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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-

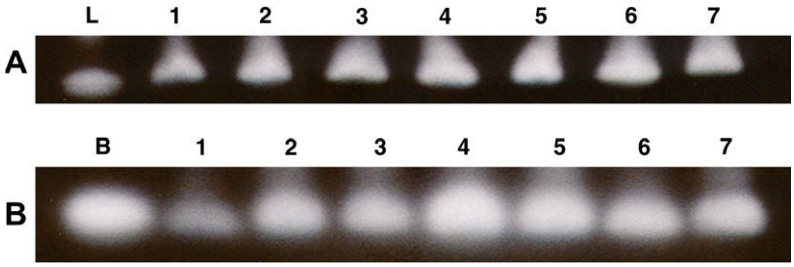

Fig. 1. Western-Blot of lunasin peptide (A) and BBI (B) from commercial soy milk samples. Lane L contains 208 ng of synthetic lunasin and lane B contains 1 μg of synthetic BBI. 1: soy milk SM-1; 2: soy milk SM-2; 3: soy milk SM-3; 4: soy milk SM-4; 5: soy milk SM-5; 6: soy milk SM-6 and 7: soy milk SM-7. Each well contains 50 μg of proteins.

Table 1
Composition, country of origin and protein concentrations of commercial soy foods.

Sample no.	Type of sample	Composition-main ingredients	Country	Protein concentration
SM-1	Enriched soy milk	Soybeans	USA	2.9[a]
SM-2	Organic original soy milk	Soybeans, malted wheat and barley extract	USA	4.6[a]
SM-3	Organic fortified soy milk	Soybeans, malted wheat and barley extract	USA	4.6[a]
SM-4	Organic plain soy milk	Soybeans	USA	2.9[a]
SM-5	Organic unsweetened soy milk	Soybeans	USA	2.9[a]
SM-6	Organic plain soy milk	Soybeans	USA	2.9[a]
SM-7	Organic original soy milk	Soybeans, rice syrup	USA	2.9[a]
SM-8	Organic plain soy milk	Soybeans, soy protein isolate	USA	4.2[a]
SM-9	Organic original soy milk	Soybeans, malt syrup	USA	2.9[a]
SM-10	Organic original soy drink	Soybeans, barley extract	USA	1.7[a]
SM-11	Fortified soy milk	Soybeans	USA	3.3[a]
SM-12	Unsweetened soy milk	Soybeans	Singapore	2.5[a]
SF-1	Soy-based formula	Corn syrup, soy protein isolate	USA	2.0[a]
SF-2	Organic soy formula	Corn syrup, soy protein	USA	2.1[a]
SF-3	Organic soy formula	Rice syrup, soy protein concentrate	USA	1.7[a]
TF-1	Soft tofu	Soybeans	USA	5.9[b]
TF-2	Soft tofu	Soybeans	USA	5.9[b]
TF-3	Silken tofu Kinugoshi	Soybeans	USA	4.4[b]
TF-4	Silken tofu	Soybeans	USA	4.4[b]
TF-5	Silken tofu	Soybeans	USA	5.9[b]
TF-6	Medium firm tofu	Soybeans	USA	8.2[b]
TF-7	Organic medium firm tofu	Soybeans	USA	9.4[b]
TF-8	Firm tofu	Soybeans	USA	8.2[b]
TF-9	Extra firm tofu Chinese style	Soybeans	USA	8.9[b]
TF-10	Baked tofu	Soybeans, soy sauce (wheat)	USA	17.6[b]
TF-11	Fried tofu	Soybean, soybean oil, soy sauce	USA	23.5[b]
TF-12	Dry tofu	Soybeans	Taiwan	16.4[b]
BC-1	Marinated bean curd	Soybeans, soy sauce	Taiwan	21.2[b]
BC-2	Marinated bean curd	Soybeans, soy sauce	USA	21.2[b]
BC-3	Salted bean curd in brine	Soybeans, wine, sesame oil	China	<18.2[b]
BC-4	Baked bean curd	Soybeans, soy sauce (wheat), soybean oil	USA	21.2[b]
BC-5	Pressed bean curd	Soybeans	USA	17.6[b]
BC-6	Soybean curd noodle	Soybeans	Taiwan	21.2[b]
TE-1	Organic soy tempeh	Soybeans, *Rhizopus oligosporus*	USA	16.5[b]
TE-2	Organic soy tempeh	Soybeans, brown rice, *Rhizopus oligosporus*	USA	19.5[b]
TE-3	Organic soy tempeh-flax	Soybeans, flaxseed, brown rice, *Rhizopus oligosporus*	USA	17.7[b]
TE-4	Organic soy tempeh-rice	Soybeans, brown rice, *Rhizopus oligosporus*	USA	15.3[b]
NT-1	Natto	Fermented soybeans (*Bacillus subtilis* natto)	Japan	15.3[b]
NT-2	Natto	Soybeans, soy sauce (wheat), *Bacillus subtilis* natto	Japan	20.0[b]
NT-3	Natto	Soybeans, soy sauce, hydrolysed protein, *Bacillus subtilis* natto	USA	19.6[b]
NT-4	Natto	Soybean sauce, *Bacillus subtilis*	Japan	14.3[b]
NT-5	Natto	Soybeans, *Bacillus subtilis*	Japan	14.0[b]
MS-1	Organic miso	Soybeans, rice, *Aspergillus oryzae*	Japan	8.3[b]
MS-2	Miso	Soybeans, rice, *Aspergillus oryzae*	Japan	11.1[b]
MS-3	Barley miso	Soybeans, barley, *Aspergillus oryzae*	USA	10.0[b]
MS-4	Miso	Soybeans, rice, *Aspergillus oryzae*	USA	13.3[b]
MS-5	Miso	Soybeans, rice, *Aspergillus oryzae*	USA	5.9[b]
SC-1	Deep fried soybean cake	Soybeans, soybean oil	USA	22.4[b]
SC-2	Baked soybean cake	Soybeans, soy sauce, sesame oil	USA	25.9[b]
SC-3	Pressed soybean cake	Soybeans	USA	22.4[b]
SJ-1	Su-jae	Soybeans, soy sauce	USA	21.2[b]

[a] Expressed as g/100 mL of soy milk or soy-based infant formula.
[b] Expressed as g/100 g soy product.

tained wheat and barley extracts in their composition and could contribute to the protein content. Soy-based formulas show protein values ranging from 4.1 and 8.5 mg protein/100 mL of formula.

Western-Blot patterns of lunasin and BBI are shown in Fig. 1. The varying intensities of the bands representing 50 μg of protein for each sample show varying amounts of lunasin and BBI in these samples. Calculated concentrations are shown in Table 2. Lunasin was found in all the soy milk samples analyzed. Soy milk is prepared after soaking the dry beans in water for several hours and heating at the boiling point for 15–20 min. The detection of lunasin in these samples confirms the stability of lunasin to heat treatment, as reported by Galvez et al. (2001). Values for lunasin vary from 10.7 to 18.9 mg/100 mL of soy milk. Differences among samples could be due to the soybean variety used in the preparation of each soy milk. Lunasin concentrations ranging from 0.5 to 8.1 mg lunasin/g flour have been reported in a number of diverse soybean genotypes grown in USA and Korea (González de Mejia et al., 2004; Jeong et al., 2007a).

BBI is present in all soy milk samples manufactured in the US. However, the presence of this polypeptide could not be detected in soy milk SM-12, imported from Singapore suggesting that the process carried out during the manufacturing of this soy milk could have caused the degradation of BBI. The BBI concentrations vary from a low of 5.5 mg BBI/g extracted protein for sample SM-10 to a high of 21.9 mg BBI/g extracted protein for soy milk SM-4. Expressing these results as mg BBI per 100 mL of soy milk, the concentrations vary from 55.9 mg BBI/100 mL to 7.2 mg/100 mL of soy milk. Wan, Lu, Anderson, Ware, and Kennedy (2000) reported BBI concentration in a commercial soy milk of 14.6 mg/100 mL of product. The soybean variety used in the elaboration of soy milk could also be responsible for the differences found in the BBI concentrations. Jeong et al. (2007a) found that the BBI concentrations in eight Korean soybean cultivars vary from 2.87 to 19.78 mg BBI/g of seed.

The infant formulas analyzed in our study contain low percentages of soy protein compared to other ingredients (i.e. 14.7% soy protein isolate labelled by the manufacturer of soy formula SF-1). Lunasin is present in the formulas analyzed, showing values of 4.2 (SF-1), 2.8 (SF-2) and 1.5 (SF-3) mg lunasin/100 mL prepared according to the manufacturer's instructions. However, BBI could not be detected in any of the three samples. Previous studies have reported values of BBI content of 0.63 mg of BBI/100 mL in a liquid soy formula (Friedman & Brandon, 2001) and lower than 0.07 μg/mg formula powder for a dehydrated infant formula (Dipietro & Liener, 1989). Drastic processes, involving evaporation, pasteurisation and/or drying are normally applied during the production of infant formulas. Heating and long storage have been found to be responsible for the Maillard reaction, affecting proteins, amino acids and carbohydrates (Contreras-Calderón, Guerra-Hernández, & García-Villanova, 2008). This reaction could also be responsible for BBI degradation in the infant formula samples.

Table 2
Extracted protein concentrations, lunasin and BBI concentrations and ratios of lunasin:BBI in US commercial soy milks and soy-based infant formulas.

Sample no.	Extracted protein (mg/mL)	Lunasin		BBI		Ratio lunasin:BBI
		mg/g protein	mg/100 mL milk	mg/g protein	mg/100 mL milk	
SM-1	31.6 ± 3.5	4.6 ± 0.6	15.7 ± 1.3	10.5 ± 1.6	33.1 ± 4.2	1:2.1
SM-2	32.3 ± 3.9	5.8 ± 0.3	18.9 ± 2.6	8.8 ± 1.6	27.1 ± 3.4	1:1.4
SM-3	33.1 ± 0.3	4.3 ± 0.3	14.2 ± 1.1	7.4 ± 1.3	24.7 ± 4.3	1:1.7
SM-4	20.9 ± 4.0	6.4 ± 0.7	13.8 ± 2.6	21.9 ± 0.4	45.7 ± 7.2	1:3.3
SM-5	27.9 ± 1.9	5.1 ± 0.7	14.4 ± 2.4	20.6 ± 1.6	55.9 ± 5.0	1:3.9
SM-6	25.9 ± 2.2	5.7 ± 0.5	14.7 ± 0.8	15.8 ± 1.3	40.0 ± 5.5	1:2.7
SM-7	21.4 ± 0.2	6.4 ± 0.4	13.7 ± 0.9	14.1 ± 1.7	30.3 ± 3.7	1:2.2
SM-8	28.0 ± 2.3	5.0 ± 0.1	13.9 ± 1.0	9.2 ± 1.0	25.9 ± 4.2	1:1.9
SM-9	25.1 ± 1.7	7.1 ± 1.0	18.3 ± 2.4	9.5 ± 1.5	23.1 ± 3.0	1:1.3
SM-10	13.0 ± 0.5	8.3 ± 0.5	10.7 ± 0.8	5.5 ± 1.2	7.2 ± 1.5	1:0.7
SM-11	26.8 ± 1.1	4.7 ± 0.2	12.3 ± 0.8	6.8 ± 1.0	18.8 ± 2.7	1:1.5
SM-12	20.8 ± 0.2	5.6 ± 0.6	11.8 ± 1.3	n.d.	n.d.	1:0
SF-1	5.5 ± 0.7	7.3 ± 0.2	4.1 ± 0.4	n.d.	n.d.	1:0
SF-2	4.1 ± 0.5	7.0 ± 0.5	2.8 ± 0.2	n.d.	n.d.	1:0
SF-3	8.5 ± 0.5	1.7 ± 0.1	1.5 ± 0.1	n.d.	n.d.	1:0

n.d.: not detected.

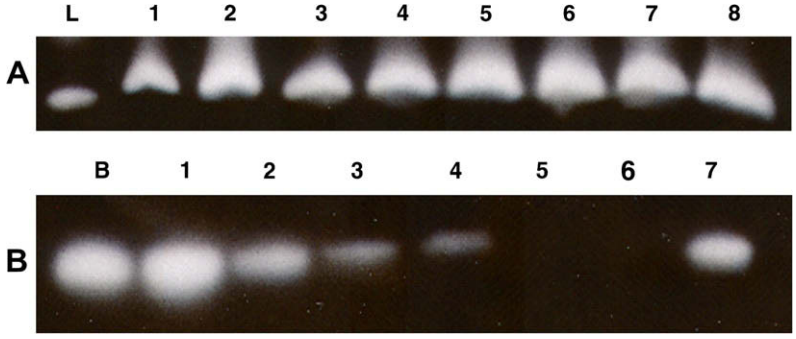

Fig. 2. (A) Western-Blot of lunasin peptide from protein extracts of commercial tofu samples. Lane L contains 208 ng of synthetic lunasin. Lane 1: tofu TF-1; Lane 2: tofu TF-2; Lane 3: tofu TF-3; Lane 4: tofu TF-4; Lane 5: tofu TF-5; Lane 6: tofu TF-6; Lane 7: tofu TF-7. Lane 8: tofu TF-8. (B) Western-Blot of BBI from commercial soy products. Lane B contains 1 μg of synthetic BBI. Lane 1: soybean cake SC-1; Lane 2: soybean cake SC-2; Lane 3: soybean cake SC-3; Lane 4: tofu TF-10; Lane 5: tofu TF-11; Lane 6: tofu TF-12; Lane 7: tofu TF-1.

3.2. Lunasin and BBI concentrations in other soy foods

Fig. 2A and B shows the Western-Blot patterns of lunasin and BBI in several commercial US soy foods. Their concentrations, expressed as mg of peptide per g of extracted protein and mg of peptide per 100 g of product, are shown in Fig. 3A–D. Lunasin is detected in all the tofu samples analyzed. Its content, expressed as mg lunasin per 100 g tofu, varies from 0.4 to 14.3 mg lunasin/100 g (Fig. 3B). No pattern can be seen in the relationship between the type of tofu and lunasin concentration. This suggests that soybean variety is likely to have a greater influence on lunasin concentration than the production process. Variety affects the quality and the physical and chemical properties of soy milk and tofu (Kim & Wicker, 2005; Min, Yu, & Martin, 2005). However, the processing of sample TF-11 (0.4 mg lunasin/100 g product), involving deep frying could be responsible for lunasin breakdown. This processing could also cause the degradation of BBI that was not detected in tofu TF-11. The BBI concentrations determined in the rest of the tofu samples analyzed ranged between 2.9 and 12.4 mg/100 g product, with the exception of tofu TF-12 (dry tofu) that did not contain this peptide. Dipietro and Liener (1989) measured BBI in two brands of firm tofu, finding values of 7 and 8 mg BBI/100 g tofu. These values are very similar to our results for the firm tofu sample (TF-8), that contains 8.2 mg of BBI/100 g product. Animal studies have indicated that dietary BBI concentrations as low as 0.01% suppress carcinogenesis. According to these results, Kennedy (1998) contended that the amount of BBI in reasonable amounts of tofu is sufficient to exert anticancer effects in humans. The lunasin:BBI ratios varied from 1:0 (TF-11 and TF-12) to 1:2.7 (TF-4) (data not shown).

Fig. 3C and D shows lunasin and BBI, expressed as mg per g of protein and mg per 100 g product, respectively, in bean curd, soybean cake, su-jae and tempeh samples. Lunasin ranges between 24.3 and 165.8 mg/g extracted protein and BBI from 4.6 to 348.1 mg/g extracted protein. Expressed in mg per 100 g product, lunasin concentrations vary from 1.1 to 10.7 mg/100 g product and BBI concentrations from 0.7 to 19.2 mg/100 g product. In the case of bean curd and soybean cake samples, both lunasin and BBI values were lower in samples that had been subjected to pressing (BC-5 and SC-3) and deep-frying (SC-1). Lunasin and BBI were absent in BC-6 (soybean noodles) and in the su-jae sample (SJ-1).

Tempeh, natto and miso are the three main fermented foods from soybeans consumed in Asian countries. Tempeh is a traditional food in Indonesia made by a natural culturing and controlled fermentation of the spores of fungus *Rhizopus oligosporus*. BBI was not found in any of tempeh samples analyzed, indicating the fermentation process caused the degradation of this protein. Handoyo and Morita (2006) have reported that this fungus rapidly degrades the different soybean proteins to amino acids and low-molecular weight peptides for its own growth. However, lunasin evidently showed more resistance to the enzymatic action of the fungus, being detected in two of four tempeh (8.2 and 6.1 mg lunasin/100 g product in TE-2 and TE-3, respectively), manufactured by the same company. Natto and miso are two traditional Japanese

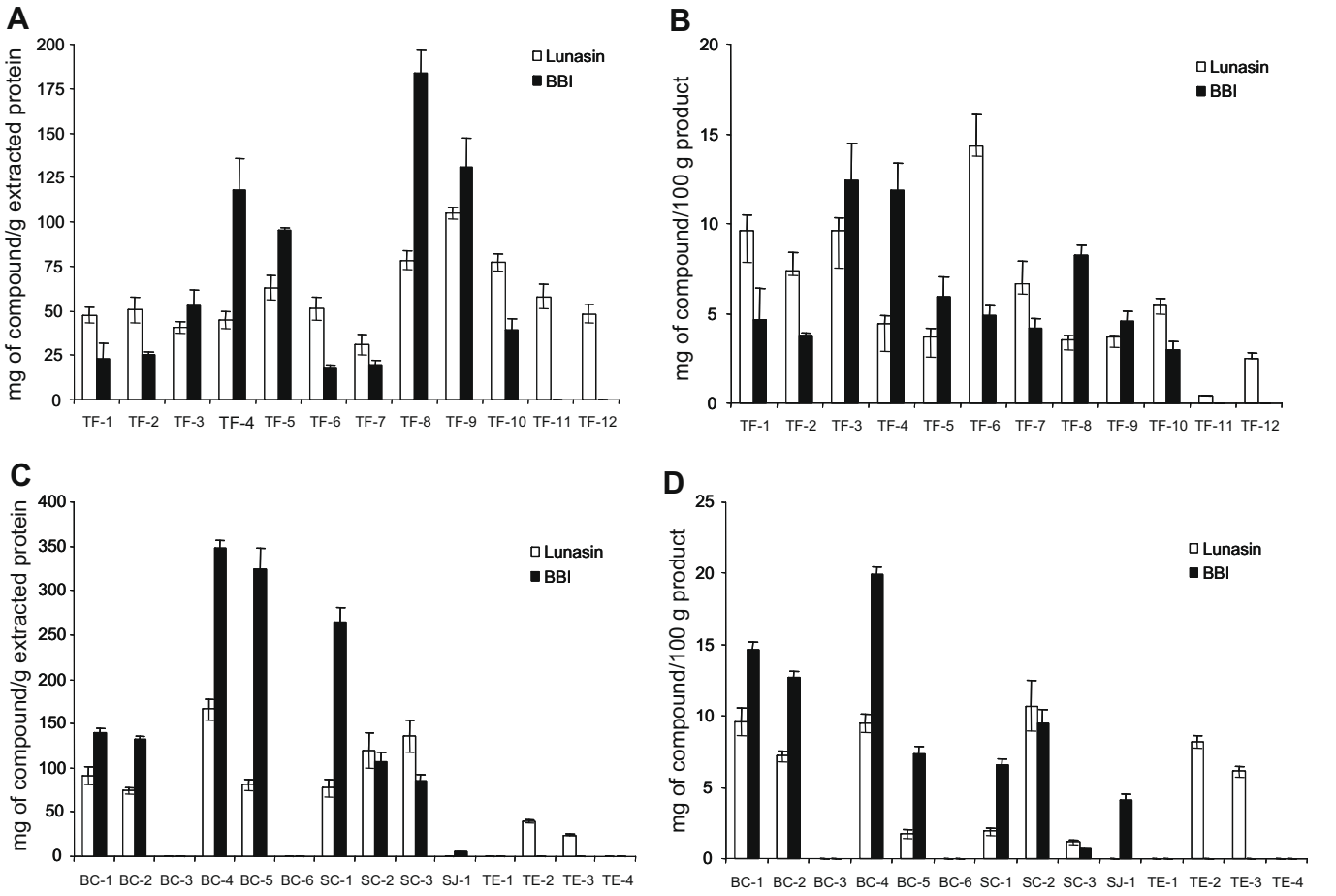

Fig. 3. Concentrations of lunasin and BBI in protein extracts of US commercial soy foods. (A and C): Concentrations expressed as mg of compound/g extracted protein. (B and D): Concentration expressed as mg of compound/100 g product. (A) and (B) correspond to tofu samples; (C) and (D) correspond to bean curd, soybean cake, su-jae and tempeh samples.

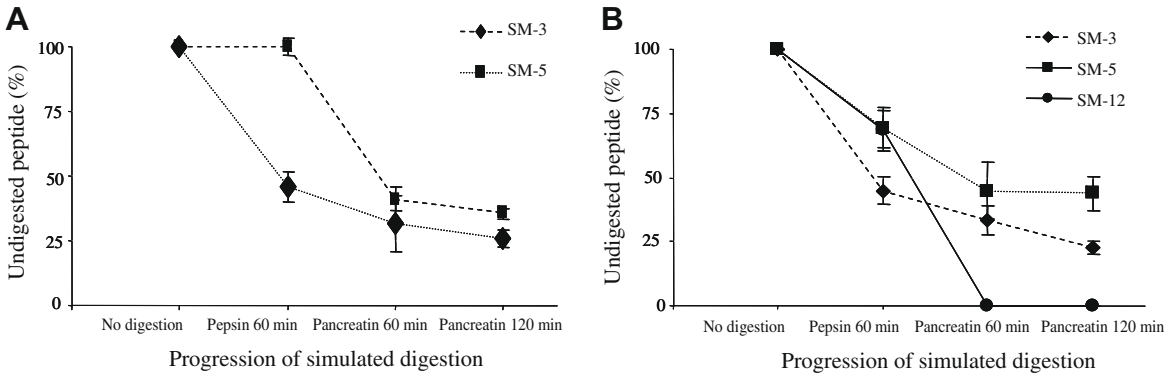

Fig. 4. Variation of (A) BBI and (B) lunasin (expressed as% relative to the original) in soy milk samples SM-3, SM-5 and SM-12 during simulated gastrointestinal digestion. Each point in the curve corresponds to an aliquot withdrawn during hydrolysis.

foods, produced by fermentation of soybeans and other ingredients with *Bacillus subtilis* and *Aspergillus oryzae*, respectively. Investigations at the protein level have revealed that the proteolytic system of these starters produce major breakdown of soybean proteins into low molecular weight peptides (Kiers, Van laeken, Rombouts, & Nout, 2000). Since lunasin and BBI could not be detected in the natto and miso samples, it can be assumed that these two peptides were degraded during the fermentation process.

3.3. Simulation of gastrointestinal digestion of soy milk

The ratios of lunasin to BBI in soy milk based on 100 mL of milk are shown in the last column of Table 2. With the exception of SM-12 that did not contain BBI, these ratios vary from 1:0.7 to 1:3.9. The ratio has been found to be relevant considering that lunasin is evidently protected from the digestion by naturally occurring soy protease inhibitors such as BBI and Kunitz trypsin inhibitor (Park, Jeong, & de Lumen, 2007). Jeong, Jeong, Kim (2007a) carried out the digestion of soybeans with lunasin:BBI ratios in the range between 1:2.9 and 1:12, demonstrating the capacity of BBI to protect lunasin in all of them. In order to evaluate the bioavailability of these two peptides after oral ingestion of soy milk, three soy milk samples (SM-3, SM-5 and SM-12) were chosen and subjected to a two-stage hydrolysis process that simulated gastrointestinal digestion. These samples were chosen based on their lunasin:BBI ratio, 1:1.7 for SM-3, 1:3.9 for SM-5 and 1:0 for SM-12. Fig. 4A and B shows soy BBI and lunasin contents, respectively, after incubation at various times with pepsin and pancreatin. The quantities of both peptides were quantified as percentage of the original material. Similar hydrolysis patterns could be observed for BBI and lunasin in soy milk SM-3. After incubation with pepsin for 60 min, 45.9% of the original BBI and 44.9% of original lunasin remained undigested. A slight increase of extent of hydrolysis was observed during incubation with pancreatin, reaching values at the end of the reaction of 26% and 22.6% of the original BBI and lunasin content, respectively (Fig. 4A and B). These results suggest that a significant degree of digestion of these peptides occurs in the stomach and to a lesser degree in the small intestine when soy milk was ingested. Similar results were found when soy protein was hydrolysed by simulated intestinal and gastric fluids (Park et al., 2007).

In the case of soy milk SM-5 that contained a higher BBI content (55.94 mg/100 mL milk) and a lunasin:BBI ratio of 1:3.9, BBI remained undigested and only 31% of lunasin was hydrolysed by pepsin. Pancreatin hydrolyses both peptides reaching final values of 35.5% of the original BBI and 43.8% of the original lunasin. These data confirm that BBI protect lunasin from the digestion (Jeong, Jeong et al. 2007b; Park et al., 2007). Further confirmation of this protective role of BBI was observed when soy milk SM-12 was subjected to the hydrolysis process. Absence of BBI in this soy milk allows pancreatin to hydrolyse lunasin that disappears totally during the first minutes of incubation (Fig. 4B). It is expected that the remaining undigested lunasin and BBI reach the tissues and organs in an intact and active form (Jeong et al., 2007a).

In summary, this paper reports for the first time comprehensive information on the concentrations of lunasin and BBI in a large number of commercially available soy foods in the US. Concentrations of these two peptides in the products seem to be determined by the soybean variety and the process used during manufacturing, indicating that these two parameters can be used to control lunasin and BBI contents. We also confirmed in soy milk the important role played by BBI in protecting lunasin from gastrointestinal digestion. Lunasin and BBI have been demonstrated previously to possess cancer chemopreventive properties in *in vitro* and *in vivo* models. Their presence in traditional soybean foods may contribute to the known beneficial properties of cancer prevention attributed to these foods.

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