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Kunitz trypsin inhibitor in addition to Bowman-Birk inhibitor influence stability of lunasin against pepsin-pancreatin hydrolysis

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ABSTRACT

Soybean contains several biologically active components and one of this belongs to the bioactive peptide group. The objectives of this study were to produce different lunasin-enriched preparations (LEP) and determine the effect of Bowman-Birk inhibitor (BBI) and Kunitz trypsin inhibitor (KTI) concentrations on the stability of lunasin against pepsin-pancreatin hydrolysis (PPH). In addition, the effect of KTI mutation on lunasin stability against PPH was determined. LEP were produced by calcium and pH precipitation methods of 30% aqueous ethanol extract from defatted soybean flour. LEP, lunasin-enriched commercially available products and KTI control and mutant flours underwent PPH and samples were taken after pepsin and pepsin-pancreatin hydrolysis. The concentrations of BBI, KTI, and lunasin all decreased after hydrolysis, but they had varying results. BBI concentration ranged from 167.5 to 655.8 μg/g pre-hydrolysis and 171.5 to 250.1 μg/g after hydrolysis. KTI concentrations ranged from 0.3 to 122.3 μg/g pre-hydrolysis and 9.0 to 18.7 μg/g after hydrolysis. Lunasin concentrations ranged from 8.5 to 71.0 μg/g pre-hydrolysis and 4.0 to 13.2 μg/g after hydrolysis. In all products tested, lunasin concentration after PPH significantly correlated with BBI and KTI concentrations. Mutation in two KTI isoforms led to a lower concentration of lunasin after PPH. This is the first report on the potential role of KTI in lunasin stability against PPH and must be considered in designing lunasin-enriched products that could potentially survive digestion after oral ingestion.

1. Introduction

Soybean is the most widely used legume throughout the world being used as a source of food, oil and petroleum replacement in plastics and other items (Singh, Kumar, Sabapathy, & Bawa, 2008). It also contains biologically active components with reported health benefits including isoflavones (Messina, 2014), saponins (MacDonald et al., 2005) and biologically active peptides (Wang & Gonzalez De Mejia, 2005). Biologically active peptides in soybean include the naturally occurring Bowman-Birk inhibitor (BBI), Kunitz trypsin inhibitor (KTI) and lunasin and peptides that are products of enzymatic hydrolysis and fermentation. Lunasin belongs to the 2S soy albumin protein with 43 amino acid residues whose biological activity is attributed to the presence of a cell adhesion motif composed of arginine-glycine-aspartic acid residues and a carboxylic acid tail composed of 8 aspartic acid residues (Galvez & De Lumen, 1999; Lule, Garg, Pophaly, Hitesh, & Tomar, 2015). Lunasin possessed different potential biological properties including anti-cancer (Shidal, Al-Rayyan, Yaddanapudi, & Davis, 2016; Jiang et al., 2016; Hsieh, Hernández-Ledesma, Jeong, Park, & de Lumen, 2010) anti-inflammatory (Dia, Wang, Oh, de Lumen & de Mejia, 2009; Cam & de Mejia, 2012; Cruz-Huerta et al., 2015; Hernández-Ledesma, Hsieh, & de Lumen, 2009) and immunomodulating properties (Yang et al., 2015; Tung et al., 2014). One potential issue on the use of lunasin as a chemopreventive and chemotherapeutic agents is its susceptibility to digestion as previous studies have shown that up to 97% of the lunasin is digested leading to low nanomolar concentrations found in human plasma after ingesting 50 g of soy protein (Dia, Torres, de Lumen, Erdman & de Mejia, 2009). One potential strategy of decreasing the digestion of intact lunasin is through the action of protease inhibitors such as BBI and KTI that are naturally present in soybean.

BBI is a protease inhibitor that belongs to cystine-rich group characterized by low molecular weight (8 kDa for BBI) and large amounts of disulfide bonds (7 for BBI) (Odani & Ikenaka, 1973). BBI protects protein digestion by inhibiting the activity of trypsin and chymotrypsin (Odani & Ikenaka, 1973). Previous studies have shown the capability of BBI to protect lunasin from hydrolysis brought up by pepsin and pancreatin (Cruz-Huerta et al., 2015; Hsieh et al., 2010). In addition, BBI has possible health benefits such as being a chemopreventative (Clemente, Marín-manzano, Arques, & Domoney, 2013) and anticarcinogenic agent (Kennedy, 1998). KTI is another protease inhibitor that is larger than BBI with molecular weight of approximately 20 kDa and contains less disulfide bridges. KTI also demonstrated different biological

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properties including anticancer, anti-HIV1 reverse transcriptase and immunoregulating properties (Fang, Wong & Ng, 2010; Inagaki et al., 2005). To date, no one has studied the role of KTI on the stability of lunasin against pepsin-pancreatin hydrolysis (PPH).

The objectives of this study were to produce different lunasin-enriched preparations (LEP) and determine the effect of protease inhibitors, Bowman-Birk inhibitor and Kunitz trypsin inhibitor, concentrations on the stability of lunasin against PPH.

2. Materials and methods

2.1. Materials

Prolia defatted soy flour was purchased from Amazon and produced by Cargill Mills (Minneapolis, MN). The commercially-available products (C-LEP) (LunaRichX, designated as L and Now, designated as N) were from Reliv (Chesterfield, MO). Control and KTI mutant soy flours were obtained as previously reported (Gillman, Kim, & Krishnan, 2015). Lunasin polyclonal antibody was raised in rabbit using the 15 amino acid corresponding to the C-terminus of lunasin (ProteinTech Group, Chicago IL), synthetic lunasin standard was synthesized by LifeTein LLC (New Jersey, USA), BBI standard was purchased from Sigma-Aldrich (St. Louis, MO) and KTI standard was purchased from VWR International (Atlanta, GA). All chemicals were purchased from ThermoFisher Scientific and VWR International unless otherwise specified.

2.2. Preparation of lunasin-enriched samples and pepsin-pancreatin hydrolysis

Lunasin-enriched samples were prepared following the procedure described earlier (Krishnan & Wang, 2015) with slight modifications. Briefly, 100 g defatted soy flour were mixed with 1-L 30% ethanol solution for 2 h at 20 to 22 °C. After centrifugation (8000 rpm, 4 °C) for 30 min, the supernatant was collected and calcium precipitation was accomplished by adding $CaCl_2$ to a final concentration of 10 mM and stirred for 10 min. After centrifugation as above the precipitate was collected and dissolved in 1 volume distilled water and divided into four parts. One part was called Ca LEP and the remaining 3 parts were split into three groups and their pH adjusted to 3 (pH 3 LEP), 4 (pH 4 LEP) and 5 (pH 5 LEP). After pH adjustment, samples were centrifuged as above and the precipitate was collected, resuspended in 10 mL of Tris-buffered saline (TBS) and the pH was readjusted to 7.5. The samples were dialyzed in a membrane with 3.5 kDa molecular weight cut-off (Spectrum Labs, Rancho Dominguez, CA) frozen and lyophilized. Samples were kept at −20 °C until analysis. PPH was performed as previously reported (Cruz-Huerta et al., 2015) with some modifications. Briefly, 30 mg of lyophilized sample was suspended in 10-mL water and pH adjusted to 2.0 and pepsin was added at 1:20 enzyme:LEP ratio. Pepsin hydrolysis was carried out for 1 h at 37 °C in a shaking water bath after which 5-mL of the mixture was taken, heated at 75 °C for 20 min to inactivate pepsin and designated as pepsin digests. The pH of the remaining mixture was adjusted to 7.5 to inactivate pepsin; and pancreatin and bile extract were added at 1:20 enzyme:LEP ratio and 1:40 bile extract:LEP ratio, respectively. The pancreatin hydrolysis was carried out for 1 h at 37 °C in a shaking water bath and pancreatin was inactivated by heating at 75 °C for 20 min and called pepsin-pancreatin digests. Digests were centrifuged at 20,000 × *g* for 30 min, supernatants were dialyzed, freeze-dried and stored at −20 °C until used.

2.3. Soluble protein concentration by Bradford assay

Ten milligrams of samples were extracted with 1-mL TBS by vortexing for 90 min at 20 to 22 °C, followed by centrifugation (20,000 × *g*, 4 °C) for 30 min and the supernatant was used for the analysis based on Bradford (1976) principle. One hundred microliters of diluted supernatant (1:50) and different concentrations of bovine serum albumin standards from 0 to 20 μg/mL were plated in 96-well plate and 100 μL of Quick Start™ Bradford dye reagent (Bio-Rad Laboratories, Hercules, CA) were added. After 5 min of incubation at 20 to 22 °C, the absorbance was read at 630 nm and total soluble proteins were calculated using the generated BSA standard curve equation.

2.4. Protein profile by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE under reducing condition was carried out by loading approximately 20 μg of protein in 4–20% Mini-Protean TGX gels (Bio-Rad Laboratories, Hercules, CA) run at 100 V for 100 min. Gels were stained with approximately 50 mL Bio-safe Coomassie Stain (Bio-Rad Laboratories, Hercules Inc.) overnight at 4 °C. Destaining was done by washing with water 3 times for 10 min each or until the background dye was removed.

2.5. Western blot analysis

After the SDS-PAGE was run, gels were equilibrated in blotting buffer (20% methanol in SDS-PAGE running buffer) for 15 min. Proteins were transferred into Amersham™Hybond™ 0.45 μm PVDF membrane (GE Healthcare, Piscataway NJ) at 110 V for 60 min at 4 °C. After the transfer, the membrane was blocked with 5% non-fat dry milk in TBST for 60 min at 20 to 22 °C. After washing with TBST three times for 10 min each, the membrane was incubated in primary antibody against lunasin, BBI (Gillman et al., 2015) and KTI (VWR International, Atlanta GA) at 1:2000 dilution overnight at 4 °C. After washing, membrane was incubated with anti-rabbit secondary antibody (ThermoFisher Scientific) at 1:2000 dilution for 2 h at 20 to 22 °C. After washing, blots were imaged by chemiluminescence using C-Digit blot scanner (Li-Cor Biosciences, Lincoln, NE).

2.6. Enzyme-linked immunosorbant assay (ELISA) for lunasin, BBI and KTI concentrations

One hundred microliters of diluted samples (1:2500), lunasin standard, BBI standard and KTI standard were plated in triplicate on immuno-96 well plate (BrandTech) and incubated for at least 14 h at 4 °C. Afterwhich, the plate was washed three times with 300 μL washing buffer (1 L PBS + 5 mL Tween 20, PBST) per well and blocked with 300 μL of 1% sodium caseinate in PBST for 1 h at 20 to 22 °C. After washing, 50 μL of the primary antibody was added to each well and incubated for 1 h at 20 to 22 °C. After incubation and washing, 50 μL of secondary antibody solution was added and incubated for 2 h at 20 to 22 °C. The secondary antibody solution was removed and the plate was washed and 100 μL of 3,3′,5,5′-tetramethylbenzidine solution was added to each well and incubated for 30 min at 20 to 22 °C in the dark. After 30 min, the reaction was stopped by adding 100 μL of 2 M H_2SO_4, and the absorbance was read at 450 nm. Lunasin, BBI and KTI concentrations were calculated using their respective standard curve.

2.7. Trypsin inhibition assay

Fifty microliters of sample (200 μg soluble protein/mL), BBI (200 μg/mL) as positive control or assay buffer (Tris buffer, pH 8.2) as blank were plated in 96-well plate followed by 50 μL trypsin working solution (160 μg/mL) and incubated for 10 min at 20 to 22 °C. After 10 min, 50 μL of *N*-α-benzoyl-D,L-arginine 4-nitroanilide trypsin substrate (0.8 mg/mL) was added and incubated for 5 min at 20 to 22 °C. The absorbance was read at 405 nm. Trypsin inhibition was calculated using the following formula: (Positive control average − sample absorbance) / (200 * 0.05) * 1000.