer the specificity and sensitivity necessary to recognize BBI in complex samples,
254 they are unable to distinguish among active or inactive forms. In addition, unusual patterns of
255 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

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

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

308 demonstrated a significant concentration- and time-dependent decrease in the growth of HT29
309 human colon adenocarcinoma cells when treated with a mixture of IBB1 and IBBD2 (Clemente et
310 al., 2010). These proteins have been shown to exert strong anti-proliferative effects of colon cancer
311 cells at concentration as low as 20 μ M and IC₅₀ values in the range 40-50 μ M; in contrast, the
312 growth of non-malignant colonic fibroblastic CCD18-Co cells was unaffected. Interestingly,
313 chemically inactivated soybean BBI did not demonstrate any significant effect of the proliferation
314 of colon cancer cells suggesting that BBI exert their anti-proliferative properties via protease
315 inhibition. In a recent study, a comparative study with rTI1B, a major pea BBI isoinhibitor
316 expressed heterologously in *Pichia pastoris*, and a related synthetic mutant derivative lacking
317 trypsin and chymotrypsin inhibitory activity was carried out (Clemente et al., 2002). Whereas the
318 proliferation of HT29 colon cancer cells was inhibited significantly by rTI1B in a dose-dependent
319 manner, the inactive mutant did not show any significant effect on colon cancer cell growth. These
320 results support the relevance of quantify active BBI in soy-foods.

321 The anti-carcinogenic properties of soybean BBI have been linked to the chymotrypsin
322 inhibitory domain, leading to the hypothesis that chymotrypsin-like proteases are potential targets
323 of BBI in anti-cancer effects (Kennedy et al., 2002). Yavelow, Collins, Birk, Troll, & Kennedy
324 (1985) reported that an enzymatically modified soybean BBI having only chymotrypsin inhibitory
325 activity was still fully effective as an inhibitor of radiation-induced transformation *in vitro*, whereas
326 the BBI form which inhibits trypsin-like proteases only was ineffective. In contrast, it has been
327 demonstrated recently that IBBD2, with ability to inhibit trypsin only, exerts anti-proliferative
328 effects on colon cancer cells (Clemente et al., 2012). This is the first indication of the involvement
329 of the trypsin inhibitory domain of BBI on health benefits and reveals that both trypsin- and
330 chymotrypsin-like proteases involved in carcinogenic processes should be considered as potential
331 targets of BBI. Due that both therapeutic targets and the action mechanism of soybean isoinhibitors,
332 IBB1 and IBBD2 remain unknown, it is difficult to recognize the relevance of differences in terms
333 of qualitative and quantitative inhibitory capacities among soymilks. Finally, recent studies suggest

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

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

344

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353

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460 **Figure Captions**

461

462 **Figure 1. A)** Elution profile of a mixture of **commercial** Bowman-Birk isoinhibitors, IBB1 and
463 IBBD2, and Kunitz inhibitor (KTI) from soybean on a MonoS 5/50 GL cation exchange column.
464 Absorbance (mAU) at 280 nm of the chromatographic elution and the linear gradient of NaCl (0-
465 0.16 M) are shown (solid and dotted lines, respectively). Using BAPNA and BTEE as specific
466 substrates, the trypsin (**▲**) and chymotrypsin (**Δ**) inhibitory activities, measured on every fraction,
467 are shown. B) SDS-PAGE under denaturing and reducing conditions of the mixture (lane **b**) and the
468 chromatographic peaks 1 (lane **c**), 2 (lane **d**) and 3 (lane **e**) that contain purified protease inhibitors.
469 Molecular weight markers are shown in lane **a**.

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

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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).

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Table 2. Amino acid sequence alignment of IBB1 and IBBD2

	1-----10-----20-----30-----40-----50-----60-----70-----
IBB1_SOYBN (GI:157830209)	<i>DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACKSCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN</i>
IBBD2_SOYBN (GI:350045)	<i>SDQSSSYDDDEYSKPCDLCMCTRSMPPOCSCEDIRLNSCHSDCKSCMCTRSOPGOCRCLDTNDFCYKPKSRDD-----</i>
	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. **P₁-P_{1'}** are the reactive peptide bond sites, in bold text. Either K or R at position P₁ determines specificity for trypsin, whereas L determines specificity against chymotrypsin. The peptides that contributed to protein identification are indicated in italics.

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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	CTKSNPPQC	CALSYPAQC	3,828 ± 209	2,917 ± 292
IBBD2	CTRSMPQC	CTRSQPGQC	4,819 ± 101	ND
KTI	SPYRIR		2,147 ± 105	78 ± 5

Specific activities values represent means ± SD from at least three independent determinations. **P₁-P₁** are the reactive peptide bond sites, in bold text. Either K or R at position P₁ determines specificity for trypsin, whereas L determines specificity against chymotrypsin. ND, not detected.

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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 BBI¹ (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

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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).

¹Total BBI is the sum of IBB1 and IBBD2.