Quantitative determination of active Bowman-Birk isoinhibitors, IBB1 and IBBD2, in commercial soymilks

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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.

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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-recognised (Deu, Verdoes, & Bogyo, 2012; Drag & Salvesen, 2010; Turk, 2006). 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.

Abbreviations: BAPNA, N-α-benzoyl-L-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; TI, trypsin inhibitor activity; TU, trypsin inhibitor units.

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Soymilk 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 (Carli et al., 2012; Clemente & Domoney, 2006; Kennedy, 1998; Magee, Owusu-Apenten, McCann, Gill, & Rowland, 2012; Safavi & Rostami, 2012). Experimental human trials utilising 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 inhibitor 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, 2008); 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 administered 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 different 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, Marin-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 (soy milk, 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 soy milk 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 inhibitory profile of soymilks was used to define the trypsin inhibitory activity (CIA) evaluation of eluted samples was 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, Jiménez, Marin-Manzano, & 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).

2.2. 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 0–16 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, Jiménez, Marin-Manzano, & 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 3500g 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 soymilk extracts 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/ionisation (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 iso inhibitors, 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 characterisation of major soybean BBI isoinhibitors, IBB1 and IBBD2

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 major 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 Fig. 1a), were identified as Bowman-Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN), Bowman-Birk type protease 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 and Berger (1967), IBBD2 showed almost identical amino acid sequences within the inhibitory domains, except for positions P2 and P4; in both inhibitory domains, the P1 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 P1 confers specific inhibition of trypsin- or chymotrypsin-like proteases, respectively. In agreement with the identity of the P1 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/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/mg of protein, respectively). When compared with the BBI isoinhibitors, KTI showed lower specific CIA (2147 ± 105 TIU/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 (Fig. 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 whereas KTI ranged from 0.59 to 9.18 mg/100 ml of soymilk. Although immunoassays offer the specificity and sensitivity necessary to recognise 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 been reported (Brandon, Bates, & Friedman, 1989). Indeed, the lack of commercially available antibodies against BBI makes it difficult to measure this protein quantitatively in soybean products (Losso, 2008). In the case of enzymatic inhibition, only functional BBI proteins with the ability to form complexes with digestive proteases, trypsin and chymotrypsin, can be evaluated (Clemente et al., 2008). Although enzymatic methods offer useful information about the overall protease inhibitory activity in complex samples, it gives no indication about which type of protease inhibitor is responsible of such activity (DiPietro & Liener, 1989). To distinguish the inhibitory activities among major soybean protease inhibitors, BBI and KTI, chromatographic fractionation of commercial soymilks prior to enzymatic inhibition measurements is strictly necessary. In view of that, we have isolated KTI and two major BBI isoinhibitors, IBB1 and IBBD2, from six commercial soymilks and quantified the corresponding amounts taken into account their specific TIA (Fig. 2). The concentrations of active BBI, considered as the sum of IBB1 and IBBD2, ranged from 0.59 to 9.18 mg/100 ml of soymilk whereas KTI ranged from 1.82 to 5.50 mg/100 ml of soymilk. The reported data reflects a significant variation on protease inhibitor concentrations.
among soymilks. The soy varieties used as well as the processing conditions might be responsible for the variability found in protease inhibitory activity among commercial soymilks. Given that beneficial effects of soybean BBI in humans seem to be dose-dependent, qualitative and quantitative differences on protease inhibitory activities among soymilks might be physiologically relevant and deserves further research.

The amounts of protease inhibitors reported in this study points out their significant resistance to processing conditions during soymilk preparation. Heat treatment, a basic step of soymilk preparation, may reduce TIA content at some extent. In a recent study, Xiao et al. (2012) reported a TIA reduction of 44.4% in soymilk when compared to that contained in whole soybean; unfortunately, no data regarding the survival rates of KTI and BBI were available. Whereas KTI is considered a heat-labile inhibitor, the ability of BBI to inhibit serine proteases seem not to be significantly diminished (Rouhana, Adler-Nissen, Cogan, & Frokiaer, 1996) except when prolonged heat treatment at high temperature is applied (Osman, Reid, & Weber, 2002; Rayas-Duarte, Bergeron, & Nielsen, 1992). The rigid structure provided by the seven intra-molecular disulfide 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 (Bateman & James, 2011; Kumar & Gowda, 2013; Trivedi, Laurence, & Siahann, 2009). 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 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

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**Table 4**

Protease inhibitory activity and quantitative determination of Bowman-Birk iso-inhibitor, IBBI and IBBD2, and Kunitz inhibitor (KTI) in six commercial soymilks.

<table>
<thead>
<tr>
<th></th>
<th>Total TIA</th>
<th>Total CIA</th>
<th>IBBI (mg)</th>
<th>IBBD2 (mg)</th>
<th>Total BBI (mg)</th>
<th>KTI (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-1</td>
<td>6853 ± 1727</td>
<td>2857 ± 382</td>
<td>0.44 ± 0.08</td>
<td>0.27 ± 0.05</td>
<td>0.71 ± 0.14</td>
<td>1.82 ± 0.58</td>
</tr>
<tr>
<td>SM-2</td>
<td>50857 ± 4895</td>
<td>15356 ± 1308</td>
<td>4.63 ± 0.62</td>
<td>4.44 ± 0.71</td>
<td>9.07 ± 1.18</td>
<td>5.50 ± 0.39</td>
</tr>
<tr>
<td>SM-3</td>
<td>11858 ± 1630</td>
<td>4913 ± 305</td>
<td>1.11 ± 0.15</td>
<td>0.67 ± 0.15</td>
<td>1.77 ± 0.23</td>
<td>1.98 ± 0.20</td>
</tr>
<tr>
<td>SM-4</td>
<td>43295 ± 6012</td>
<td>14368 ± 2888</td>
<td>5.20 ± 0.82</td>
<td>3.54 ± 0.46</td>
<td>8.74 ± 1.25</td>
<td>2.96 ± 0.17</td>
</tr>
<tr>
<td>SM-5</td>
<td>8495 ± 1364</td>
<td>2835 ± 171</td>
<td>0.49 ± 0.03</td>
<td>0.11 ± 0.03</td>
<td>0.60 ± 0.05</td>
<td>2.84 ± 0.48</td>
</tr>
<tr>
<td>SM-6</td>
<td>12839 ± 1408</td>
<td>3198 ± 251</td>
<td>0.80 ± 0.11</td>
<td>0.33 ± 0.04</td>
<td>1.12 ± 0.11</td>
<td>3.74 ± 0.44</td>
</tr>
</tbody>
</table>

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).

* Total BBI is the sum of IBB1 and IBBD2.

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![Fig. 2](image-url)
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 BBIC 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 IB1B 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 IC50 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 BBIC did not demonstrate any significant effect of the proliferation of colon cancer cells suggesting that BBIC exert their anti-proliferative properties via protease inhibition. In a recent study, a comparative study with rTI1B, a major pea BBIC isoinhibitor expressed heterologously in P. pastoris, and a related synthetic mutant derivative lacking trypsin and chymotrypsin inhibitory activity was carried out (Clemente et al., 2012). 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 quantifying active BBIC in soy-foods.

The anti-carcinogenic properties of soybean BBIC have been linked to the chymotrypsin inhibitory domain, leading to the hypothesis that chymotrypsin-like proteases are potential targets of BBIC in anti-cancer effects (Kennedy et al., 2002). Yavelow, Collins, Birk, Troll, and Kennedy (1985) reported that an enzymatically modified soybean BBIC having only chymotrypsin inhibitory activity was still fully effective as an inhibitor of radiation-induced transformation in vitro, whereas the BBIC 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 BBIC on health benefits and reveals that both trypsin- and chymotrypsin-like proteases involved in carcinogenic processes should be considered as potential targets of BBIC. Due to both therapeutic targets and the action mechanism of soybean isoinhibitors – IB1B and IBBD2 – remain unknown, it is difficult to recognise the biological relevance of differences in terms of qualitative and quantitative inhibitory capacities among soymilk. Finally, recent studies suggest that BBIC 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 (Hernández-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 BBIC isoinhibitors, present in commercial soymilks. The results obtained in this study suggest that soymilk might be considered as a rich source of active BBIC to exert potential health benefits. Research is needed to investigate the bioavailability of active BBIC present in soymilks and to study their contribution in chronic disease prevention in healthy subjects.

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