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Lunasin and Bowman-Birk protease inhibitor (BBI) in US commercial soy foods

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ABSTRACT

The inverse association between the intake of soybean foods and cancer incidence and mortality rates supported by published literature has led to studies on identifying bioactive components. The cancer preventive properties of the soybean peptides lunasin and Bowman-Birk protease inhibitor (BBI) have been demonstrated by *in vitro* and *in vivo* assays. Since there is no comprehensive information on the concentrations of these two peptides, US commercially available soy foods, including soy milk, soy-based infant formula, tofu, bean curd, soybean cake, tempeh, natto, miso and su-jae samples, were analyzed for lunasin and BBI. Both peptides were present in most of the products, in varying concentrations, depending mainly on the soybean variety and the manufacturing process. Lunasin and BBI were absent in the fermentation products natto and miso, suggesting that fermentation destroys both peptides. To study the bioavailability of lunasin and BBI, three soy milk samples with different concentrations of these peptides were subjected to an enzymatic hydrolysis process simulating physiological digestion. The results confirm the important role BBI plays in the protection of lunasin from digestion by pepsin and pancreatin.

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1. Introduction

Soybean (*Glycine max*) is one of the most cultivated plants in the world with a well known nutritional value. It contains a high concentration of proteins (40–50%), lipids (20–30%) and carbohydrates (26–30%). Soybean is currently consumed worldwide, but has been a staple among Asian populations in which the daily average consumption is 20 to 80 g soy protein. These populations have used soybeans traditionally to prepare non-fermented soy foods, mainly soy milk and tofu, sometimes known as bean curd, and their fried, baked and pressed forms as well as fermented soy foods such as natto, miso and tempeh. In the last decades, soybean foods have generated a lot of interest as a result of evidence that its consumption may alleviate menopausal symptoms (Messina, 2000), and reduce the risk of osteoporosis and some chronic diseases, most notably coronary heart disease and cancer (McCue & Shetty, 2004; Messina & Barnes, 1991). Epidemiological studies, animal experiments and human trials have demonstrated an inverse association between diets containing high amounts of soybean products and low cancer incidence and mortality rates, particularly breast, colon and prostate cancer (Fournier, Erdman, & Gordon, 1998). Although the specific components that are responsible for this chemopreventive activity remain to be identified, several constituents isolated from soybeans have demonstrated biological activities that are consistent with efficacy in cancer prevention (Isanga & Zhang, 2008; Messina & Flickinger, 2002). Isoflavones have been extensively studied and their chemopreventive effects have been mainly attributed to their long-term estrogenic effects and their antioxidant activity (McCue & Shetty, 2004). However, the capacity of soybean proteins and peptides for preventing cancer and other age-related disorders is recently receiving more attention (Omoni & Aluko, 2005).

Bowman-Birk protease inhibitor (BBI) from soybean is a 8 kDa polypeptide containing 71 amino acids and two separate protease inhibitor sites, one each for trypsin and chymotrypsin (Odani & Ikenaka, 1973). Its chymotrypsin inhibitory activity has been found to be essential for anticarcinogenicity (Yavelow, Collins, Birk, Troll, & Kennedy, 1985). BBI has been demonstrated to be effective in preventing or suppressing radiation- and chemical carcinogen-induced transformation in a wide variety of *in vitro* assays (Kennedy, 1998). *In vivo*, BBI has also been found to inhibit carcinogenesis in the colon, oesophagus, liver, lung and the oral cavity (see review of Losso, 2008). Clinical trials using a soybean extract enriched in BBI, called BBI concentrate (BBIC), are currently underway. Preliminary findings show that BBIC induces the regression of oral leukoplakia in human subjects and has effects on potential cancer biomarkers (Armstrong, Wan, Kennedy, Taylor, & Meyskens, 2003). Lunasin is another peptide that has received special attention during the last few years for its promising cancer preventive effects (Galvez, Chen, Macasieb, & de Lumen, 2001; Galvez, Revilleza, & de Lumen, 1997). The capacity of lunasin, a 43-amino acid peptide, to prevent transformation of mammalian cells caused by chemical carcinogens and viral oncogenes has been reported (Galvez et al., 2001; Jeong, Park, Lam, & de Lumen, 2003; Lam, Galvez, & de Lumen, 2003). In the first animal model, lunasin reduced skin tumour incidence and

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multiplicity after its topical administration to SENCAR mice (Galvez et al., 2001).

BBI was quantified in 1989 by Dipietro and Liener in some soy products, such as soy milk dehydrated and frozen, infant formula and tofu (Dipietro & Liener, 1989). Lunasin has been found in all the soybean varieties analyzed, as well as in a number of commercial soy proteins and isoflavones-enriched products (González de Mejia, Vásconez, de Lumen, & Nelson, 2004; Jeong, Jeong, Kim, & de Lumen, 2007a). However, there is no comprehensive information on the concentration of these two peptides in the traditionally consumed soybean foods in the US.

The aim of this study was to analyze the concentrations of lunasin and BBI in different US commercially available non-fermented and fermented soy foods. Furthermore, soy milk was subjected to an enzymatic hydrolysis process simulating physiological digestion to study the bioavailability of these two peptides.

2. Materials and methods

2.1. Samples and preparation of sample extracts

Twelve soy milks (SM-1 to SM-12), three soy-based infant formula (SF-1 to SF-3), twelve tofu (TF-1 to TF-12), six bean curd (BC-1 to BC-6), four tempeh (TE-1 to TE-4), five natto (NT-1 to NT-5), five miso (MS-1 to MS-5), three soybean cake (SC-1 to SC-3) and one su-jae (SJ-1) samples were purchased from a number of San Francisco Bay Area stores. The composition (main ingredients) and the protein concentration provided by the manufacturers are shown in Table 1.

Twenty grams of soy food samples were added to 200 mL of distilled water, blended and magnetically stirred for a period of 3 h. The samples were centrifuged at 15,300*g* for 30 min and the supernatants were collected. Soy-based infant formula was directly analyzed without carrying out the centrifugation step. The protein concentration of the soy milk and soy-based infant formula samples and the supernatants obtained from the other soy products was determined according to the Bradford method, using bovine serum albumin (BSA) as the standard protein.

2.2. Lunasin identification and quantification

A volume of 100 μL of soy milk, soy foods-derived extracts or synthetic lunasin (American Peptide Co, Sunnyvale, CA, USA) (33 μM) was added to 200 μL of tricine sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and heated at 100 °C for 5 min. After the samples and standard had cooled to room temperature, they were loaded onto 16.5% Tris–tricine polypeptide gels (Bio-Rad). The gels were run in Mini Protean-2 Cells (Bio-Rad) using Tris–tricine–SDS buffer as the running buffer. The conditions were set at 100 V constant, and the gels were run for 100 min. An Immun-Blot PVDF membrane (Bio-Rad) was prepared for transfer by soaking in 100% methanol and rinsing with distilled water. The proteins on SDS–PAGE gel were transblotted to the membrane for 60 min at 100 V and 4 °C. Upon completion of transfer, the nonspecific binding sites were blocked by immersing the membrane for 1 h in 5% nonfat dry milk dissolved in Tris-buffered saline 1% Tween 20 (TBS-1T). The membrane was washed with fresh changes of the TBS-1T at room temperature and incubated with lunasin monoclonal primary antibody (diluted 1:5000 in 3% nonfat dry milk in TBS-1T) for 1 h at room temperature. After washing with TBS-1T, the membrane was incubated for 1 h with an anti-mouse horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:3000 dilution in 3% nonfat dry milk in TBS-1T. The membrane was washed three times with TBS-1T, detected using the detection agent (Amersham Biosciences, Piscataway, NJ, USA) and immediately developed using 667 Polaroid films. Lunasin content of the samples was calculated by comparing the band intensities with those of known lunasin standards run under the same conditions. The intensities of the bands were quantified using the software *Un-SCAN-IT gel* version 5.1 (Silk Scientific, Inc. Orem, UT, USA). Samples obtained from the two extraction processes were analyzed in duplicates. Results are expressed as the mean of the four obtained values ± standard deviation.

2.3. BBI identification and quantification

One hundred microlitres of soy milk, soy foods-derived extracts or commercially prepared BBI (Sigma Chemical, St. Louis, MO, USA) (38 μM) were added to 100 μL of Laemmli sample buffer (Bio-Rad) and heated at 100 °C for 5 min. The samples were loaded into 15% Tris–HCl gels (Bio-Rad) that were run at 200 V for 40 min, using Tris–Glycine–SDS buffer as running buffer and the same equipment described above. The proteins were then transblotted to the PVDF membrane for 60 min at 100 V and 4 °C. Western-Blot was carried out using the mouse One-Step Western™ Basic Kit (GenScript Corp. Piscataway, NJ, USA), following the manufacturer's specifications. The primary antibody against BBI (Agdia Inc. Elkhart, IN, USA) was diluted 1:3000. The membrane was detected and developed and BBI content was calculated using synthetic BBI as standard and the same protocol described for lunasin quantification. Samples from two extraction processes were analyzed in duplicates. Results are expressed as the mean of the four obtained values ± standard deviation.

2.4. Simulation of gastrointestinal digestion

Three soy milk samples (SM-3, SM-5 and SM-12) were selected for simulated gastrointestinal digestion. Hydrolysis was carried out according to the method of Hernández-Ledesma, Quirós, Amigo, and Recio (2007), with some modifications. The samples (5 mL) were first hydrolysed with pepsin (E.C. 3.4.23.1; 1:10,000, 859 U/mg protein; Sigma, Saint Louis, MI, USA) (58 mg/g protein) for 60 min at 37 °C at a pH of 1.5, and stirring speed of 150 rev/min. The pH of the digests was adjusted to 7.5 with 1 M NaOH to stop hydrolysis. Pancreatin from porcine pancreas (Sigma) was added at a concentration of 58.8 mg/g protein and the samples were incubated for 120 min at 37 °C with stirring. Aliquots were drawn after hydrolysis with pepsin and after 60 and 120 min of incubation with pancreatin, and the protein concentration was measured. 100 μL of these aliquots were added to 200 μL of Laemmli buffer, and the reaction was immediately stopped by placing tube in a boiling water bath for 5 min. Digestion of samples was carried out in triplicates.

The digests (20 μL) were loaded in SDS–PAGE and analyzed by Western-blotting according to the procedures described above for lunasin and BBI quantification.

3. Results and discussion

3.1. Lunasin and BBI concentration in soy milk and soy-based infant formula

Table 2 shows the results of extracted protein concentration, lunasin and BBI concentration and ratios of lunasin:BBI obtained after analyzing 12 commercial soy milk samples (SM-1 to SM-12) and three soy-based infant formula (SF-1 to SF-3). The protein contents range from 13.0 to 33.1 mg protein/mL of milk. The lowest protein concentration was found in sample SM-10, labelled by the manufacturer as a soy drink. Highest values were found in samples SM-2 (32.3 mg/mL) and SM-3 (33.1 mg/mL) which con-