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

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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-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. 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 (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 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, 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

Abbreviations: 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.

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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, Jiménez, 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 serine 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, Jiménez, Marín-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 3500*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/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 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