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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.

2.8. Size-exclusion chromatography

The molecular weight profiles of the samples were analyzed by size exclusion chromatography on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler (G1329A), a quaternary pump (G1311A), a vacuum degasser (G1322A), a temperature controlled column (G1316A) and a diode array detector (G1315D). The separation was performed on a BioSep-SEC-S2000 column (300 × 7.80 mm, Torrance, CA). The mobile phase was 45% acetonitrile in water with 0.1% trifluoroacetic acid at flow rate of 1.0 mL/min. The injection volume was 20 μL with 20 min run time. The detector was set at 214 nm and the analysis was performed at ambient temperature.

2.9. Antioxidant capacity

2.9.1. Oxygen radical antioxidant capacity (ORAC) assay

ORAC assay was done as previously described (Mojica, Meyer, Berhow, & de Mejia, 2015) briefly 20 μL of sample, standard (240 to 4 μM Trolox), and blank were added to 96-well plate in triplicate followed by 120 μL of fluorescein (70 nM final concentration) and incubated at 37 °C in the dark for 15 min. Afterwhich, 60 μL of 2,2′-azobis-(2-methylpropionamidine) (12 mM) was added to each well and the plate was read at excitation of 485 nm and emission of 582 nm with a sensitivity of 60 every two minutes for 120 min. ORAC values were calculated from Trolox standard curve and reported as μM Trolox equivalent (TE).

2.9.2. Nitric oxide radical scavenging assay

Nitric oxide scavenging assay was performed as previously described (Mojica et al., 2015), briefly 50 μL of samples and control were added in triplicate to two 96 well plates, and 50 μL of water was added to each well. One plate received 25 μL of 100 mM sodium nitroprusside and the other received 25 μL of water, and both plates were incubated for 2 h at 20 to 22 °C in the dark. 100 μL of Griess reagent (1:1 1% sulfanillic acid in 5% phosphoric acid, 0.1% *N*-(1-napthyl)-ethylenediamine dihydrochloride) was added to each well and incubated for 15 min at 20 to 22 °C in the dark. Absorbance was read at 550 nm.

2.9.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging was performed as previously described (Dia, Pangloli, Jones, McClure, & Patel, 2016), briefly 100 μL of samples and control were plated in triplicate in a 96 well plate and 100 μL of DPPH solution (2 mg DPPH dissolved in 50 mL methanol) was added and incubated at 20 to 22 °C in the dark for 30 min. The absorbance was read at 517 nm.

2.10. KTI mutant flour

The preparation of the KTI mutant flours was done by adding 30 mg of flour per 10 mL of water and stirred for 90 min at 20 to 22 °C (unhydrolyzed). The hydrolyzed samples contained 30 mg of flour per 10 mL of water and the hydrolysis process was performed as described earlier. After the preparation of pre and post-hydrolysis samples, all samples underwent soluble protein concentration, SDS-PAGE, ELISA, trypsin inhibition, size exclusion chromatography and ORAC assays as previously described.

2.11. Statistical analysis

All experiments were performed in at least three independent replicates. Data were analyzed using PROC GLM procedure of SAS Version 9.4 and means were separated using Tukey posthoc test and significance was reported at $P < 0.05$.

3. Results

3.1. Protein concentration and protein profile of lunasin-enriched samples

Fig. 1a shows that C-LEP (N, L) have less soluble protein concentration (43.7 mg/g for N) than LEP (108.4 mg/g for pH 4). LEP at pH 5 contained more soluble proteins (58.4 mg/g) after hydrolysis compared to C-LEP (23.6 mg/g). Fig. 1b shows the protein profile of samples before and after hydrolysis; the large molecular weight proteins were hydrolyzed indicated by disappearance of the higher molecular weight bands after PPH. Fig. 1c shows western blot bands for BBI, KTI, and lunasin indicating the presence of these proteins in the samples and subsequent reduction of signal after PPH.

3.2. Concentrations of protease inhibitors, BBI and KTI, are higher in LEP than C-LEP

Fig. 2a shows the concentration of BBI present in lunasin-enriched products before and after hydrolysis. N sample did not contain any BBI while the other samples have BBI concentrations ranging from 167.5 to 655.8 μg/g prior to PPH. In general, PPH led to reduction in BBI concentrations in all products. Fig. 2b shows the concentration of KTI present in lunasin-enriched products before and after PPH. KTI concentrations ranged from 0.32 to 122.3 μg/g before PPH and was generally reduced after PPH. Fig. 2c shows the capability of different products to inhibit the activity of trypsin. N sample did not inhibit the activity of trypsin while Ca sample exhibited the highest trypsin inhibition by 50.6 AU/mg protein before PPH. After PPH, the capability of these samples to inhibit trypsin activity was reduced with Ca sample inhibition at 12.0 AU/mg protein.

3.3. Lunasin concentration after hydrolysis correlates with BBI and KTI concentrations in different lunasin-enriched products

Fig. 3a shows the concentration of lunasin present in lunasin-enriched products before and after hydrolysis. In general, PPH led to the reduction of lunasin concentrations. Prior to PPH, lunasin concentrations ranged from 8.5 (N) to 71.0 μg/g (pH 3) and from 4.0 (L) to 13.2 μg/g (pH 3) after hydrolysis. Fig. 3b shows a positive correlation of BBI and KTI concentrations before hydrolysis and the percentage residual lunasin concentrations after hydrolysis. Fig. 3c shows the Pearson correlation indicating that the correlations between lunasin stability against PPH and BBI and KTI concentrations pre-hydrolysis are highly significant ($P < 0.0001$).

3.4. Molecular weight profile of lunasin-enriched products as determined by size exclusion chromatography

Fig. 4a shows the chromatogram of pH 3 precipitated lunasin-enriched product (non-hydrolyzed, pepsin digest, pepsin-pancreatin digest, top to bottom) and Fig. 4b shows table inset of all lunasin-enriched products in the various stages of hydrolysis. As indicated, pepsin and pepsin-pancreatin hydrolysis led to a reduction in higher molecular weight molecules which is in agreement with the protein profile as shown by the SDS-PAGE gels in Fig. 1b. For instance, pH 3 sample molecular weight distribution before PPH was 10.6% < 1 kda, 3.5% 1–5 kda, 6.0% 5–10 kda, 79.8% > 10 kda. After PPH, this distribution was changed to 29.3% < 1 kda, 18.9% 1–5 kda, 15.6% 5–10 kda, 36.1% > 10 kda.

3.5. Antioxidant capacity of lunasin-enriched products

Fig. 5a shows the ORAC values for LEP and C-LEP with values ranging from 79.41 to 176.92 μM TE. ORAC values indicated similar capability of samples to scavenge oxygen radicals with only L significantly lower than the rest of the samples. Fig. 5b and c show antioxidant scavenging potential by Nitric Oxide and DPPH, respectively. In general, lunasin-

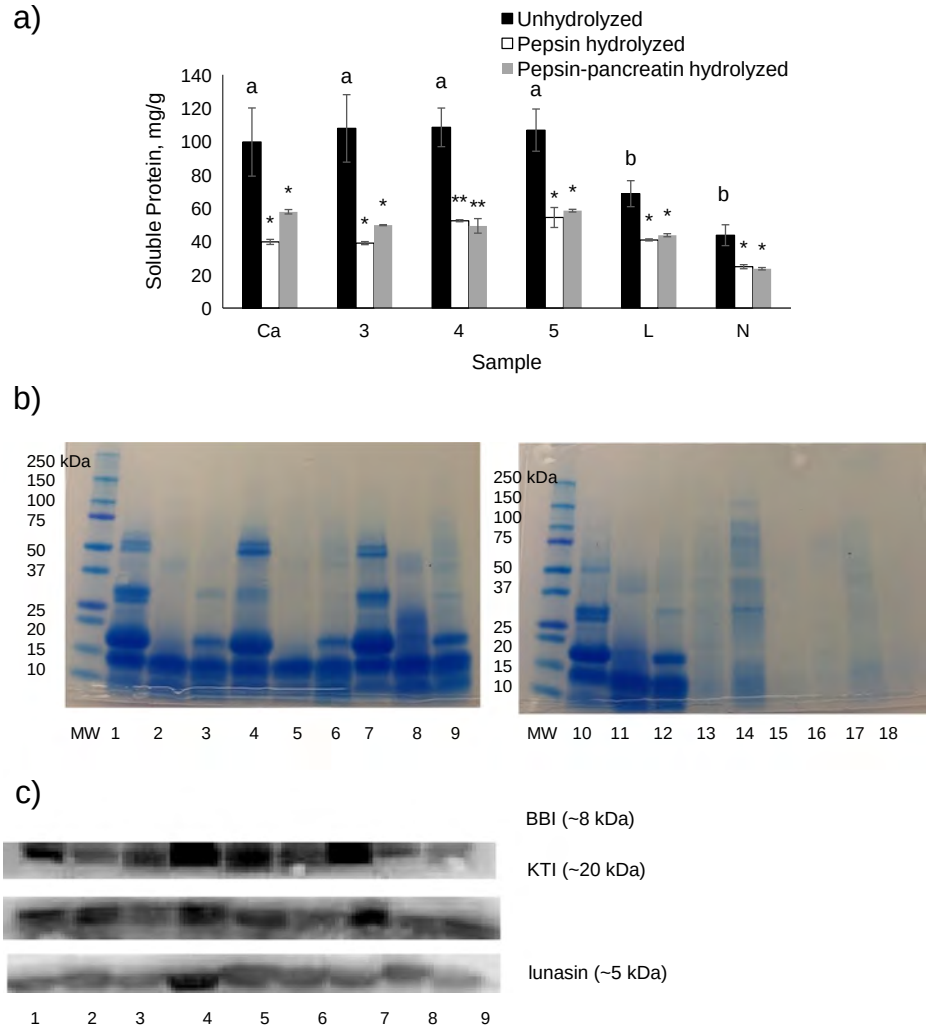


Fig. 1. A) Commercially available products have less soluble protein concentration than laboratory-prepared lunasin-enriched products. Bars with different letter are significantly different from each other ($P < 0.05$, $n = 3$). Pepsin-pancreatin hydrolysis led to a significant reduction in the protein concentration of lunasin-enriched preparation as designated by *($P < 0.05$) and **($P < 0.001$). B) Protein profile of lunasin-enriched preparations as affected by pepsin and pepsin-pancreatin digestion. Lanes 1 (Ca ppt), 2 (Ca-pepsin hydrolyzed), 3 (Ca-pepsin-pancreatin hydrolyzed), 4, (pH 3 ppt), 5 (pH 3 ppt-pepsin hydrolyzed), 6 (pH 3 ppt-pepsin-pancreatin hydrolyzed), 7 (pH 4 ppt), 8 (pH 4 ppt-pepsin hydrolyzed), 9 (pH 4 ppt-pepsin-pancreatin hydrolyzed) Lanes 10 (pH 5 ppt), 11 (pH 5-pepsin hydrolyzed), 12 (pH 5-pepsin-pancreatin hydrolyzed), 13 (LunarichX, L), 14 (LunarichX, L-pepsin hydrolyzed), 115 (LunarichX, L-pepsin-pancreatin hydrolyzed), 16 (Now, N), 17 (Now, *N*-pepsin hydrolyzed), 18 (Now, *N*-pepsin-pancreatin hydrolyzed) and MW: molecular weight marker. C) Western blot profile showing the presence of BBI, KTI and lunasin in different lunasin preparations and commercially available products as affected by pepsin-pancreatin digestion. Lanes 1 (Ca ppt), 2 (Ca-pepsin hydrolyzed), 3 (Ca-pepsin-pancreatin hydrolyzed), 4, (pH 3 ppt), 5 (pH 3 ppt-pepsin hydrolyzed), 6 (pH 3 ppt-pepsin-pancreatin hydrolyzed), 7 (pH 4 ppt), 8 (pH 4 ppt-pepsin hydrolyzed), 9 (pH 4 ppt-pepsin-pancreatin hydrolyzed).

enriched products were not capable of scavenging both NO and DPPH radicals.

3.6. Effect of KTI mutations on the concentrations of bioactive peptides in soybean and their stability against pepsin-pancreatin digestion

Fig. 6a shows the protein profile of the control, KTI-1 mutant, and KTI-1,3 mutant soybean flour before and after PPH. Large molecular weight bands disappeared after PPH. Samples had protein concentrations ranging from 47.2 (KTI-1,3) to 89.7 mg/g (KTI-1) prior to PPH and from 16.5 (KTI-1,3) to 21.2 mg/g (KTI-1) after hydrolysis (Fig. 6b) indicating reduction of protein concentrations after PPH. Fig. 6c shows BBI concentrations of samples ranging from 46.4 (KTI-1,3) to 83.6 μg/g (KTI-1) prior to PPH and from 6.2 (KTI-1,3) to 7.2 μg/g (KTI-1) after PPH and Fig. 6d shows the reduction of KTI in KTI mutant flours after hydrolysis with KTI concentrations ranged from 34.4 (KTI-1,3) to 52.9 μg/g (Control) before PPH and from 5.4 (KTI-1) to 8.4 μg/g (Control) after PPH. Control, KTI-1, and KTI-1,3 flour contained lunasin 32.5, 35.1, and 32.7 μg/g prior to hydrolysis and reduced to 4.0, 3.3 and 2.8 μg/g, respectively, after hydrolysis (Fig. 6e). Fig. 6f shows trypsin inhibitory capability of the samples ranging from 59.1 AU/mg protein (KTI-1) to 64.9 (KTI-1,3) pre-hydrolysis. ORAC values ranged from 193.2 μM TE (KTI-1) to 237.0 μM TE (control) as shown in Fig. 6g. After PPH, ORAC values ranged from 145.4 μM TE for control to 147.3 μM TE for both of the KTI mutant flours. Fig. 7 shows the representative chromatograms of KTI-1 mutant flour (unhydrolyzed, pepsin-hydrolyzed and pepsin-pancreatin-hydrolyzed; from top to bottom) and the molecular weight distribution for all three sample before and after PPH. PPH led to reduction in high MW molecules in all samples.

4. Discussion

Soy is one of the most abundantly produced crops in the U.S., with 3.93B barrels being produced stateside (USDA National Agricultural Statistics Service, 2016). Previous studies have shown the effects of mutations on the KTI gene within soy (Gillman et al., 2015) and the effects of the Bowman-Birk Inhibitor on the stability of lunasin (Cruz-Huerta et al., 2015), but to our knowledge our study is the first to demonstrate the

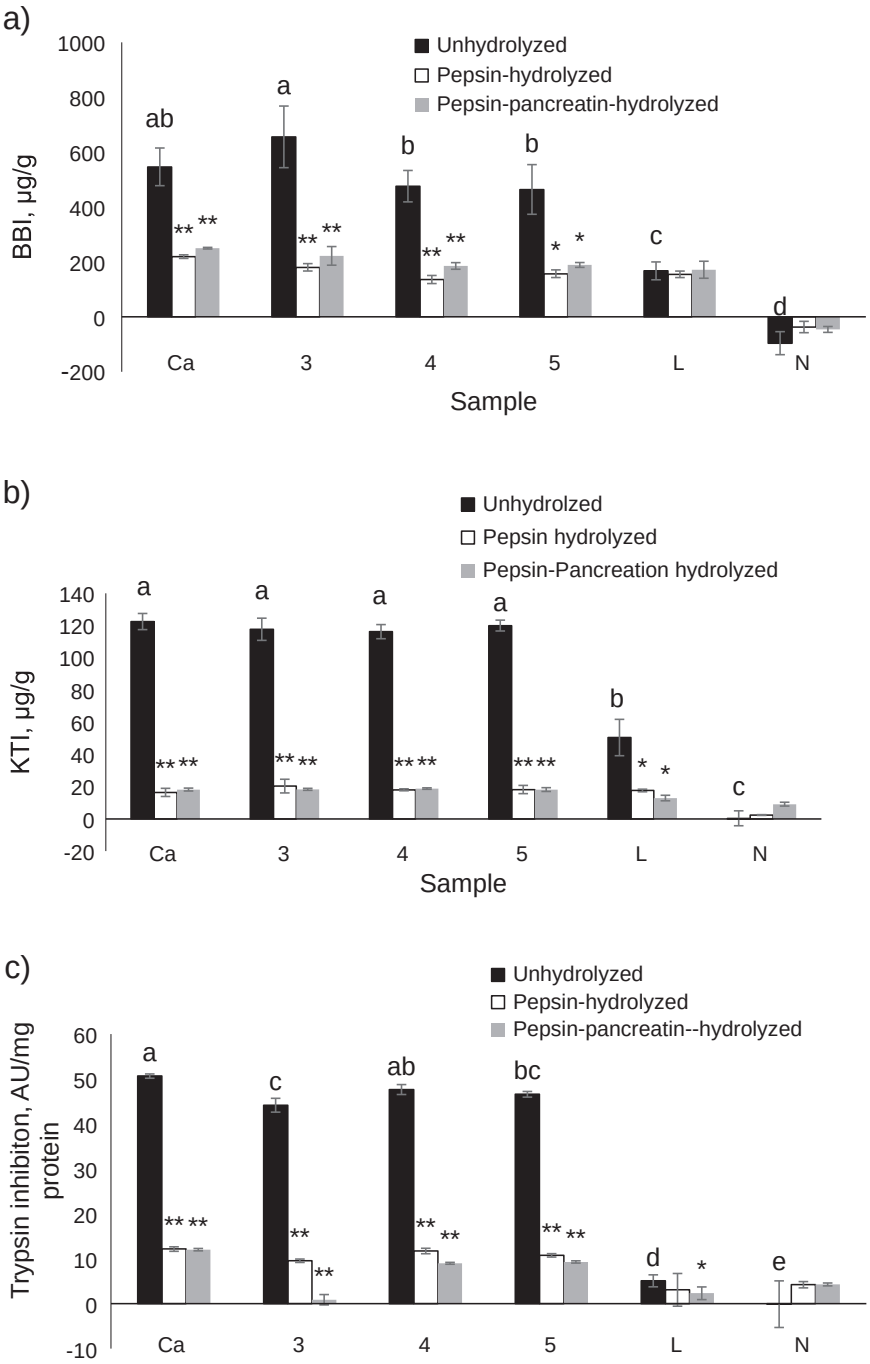
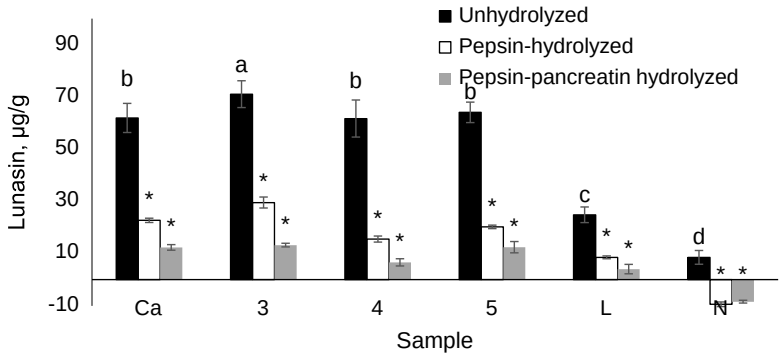


Fig. 2. Pepsin-pancreatin hydrolysis affected the concentrations of protease inhibitors BBI and KTI as well as trypsin inhibitory activity of different lunasin preparations and commercially available products. A) Commercially available products have less BBI concentrations than laboratory-prepared lunasin-enriched products. Bars with different letter(s) are significantly different from each other ($P < 0.05$, $n = 3$). BBI was not detected in N product. Pepsin-pancreatin hydrolysis led to a significant reduction in the BBI concentrations of lunasin-enriched preparation as designated by *($P < 0.001$) and **($P < 0.0001$) except for L product. B) Commercially available products have less KTI concentrations than laboratory-prepared lunasin-enriched products. Bars with different letter(s) are significantly different from each other ($P < 0.05$, $n = 3$). KTI was not detected in N product. Pepsin-pancreatin hydrolysis led to a significant reduction in the KTI concentrations of lunasin-enriched preparation as designated by *($P < 0.001$) and **($P < 0.0001$). C) Commercially available products have less trypsin inhibitory capacity than laboratory-prepared lunasin-enriched products. Bars with different letter(s) are significantly different from each other ($P < 0.05$, $n = 3$). Pepsin-pancreatin hydrolysis led to a significant reduction in the trypsin inhibitory capacity of lunasin-enriched preparation as designated by *($P < 0.05$) and **($P < 0.0001$) except for commercially available N product.

effect of Kunitz trypsin inhibitor and Bowman-Birk inhibitor on the stability of the bioactive peptide lunasin. Lunasin has been shown to have multiple health benefits, hence ways of protecting it from the action of digestive enzymes is one way to increase the potential biological effects of lunasin in the human body. It has been shown in previous studies that lunasin can be found in the plasma after eating simple soy products (Dia, Torres, de Lumen, Erdman & de Mejia, 2009), not LEP, indicating that consumption of LEP with optimum level of protease inhibitor could lead to even higher bioavailability of this bioactive peptide. In this study, we used pH precipitation method to potentially concentrate lunasin in a previously reported lunasin-enriched preparation using calcium precipitation (Krishnan & Wang, 2015). The calcium-precipitated sample was adjusted to pH 3, 4 and 5 owing to the isoelectric point of soy proteins (~4.5 pH) causing different proteins and different

a)



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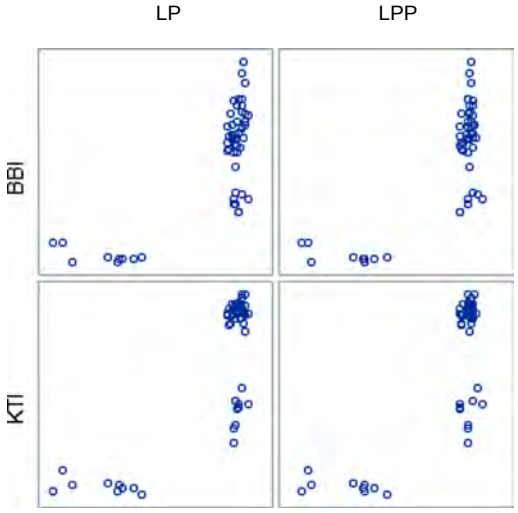


c)

Pearson correlation coefficient, r, between protease inhibitor concentrations and residual (%) lunasin concentrations after pepsin and pepsin-pancreatin digestion.

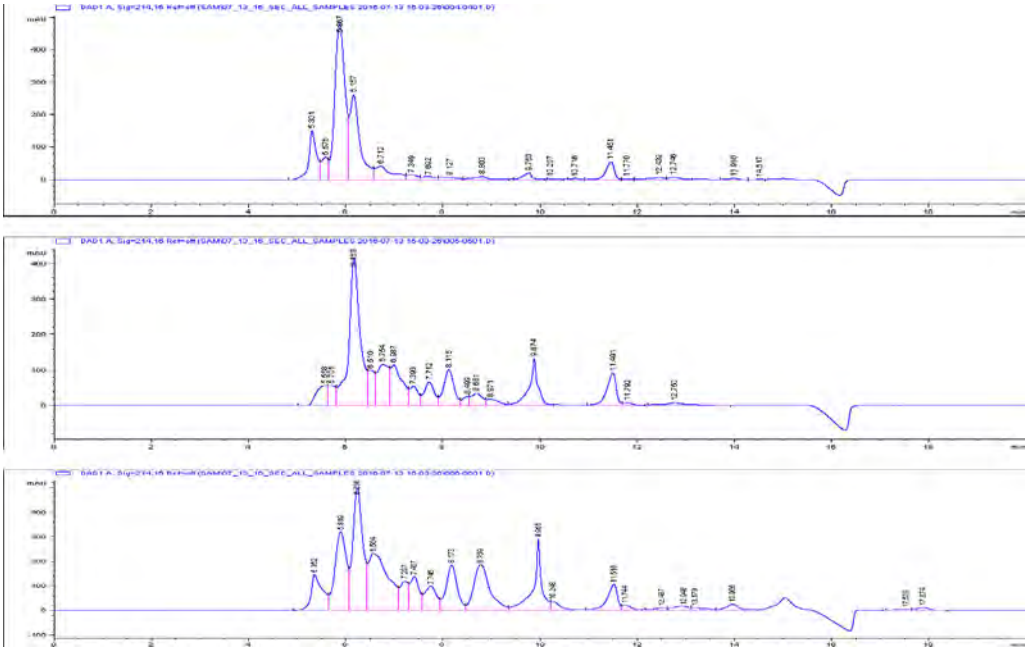
	Parameter	
Parameter	LP	LPP
BBI	0.77 (P < 0.0001)	0.76 (P < 0.0001)
KTI	0.80 (P < 0.0001)	0.80 (P < 0.0001)

BBI: Bowman-Birk inhibitor concentration in unhydrolyzed samples; KTI: Kunitz trypsin inhibitor concentration in unhydrolyzed samples; LP: lunasin concentration after pepsin digestion; LPP: lunasin concentration after pepsin-pancreatin digestion

Fig. 3. Lunasin stability against pepsin-pancreatin digestion is affected by the concentrations of protease inhibitors BBI and KTI in different lunasin-enriched preparations and commercially available products. A) Commercially available products have less lunasin concentrations than laboratory-prepared lunasin-enriched products. Bars with different letter(s) are significantly different from each other ($P < 0.05$, $n = 3$). Pepsin-pancreatin hydrolysis led to significant reduction in the lunasin concentrations of lunasin-enriched preparation as designated by *($P < 0.0001$). B) Concentrations of protease inhibitors BBI and KTI positively correlated with the percentage residual lunasin concentrations after pepsin and pepsin-pancreatin digestions indicating that BBI and KTI have protective effects on lunasin against enzymatic digestion. C) An inset table shows the correlation coefficient and *P*-values for lunasin percentage residual concentrations after pepsin and pepsin-pancreatin digestion as affected by BBI and KTI concentrations.

concentrations of proteins to precipitate (Gennadios, Brandenburg, Weller, & Testin, 1993). Though there is an increase in lunasin concentration in pH 3 sample, the protein profile of the three pH precipitated samples did not differ with the protein profile of the Ca precipitated sample indicating that there is no increase in lunasin purity. We then compared the concentrations of lunasin, BBI and KTI of our LEP with C-LEP, LunarichX and Now. Results showed that our LEP have higher concentrations of these bioactive peptides than C-LEP. Notably, the concentrations of BBI and KTI in C-LEP are very low indicating the potential effect of processing methods used in production of C-LEP. Previous studies have shown that processing could lead to reduction in the concentrations of BBI and KTI. Heat-induced protein aggregation led to reduction in KTI and BBI in soymilk (Chen, Xu, Zhang, Kong, & Hua, 2014) while germination and hydrolysis led to modification of these protease inhibitors (Dia et al., 2012). As expected, pepsin and pepsin-pancreatin hydrolysis led to reduction in the concentration of these bioactive peptides (Figs. 2 and 3) leading to production of low molecular weight peptides as evidenced in SDS-PAGE profile (Fig. 1b) and molecular weight distribution profile as determined by size exclusion chromatography (Fig. 4) of the samples. The low concentrations of KTI and BBI led to lower percentage retention in C-LEP as compared to LEP as shown by strong correlations between BBI concentration and

a)



b)

Molecular weightdistribution after pepsin and pepsin-pancreatin hydrolysis of different lunasin-enriched preparations and commercially available products

Sample*	Molecular weight range (kDa)			
	< 1	1 – 5	5 – 10	> 10
$CaCl_2$	9.4	2.9	4.1	83.4
$CaCl_2$-P	19.8	12.8	21.9	45.3
$CaCl_2$-PP	19.3	15.1	10.5	55.0
pH 3	10.6	3.5	6.0	79.8
pH 3-P	22.1	15.1	18.5	44.2
pH 3-PP	29.3	18.9	15.6	36.1
pH 4	13.4	9.5	6.0	70.9
pH 4-P	19.2	7.7	15.3	57.6
pH 4-PP	28.6	17.5	0	53.8
pH 5	12.2	9.5	5.7	72.5
pH 5-P	18.7	13.1	17.2	50.9
pH 5-PP	21.3	14.9	9.8	53.7
LunaRich	42.2	3.2	6.1	48.3
Lunarich-P	49.8	35.8	11.0	3.3
LunaRich-PP	65.3	27.9	6.6	0
Now	87.7	8.2	2.9	0.9
Now-P	40.8	34.5	12.4	12.1
Now-PP	64.1	27.7	8.0	0.06

Fig. 4. Molecular weight distribution of lunasin enriched samples. A) Chromatogram of pH 3 precipitated lunasin-enriched product (non-hydrolyzed, pepsin digest, pepsin-pancreatin digest in descending order), and B) Table inset of all lunasin-enriched products in the various stages of hydrolysis (sample name indicates no hydrolysis, P = Pepsin digest, PP = pepsin-pancreatin digest) which shows a reduction in higher weight molecules after hydrolysis.

percentage residual lunasin after pepsin and pepsin-pancreatin hydrolysis and KTI concentration and percentage residual lunasin after pepsin and pepsin-pancreatin hydrolysis (Fig. 3b and c). Interestingly, in Now (Fig. 4b) and control, KTI-1 and KTI-1,3 (Fig. 7b) samples, the percentage of molecules with <1 kDa prior to hydrolysis is higher than pepsin digests. This could be explained by the starting materials used, Now is a commercially available product containing vitamins, minerals and a proprietary blend of different plant extracts while control, KTI-1 and KTI-1,3 samples are prepared from whole soybean flour. On the other hand, LEP samples were obtained from defatted soybean flour. Looking at the molecular weight distribution of molecules with molecular weight <5 kDa, pepsin digests consistently showed lower percentage as compared to pepsin-pancreatin digests, for instance pepsin-hydrolyzed Ca has 32.6% of <5 kDa molecules while pepsin-pancreatin hydrolyzed Ca has 34.4% of <5 kDa molecules. This is true for other LEP samples: 37.2 vs 48.2% for pH 3; 26.9 vs 46.1% for pH 4; 31.8 vs 36.2% for pH 5, 84.6 vs 93.2% for LR and 75.3 vs 91.8% for Now (Fig. 1b and Fig. 4b). The high percentage of these low molecular weight molecules in C-LEP than our LEP further shows the importance of BBI and KTI in protecting high molecular weight compounds from protease digestion. The increase in the percentage of >10 kDa in Now sample after pepsin hydrolysis may be explained by potential crosslinking effect as

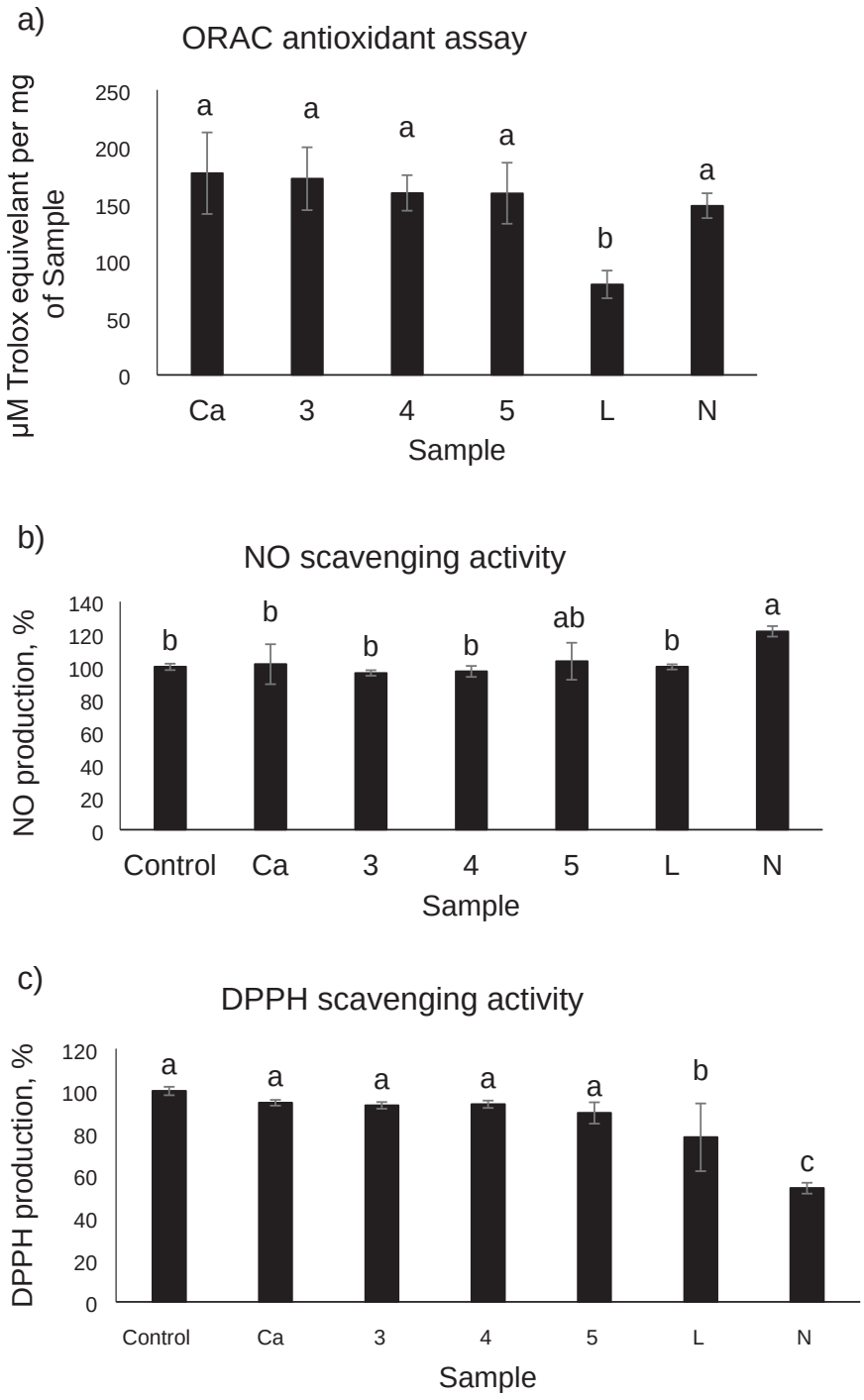


Fig. 5. Antioxidant activity of lunasin-enriched preparations is associated with oxygen radical scavenging but not to nitric oxide and DPPH radicals scavenging. A) ORAC values for lunasin-enriched preparations are significantly higher than LunaRichX but similar to Now. B) Lunasin-enriched preparations do not possess the capability to scavenge NO radicals and C) Lunasin-enriched preparations do not possess the capability to scavenge DPPH radicals.

mediated by other ingredients present in Now such as a proprietary blend plant extract that may contain certain polyphenols. Procyanidine increased the ultimate tensile strength and elongation of gelatin membranes (Chen, Wang, & Jiang, 2012) while soy protein isolate-ogaja fruit extract reinforced crosslinking of protein networks in the development of gluten-free rice noodle (Lee et al., 2016). The pancreatin used in this study is composed of trypsin, amylase, lipase, ribonuclease and other proteases as such will be able to hydrolyze proteins, fats and starch. Other peptidases with potential hydrolytic activity found in human plasma and gastrointestinal tract include kallikrein, elastase, collagenase, gelatinase, carboxypeptidase and aminopeptidase. Kunitz-type trypsin inhibitor isolated from seeds of *Enterolobium contortisiliquum* was capable of inhibiting human plasma kallikrein and human neutrophil elastase, which could potentially explain their capability to inhibit the growth of different human cancer cells (Nakahata et al., 2011). On the other hand, HcPI inhibitor isolated from *Hermodice carunculata* was able to inhibit pyroglutamyl aminopeptidase II but not the activities of serine, cysteine, aspartic and other metallo proteinases (Pascual et al., 2004). These previous studies demonstrated the specificity of protease inhibitors which could have potential impact on preserving the structure and biological activities of bioactive peptides such as lunasin. Previous studies indicated the protective role of BBI on lunasin digestibility

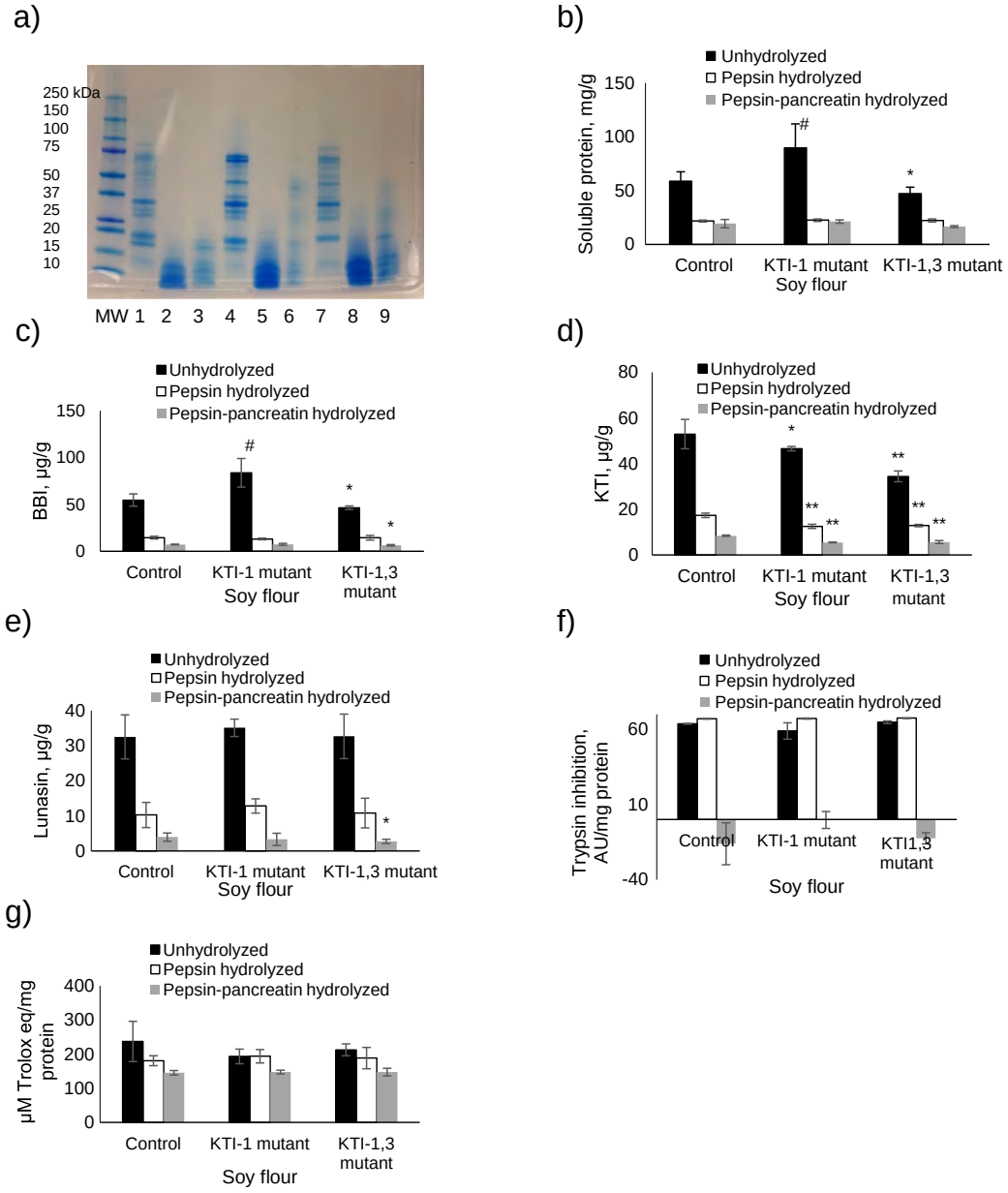
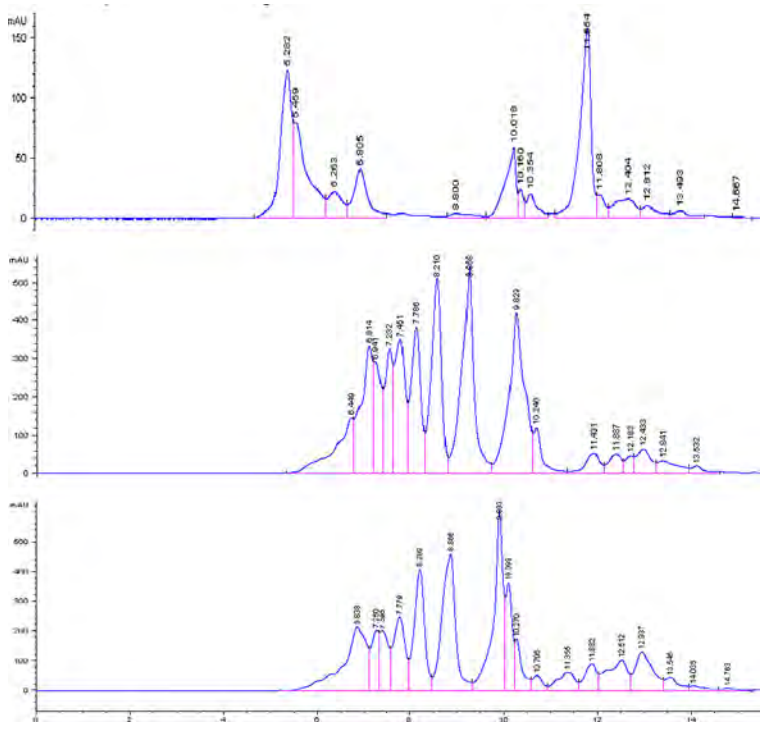


Fig. 6. Effect of KTI mutations on the concentrations of bioactive peptides in soybean and their stability against pepsin-pancreatin digestion. A) Electrophoresis profile showing degradation of proteins after pepsin and pepsin-pancreatin digestion Lanes MW: molecular weight marker, 1: control soy flour, 2: control after pepsin hydrolysis, 3: control after pepsin-pancreatin hydrolysis, 4: KTI-1 mutant soy flour, 5: KTI-1 mutant soy flour after pepsin hydrolysis, 6: KTI-1 mutant soy flour after pepsin-pancreatin hydrolysis, 7: KTI-1,3 mutant soy flour, 8: KTI-1,3 mutant soy flour after pepsin hydrolysis, 9: KTI-1,3 mutant soy flour after pepsin-pancreatin hydrolysis; B) KTI-1 mutant soy flour has higher soluble protein concentration than control flour (#, $P = 0.0099$) while KTI-1,3 mutant soy flour has lower soluble protein concentration than control flour (*, $P = 0.0250$). After pepsin and pepsin-pancreatin hydrolysis, KTI mutation did not affect soluble protein concentration; C) BBI concentration of KTI-1 mutant soy flour is significantly higher than control soy flour (#, $P = 0.0016$) while BBI concentration of KTI-1,3 mutant soy flour is significantly lower than control soy flour (*, $P = 0.0153$). KTI-1,3 mutation led to a significantly lower residual BBI concentration than control soy flour after pepsin-pancreatin hydrolysis. D) KTI mutation led to a significantly lower KTI concentration in KTI-1 mutant flour (*, $P = 0.0366$) and KTI-1,3 mutant flour (**, $P < 0.0001$) than control soy flour. Pepsin and pepsin-pancreatin hydrolyzates of KTI mutant flours have lower residual KTI concentrations that control flour (**, $P < 0.0001$); E) KTI mutation did not affect the concentration of lunasin in unhydrolyzed and pepsin-hydrolyzed soy flour while KTI-1,3 mutation led to a significantly lower residual lunasin concentration after pepsin pancreatin hydrolysis than control soy flour (*, $P = 0.0474$); F) KTI mutation did not affect the capability of soy flour to inhibit trypsin activity and G) KTI mutation did not affect the antioxidant capacity of soy flour extract as measured by ORAC. Pepsin-pancreatin hydrolysis led to significant lower concentrations of all parameters measured.

but to our knowledge this is the first report on the potential protective role of KTI on lunasin digestibility. To further investigate the role of KTI in lunasin stability against pepsin-pancreatin hydrolysis, two soybean flours with mutations in KTI gene was used and compared their behavior with a control soybean flour. KTI mutation in KTI-1 isoform did not lead to significantly different residual lunasin concentrations while mutation in KTI-1 and KTI-3 isoforms led to significantly lower residual lunasin concentrations than the control soybean flour. After pepsin-pancreatin hydrolysis, the percentage residual lunasin in control soybean flour is 12.3% which is higher than the percentage residual lunasin in KTI-1 mutant flour of 9.4% and KTI-1,3 mutant flour of 8.6%. There results for the first time demonstrated the possible role of KTI in the stability of lunasin against pepsin-pancreatin hydrolysis. Measurement of the antioxidant activity of the samples showed no potential effect of KTI mutations nor pepsin-pancreatin hydrolysis on the ORAC values of the samples.

In summary, we report here the effect of protease inhibitor concentrations on the stability of lunasin against pepsin-pancreatin hydrolysis.

a)



b)

Molecular weight distribution after pepsin and pepsin-pancreatin hydrolysis of control, KTI-1mutant and KTI-1,3 mutant soy flours.

Sample*	Molecular weight range (kDa)			
	< 1	1 – 5	5 – 10	> 10
Control	50.7	0.1	7.0	42.3
Control-P	42.7	36.3	19.0	2.1
Control-PP	61.9	25.5	10.5	2.1
KTI-1	53.2	0.2	7.6	39.1
KTI-1-P	42.6	36.6	14.4	6.4
KTI-1-PP	60.8	26.8	12.4	0
KTI-1,3	56.8	2.1	7.0	34.1
KTI-1,3-P	43.6	35.8	20.5	0.1
KTI-1,3-PP	63.5	26.0	8.7	1.8

Fig. 7. Molecular weight distribution of control, KTI-1 mutant and KTI-1,3 mutant flours as affected by pepsin and pepsin-pancreatin hydrolysis. A) Representative chromatograms of KTI-1 mutant flour (unhydrolyzed, pepsin-hydrolyzed and pepsin-pancreatin-hydrolyzed; from top to bottom) and B) Table inset showing the molecular weight distribution for unhydrolyzed, pepsin-hydrolyzed (P) and pepsin-pancreatin hydrolyzed (PP) soy flours.

BBI concentration positively correlated with lunasin stability. For the first time, we demonstrated that in addition to BBI, KTI also play an important role in protecting lunasin from pepsin-pancreatin hydrolysis. These factors must be considered in the production of lunasin-enriched dietary supplements in order to ascertain the consumer of the potential health-benefits associated with intake of such supplement.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work is supported by the USDA National Institute of Food and Agriculture, HATCH 1010230 to VPD. Product names are necessary to report factually on available data; however, USDA neither guarantees nor warrants the standard of product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

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