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Comparative Chemical Characterization of Lunasin-enriched Preparations and Modifications of the Inflammasomes *In Vitro*

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I am submitting herewith a thesis written by Samuel James Price entitled "Comparative Chemical Characterization of Lunasin-enriched Preparations and Modifications of the Inflammasomes *In Vitro*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Comparative Chemical Characterization of Lunasin-enriched Preparations and Modifications of the Inflammasomes *In Vitro*

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Samuel James Price
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DEDICATION

To my mother and father, thank you for your support.

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ABSTRACT

Soybean (*Glycine max)* is one of the most cultivated crops in the world providing the population with large amounts of protein and oil. In addition to its nutritional composition, soybean also contains biologically active compounds with potential health-promoting properties. The presence of these bioactives may be responsible for the lower incidence of chronic diseases in populations that consume a significant portion of soybeans in their diet. One group of soybean-derived bioactives are bioactive peptides and proteins including lunasin, Bowman-Birk inhibitor (BBI) and Kunitz-type trypsin inhibitor (KTI). The overall objective of this research was to develop a method of preparing lunasin-enriched material and evaluate the ability of lunasin-enriched material to inhibit activation of the inflammasomes *in vitro.* Lunasin-enriched materials were prepared using calcium chloride and pH precipitation methods and compared with two commercially-available lunasin-enriched products. The stability of lunasin against pepsin-pancreatin hydrolysis was evaluated in these materials and the effect of BBI and KTI concentrations were analyzed. Lunasin concentrations ranged from 8.5 to 71.0 µg [microgram]/g pre-hydrolysis and 4.0 to 13.2 µg/g after hydrolysis. In all products tested, lunasin concentration after pepsin-pancreatin hydrolysis (PPH) significantly correlated with BBI and KTI concentrations. One lunasin-enriched preparation was evaluated for its ability to modify activation of the inflammasomes *in vitro* using THP-1 human macrophages. Aberrant activation of the inflammasomes is associated with development of human diseases such as

cancer, diabetes and inflammatory bowel diseases. The activation of the inflammasomes in THP-1 human macrophages was accomplished by priming with lipopolysaccharide followed by adenosine triphosphate. Lunasin-enriched material was added during the priming step at concentrations ranging from 0.0625 to 0.25 mg/mL. Addition of lunasin-enriched preparation led to reduction of intracellular reactive oxygen species (ROS) which correlated with reduction in the amount of pro-inflammatory cytokines interleukin-1β(beta) and interleukin-18. These results indicate that ROSs play an integral role in lunasin's ability to inhibit inflammation and inflammasomes' activation. This research is the first to report on the role of Kunitz-type trypsin inhibitor on the stability of lunasin against PPH and potential of lunasin-enriched preparations as chemopreventive agent against diseases associated with aberrant activation of the inflammasomes.

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CHAPTER I
INTRODUCTION

Soy is commonly consumed throughout the world, especially in Asian countries, because it contains high amounts of protein and many essential amino acids that are not commonly found in other plants (Michelfelder, 2009). Soy was used as far back as 9000 B.C in China and 7000 B.C in Japan (Lee et al.,, 2011)as a staple food. Soybeans are still an important part of Asian cuisine with multiple food products produced such as soymilk, soy sauce, tempeh, and tofu (Singh et al., 2008). Soy is used in Asian cultures because it contains large amounts of protein so it is a suitable meat substitute for people with vegetarian diets. In modern times, soybeans have become the most widely used legume because of its use in food and oil production (Singh et al., 2008). In 2015, the United States produced a total of 3.93 billion bushels of soy (USDA National Agricultural Statistics Service, 2016), showing its importance as a crop. Although the United States produces large amounts of soy, it is not used in human food products as much as it is in Asia.

In countries that have higher rates of soy consumption (especially Asia), it has been shown that there are decreased risks for certain types of diseases such as cardiovascular disease, obesity and cancer (Wu et al., 1998). For instance, Asian women have reduced risks of breast and other types of cancers compared to women in western countries due to their increased intake of soy (Wu et al., 2008). This has prompted studies into the composition of soy to understand this

observation. The recent data suggest that bioactive compounds in soy are responsible for the reduced risks of certain cancers. However, the exact reasons of how bioactive compounds have these protective effects is largely unknown.

One group of bioactive compounds are peptides which can be either naturally occurring or produced by enzymatic hydrolysis and fermentation. Food-derived bioactive peptides in addition to their nutritional value exert a physiological effect in the body acting as potential metabolism modulators and regulatory compounds (Shahidi & Zhong, 2008). Some of the bioactive peptides found in soy include Bowman-Birk Inhibitor (BBI), Kunitz-Trypsin Inhibitor (KTI), soymetides, soymorphins, and lunasin (Wang & Gonzalez De Mejia, 2005). Previous studies have shown the chemopreventive property of these bioactive peptides associated with their anti-inflammatory effects. BBI inhibited autoimmune inflammation and neuronal loss in a mouse model of multiple sclerosis (Touil et al., 2008) while KTI suppressed lipopolysaccharide-induced inflammation by reducing expressions of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6. In addition, lunasin attenuated obesity related inflammation by reducing production of pro-inflammatory cytokines monocyte chemoattractant protein-1 and IL-1β in a co-culture model of RAW 264.7 macrophages and 3T3-L1 adipocytes (Hsieh et al., 2017).

Cytokines are small secreted proteins released by immune cells in response to a variety of stimuli and have diverse effects on the interactions of

cells in the body(Tilg et al., 2016). The IL-1 family has been implicated in different pathogenesis associated with their pro-inflammatory effects such as Parkinson's disease (Mao et al., 2017), multiple sclerosis (Barclay & Shinohara, 2017) and inflammatory bowel diseases (Peyrin-Biroulet, Lémann, 2011; Ruffolo et al., 2010). The release of IL-1 family of cytokines such as IL-1β and lL-18 is controlled by activation of macromolecular protein complex called the inflammasomes(Domiciano et al., 2017). Inflammasomes are multiprotein complexes that are formed within macrophages after danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) stimulation which induce the production of IL-1β and lL18 as well as the inflammatory caspase family subtypes (Lu & Wu, 2015). Chronic inflammation by overexpression of the inflammasomes and the associated pro-inflammatory cytokines have been linked to multiple conditions, such as metabolic disorders (atherosclerosis, Type II diabetes) as well as mucosal immune responses that can lead to inflammatory bowel syndrome (IBS) (Strowig et al., 2012).

To date there is a lack of information on how soybean-based products, such as lunasin-enriched material, can modify inflammasomes activation. If soybean-based products, especially lunasin-enriched materials, can inhibit the inflammasome, then this could lead to more studies to determine if lunasin could be a viable chemopreventive option in diseases associated with chronic inflammation, as well as for other potential uses. If future studies are able to conclude that lunasin and other soy-based products are able to be used as

treatments, then a proposal to the FDA to grant this health promoting claim would be the next step.

Hypothesis and Objectives

Objectives

The overall objectives are to develop a method of preparing lunasin-enriched material and evaluate the ability of lunasin-enriched material to inhibit activation of the inflammasomes *in vitro.*

Central Hypothesis

Lunasin-enriched material prepared from defatted soybean flour can inhibit activation of the inflammasomes by reducing reactive oxygen species.

Aims

Aim 1. To develop a method of preparing lunasin-enriched material and determine the effect of soybean protease inhibitors in the stability of lunasin against pepsin-pancreatin hydrolysis

Hypothesis: The concentrations of soybean protease inhibitors BBI and KTI will correlate to the stability of lunasin against pepsin-pancreatin hydrolysis in different lunasin-enriched preparations.

1.1 To develop a method of producing lunasin-enriched preparations using $CaCl_2$ and pH precipitation methods;

1.2 To compare the concentrations of lunasin, BBI and KTI among lunasin-enriched preparations and two commercially available lunasin-enriched products;

1.3 To determine the effect of BBI and KTI on the stability of lunasin against pepsin-pancreatin hydrolysis; and

1.4 To measure the antioxidant activities of lunasin-enriched preparations and their pepsin-pancreatin hydrolysis products.

Aim 2. To evaluate the potential of lunasin-enriched material to modify activation of the inflammasomes in THP-1 human macrophages in vitro.

Hypothesis: Lunasin-enriched material can inhibit activation of the inflammasomes in THP-1 human macrophages in vitro

2.1 To investigate the effect of lunasin-enriched material in the production of IL-1β and IL-18 in THP-1 human macrophages primed and activated by lipopolysaccharide and adenosine-triphosphate, respectively;

2.2 To determine the mechanism of inactivation of the inflammasomes by the lunasin-enriched material *in vitro*; and

2.3 To determine the effect of pepsin-pancreatin hydrolysis on the ability of lunasin-enriched material to inhibit activation of the inflammasomes *in vitro.*

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CHAPTER II
LITERATURE REVIEW

Soybeans and soybean proteins

Soybeans (*Glycine max*) are an important legume because they contain large amounts of protein, and they were used for ~68% of global protein meal production in 2006 (Krishnan et al., 2009). Soybeans contain approximately 40% protein per seed (USDA National Agricultural Statistics Service, 2016) and the majority of these proteins are one of two storage proteins, the 11s glycinin and 7s ß-conglycinin families, which make up 40 and 30% of these proteins, respectively (Utsumi et al., 2002). Another study was able to identify 80 unique proteins within the glycinin and ß-conglycinin family, falling under four distinct categories based on solubility (Natarajan et al., 2006). The categories are water-soluble albumins, salt-soluble globulins, weak acid/base-soluble glutelins, and alcohol-soluble prolamins (Ferreira et al., 1999). Other proteins found within soybean include γ-conglycinin, lipoxygenase, ß-amylase, basic 7s globulin, agglutinin, and 2s albumins from the 2s seed storage protein family (El-moneim et al., 2011). Proteins are classified under sedimentation coefficients, or Svedburg units, such as 7s and 11s, which uses the weight and shape of the protein to determine the coefficient (Smith, 1988). Albumins are classified in the globular protein family due to their shape, but differ categorically from globulins because they are soluble in water (Masuelli, 2013), whereas globulins are soluble with low salt concentrations (Sirison et al., 2017).

Bioactive proteins and peptides from soybean

Glycinin and ß-conglycinin have been found to contain proteins that induce allergic reactions (Holzhauser et al., 2009), but other peptide sections contained within both of these proteins have bioactive properties, such as improved glucose and lipid metabolism from soymorphins (Yamada et al., 2012), and reduced cholesterol absorption from soystatins (Nagaoka et al., 2010). There are other bioactive peptides found in soy that come from sources other than the glycinin and ß-conglycinin, such as lunasin from 2s albumins, (Galbas et al., 2013) and bioactive proteins such as protease inhibitors (Gillman et al., 2015).

Lunasin. Lunasin is a peptide naturally found in soy and other grains such as barley and quinoa (Ren & Zhu, 2017). Soy is the most commonly used source for the extraction of lunasin, because it contains large amounts of protein, comprising approximately 41% protein, 90% of which are globulins and the remaining 10% are albumins (Singh et al., 2008). Lunasin is a 2S albumin with a 43 amino acid sequence: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD (Cruz-Huerta et al., 2015). Because there are very small amounts of lunasin in soy, compared to other proteins, new methods have been implemented in order to increase the yields and purity of lunasin directly extracted from soy. The newest method uses 100 grams of de-fatted soy flour in one liter of an aqueous 30%

ethanol solution to extract proteins from the soy flour, and it is then centrifuged and the supernatant fluid is precipitated with $CaCl_2$ and further centrifuged before being lyophilized (Krishnan & Wang, 2015).

Several studies have shown the chemopreventive effect of lunasin extracted from soybean using different *in vitro* and *in vivo* experiments. Oral administration of lunasin showed that lunasin was able to spread throughout the body and reach areas that are most commonly associated with cancer (i.e. colon, breast, lung), meaning that it is possible to reach a target site through oral administration only (Hsieh et al., 2010). Another study showed that lunasin was found in the blood plasma of men after ingesting soy (Dia et al., 2009) indicating that lunasin is able to be absorbed and transported throughout the body. In the study that used intravenous and intraperitoneal administration, it was shown that lunasin administered at 30 mg/kg body weight in mouse xenograft model reduced tumor weight of A375 melanoma cell line, with the intravenous injection reducing 35% of total weight and the intraperitoneal injection reducing 46% of the total weight (Shidal et al., 2016).

Though oral administration of lunasin is the best route for use as a chemopreventive agent, lunasin might become digested and proteolyzed if not protected by a protease inhibitor such as the Bowman-Birk protease inhibitor (BBI). If there is no protease inhibitor, then pepsin hydrolyzed up to 97% of the lunasin, which was the result when the gastric process was simulated *in vitro* (Cruz-Huerta et al., 2015). Once the peptide has been absorbed into the blood

stream, it will reach the target cells and eventually collect in the liver and kidneys (Galbas et al., 2013), but there have been studies that indicate it does not affect the functionality of the liver or kidney cells (Al-Rayyan et al., 2014).

There are many health benefits for lunasin, ranging from anti-cancer(Cruz-Huerta et al., 2015), anti-obesity (Liu et al. 2014), prevention from cardiovascular disease (Cam & de Mejia, 2012), and anti-inflammatory properties (Dia et al. 2009). The majority of recent studies have focused on the use of lunasin as an anti-tumor and cancer treatment through various means, with the most prominent being the arginine-glycine-aspartic acid (RGD) motif found along the 32^{nd}-34^{th} amino acids in the sequence (Setrerrahmane et al., 2014). Peptides that contain the RGD motif have been found to suppress inflammation and promote apoptosis in different cancer cell lines(Dia & de Mejia, 2010). RGD motif containing peptides are able to cause apoptosis through the activation of pro-caspase-3, a protein that contains an aspartate-aspartate-methionine site that binds with the RGD, which triggers integrin activation and causes the pro-caspase-3 to activate, and induces apoptosis among cells by allowing caspase-3 to penetrate the cell walls and activate the enzymatic activity of the protein (Buckley et al., 1999). Lunasin is also able to amplify the effects of other treatments such as amplifying the amount of Interferon Gamma (IFN-γ) produced by natural killer (NK) cells in post-transplant lymphoma patients, where IFN-γ is an interferon used for anti-tumor immunity by enhancing tumor immunogenicity for antigen presentation, producing apoptosis in tumor cells, promoting T helper 1 cell differentiation, and

enhancing cytotoxicity of $CD8^+$ cytotoxic T lymphocytes (Chang et al., 2014). Once the IFN-γ is able to be produced again, the immune system will be able to function on an improved level. In addition, lunasin has been shown to be anti-inflammatory through the inhibition of production of inflammatory responses including nitric oxide, prostaglandins, and cytokines(de Mejia & Dia, 2009). For instance, lunasin was able to allergic airway inflammation important in asthma therapy and allergy immunotherapy (Yang et al., 2015) and lunasin showed potent radical scavenging activities in intestinal CaCo-2 cells indicating its promising activity to preserve intestinal mucosal integrity against oxidative damage (Garcia-Nebot et al., 2014).

Protease inhibitors*.* Protease inhibitors are molecules (typically proteins) that are able to inhibit proteolytic enzymes. Because there are so many different types of protease inhibitors, they have been broken down into "families" and "clans" to help in differentiating them through means such as inhibitory sites and proteases inhibited (Rawlings et al., 2004). Proteases are divided into seven different families and are all based on the catalytic residues, the site that either binds to a peptide bond or utilizes an acid to hydrolyze the peptide bond (Oda, 2012). Six families of proteases use different amino acids as the catalytic triad in the active site, with the seventh being a metalloprotease. The largest protease family is the serine protease family, which accounts for approximately one third of proteases (Di Cera, 2009). Two proteases that belong to a subfamily of the

serine protease family are chymotrypsin-like which cleaves the hydrophobic amino acids, and trypsin like which cleaves after arginine and lysine (Rawlings et al. 2016). Two protease inhibitors found in soy come from two different families, the BBI family and the Kunitz-type Trypsin Inhibitor (KTI) family. The BBI found in soy has two binding sites, one for trypsin and one for chymotrypsin. It is able to inhibit chymotrypsin and trypsin by the standard, or Laskowski method. The Laskowski method is described as an inhibitor having a specific peptide bond corresponding to the protease catalytic site, thus binding with it and at a neutral pH does not completely hydrolyze, instead forming an equilibrium between hydrolyzed and non-hydrolyzed peptide bonds and stable enzyme-substrate complex (Laskowski & Kato, 1980; Rawlings et al., 2016). BBI is able to inhibit one trypsin molecule and one chymotrypsin molecule per molecule of BBI using a lysine-leucine peptide bond, with lysine binding to trypsin and leucine binding to chymotrypsin (Rawlings et al., 2016). The KTI found in soy has a high affinity for trypsin, and is responsible for approximately 76% of the trypsin inhibition (Chen et al., 2014). KTI also uses the Laskowski method, but uses an arginine-isoleucine peptide bond to strongly bind to trypsin (Wasmuth & Lima, 2016), as well as protruding loop that is inserted into the active site of enzymes (Azarkan et al., 2011). Since these protease inhibitors are found in soy, they can be concentrated along with lunasin to potentially prevent its digestion upon oral administration.

Bowman-Birk Inhibitor. The BBI is found within soy and other seeds of legumes. The species found in soybeans has110 amino acids with the following sequence:

MVVLKVCLVLLFLVGGTTSANLRLSKLGLLMKSDHQHSNDDESSKPCCDQCAC TKSNPPQCRCSDMRLNSCHSACKSCICALSYPAQCFCVDITDFCYEPCKPSED DKEN(Wasmuth & Lima, 2016). Each source of BBI contains protease inhibitors that can differ slightly (as is the case in horse gram and soy which differs only by a few amino acids) or greatly (as is the case of eudicots BBI which are typically 8 kDa with two proteolytic sites versus monocots BBI is either 8 kDa with one proteolytic site, or 16 kDa with two proteolytic sites) (Prakash et al., 1996). This specific family of protease inhibitors are a cysteine-rich group, and characterized by a lower molecular weight (8 kDa for soy-based) and large amounts of disulfide bonds, 7 of which is present in soybean BBI (Odani et al., 1973). The purpose of a protease inhibitor is to protect against enzymes that proteolyze proteins, and BBI protects against the proteolytic enzymes trypsin and chymotrypsin (Odani et al., 1973). The trypsin and chymotrypsin inhibitory properties are due to multiple disulfide bonds creating two inhibition sites (Werner & Wemmer, 1992). In soy processing, the protease inhibitors found are considered to be disadvantageous because protein digestibility could be affected (Lu et al., 2015), stemming from the protease inhibitors present within soy products that may not be subject to extreme heat or pH change such as soy flour and soy milk. In previous studies protein digestibility of soy was linked to pancreatic enlargement and possible

disease (Liener & Hasdai, 1986) and led to BBI being thought of as an antinutrient, but this was later proven inaccurate as multiple studies showed that in smaller concentrations, BBI could prove beneficial (Armstrong et al., 2000; Losso, 2008). In addition, phase I randomized controlled trials on the safety of BBI concentrate showed that the inhibitor concentrate was well tolerated by male participants with no apparent toxicity and changes in hematological and biochemical markers (Lin et al., 2014). BBI has been linked to chemoprevention against different types of cancer such as colorectal cancer (Clemente, Marín-manzano, Arques, & Domoney, 2013), oral cancer (Armstrong et al., 2013; William & Papadimitrakopoulou, 2013) and prostate cancer (Kennedy & Steven Wan, 2002; Tang et al., 2009). In addition, BBI showed activity against aphid parasitoid Aphidius ervi Haliday (Azzouz et al., 2005) and anti-viral properties against human immunodeficiency virus? (Ma et al., 2016).

Kunitz Trypsin Inhibitor. The KTI family of protease inhibitors are larger than BBI at around 20 kDa and contain fewer cysteine and disulfide bridges. KTI family comes from different sources, first described from bovine pancreas and then from soybeans (Kunitz, 1947). The soy-based KTI is defined by primarily inhibiting serine-based proteases including trypsin by using the standard mechanism (Laskowski & Kato, 1980). In soybean, KTI accounts for approximately 74% of trypsin inhibition, and even though its inhibition site is able to bind to trypsin and chymotrypsin (Bosterling & Quast, 1981), it does not bind

as well to chymotrypsin (Chen et al., 2014), which is why the majority of studies done thus far focus on BBI. Some of the proposed health benefits of KTI include the inhibition of HIV reverse transcriptase (Fang, Wong, & Ng, 2010), which could prove to be beneficial to HIV patients, and a reduction in inflammation caused by elastase by inhibiting the enzyme (Ribeiro, Cunha, Fook, & Sales, 2010). In addition, soybean KTI showed different mechanisms of chemopreventive properties including reduction of TNF-α by inhibiting nuclear translocation of NF-κB (Kobayashi et al., 2005) and suppressing c-JUN N-terminal kinase signaling (Kobayashi et al., 2005). Anti-cancer properties of KTI are associated with its ability to suppress urokinase expression leading to reduced invasion and metastasis of cancer cells (Inagaki et al., 2005; Kobayashi et al., 2004).

Inflammation and inflammasomes

Inflammation is a result of the innate immune system in response to dangers to the host, such as cell injury, infection, and recognition of danger-associated molecular pathways (DAMPs) and pathogen-associated molecular pathways (PAMPs) (Ferrero-Miliani et al., 2007). Another name for this immune response is acute inflammation, which is defined by pro-inflammatory molecules activating the immune response and then switching to anti-inflammatory once the insult is no longer a threat (Levy et al., 2001). If these pro-inflammatory molecules are

not switched off and the host system returns to a state of homeostasis, then this results in chronic inflammation (Monteiro & Azevedo, 2010). Chronic inflammation is a major problem within the host system because homeostasis is not available, and this has been linked to metabolic diseases such as obesity and type II diabetes (Hsieh et al., 2017), as well as neurodegenerative diseases such as Parkinson's and Alzheimer's (Mao et al., 2017). One of the leading causes of chronic inflammation is the inflammation cascade associated with sterile stressors that are released by intracellular oligomers called inflammasomes (Abais et al., 2015). The inflammasome is activated through a complex pathway that is started with the recognition of a DAMP or PAMP which activates the Toll-like receptor protein 4 (TLR4), which activates the NF-κB that is responsible for the translation of inflammasome associated proteins (Walsh, Muruve, & Power, 2014), and this portion is known as "priming". Once the inflammasome is primed, a stimulant such as ROS or ATP is needed to "activate" the inflammasome, which converts pro-caspase into caspase-1 through thioredoxin-interacting protein (TXNIP) binding to the inflammasome allowing cleavage just before the active caspase-1 (Zhou et al., 2010). Once caspase-1 is activated, it cleaves the immature pro-inflammatory cytokines interleukin-1ß and interleukin-18 into their active forms, which are then expressed out of the cell and initiate the priming step as before, creating a cascade of inflammation in neighboring cells (Tőzsér & Benko, 2016).

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CHAPTER III
KUNITZ TRYPSIN INHIBITOR IN ADDITION TO BOWMAN-BIRK INHIBITOR INFLUENCE STABILITY OF LUNASIN AGAINST PEPSIN-PANCREATIN HYDROLYSIS

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SJP performed the experiments, analyzed and interpreted the data and wrote the manuscript; PP helped in the experiments and edited the manuscript, HBK provided the BBI antibody, generated KTI-mutant soybean and edited the manuscript, VPD designed the experiments, helped in the analysis and interpretation of data and edited the manuscript. Minor formatting changes were done to follow formatting guidelines per UTK Graduate School requirements.

Abstract

Soybean contains several biologically active components and one of this belong 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.
Keywords: soybean, BBI, KTI, lunasin, pepsin-pancreatin hydrolysis, protease inhibitors

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 & Lumen, 1999; Lule, Garg, Pophely, Hitesh & Tomar, 2015). Lunasin possessed different potential biological properties including anti-cancer (Shidal, Al-Rayyan, Yaddanapudi & Davis, 2016; Jiang, Pan, Cheng, Li, Liu & Li, 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 & Bowman-Birk, 1973). BBI protects protein digestion by inhibiting the activity of trypsin and chymotrypsin (Odani et al., 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 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.

Materials and Methods

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.

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, 100g defatted soy flour were mixed with 1-L 30% ethanol solution for 2 h at 20 to 22 °C. After centrifugation (8,000 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 x g for 30 min, supernatants were dialyzed, freeze-dried and stored at -20 °C until used.

Soluble protein concentration by Bradford Assay

Ten mg of samples were extracted with 1-mL TBS by vortexing for 90 min at 20 to 22 °C, followed by centrifugation (20,000 x g, 4 °C) for 30 min and the supernatant was used for the analysis based on Bradford (1976) principle. One hundred µL 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.

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 volts for 100 minutes. 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 minutes each or until the background dye was removed.

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™ 0.45µm PVDF membrane (GE Healthcare, Piscataway NJ) at 110 volts for 60 minutes 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 minutes each, the membrane was incubated in primary antibody against lunasin, BBI (Gillman et al., 2015) and

KTI (VWR International, Atlanta GA) at 1:2,000 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).

Enzyme-Linked Immunosorbant Assay (ELISA) for lunasin, BBI and KTI concentrations

One hundred µL 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 (1L PBS + 5 mL Tween 20, PBST) per well and blocked with 300µl 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 100µl 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 100µl of 2 M H_2SO_4, and the absorbance was read at 450nm. Lunasin, BBI and KTI concentrations were calculated using their respective standard curve.

Trypsin Inhibition Assay

Fifty µL 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 minutes, 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 405nm. Trypsin inhibition was calculated using the following formula: (Positive control average - sample absorbance)/(200*0.05)*1000

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 X 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 20µl with 20 min run time. The detector was set at 214 nm and the analysis was performed at ambient temperature.

Antioxidant Capacity

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 96-well plate in triplicate followed by 120 µL of fluorescein (70nM final concentration) and incubated at 37°C in the dark for 15 minutes. Afterwhich, 60 µL of 2,2'-azobis-(2-methylpropionamidine) (12mM) 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 minutes. ORAC values were calculated from Trolox standard curve and reported as µM Trolox equivalent (TE).

Nitric Oxide Radical Scavenging Assay. Nitric oxide scavenging assay was performed as previously described (Mojica, Meyer, Berhow & de Mejia, 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 hours 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.

2,2-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 minutes. The absorbance was read at 517nm.

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.

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 $P < 0.05$.

Results

Protein concentration and protein profile of lunasin-enriched samples

Fig. 3.1a (all figures are listed at the end of this chapter as an appendix) 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. 3.1b** shows the protein profile of samples before and after hydrolysis; the large molecular weight proteins were hydrolyzed indicated by disappearance of the higher weight bands after PPH. **Fig. 3.1c** 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.

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

Fig. 3.2a 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. 3.2b** 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. 3.2c** 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.

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

Fig. 3.3a 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. 3.3b** shows a positive correlation of BBI and KTI concentrations before hydrolysis and the percentage residual lunasin concentrations after hydrolysis. **Fig. 3.3c** is shows the Pearson correlation indicating that the correlations between lunasin stability against PPH and BBI and KTI concentrations pre-hydrolysis are highly significant ($P < 0.0001$).

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

Fig. 3.4a shows the chromatogram of pH 3 precipitated lunasin-enriched product (non-hydrolyzed, pepsin digest, pepsin-pancreatin digest, top to bottom) and **Fig. 3.4b** 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. 3.1b**. For instance, pH 3 sample molecular weight distribution before PPH was 10.6% <1kda, 3.5% 1-5kda, 6.0% 5-10kda, 79.8% >10kda. After PPH, this distribution was changed to 29.3% <1kda, 18.9% 1-5kda, 15.6% 5-10kda, 36.1% >10kda.

Antioxidant capacity of lunasin-enriched products

Fig. 3.5a 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. 3.5b and 3.5c** show antioxidant scavenging potential by Nitric Oxide and DPPH, respectively. In general, lunasin-enriched products were not capable of scavenging both NO and DPPH radicals.

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

Fig. 3.6a 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 adter 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. 3.6b)** indicating reduction of protein concentrations after PPH. **Fig. 3.6c** 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. 3.6d** 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. 3.6e)**. **Fig. 3.6f** 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. 3.6g.** 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. 3.7** 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.

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, Kim, & Krishnan, 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 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 protein (~4.5 pH) causing different proteins and different 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, Gomez, Vernaza, Berhow, Chang & de Mejia, 2012). As expected, pepsin and pepsin-pancreatin hydrolysis led to reduction in the concentration of these bioactive peptides (**Fig. 3.2 and 3.3)** leading to production of low molecular weight peptides as evidenced in SDS-PAGE profile (**Fig. 3.1b)** and molecular weight distribution profile as determined by size exclusion chromatography (**Fig. 3.4)** 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 percentage residual lunasin after pepsin and pepsin-pancreatin hydrolysis

and KTI concentration and percentage residual lunasin after pepsin and pepsin-pancreatin hydrolysis (**Fig. 3.3b and 3.3c**). Interestingly, in Now (**Fig. 3.4b**) and control, KTI-1 and KTI-1,3 (**Fig. 3.7b**) samples, the percentage of molecules with less than 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 less than 5 kDa, pepsin digests consistently showed lower percentage as compared to pepsin-pancreatin digests, for instance pepsin-hydrolyzed Ca has 32.6% of less than 5 kDa molecules while pepsin-pancreatin hydrolyzed Ca has 34.4% of less than 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. 3.1b and Fig. 3.4b**). 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 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, Kim, Song, Lee, Lee & Yoo, 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 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. 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.

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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.

Conflict of Interest

The authors declare no conflict of interest.

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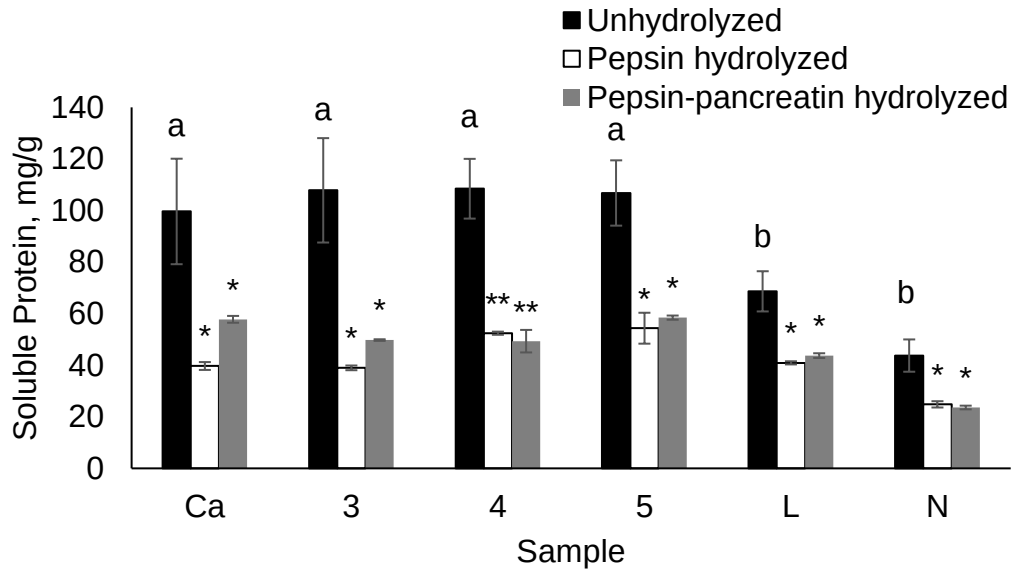
Appendix

Figures

Fig. 3.1. 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).

Fig. 3.1. continued

a)



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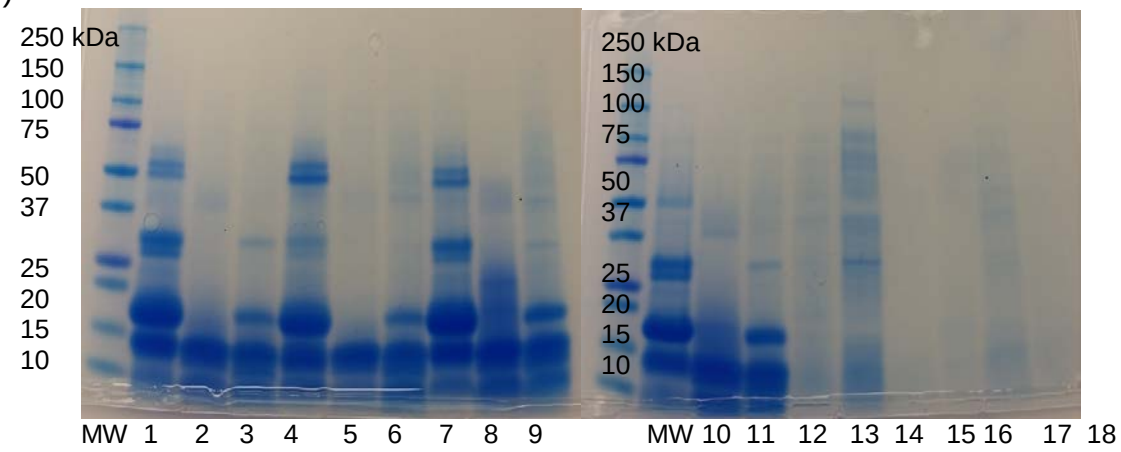

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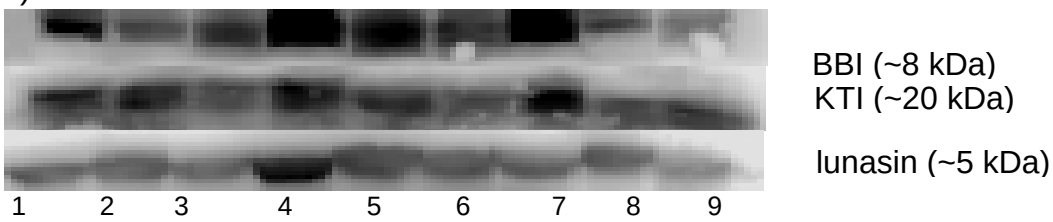
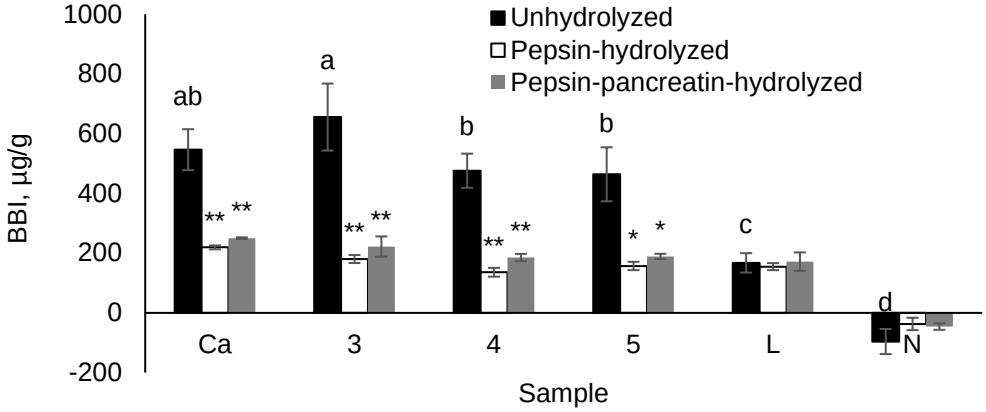


Fig. 3.2. Pepsin-pancreatin hydrolysis affected the concentrations of protease inhibitors BBI and KTI as well as trypsin inhibitory activity of different lunasin preparations and commercially available products. a) Commercially available products have less BBI concentrations than laboratory-prepared 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.

Fig. 3.2. continued

a)



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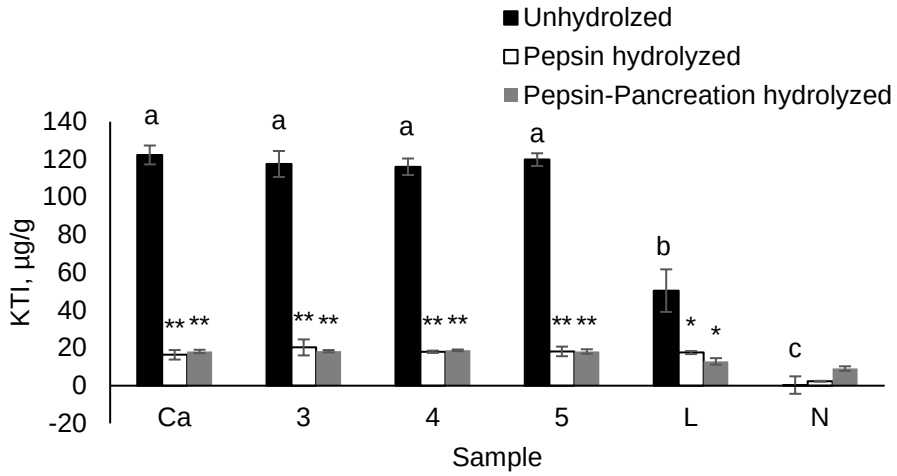

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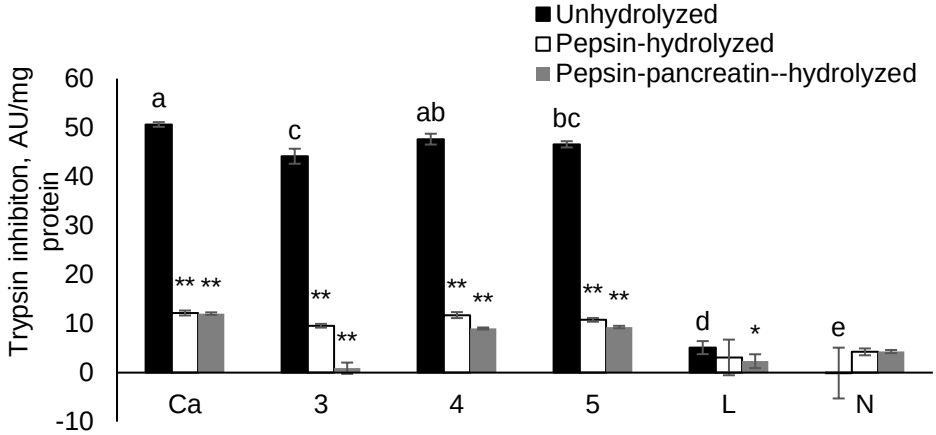
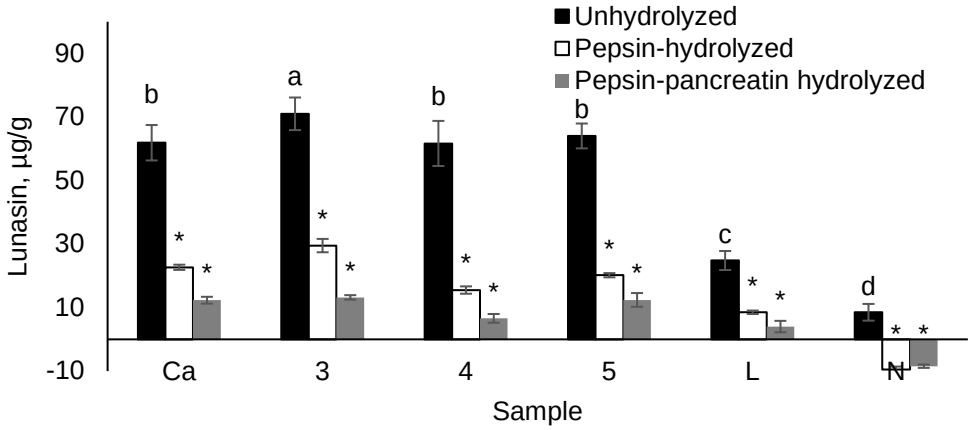


Fig. 3.3. Lunasin stability against pepsin-pancreatin digestion is affected by the concentrations of protease inhibitors BBI and KTI in different lunasin-enriched preparations and commercially available products. a) Commercially available products have less lunasin concentrations than laboratory-prepared 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.

Fig. 3.3. continued

a)



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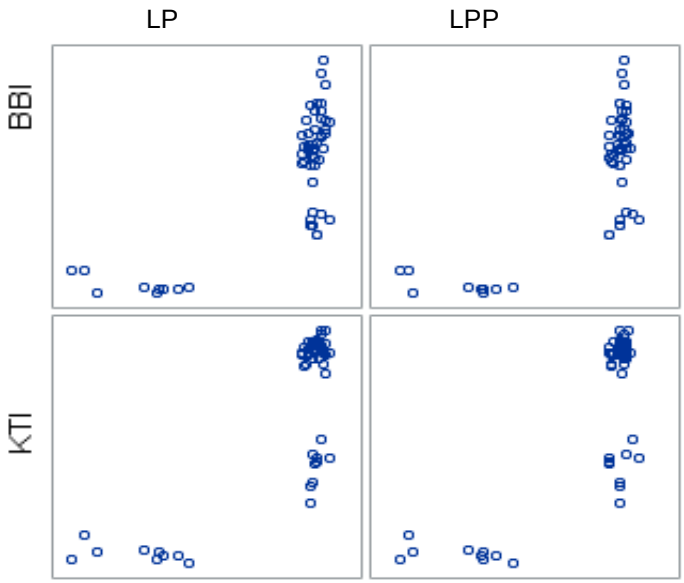


c)

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

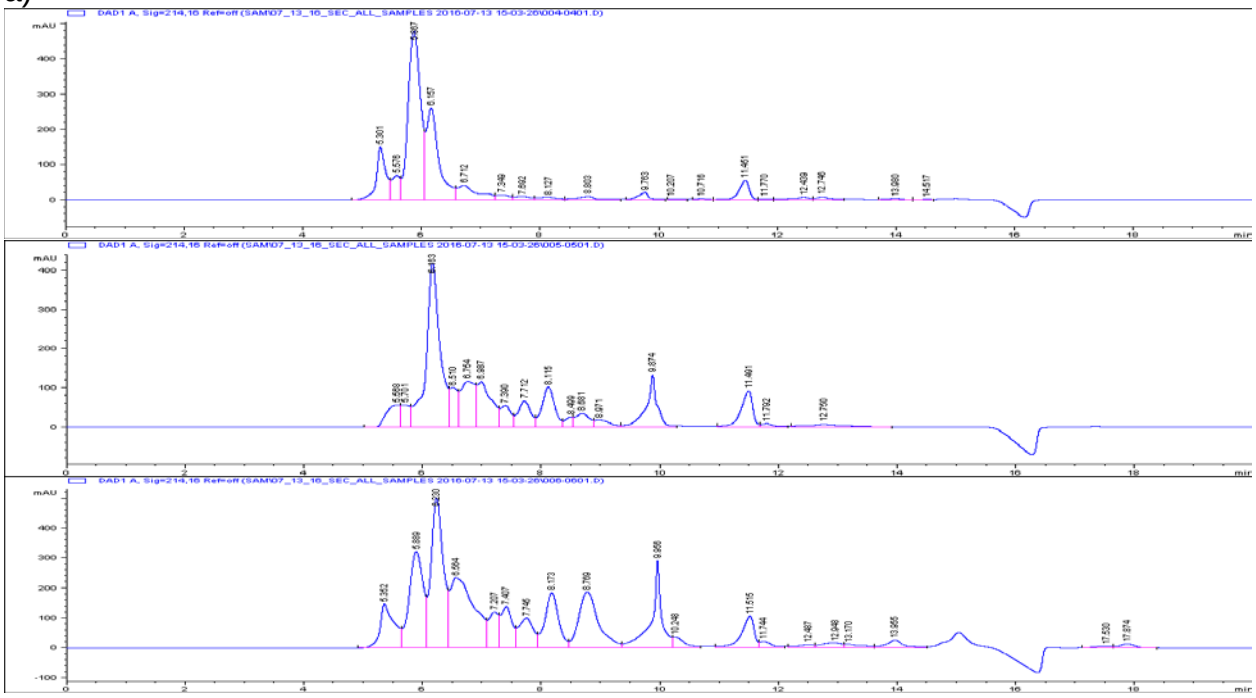
	Parameter	
Parameter	LP	LPP
BBI	0.77 (P < 0.0001)	0.76 (P < 0.0001)
KTI	0.80 (P < 0.0001)	0.80 (P < 0.0001)

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. **3.4**. 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.

Fig. 3.4. continued

a)



b)

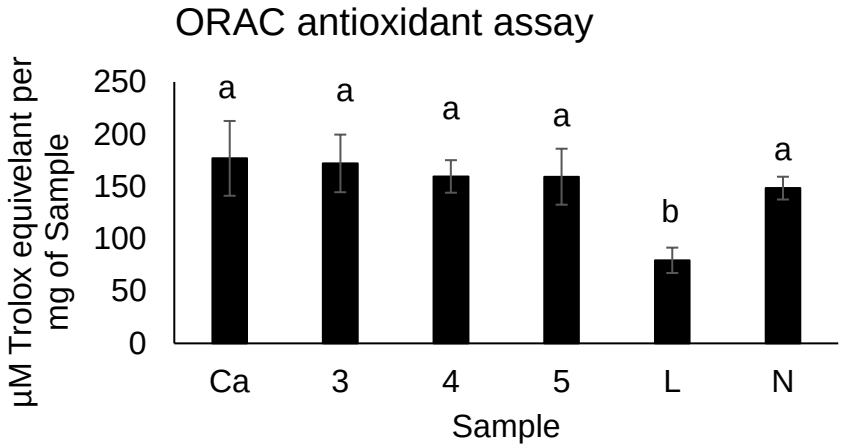
Molecular weight distribution after pepsin and pepsin-pancreatin hydrolysis ofdifferent lunasin-enriched preparations and commercially available products

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. 3.5. 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.

Fig. 3.5. continued

a)



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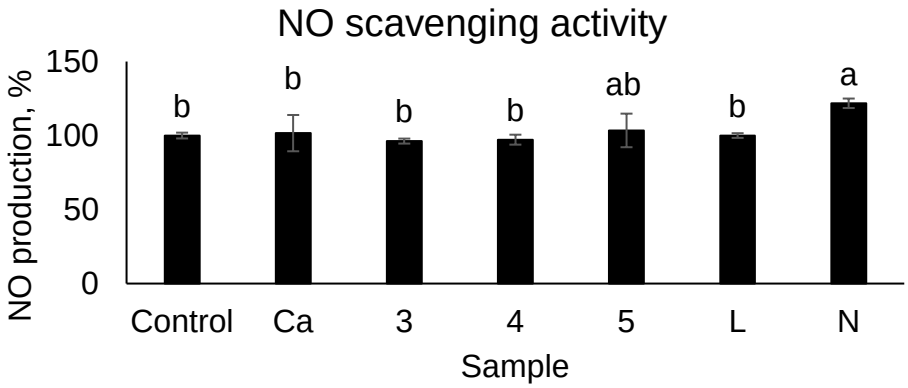

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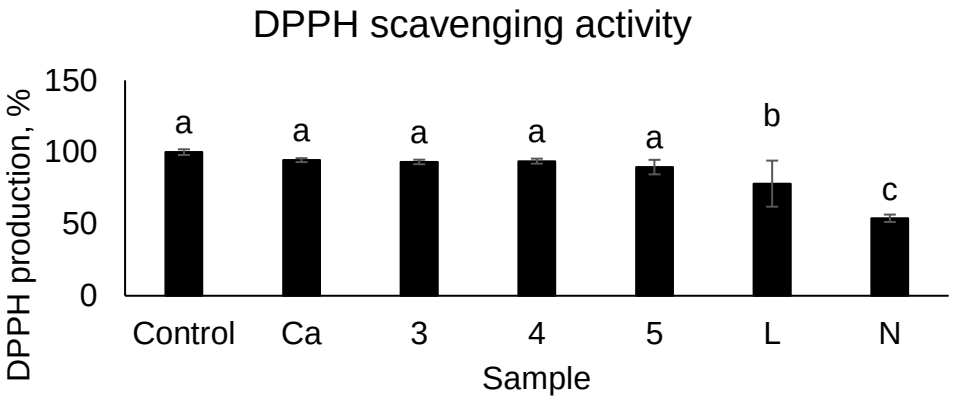
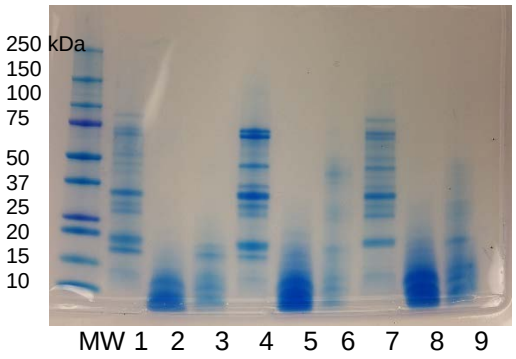


Fig. 3.6. Effect of KTI mutations on the concentrations of bioactive peptides in soybean and their stability against pepsin-pancreatin digestion. a) Electrophoresis profile showing degradation of proteins after pepsin and pepsin-pancreatin digestion Lanes MW: molecular weight marker, 1: control soy flour, 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

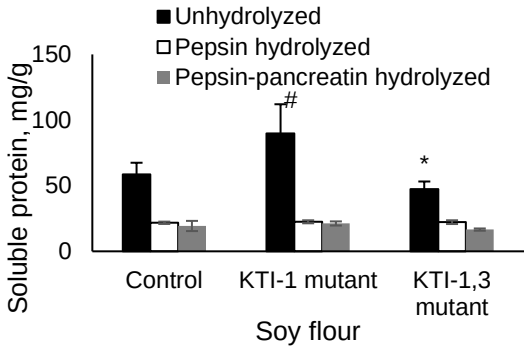
while KTI-1,3 mutation led to a significantly lower residual lunasin concentration after pepsin pancreatin hydrolysis than control soy flour (*, P = 0.0474); f) KTI mutation did not affect the capability of soy flour to inhibit trypsin activity and G) KTI mutation did not affect the antioxidant capacity of soy flour extract as measured by ORAC. Pepsin-pancreatin hydrolysis led to significant lower concentrations of all parameters measured.

Fig. 3.6. continued

a)



b)



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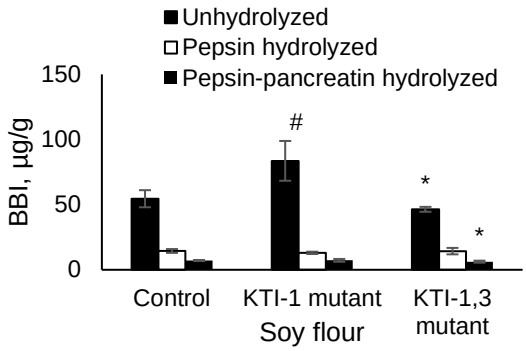

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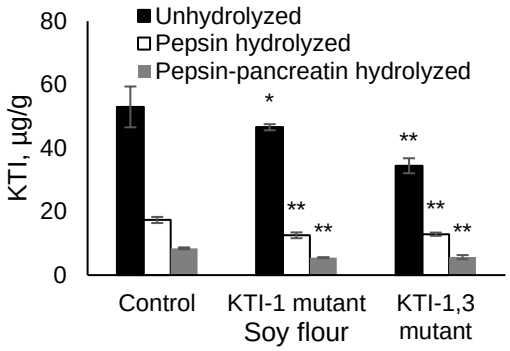

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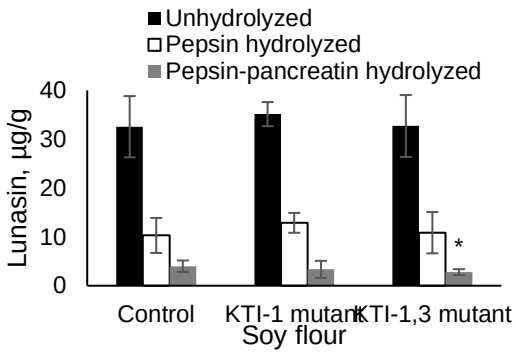

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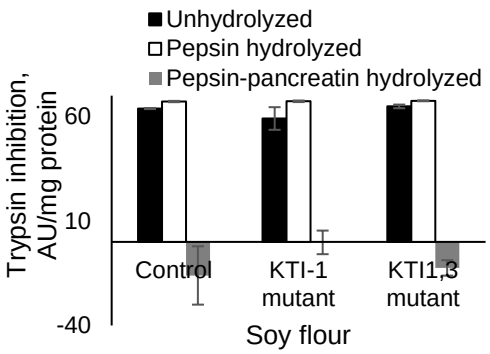


g)

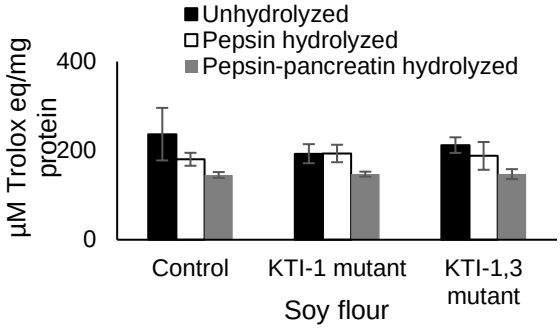
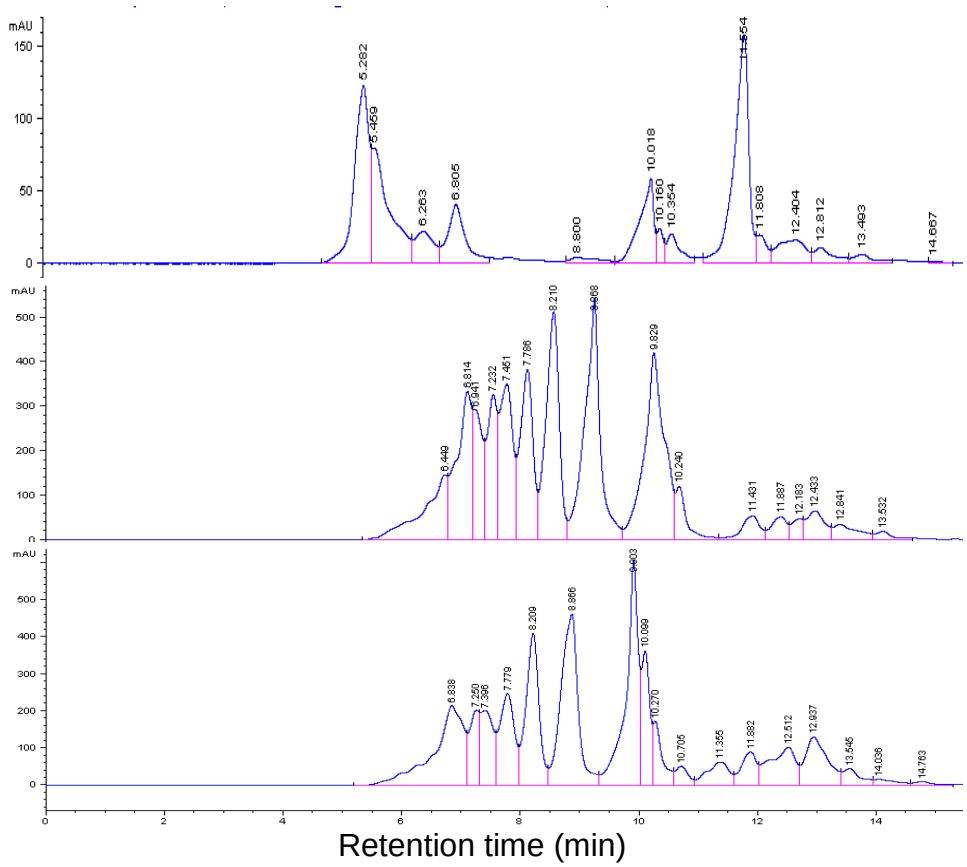


Fig. 3.7. Molecular weight distribution of control, KTI-1 mutant and KTI-1,3 mutant flours as affected by pepsin and pepsin-pancreatin hydrolysis. a) Representative chromatograms of KTI-1 mutant flour (unhydrolyzed, pepsin-hydrolyzed and pepsin-pancreatin-hydrolyzed; from top to bottom) and b) Table inset showing the molecular weight distribution for unhydrolyzed, pepsin-hydrolyzed (P) and pepsin-pancreatin hydrolyzed (PP) soy flours.

Fig. 3.7. continued

a)



b)

Molecular weight distribution after pepsin and pepsin-pancreatin hydrolysis of control, KTI-1 mutant 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

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CHAPTER IV
LUNASIN-ENRICHED MATERIAL INHIBITS ACTIVATION OF THE INFLAMMASOMES IN THP-1 HUMAN MACROPHAGES BY REDUCING REACTIVE OXYGEN SPECIES

Abstract

Inflammation caused by the NLRP3 inflammasome has been linked to many diseases, and multiple pathways to activate the inflammasome have been described using different signals including the production of reactive oxygen species.Lunasin, a bioactive peptide in soybean, was previously reported to inhibit nuclear factor of kappa B-mediated transcription, first step in inflammasomes activation. The objective was to determine the ability of lunasin-enriched preparation (LEP) to inhibit inflammasomes activation *in vitro* using THP-1 human macrophages. THP-1 human monocytes were differentiated into macrophages with cells using phorbol 12-myristate 13-acetatebefore being treated with different concentrations of LEP (0.0625 to 0.25 mg/mL) in the presence of 1 µg/mL lipopolysaccharide as the priming signal for 6 hours. After priming, inflammasomes were activated by adding 5 mMadenosine triphosphate. Unhydrolyzedd LEP treatment at 0.25 mg/mL led to inhibition of IL-1β and IL-18 release in the cell culture supernatant by 52.0 and 49.6%, respectively. In addition, LEP treatment inhibited the production of intracellular reactive oxygen species (ROS) in THP-1 human macrophages without affecting the expressions of NLRP3 and ASC proteins involved in activation of inflammasomes. Pepsin-pancreatin hydrolysis reduced the ability of LEP to inhibit production of intracellular ROS. Our results showed that LEP inhibited activation of inflammasomes by reducing intracellular ROSs *in vitro.*

Introduction

Inflammation occurs when pattern recognition receptors (PRRs) identify exogenous pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Abais, Xia, Zhang, Boini, & Li, 2015). PAMPs are molecules associated with pathogens and infection, such as lipopolysaccharide (LPS), an endotoxin associated with gram-negative bacteria (Kulp & Kuehn, 2012). LPS is recognized by the pattern recognition receptor, PRR Toll-Like Receptor 4 (TLR4) which activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Lu, Yeh, & Ohashi, 2008). DAMPs are molecules that are associated with internal cellular damage and activate NF-κB (Rubartelli & Lotze, 2007). A novel theory is that mitochondrial reactive oxygen species (ROSs) are produced after the cell has been damaged, and induce the priming step of inflammasome activation in which NF-κB transcribes the components of the inflammasome, particularly pro-interleukin-1ß (pro-IL-1ß) and nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) (Tőzsér & Benko, 2016). After priming, DAMPs and PAMPs cause NLRP3 oligomerization and bind to apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) which then binds to pro-cysteine-aspartic protease-1 (pro-caspase-1), resulting in a large oligomer that activates caspase-1 (Tschopp & Schroder, 2010). Once activated, caspase-1 is able to cleave pro-IL-1ß, resulting in the mature IL-1ß which is then

expressed out of the cell and acts as a cytokine, resulting in a cascade of inflammation (Haneklaus, O'Neill, & Coll, 2013). Inflammasomes are associated with chronic inflammation which can damage the integrity of cells, resulting in diseases such as Alzheimer's, type II diabetes, atherosclerosis, and gout (Abais et al., 2015).

Lunasin is a bioactive peptide found in soy with a unique amino acid sequence that includes an arginine-glycine-aspartic acid motif(Lule, Garg, Pophaly, Hitesh, & Tomar, 2015). Previous studies have shown the potential of lunasin to act as a chemopreventive agent (Dia & Mejia, 2010; Shidal, Al-Rayyan, Yaddanapudi, & Davis, 2016;Cruz-Huerta et al., 2015; Hernández-Ledesma, Hsieh, & de Lumen, 2009; Hsieh, Chou, & Wang, 2017). This chemopreventive property of lunasin may be associated with its ability to inhibit inflammation through NF-κB signaling in THP-1 human macrophages (Cam and de Mejia, 2012), the priming step in inflammasome activation. This led us to ask if lunasin-enriched preparation (LEP) can inhibit inflammasomes activation in THP-1 human macrophages.

This study aims to evaluate the ability of LEP to inhibit activation of the inflammasomes *in vitro* and determine the potential mechanism of action. In addition, the effect of pepsin-pancreatin hydrolysis in LEP bioactivity was evaluated.

Materials and Methods

Materials

Prolia defatted soy flour was purchased from Amazon and produced by Cargill Mills (Minneapolis, MN). THP-1 human leukemia monocyte cells were obtained from American Type Culture Collection (Manassas, VA).Roswell Park Memorial Institute-1640 (RPMI) growth media was obtained from Corning Inc. (Conring, NY). Fetal Bovine Serum (FBS) was obtained from Life Tech (Carlsbad, CA). LPS and Phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Primary antibodies (ASC 10500-1-AP, Beta-actin 60008-1-I9, caspase-1 22915-1-AP, and IL-1ß 60136-1-Ig) were obtained from Proteintech Group Inc. (Chicago, IL). Secondary antibodies (Goat anti-mouse 926-80010, goat anti-rabbit 926-80011) were obtained from Li-Cor Biosciences Inc.(Lincoln, NE). ELISA kit for IL-1ß was obtained from BioLegend Inc. (San Diego, CA) and IL-18 kit was purchaseded from Research and Diagnostics Systems Inc.(Minneapolis, MN).

LEP preparation was done by stirring 100g defatted soy flour with 1-L 30% ethanol solution for 2 h at 20 to 22 °C, then followed by centrifugation (8000 rpm, 4 °C) for 30 min. The supernatant was then 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 before dialysis with a 3.5kDa molecular

weight cutoff membrane (Spectrum Labs, Rancho Dominguez, CA) then frozen and lyophilized.

Protein profile by size-exclusion chromatography and high-performance liquid chromatography

The molecular weight profile of the sample was 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 used 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

Cell culture and treatment

THP-1 human leukemia monocyte cells were cultured using RPMI 1640 that contained 10% FBS, 1% penicillin-streptomycin, 1% sodium pyruvate, 10mM HEPES, and 50µM ß-mercaptoethanol. After the cells were allowed to

proliferate, they were treated with 162nM of PMA to allow differentiation into macrophage-like cells as previously described (Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014) with slight modification. Cells were seeded into 6 and 12-well plates at a concentration of 500,000 cells/mL and differentiation occurred for 24h at 37°C in a 5% CO_2/95% air incubator. After differentiation, the cells were treated with 0.25, 0.125, and 0.0625mg/ml LEP in the presence of 1 µg/mL LPS for 6 h. After 6 h, ATP was added at 5 mM to treat cells for another hour. Negative control cells were left in plain RPMI. The concentration of LEP did not affect viability of THP-1 cells as determined by MTS assay.

Whole cell lysates preparation and cell culture supernatant collection

After the cells were treated, the plates were placed on ice while the supernatant was collected in microcentrifuge tubes, then the cells were washed with ice cold PBS twice to remove cell debris. After washing, 100 µL of RIPA lysis buffer (protease inhibitor cocktail to RIPA 1:100) was added to each well and incubated for 5min at 4°C. Cells were harvested using cell scrapers and added to pre-chilled microfuge tubes, then vortexed for 5min at 4°C before being centrifuged at 14,000 x *g* for 10min at 4°C. The supernatant was then collected and the pellet discarded. Bradford protein analysis assay was performed to determine protein

concentration using bovine serum albumin as the standard, before denaturation with Laemmli buffer containing 5% ß-mercaptoethanol and boiling for 5 minutes.

Western blot for NLRP3 and ASC

SDS-PAGE under reducing condition was carried out by loading approximately 10 µg of protein in 4–20% Mini-Protean TGX gels (Bio-Rad Laboratories, Hercules, CA). After the SDS-PAGE was runat 200 V for 35 min, 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 polyvinylidene fluoride 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 againstNLRP3 (NOD-like receptor family pyrin domain containing 3), ASC (Apoptosis-associated speck-like protein containing a CARD) at a 1:2000 dilution, and Beta-actin at a 1:5000 (Proteintech Group, Chicago IL) for 3 h at 20 to 22 °C. After incubation, the membrane was washed as described previously, and incubated with secondary antibodies at a 1:1000 for 1.5 h at 20 to 22 °C(goat anti-rabbit for ASC and NLRP3, and goat anti-mouse for Beta-actin). After washing as above, 300µl of a 1:1 mixture of WesternSure Premium stable peroxide and luminol enhancer

was added and the membrane was imaged by chemiluminescence using C-Digit blot scanner (Li-Cor Biosciences, Lincoln, NE). Membranes were stripped for reprobing with β-actin using a mild stripping buffer twice for 10 min, followed by a wash with PBS twice for 10 min, then washed with TBST twice for 5 min before being blocked as described previously.

Measurement of intracellular reactive oxygen species

Fluorescence microscopy. THP-1 cells were treated as described previously. After the treatment, the plate was placed on ice and the supernatant was removed by pipetting before the cells were washed with 500 µL ice-cold PBS twice. After washing, 1 mL of 10 µM 2'7'-dichlorofluoroscein diacetate was added to each well before being incubated at 37°C for 10 min in a 5% CO_2 incubator. After incubation, the dye was removed and the cells were washed with PBS twice before pictures were taken using a 20x magnification green fluorescent protein (GFP) channel on an Evos microscope (Thermo Fisher Scientific, Waltham MA).

Fluorescence spectrophotometry. THP-1 cells were treated and harvested as described previously, and after the wash step, 200 µL of a 0.25% trypsin in 1mM ethylenediaminetetraacetic acid was added to each well and incubated for 5 min to detach cells from the plate. After incubation, 800 µL of complete RPMI was added to inactivate trypsin, and the media was collected and centrifuged at 500 x

g for 5 min. The supernatant was then discarded and the pellet was re-suspended in 500 µL of PBS containing 10 µM 2'7'-dichlorofluoroscein diacetate and incubated for 30 min at 37°C in a 5% CO_2 incubator before being vortexed and 200 µL of the suspension was plated in duplicate on a 96-well plate and read with a fluorescence intensity at 485 nm/528 nm.

Flow cytometry. THP-1 cells were treated the same as described in fluorescence spectrophotometry*,* except the number of cells with green fluorescence and intensity were measured using an FL 1 channel (FITC) on a MACSQuant flow cytometer from (Miltenyi Biotec Inc., Bergisch Gladbach, Germany).

Measurement of released interleukin-1ß and interleukin-18 in the cell culture supernatant by enzyme-linked immunosorbent assay (ELISA)

Interleukin-1ß. Measurement ofextracellular IL-1ß that was released into the supernatant was performed by ELISA, using a Human IL-1ß ELISA MAX™ Deluxe Set obtained from BioLegend Inc. (San Diego, CA). One hundred µL of detection antibody was added to an uncoated 96-well ELISA plate and incubated for at least 14 h at 4°C. After incubation, the plate was washed with 300 µL of washing buffer (0.5% Tween 20 in PBS) four times, then blocked by adding 200 µL of assay diluent A and incubated for 1 h on an orbital shaker at approximately 50rpm at 20 to 22 °C. After blocking, the plate was washed as previously described, and 50 µL of Assay Buffer D and 50 µL of diluted samples (1:50) and

standard were added in duplicate and incubated for 2 h under the same conditions. After incubation with the samples, the plate was washed again and 100 µL of diluted Detection Antibody were added to each well and incubated for 1 h under the same conditions. After washing, 100 µL of diluted avidin-horseradish peroxidase solution was added to each well and incubated for 30 min under the same conditions. Then, the plate was washed 5 times with 30 s of soaking between each wash, and 100 µL of substrate solution F was added and incubated in the dark for 20 min before adding 100 µL of 2 N H_2SO_4 to stop the reaction. The plate was immediately read at 450 nm using a Cambrex microplate reader (BioTek Inc, Winooski, VT).

Interleukin-18. Measurement of interleukin-18 that was released into the supernatant was performed using an ELISA kit obtained from R&D Systems (Research and Diagnostics Systems Inc., Minneapolis MN) and slight modifications to the IL-18 method: the detection antibody was incubated for 2 h, and streptavidin-HRP was incubated for 30 minutes.

Statistical Analysis

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

Results

Characterization of lunasin-enriched preparation

Fig. 4.1a (all figures are listed in the appendix of this chapter) shows the size exclusion chromatography profile of LEP before and after pepsin-pancreatin hydrolysis. As shown, pepsin-pancreatin hydrolysis led to digestion of high molecular weight compounds to low molecular weight molecules. In addition, HPLC analysis (**Fig. 4.1b**) showed that pepsin-pancreatin hydrolysis led to reduction of intact lunasin in LEP.

Effect of lunasin-enriched preparation on secretion of IL-1β and IL-18 in cell culture supernatant

Fig. 4.2a shows the effect of different treatment regimens on the production of IL-1ß by THP-1 human macrophages. Compared to untreated cells producing a small amount of IL-1ß, addition of LPS led to a significant increase in the amount of IL-1ß produced. Addition of ATP led to further increase in IL-1ß indicating activation of inflammasome. LEP treatment led to a significant reduction of IL-1ß secretion indicating the ability of LEP to inhibit inflammasome activation. Unhydrolyzed LEP at 0.25 mg/mL reduced IL-1ß secretion by 52.0% while hydrolyzed LEP at the same concentration reduced IL-1ß secretion by 75.9%.

Fig. 4.2b shows the effect of different treatments on IL-18 production by THP-1 human macrophages.At all LEP concentrations, unhydrolyzed LEP significantly

reduced IL-18 production ranging from 49.7% to 63.6%. On the other hand, only 0.25 mg/mL LEP-hydrolyzed treatment led to significant decrease in IL-18 production by 49.6%.

Effect of lunasin-enriched preparation did not affect the expression of NLRP3 and ASC proteins involve in inflammasomes in THP-1 human macrophages

Fig. 4.3 indicates that LEP treatment, whether unhydrolyzed or hydrolyzed, did not affect expression of NLRP3 and ASC proteins involve in inflammasome.

Effect of lunasin-enriched preparation on production of intracellular reactive oxygen species in THP-1 human macrophages

THP-1 macrophage like cells after treatment with LEP, LPS, and LPS + ATP were incubated with 2'7'-dichlorofluoroscein diacetate, a non-fluorescent dye that fluoresces after oxidation caused by ROSs(Owusu-Ansah, Yavari, & Banerjee, 2008). Images show increased fluorescence with LPS and LPS + ATP (**Fig. 4.4)** compared to the control, as well as a significant decrease in fluorescence with increased concentrations ofunhydrolyzed LEP. On the other hand, slight reduction was observed in cells treated with hydrolyzed LEP. To confirm this observation, quantitative fluorescent spectrophotometry and flow cytometry were

conducted.At all concentrations used, unhydrolyzed LEP led to a significant reduction in mean fluorescence intensity as compared to LPS + ATP-treated cells (**Fig. 4.5a)**. At 0.25 mg/mL unhydrolyzed LEP, the mean fluorescence intensity was reduced from 723.25 (LPS + ATP) to 362.25 (LPS + ATP + 0.25 mg/mL unhydrolyzed LEP) which is equivalent to 49.9%. On the other hand, hydrolyzed LEP significantly reduced the production of intracellular ROS only at the highest concentration used (0.25 mg/mL), by 30.9%. **Fig. 4.5b** shows the mean and median fluorescence intensity from flow cytometry compared with the percentage of stained cells in both unhydrolyzed and hydrolyzed LEP treatments, as well as the histogram plots of the observed FITC-A fluorescence of the different treatments. In both treatments, addition of LPS alone and LPS + ATP led to a significant increase in the percentage of cells stained as well as mean and median fluorescence intensities. In THP-1 cells treated with unhydrolyzed LEP, the highest LEP concentration treatment at 0.25 mg/mL led to significant reduction in the percentage of stained cells, mean fluorescence intensity and median fluorescence intensity by 40.8%, 31.4% and 45.4%, respectively. On the other hand, hydrolyzed LEP at 0.25 mg/mL only reduced the percentage of stained cells and mean fluorescence intensity by 19.5% and 33.9%, respectively. The observations from flow cytometry are in agreement with the fluorescence microscopy and fluorescence spectrophotometry experiments. Representative histograms for all treatments are also shown in **Fig. 4.5.** The right shift from the treatment of LPS alone to the LPS + ATP treatment indicating the increased

fluorescence was counteracted by addition of 0.25 mg/mL LEP, both hydrolyzed and unhydrolyzed, in the LPS + ATP treatment.

Discussion

Inflammation is linked to multiple diseases, such as in neurodegenerative disorders (Gao & Hong, 2008; Guo, Callaway, & Ting, 2015; Mao et al., 2017), type II diabetes (Strowig, Henao-Mejia, Elinav, & Flavell, 2012), inflammatory bowel disease (Peyrin-Biroulet, Lémann, 2011; Ruffolo et al., 2010), and cancer (Balkwill & Mantovani, 2001; Mantovani, Allavena, Sica, & Balkwill, 2008; Simone Reuter, 2011). Inflammation, and complications associated with it, account for approximately 15% of cancer-related deaths (Mantovani et al., 2008). If chronic inflammation can be inhibited without disruption of normal cell functions, this could lead to breakthroughs in possible treatments and preventative measures for these diseases. One possible mechanism for inhibiting chronic inflammation is inhibiting the inflammasome, a multi-protein oligomer that is responsible for the production of pro-inflammatory cytokines, and only activated under specific conditions (Abais et al., 2015; Martinon, Burns, & Tschopp, 2002).

Lunasin has been reported to have anti-cancer and anti-inflammatory properties (Hernández-Ledesma et al., 2009; Hsieh et al., 2017), and ROSs have been linked to activation of the inflammasome (Abais et al., 2015; Guo et al., 2015), but to our knowledge no studies have been done to determine if lunasin

can inhibit the inflammasome by reduction of ROSs within the cell. This study used lunasin-enriched preparation that was obtained by calcium chloride precipitation (Price et al., 2016) to study its effect on inflammasome activation *in vitro* using THP-1 human macrophages as well as LPS and ATP signals, known initiators of inflammasome activation (Tschopp & Schroder, 2010). Inflammasomes require a two-step process in order to activate, first is priming with LPS which acts as an upstream regulator of NF-κB which transcribes pro-IL-1β and NLRP3 (Ghonime et al., 2014), and then activation with ATP, which releases ROS within the cell, which is critical for the binding sites of the NLRP3 to form a disulfide bond allowing oligomerization with ASC and pro-caspase-1 to form the inflammasome (Abais et al., 2015; Bae & Park, 2011). ROSs have also been shown to be crucial in the activation of active caspase-1 by oxidizing thioredoxin (TRX), a redox protein, releasing thioredoxin-interacting protein (TXNIP) which binds to the NLRP3 portion of the inflammasome and has been proven to be a key step in activation (Abais et al., 2015; Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010). One of the most detrimental effects of this immune response is the cascade of pro-inflammatory cytokines, which act as the priming step like LPS, and the resulting cell and tissue death associated with sterile inflammation (Rock, Latz, Ontiveros, & Kono, 2010). Cells treated with LEP showed significant reduction in pro-inflammatory cytokine production expressed into the supernatant, indicating there was an effect on the inflammasome pathway, leading us to study the mechanism of action involved. Since there was

a reduction in cytokine expression, but NLRP3 and ASC were not affected by LEP indicating a different mechanism of action.Our study showed the effect of LEP in the production of intracellular ROS (**Fig. 4.4 and 4.5**) which could potentially explain the observed reduction in the production of pro-inflammatory cytokines IL-1β and IL-18 (**Fig. 4.2**). Previous studies have shown the ability of lunasin to act as antioxidant leading to reduced inflammation(Hernández-Ledesma, Hsieh, & de Lumen, 2009). In addition, lunasin prevented galactose-induced cataract formation in rats by attenuating oxidative damage through upregulation of antioxidant enzymes (Dai et al,, 2016) and protected DNA by suppressing generation of hydroxyl radical via blockage of the Fenton reaction (Jeong, de Lumen & Jeong, 2010). Our current study supported these previous studies showing the ability of LEP to reduce intracellular ROSs in THP-1 human macrophages involved in inflammasome activation.

We also observed that pepsin-pancreatin hydrolysis led to lower ability of LEP to reduce intracellular ROS in THP-1 human macrophages treated with LPS and ATP. This may be attributed to reduction in amount of bioactive peptides present in hydrolyzed LEP as our previous study showed reduction in the concentrations of lunasin, Bowman-Birk inhibitor and Kunitz-type trypsin inhibitor after pepsin-pancreatin hydrolysis (Price, Pangloli, Krishnan, & Dia, 2016). In the previous study, more intact lunasin was associated with higher anti-proliferative effects in colon cancer cells (Cruz-Huerta et al., 2015) and addition of BBI complemented the anticancer effect of lunasin in xenograft model of breast

cancer (Hsieh, Hernandez-Ledesma, Jeong, Park & de Lumen, 2010). This indicated the importance of protecting lunasin from digestion to exert its potential chemoprevetive effect. **Fig. 4.6** shows the proposed mechanism that lunasin uses to inhibit the production of pro-inflammatory cytokines IL-1ß and IL-18. Since lunasin was able to reduce cytokine expression and intracellular ROSs, but not inflammasome associated proteins, the two proposed mechanisms are blocking ROSs from activating the inflammasome, and inhibiting activation of the pro-interleukins.

In conclusion, lunasin-enriched products are able to inhibit inflammasome activation in THP-1 human macrophages by reducing intracellular ROS,indicating its potential use in aiding relief of chronic inflammation. Since the NLRP3 and ASC proteins were not affected by LEP treatment in LPS+ATP inflammasome-activated THP-1 human macrophages, further studies are needed to determine the exact mechanism lunasin uses to inhibit inflammasome.

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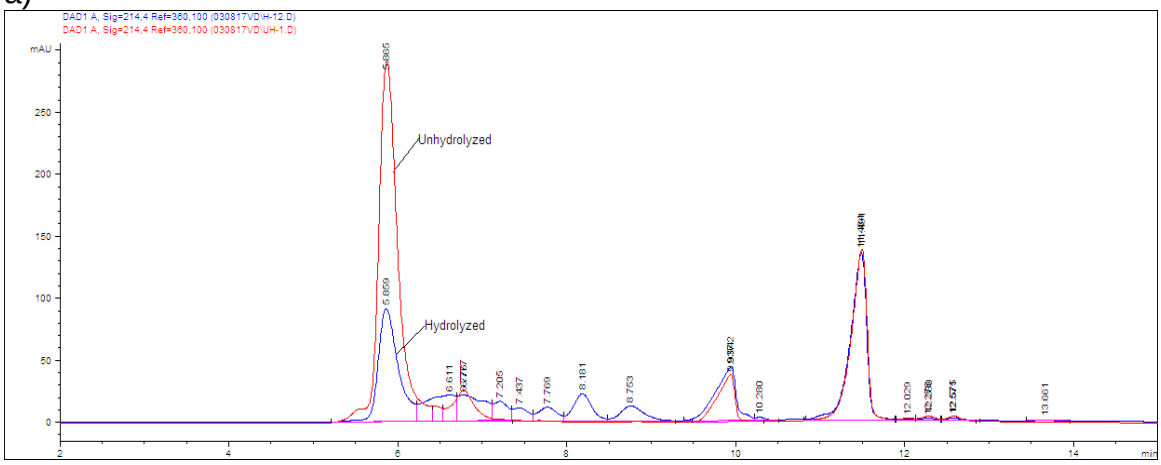
Appendix

Figures

Fig. 4.1. Protein profile of lunasin-enriched material prepared by calcium chloride precipitation methods. Figure 4.1a shows size-exclusion chromatography of $CaCl_2$ lunasin-enriched product, chromatogram overlays hydrolyzed (blue) and unhydrolyzed (red) samples. Unhydrolyzed sample contains large peak associated with high molecular weight compounds which were reduced after pepsin-pancreatin hydrolysis. Figure 4.1b shows high-performance liquid chromatography results of hydrolyzed sample (red), unhydrolyzed (green), and lunasin standard (blue). Results show a reduction of peaks in hydrolyzed sample, correlating to reduction of peptides during hydrolysis.

Fig. 4.1. continued

a)



b)

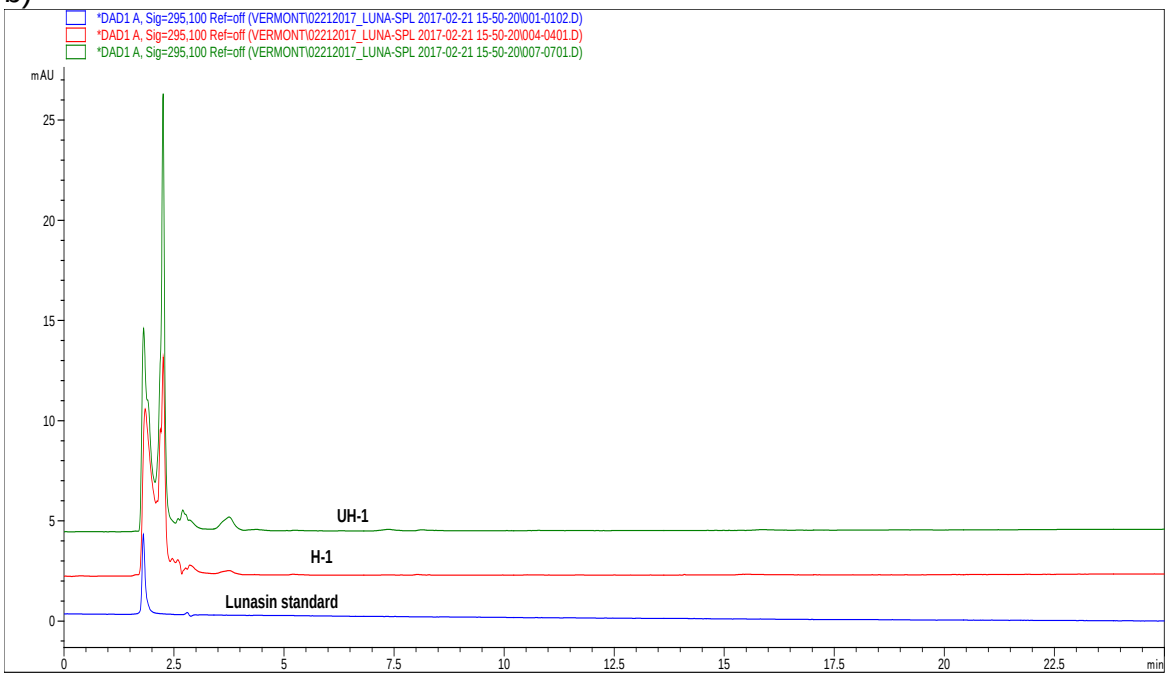
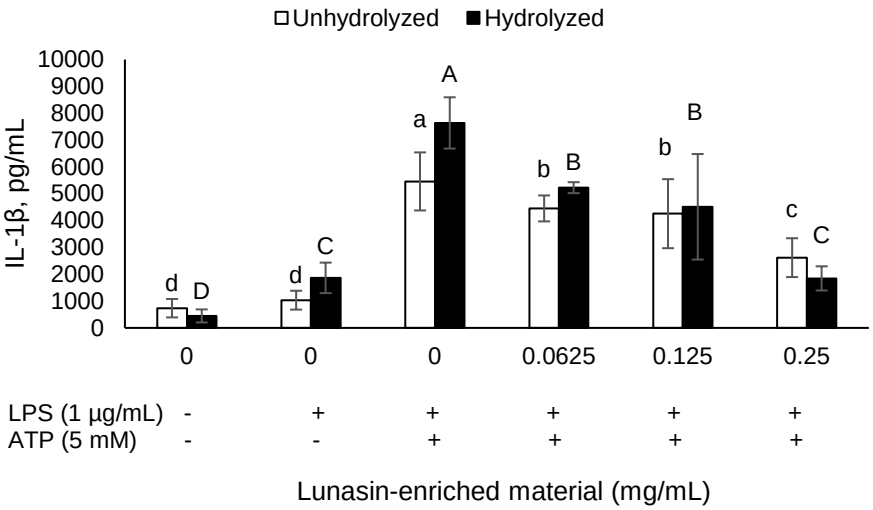


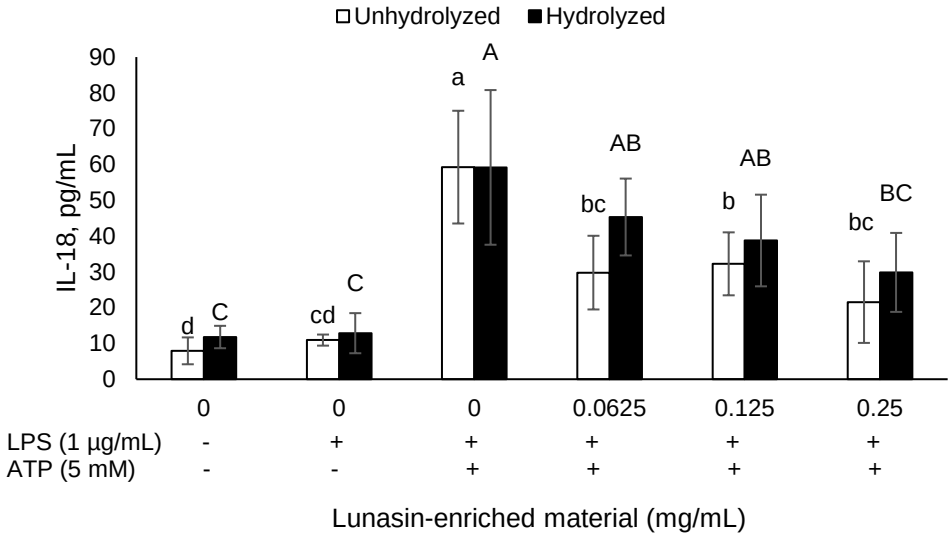
Fig. 4.2. Lunasin-enriched preparation reduced the production of IL-1β and IL-18 in THP-1 human macrophages treated with LPS and ATP for inflammasome activation. Figure 4.2a shows an overall reduction of IL-1β expression in LEP treated cells compared to the positive control of the LPS + ATP treated cells. Bars with different letter(s) are significantly different from each other ($P<0.05$). Figure 4.2b shows the reduction in IL-18 secretion by THP-1 human macrophages when treated with LEP. Bars with different letters were significantly different from each other ($P<0.05$, $n = 6$).

Fig. 4.2. continued

a)



b)



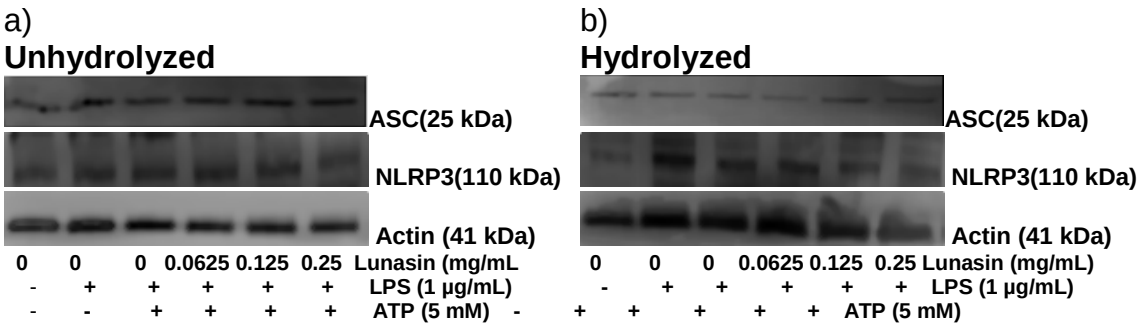


Fig. 4.3. Lunasin-enriched preparation did not affect the expression of NLRP3 and ASC proteins involved in the formation of inflammasome macromolecular complex in THP-1 human macrophages.

Fig. 4.4. Lunasin-enriched preparation reduced production of intracellular reactive oxygen species as measured by fluorescence microscopy. Unhydrolyzed LEP (a) is more effective in lowering ROS production than hydrolyzed LEP (b).

Fig. 4.4. continued

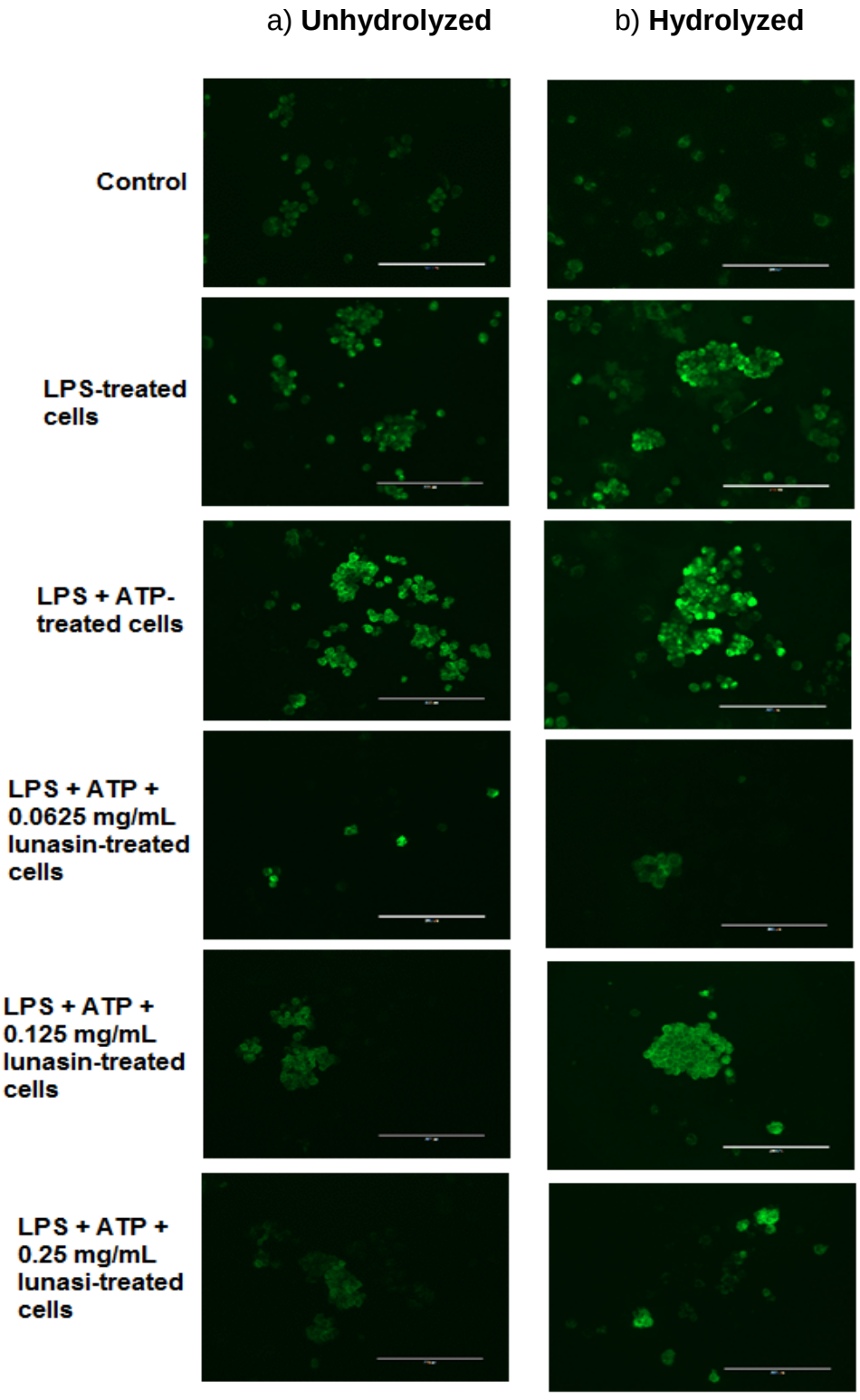
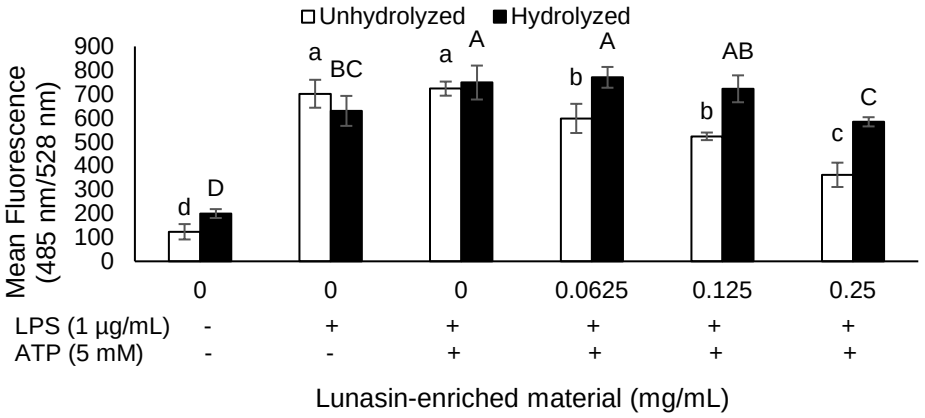


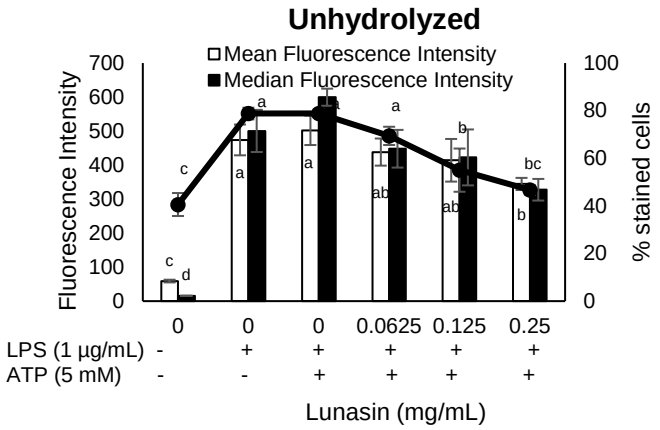
Fig. 4.5. Measurement of intracellular ROS in THP-1 macrophages by fluorescence spectroscopy and flow cytometry shows that lunasin-enriched treatment reduced intracellular reactive oxygen species production in THP-1 human macrophages treated with LPS and ATP. a) Mean values of fluorescence spectroscopy indicate intracellular ROS was produced after treatment with LPS and ATP, with all values differing significantly from the negative control. The highest concentration of hydrolyzed and unhydrolyzed LEP treatment (0.25mg) differ significantly from LPS + ATP treatment. The three concentrations of unhydrolyzed LEP differ significantly from both LPS and LPS + ATP treatment, but the two lower concentrations of hydrolyzed LEP did not significantly reduce ROS compared to the LPS and LPS + ATP treatments. b) Mean and median fluorescence intensity of flow cytometry compared with percentage of cells that were stained. Highest concentration of hydrolyzed and unhydrolyzed LEP treated cells differ significantly from LPS and LPS + ATP treated cells. Flow cytometry histogram shows a decrease in FITC-A corresponding to LEP treatment concentrations. Means with different letter(s) are significantly different from each other ($P<0.05$, $n = 4$).

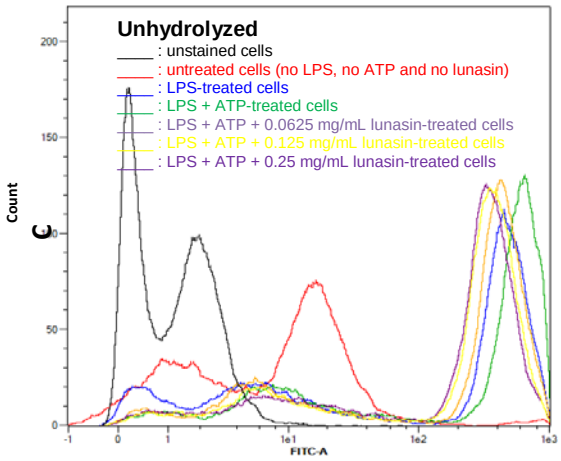
Fig. 4.5. continued

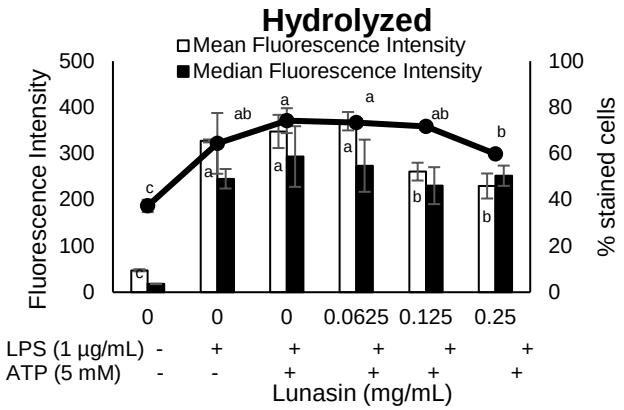
a)



b)







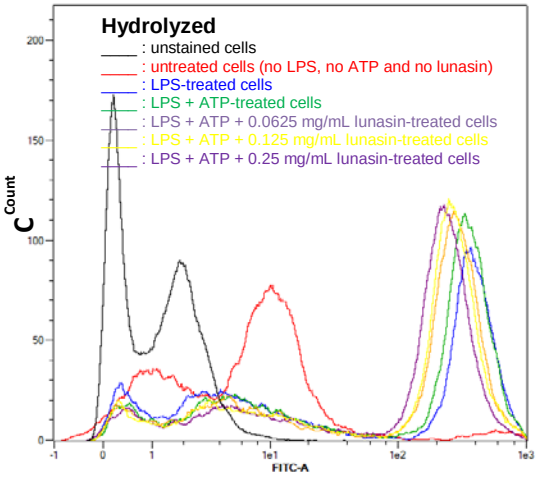
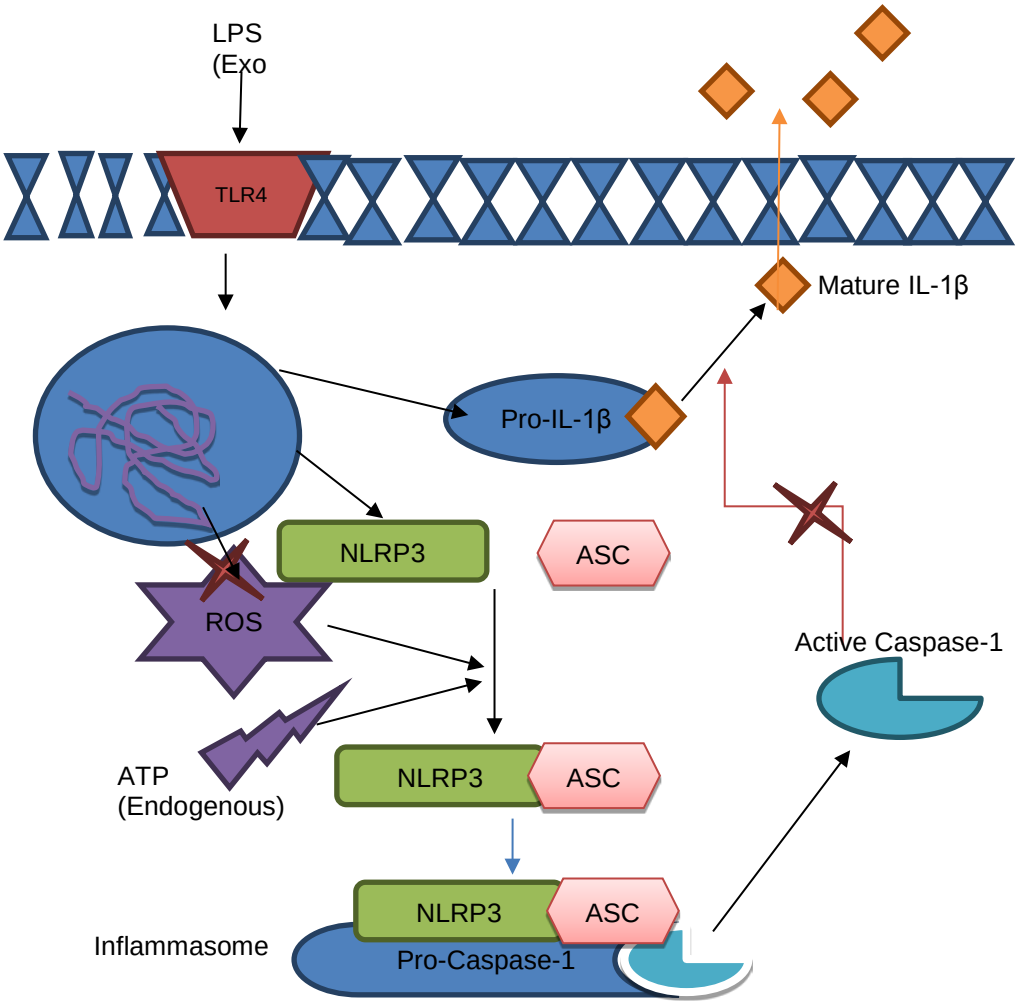


Fig. 4.6. Proposed mechanism by which lunasin-enriched preparation (LEP) inhibit activation of inflammasomes in THP-1 human macrophages by LPS and ATP. Treatment of LPS and ATP lead to increase production of intracellular reactive oxygen species (ROS) which is capable of promoting oligomerization of NLRP3 and ASC components of inflammasome. This oligomer will then bind to pro-caspase-1 resulting in processing and activation of pro-caspase-1 to caspase-1. Active caspase-1 is responsible for the maturation of pro-inflammatory cytokines IL-1β and IL-18 which are responsible for chronic inflammation. LEP treatment (✗) led to reduced production of these pro-inflammatory cytokines by reducing the amount of intracellular ROS induced by LPS and ATP treatment.

Fig. 4.6. continued



CHAPTER V
CONCLUSIONS

Production of lunasin-enriched materials was accomplished by both calcium chloride and pH precipitation methods. Bowman-Birk and Kunitz-Trypsin inhibitor played an important role in stabilizing lunasin against pepsin-pancreatin hydrolysis, and the use of KTI-mutant soybean study proved that KTI played a critical role in the stabilization of lunasin. Treatment of THP-1 human macrophagesprimed and activated by LPS and ATP for inflammasome activation with lunasin-enriched material led to reduction of intracellular reactive oxygen species which resulted in reduced pro-inflammatory cytokines. This research is the first to report the following:

- Importance of Kunitz trypsin inhibitor in the stability of lunasin against pepsin-pancreatin hydrolysis; and
- Ability of lunasin-enriched material to inhibit activation of inflammasomes *in vitro*

Determination of the exact component present in lunasin-enriched material responsible for the inhibition of inflammasomes is warranted. In addition, other mechanism of action involved in the inhibition of the inflammasomes must be studied. An *in vivo* study would be beneficial in determining if lunasin has the potential to be used as a chemopreventive agent against inflammation associated diseases.

VITA

Samuel James Price attended Pellissippi State Community College (Knoxville, TN) for Hospitality Business and Culinary Arts from Fall of 2009 to Spring of 2012. After that, he attended the University of Tennessee where he obtained a Bachelor of Science degree in Food Science in Spring 2015. He continued to pursue a Master of Science degree in Food Chemistry from the Fall of 2015 to the Spring of 2017. While pursuing his M.S degree, he published his research in *Food Research International*. While pursuing his B.S degree, he became a member of FarmHouse Fraternity, where he held multiple chairs and leadership positions. He has worked in various positions while obtaining his degrees, at places such as Husk Nashville, House of Thaller, and The Irish Times.