Anti-inflammatory and chemopreventive activity of lunasin from tofu whey for the management of gastrointestinal diseases

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DEDICATION

To my family and husband-to-be, the engine of my life and the best reason to dream, try, and achieve anything.

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ABSTRACT

Gastrointestinal diseases such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC) are pathological conditions associated with chronic inflammation, characterized by intestinal damage, debilitating symptoms, and detrimental health consequences. The increased risk of CRC in IBD patients, and the adverse effects associated with current therapeutic strategies, point out the need for safer alternatives to reduce chronic inflammation in the bowel. Lunasin is a bioactive peptide naturally occurring in soybeans, with chemopreventive and anti-inflammatory properties demonstrated in several extra-intestinal diseases. However, to date, there is no evidence of the biological activity of lunasin on the gastrointestinal tract as a target site. The objective of this research was to investigate the biological activity of lunasin against bowel-related diseases using *in vivo* models. First, lunasin was isolated from tofu wastewater applying a designed process that combines precipitation techniques and chromatographic purification. The three-step isolation process was applied to industrially produced tofu wastewater yielding 773mg lunasin of 80% purity per kg dry tofu whey, with anti-inflammatory properties as tested *in vitro* using activated murine macrophages. Then, the anti-inflammatory activity of lunasin in the gut was determined using an *in vivo* model of spontaneous colitis. Oral administration of lunasin reduced pro-inflammatory cytokines throughout the small and large intestines and decreased the frequency of clinical manifestations of IBD in IL-10$^{-/-}$ mice. *In vitro* analysis indicated that the mechanism of action is partially

associated with the ability of lunasin to modulate the activation of the NLRP3 inflammasome. Finally, the chemopreventive activity of lunasin in the bowel was evaluated using an *in vivo* model of colitis-associated CRC. Oral administration of lunasin decreased the impact of inflammation over the colon, facilitated recovery from episodes of mild and moderate inflammation, reduced tumor burden, and mitigated the progression of malignant features. The results of this study demonstrate: 1) the presence of bioactive lunasin in tofu wastewater, 2) the anti-inflammatory and chemopreventive activity of lunasin in the bowel, and 3) the modulatory activity of lunasin over the NLRP3 inflammasome. This research can serve as a basis for further investigation on the active incorporation of lunasin for the management of IBD.

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CHAPTER 1. INTRODUCTION

1.1 Abstract

Inflammatory bowel disease (IBD) is a debilitating health condition that can lead to detrimental and life-threatening consequences. Genetic susceptibility, environmental conditions, a compromised intestinal epithelial barrier, and dysregulation of the intestinal microbiota, are all risk factors that contribute to the impairment of intestinal homeostasis. When this happens, the immune system is triggered, and cytokines come into play to drive the initiation and control progression and maintenance of a state of chronic inflammation in the bowel. Diet has been suggested as one of the main factors contributing to IBD, with bioactive compounds from plant-based foods potentially providing a protective effect. The widely recognized bioactive properties of the soybean peptide lunasin, make it a potential candidate for the management of inflammation-related diseases. However, lunasin research has been focused on extraintestinal conditions, and to date the knowledge about the interaction of lunasin with the gastrointestinal tract is incipient. Therefore, studying the biological activity of lunasin in the bowel, and particularly in the context of inflammation, is essential to identify its potential utilization for the management of bowel-inflammation related diseases.

1.2 Literature Review

Exposure to potentially harmful elements is inherent to the interaction with the environment around us. Inflammation, when properly regulated and resolved, is one of the most efficient mechanisms of defense that our body has developed to ensure our survival and maintain our integrity. However, when inflammation is subnormal, excessive, or fails to resolve, it becomes harmful to the host and results in negative consequences. Non-resolved inflammation is currently considered a major driver of diseases and has directly been linked to diverse chronic conditions such as asthma, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, obesity, IBD, and cancer [1].

1.2.1 The gastrointestinal tract and inflammatory bowel disease

The gastrointestinal tract (GIT) is one of the most complex entities in the human body. In the GIT, the symbiotic relationship between the intestinal epithelium and a wide diversity of commensal microbiota orchestrate the processes required for nutrition, mediating the breakdown of food components, the selective absorption of nutrients, and the exclusion of waste and harmful compounds from the body [2]. With about 5 meters in length and 32 m^2 surface area [3], the GIT provides one of the largest interfaces between our body and the external environment. A thick layer of mucus, secretory immunoglobulins, enzymes, and antimicrobial peptides, among other components, contribute to the physical barrier created by the intestinal epithelium, and in conjunction with the immune system, regulate the safe interaction with the microbiota and the

components in the luminal content [2]. While in healthy individuals anti-inflammatory mechanisms prevent spontaneous inflammation and contribute to the resolution of episodes of acute inflammation, facilitating intestinal homeostasis, dietary and environmental factors leading to persistent inflammatory stimuli, abnormal immune response, and inappropriate anti-inflammatory regulation, contribute to the initiation and maintenance of non-resolved inflammation [1].

IBD is a debilitating condition of chronic inflammation of the gastrointestinal mucosa [4], that not only affects the well-being and impairs the quality of life of the patients, but can lead to life-threatening conditions such as bowel perforation, toxic megacolon, and cancer [4,5]. More than 6.8 million people are estimated to live with IBD worldwide [6], and newly industrialized countries in Africa, Asia, and South America show an accelerating incidence as their societies become more westernized [7]. In the USA, patients with IBD incur over three-fold higher direct costs (around $23,000 per member per year) than people not affected by IBD due to health care utilization, treatment with specific therapeutics, out-of-pocket expenditures, and workplace productivity losses [8].

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of IBD. Although CD is more common in areas between the terminal ileum and the perianal area, it can affect the entire thickness of the bowel in any section of the GIT, in a characteristic discontinuous pattern in which healthy regions of the intestine are mixed with inflamed areas [9]. In contrast, UC only affects the

innermost lining of the intestinal wall (the mucosa), begins in the most distal region of the bowel, the rectum, and progresses continuously toward the ascending, transverse and descending colon, being limited to the large intestine [9]. Symptoms of IBD depend on the localization of the affected area and the level of involvement, but the most common clinical manifestations include diarrhea, rectal bleeding, loss of body weight, abdominal pain, appetite changes, nausea, vomiting, and in the most severe cases physiological manifestations associated with bowel obstruction, infection, malnutrition, anemia, among others can occur [4]. But the effects of inflammation in IBD are not only limited to the gut. Extraintestinal manifestations are commonly observed in 25 to 40% of IBD patients and involve the skin, eyes, skeletal muscle, liver, kidneys, and the metabolic system [10].

Cardiovascular disease and colorectal cancer (CRC) account for the most common causes of death among IBD patients [5]. Indeed, increased circulating levels of pro-inflammatory mediators such as TNF-α and nitric oxide, bacterial endotoxins reaching systemic circulation due to disruption of the intestinal barrier, resulting from the inflammatory burden in the bowel, have been associated with arterial stiffening, venous thromboembolism, and atherosclerosis, events that contribute to an increased risk of cardiovascular failure [11]. The correlation between IBD and cancer has been largely studied. As reviewed by Chang and coworkers, IBD patients are at increased risk of developing intestinal and extra-intestinal malignancies. When compared to the regular population:

patients with extensive UC have a 19 times higher risk of CRC, and CD patients 20- to 30-fold increased risk of small bowel cancer, while hematological and skin cancers, and to a lower extent cervical and urinary cancers are potential consequences of the immunosuppressive effects of therapeutics for the treatment of IBD [12]. Although the specific risk of colitis-associated CRC, the most prevalent type of cancer in IBD, depends on the characteristics of the studied population, it is commonly recognized that young age at the onset of colitis, anatomical location, long duration of the disease, and family history of CRC are the major risk factors [13].

Management of IBD is personalized based on disease severity, anatomic location, underlying complications, potential adverse effects, and previous response to medication [4,14]. Current pharmacological alternatives include short interventions with corticosteroids to induce remission in periods of active flares, and individual or combined therapy with aminosalicylates, immunomodulators, antimetabolites, and biologics to maintain remission, which are regularly accompanied by parenteral nutrition and nutrition supplementation to overcome malnutrition complications [15]. These therapeutics act as immunosuppressants, resulting in an inherently increased risk of opportunistic infections, and have been associated with adverse effects such as peptic ulcers, renal dysfunction, hepatotoxicity, pancreatitis, pulmonary fibrosis, hypertension, and increased risk of other malignancies [16,17].

1.2.2 Risk factors contributing to the pathogenesis of IBD.

Although no agreement has been reached about the specific cause of IBD, important signs of progress have been made revealing the interplay between internal and external factors that include genetic susceptibility of the host, environmental triggers, intestinal microbiota, the immune system, and the intestinal epithelial barrier [18].

1.2.2.1 Genetic susceptibility

As reviewed by De Lange and Barret, 163 susceptibility loci associated with the epithelial barrier (*CDH1, ERRF11*), innate mucosal defense (*CARD9, NOD2*), autophagy (*CUL2, DAP*), apoptosis (*MST1, PUS10*), activation and regulation of adaptive immune response (*JAK2, STAT3, NFKB1, REL*), as well as those involved in cytokines production (*TNF-α, IL-12*) and IL-10 signaling, just to name a few, have been identified as directly implicated with the disease. Among the identified genes, 30 variants are specific for CD, 23 for UC, and the remaining 110 were associated with the two phenotypes, indicating a high level of overlap in the biological mechanisms involved [14]. Although common variants can only explain 19% and 26% of the inheritability of UC and CD respectively [19], the finding of low frequency or rare variants strongly associated with chronic bowel inflammation, such as the X-linked inhibitor of apoptosis (XIAP) [20], and the successful reproduction of some features of the disease in genetically modified animal models [21], keep pointing out genetic susceptibility

as one of the potential underlying conditions for IBD as they affect the host and the interaction with microbial and environmental factors [22].

1.2.2.2 Dysregulated gut microbiota

Microbial cells in the gut exceed about 10 times the number of host cells and can contain between 1000 and 5000 different species, among which firmicutes, bacteroidetes, actinobacteria, and proteobacteria are recognized as predominant [9]. The microbiota is a community that has co-evolved in a homeostatic relationship with the host [22], and acts as an acquired functional organ that contributes to the metabolism of indigestible nutrients, defense against pathogens, angiogenesis, proliferation, differentiation of epithelial cells, pH, and energy balance, and development and modulation of the mucosal immune system [18]. Although there is no agreement on the definition of a healthy gut microbiota, loss of the homeostatic equilibrium resulting from alterations in the stability, diversity, and dynamics of this community can impact gut health and triggers autoimmune disorders such as IBD [18]. Studies of the intestinal microflora in IBD patients have shown decreases in firmicutes, bifidobacterium, ruminococcaceae, and bacteroides, with concurrent increases in the pathogenic consortium [23]. Some particular pathogens such as *Escherichia coli, Diplostreptococcus, Clostridium difficile, Fusobacterium necroporum, Shigella sp., Helicobacter hepaticus, RNA viruses, Bacteroides vulgatus,* and *Yersinia sp*. have been suggested as potentially associated with UC [24]. Whether dysregulated microbiota is a cause of the disease, or a consequence of other

physiological changes in the gut, and/or of the therapeutics used for management of inflammation, is still a matter of debate [14], but the positive results observed when using probiotics or performing transplantation of fecal microbiota, have created a new potential strategy for the treatment of the disease by pursuing the repopulation of the gut microbial community [23].

1.2.2.3 Disruption of the intestinal epithelial barrier

Intestinal epithelial cells (IECs) and non-epithelial cells constitute the primary physical barrier against microbial cells and components of the luminal content [18]. The adherent and tight junctions between IECs regulate the selective penetration of nutrients, fluids, and microorganisms [9], and mucins and antibacterial peptides form a mucus coating that confers an additional layer of protection while allowing the exchange of molecules [18]. Impairment of the mucosal barrier leads to an increase in intestinal permeability and facilitates the free passage of microbes and their products across the intestinal membrane, triggering the immune response, which is further exacerbated by mucosal penetration of enteric pathogens and dysbiosis, marking the onset of the disease [18,25]. Diarrhea, one of the most common clinical manifestations of the disease, is the consequence of inappropriate expression or function of epithelial ion transporters, that lead to electrolyte retention and accumulation of water in the lumen [25]. The truncated function of the gene *CDH1*, which encodes for the adherent junction E-cadherin, is one of the IBD susceptibility loci encountered by genetic wide association studies [14] and has been found in intestinal biopsies

from CD patients, in which the protein presented a cytosolic localization [26], as opposed to the membranous localization expected in healthy individuals . Several variables affecting different components of the intestinal epithelial barrier such as goblets cells loss, mucodepletion and mutation in mucin proteins [27], degradation of E-cadherin by neutrophils elastase that contributes to tissue damage [28], extensive apoptosis of IECs triggered by pro-inflammatory mediators [29], altered expression and delocalization of tight junction proteins [30], among others, have been found to be correlated with IBD.

1.2.2.4 Environmental factors

Environmental factors have a direct impact on the gut microbiota and the immune system, and their differential contribution to IBD adds a substantial level of complexity to understanding the disease. Several studies have tried to identify potential patterns between exposure to environmental conditions and the disease, but results are widely diverse and, in many cases, controversial. According to a recent review by Ananthakrishnan *et al*. (2018) particulate matter and other compounds present in air pollution can trigger the immune system making the disease more common in urban centers. The use of medications can trigger or perpetuate IBD, as antibiotics decrease the diversity of gut microbiota creating dysbiosis, while non-steroidal anti-inflammatory drugs (NSAIDs) are associated with gastric and duodenal ulcerations and increased mucosal permeability [31]. Behavioral patterns such as stress, anxiety, depression, impaired sleep, and excessive sanitization contribute to IBD, while smoking has a

controversial role as it seems to worsen CD by increasing the relapse rate, but appears to be protective in UC, involving effects over the gut microbiota, mucosal membrane integrity, and alteration in endothelial functions mediated by nitric oxide [18].

1.2.3 Dysregulated immune response: the central role of cytokines in IBD

The intestinal immune response is the outcome of the interaction between the immune cells, gut microbiota, and the intestinal epithelial barrier, shaped by the contribution of environmental and genetic factors [18]. Transgression of the epithelial barrier by microbial antigens activates mucosal immune cells and triggers the release of pro-inflammatory signals, initiating the inflammatory response. In healthy individuals, anti-inflammatory signals and tissue repair mechanisms mediate the resolution of inflammation facilitating the recovery of intestinal homeostasis. However, when the inflammatory response is aberrant, or regulatory mechanisms fail, non-resolved inflammation becomes chronic [32]. This results in a large number of T cells, B cells, macrophages, neutrophils, and dendritic cells infiltrating the lamina propria, producing high levels of pro-inflammatory cytokines and generating tissue damage [9].

Cytokines have a central role in the pathogenesis and development of IBD as they: i) govern the initiation, evolution, and resolution of the inflammatory response, ii) drive intestinal inflammation and associated symptoms, iii) regulate systemic effects that contribute to extra-intestinal manifestations, and iv) are involved in the progression of destructive forms of the disease such as the

development of colitis-associated CRC [32]. Depending on the nature of the trigger, T helper cells (Th) help to polarize the response of other immune cells. CD and UC differ in the subset of Th cells involved in the inflammatory response. CD is characterized by a more inflammatory Th1/Th17 response in which IFN-γ, IL-17, IL-21, and IL-22 are the main cytokines produced, while UC is characterized by a Th2-like response that involves IL-13, IL-5, and the expansion of natural killer T cells [33]. These cytokines to different extent mediate pro-inflammatory functions such as the neutrophils recruitment, release of matrix metalloproteinases, and the upregulation of TNF-α, IL-1β, IL-6, and IL-8, however, results of clinical trials using antibodies against IFN-γ [34], IL-17A [35], and IL-13 [36], have been disappointing [32].

1.2.3.1 Crucial pro-inflammatory cytokines

The pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 are produced in large amounts by dendritic cells and macrophages, activate the pro-inflammatory pathway NF-κB, and participate in upstream and downstream events associated with both forms of the disease [33]. They induce the secretion of chemokines, which recruit leukocytes by activating integrin receptors, then maintaining a constant supply of infiltrating immune cells in the site of inflammation [37]. These cytokines are not only considered the most important markers of inflammation [18], but also crucial players in the development of inflammation-related cancer [38,39].

- IL-1 family

IL-1β and IL-18 belong to the IL-1 family and are produced as inactive zymogens that require the cleavage and activation by the IL-1 converting enzyme caspase-1 mediated by the inflammasomes, to act as pro-inflammatory signals [40]. IL-1β promotes innate immune pathology by boosting the recruitment of neutrophils and the activation of innate lymphoid cells, while IL-1 receptor signaling in T cells is involved in the early accumulation and survival of pathogenic CD4+ T cells in the colon [32]. Inhibition of the NLRP3 inflammasome and blockade of caspase-1 has shown beneficial effects in several murine models of colitis [41,42].

- IL-6

IL-6 acts as a pro-inflammatory signal by activating multiple target cells such as antigen presenting cells and T cells and inhibiting the apoptosis of T-cells. Although response in a subset of CD patients has been observed by the blockade of IL-6 signaling, its clinical application requires careful attention as IL-6 also has important homeostatic functions by promoting the proliferation and expansion of IECs [32].

- TNF-α

TNF-α is the most important cytokine mediating intestinal inflammation [43]. In addition to innate immune cells, adipocytes, fibroblast and T cells from patients with IBD produce considerable amounts of TNF-α [32]. The pleiotropic effect of this cytokine involves the activation of macrophages and T cells which results in increased production of IL-1β and IL-6, induction of apoptosis in IECs, and the

production of matrix metalloproteinases, aggravating epithelial erosions, ulcers, and having a detrimental effect on the intestinal epithelial barrier, promotion of angiogenesis, and pro-coagulant factors [32,44]. The positive clinical improvement and macroscopic healing observed upon TNF-α blockade by specific antibodies, points out the crucial role of this cytokine in IBD and makes anti-TNF-α biologics a cornerstone in the treatment of IBD [45].

1.2.4 The potential role of diet in IBD

Diet is considered one of the most important factors impacting gut health and it is potentially one of the main environmental factors involved in IBD, as it greatly affects gut microbiota and the mucosal immune system. Evidence suggests an inverse association between being breastfed as an infant and the incidence of CD and UC as an adult [46]. Breastfeeding has a strong and durable effect on the composition of the microbial community of the infant, exerts a protective effect by facilitating the transference of antimicrobial components, and contributes to the development of tolerance to dietary and microbial antigens [31]. Short-term consumption of diets composed entirely of plant or animal products rapidly altered microbial communities, mirroring the characteristic differences between carnivorous and herbivorous diets, particularly in microbial functions associated with carbohydrates and protein fermentation [47]. Mice fed a diet high in saturated milk-derived fat, imitating a “western diet”, not only exhibited a microbial community with an increased proportion of Bacteroidetes and decreased in Firmicutes, but promoted the bloom of a minor pathobiont

component of the gut microbiota (*Bilophila wadsworthia*), which promoted a Th1-mediated immune response and the development of colitis in IL-10 deficient mice (IL-$10^{-/-}$) [48]. Low fruit and vegetable intake, and high consumption of total protein, fat, and refined sugar are more common among IBD patients [18,24].

Epidemiological studies demonstrate a higher prevalence of IBD in developed nations, and an accelerated growing incidence in developing countries acquiring a westernized culture [7]. Western diet is high in fat and refined carbohydrates, low in fiber and plant polysaccharides, and incorporates a large proportion of animal-derived foods as a source of protein, while in contrast, the Asian diet is mainly plant-based, with soybean serving as one of the main ingredients [49]. Soybean and soy-derived compounds such as saponins, phytosterols, phosphatidylcholine, lectin, isoflavones, proteins, and peptides have received increasing attention over the last decades due to the plethora of bioactive properties that have been identified [50], particularly after the FDA authorized a health claim about the reduction of coronary diseases linked to a soy protein consumption of 25 g per day [50]. A recent study revealed that replacement of animal protein by soy-pea protein in an “American diet” reduced the severity of experimental IBD in animal models, and had a better impact than replacing refined carbohydrates with polysaccharides, or changing the polyunsaturated fatty acids ratio [51]. In similar approaches, reviewed by Juritsch and Moreau, animal models of IBD have shown improvement in histological changes and pro-inflammatory markers when using soy protein concentrates, or

soy as the only source of protein in the diet [52]. However, the incorporation of soybeans, soy-product, or soy protein in the diet needs careful consideration, as certain subsets of the population may suffer from an adverse reaction to soy components. For instance, soy is one of the triggers that contribute to food protein-induced enterocolitis syndrome, a non-IgE mediated food allergic disorder in infants [53]. Soybean is officially recognized as one of the foods that account for most serious allergic reactions in the United States [54,55], with at least 16 IgE-binding proteins, including glycinin, β-conglycinin, and Kunitz trypsin inhibitor, that could be involved in clinical allergy [56]. Therefore, the study and utilization of individual soy components could be a more ideal approach to take advantage of those with potential beneficial biological activity over IBD.

The utilization of non-protein compounds naturally present in plant materials in the context of IBD has also been widely explored and reviewed. Anthocyanins, flavan-3-ols (green tea polyphenols), soy isoflavones (genistein, daidzein, and equol), proanthocyanidins (oligonol, cocoa and apple extracts), flavanones (hesperidin and naringenin), flavonones (quercetin, rutin, kaemferol, and myricitrin), stilbenes (resveratrol, piceatannol), curcuminoids, and hydroxycinamic acids, among other plant derived components have been tested in animal models of IBD but with controversial results, as in some cases their administration can induce improved outcomes, while in others lead to worsening of symptoms or particular markers of the disease depending on the dose, mode of administration, nature of the compound (crude extract, purified , enriched food

matrix, whole food), and the particular mechanism used to induce bowel inflammation in the animal model [117, 118].

1.2.5 Lunasin, a soybean peptide with potential activity against IBD

Lunasin is a soybean peptide that for the past 20 years has been extensively investigated due to its high potential activity against diverse chronic disorders as a result of its antioxidant, anti-inflammatory, and anticancer properties [57]. The observed biological activity in mammalian cell lines and animal models, has raised the interest on its amino acids sequence, its mode of action, and the potential presence of the peptide in other plant sources.

Although initial studies characterized lunasin as a 43 amino acids peptide with an expected mass of 5025.23 Da, recent investigations have reported an additional asparagine residue at the C-terminal, conferring a final structure of 44 amino acids with a mass of 5139.25 Da [58]. The structure of lunasin contains three well characterized regions that have been associated with its biological activity (Figure 1.1): a polyaspartic acid tail that interacts with core histones H3 and H4 and affects their acetylation, a cell adhesion domain composed of arginine-glycine-aspartic acid that mediates integrin signaling and cellular internalization, and a chromatin binding region that contributes to the immunomodulatory function [59]. It was recently suggested that a fourth region ^{11}RKQLQGVN^{18} can be involved in transepithelial transport across the intestinal wall via the paracellular route [60].

In soybeans, lunasin is encoded within the 2S albumin *GM2S-1* gene [61] and has been found in concentrations ranging from 0.1 to 1.33 g/100g flour in ancestral lines and modern cultivars [62]. Evaluation of commercially available soybean derived products via immunoassays suggests that lunasin remains present in soy flakes, soy protein isolates, concentrates and hydrolysates, tofu, soymilk, and soymilk based products at levels ranging between µg to mg/100g [62,63]. Several studies have reported the presence of lunasin in different sources such as rye [64], oats [65], triticale [66], barley [67], wheat [68], *Solanum nigrum* [69], quinoa [70], amaranth [71], millet [72], lupinus species [73] and other sources. However, searches of transcriptome and DNA sequences databases, chemical, and molecular approaches have failed to find regions encoding for the peptide or a precursor protein [74,75], pointing out the question about the origin of the reported peptides in these sources. An immunological approach revealed that the peptide isolated from some cereals and seeds react with antibodies specific for the N-terminal but not for the C-terminal sequence [76], suggesting that lunasin-like peptides, but non-identical to the one found in soybeans, are present in these plant materials. Several methods have been designed for the isolation of lunasin using soybeans [77] and mostly defatted soy flour [58,63,78]. However, to date, there have been no attempts to identify or recover lunasin from other byproducts of the soybean industry, such as okara and tofu whey, which are produced in large quantities during soymilk and tofu manufacturing.

1.2.6 Biological activity of lunasin

Several biological properties are attributed to lunasin. Among them, anti-cancer and anti-inflammatory properties have been the most widely investigated.

1.2.6.1 Anti-cancer properties

In cell cultures, lunasin was shown to prevent the malignant transformation of mammalian cells induced by E1A-oncogene [79,80] and ras-oncogene [81], as well as by the chemical carcinogens 7,12-dimethylbenz [a]anthracene and 3-methylcholanthrene [82], acting as a chemopreventive agent. Lunasin has been shown effective in reducing proliferation, inducing cytotoxicity, reducing migratory as well as colony and tumor forming capacity in diverse i*n vitro* models of cancer including leukemia [83], colon [84–86], breast [87], melanoma [88,89], and non-small cell lung cancer [90]. These chemotherapeutic effects seem to be mainly mediated by the induction of cell cycle arrest in the G1/S and G2/M phases caused by the upregulation of the p27 and p21 cyclin-dependent kinase inhibitors [83,84,86,90], and in some cases by the promotion of apoptosis via the mitochondrial pathway [83,84], mediated by PTEN [87], PARP [86], Bcl-2, Bax, and cytochrome C [85]. In non-small lung cancer cells, cycle arrest was observed without inducing apoptosis but accompanied by a reduction in phosphorylated Akt and the sequential phosphorylation of the retinoblastoma protein [90]. The reduced invasive potential is possibly associated with the altered expression of extracellular matrix

and adhesion genes downregulating integrins α5, β2, and matrix metalloproteinase 10 (MMP-10) [85].

In vivo, topical administration of lunasin reduced tumor incidence and delayed the appearance of tumors by two weeks in a model of skin tumorigenesis, by inhibiting histones H3 and H4 acetylation with the consequent induction of apoptosis and cell cycle arrest [80]. Intraperitoneal injection of lunasin decreased breast tumor incidence [91], and reduced tumor volume in non-small cell lung cancer [90]. A study on melanoma reported that lunasin induced the expression of differentiation markers and reduced the expression of stemness factors in the subpopulation of cancer initiating cells, leading to a reduction in the colony and oncosphere formation, as well as decreased tumor growth and pulmonary colonization [88,89].

1.2.6.2 Anti-inflammatory properties

The role of chronic inflammation in different degenerative disorders has raised the interest in lunasin as an anti-inflammatory agent. In terms of the immune cells, research has been focused on the effect of the peptide over macrophages in an inflammatory state. In lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophages and human THP-1 macrophages lunasin attenuated the inflammatory response by reducing the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, as well as nitric oxide (NO), and prostaglandin E2 (PGE_2). Attenuation was the result of reduced expression of iNOS and COX-2, through the inhibition of the NF-κB signaling pathway by

preventing the activation of phosphorylated Akt and the translocation of p50 and p65 sub-units of NF- κB in the nucleus [78,92,93]. Further investigation revealed that cellular uptake of lunasin is enhanced by inflammatory signals, and mediated by endocytic mechanisms that involve macropinosomes, integrin signaling (αVβ3), and clathrin-coated structures [94].

Investigation of the biological activity of lunasin from the perspective of inflammation-related diseases is still incipient. In synovial fibroblast isolated from patients with rheumatoid arthritis, lunasin inhibited cell proliferation, induced apoptosis, reduced the production of IL-6, IL-8, and MMP-3, and suppressed NF-κB [95]. In an *in vitro* model of osteoarthritis, lunasin enhanced the expression of the tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2, and suppressed expression of MMP-3 and MMP-13, protecting type II collagen from degradation in IL-1β-stimulated chondrocytes, by attenuating JAK2/STAT1/IRF-1 pathway activation [96]. Using a model of obesity-induced inflammation, Hsieh and coworkers demonstrated that in high fat-diet fed mice intraperitoneal administration of lunasin decreased serum levels of leptin and malondialdehyde, and reduced levels of MCP-1, IL-1β, and TNF-α in peritoneal macrophage from female mice, while in males it prevented spleen enlargement, inhibited IL-6 and promoted IFN-γ and IL-2 production in the immune cells isolated from the spleen, inducing Th1-type response [97]. In the context of asthma, lunasin administration to an *in vivo* model of allergic airway inflammation reduced eosinophil infiltration, goblet cell metaplasia, IL-4 production, and expression of the proinflammatory

gene *FIZZ1*, while promoting the accumulation of regulatory T cells in the lung [98].

1.2.6.3 Other biological properties

Lunasin also acts as an antioxidant. It inhibited linoleic acid oxidation as well as the production of reactive oxygen species in LPS-induced murine macrophages [99], and protected intestinal epithelial cell lines from oxidative damage and the cytotoxic effect of chemical oxidants such as hydrogen peroxide and tert-butylhydroperoxide [100], mainly acting as a free radical scavenger [70]. Other biological effects have been reported on cholesterol regulation, Alzheimer like-neuropathology, bone regeneration, cataracts, and other cardiovascular and central nervous systems conditions [57,61].

Some evidence suggests subtle differences in the biological activity of lunasin chemically synthesized and the one isolated from natural sources. Naturally occurring lunasin has higher colony formation inhibitory activity, [81], and increased binding affinity for histone H4 than the synthetic peptide [58]. Differences in the induction of cell cycle arrest and apoptosis between similar studies have been also associated with this difference in origin [59]. Interestingly, lunasin from oat showed similar activity to synthetic lunasin [65], although evidence indicates the absence of the peptide in cereals [74,76]. It is possible that amino acid sequences of the peptide, also found in non-soybean sources, are responsible for the biological activity observed, but structural differences

explain the decreased effect of the synthetic peptide when compared with the soy derived lunasin.

1.2.7 Lunasin in the gastrointestinal tract

Table 1.1 summarizes *in vivo* models reported for the anticancer and anti-inflammatory activity of lunasin. Despite multiple studies promoting the incorporation of soybean products in the diet, as a source of lunasin, *in vivo* models that support biological activity upon oral ingestion are scarce.

1.2.7.1 Protection of lunasin during gastrointestinal digestion

Lunasin is known to suffer a proteolytic breakdown in the gastrointestinal tract. Exposure of purified lunasin from soybeans and synthetic lunasin to simulated gastrointestinal fluid showed that the peptide was digested after two minutes of exposure [101]. In this same study, evaluation of Bowman-Birk inhibitor (BBI), a bioactive peptide also present in soybeans, indicated that this peptide, in contrast, was resistant to digestion and conferred some protective effect to lunasin when combined [101]. Another study demonstrated that 25% of lunasin survived pepsin digestion after 3 h, and 6% of the peptide was still detectable after 6 h of sequential digestion by pepsin-pancreatin when lunasin was part of soy flour [83], suggesting that the food matrix exerted a protective effect over the stability of the peptide during gastrointestinal digestion. Upon these findings, more attention was paid to the interaction between lunasin, and the protease inhibitors present in soybeans, BBI, and Kunitz trypsin inhibitor (KTI). The complementary role of these protease inhibitors and lunasin, in cancer

prevention, has been investigated. The study performed by Hsieh and coworkers in an *in vivo* model of breast cancer showed that intraperitoneal injection of soybean lunasin, but not BBI, reduced tumor incidence and size, and delayed tumor generation, suggesting that lunasin was the bioactive cancer preventive agent in Bowman-Birk Inhibitor concentrate (BBIC), a crude soybean extract attributed with chemo-preventive properties [102]. In the same study, the exposure of lunasin to pancreatin in the presence or absence of BBI or KTI indicated that both exert a protective effect over lunasin. Our research group reported that lunasin concentration, after pepsin-pancreatin hydrolysis of lunasin enriched preparations, was significantly correlated with BBI and KTI concentrations, and mutations in KTI isoforms can result in a lower protective effect to the peptide [103].

Mice and rats were used as *in vivo* models to investigate the bioavailability of lunasin when orally administered as part of a lunasin-enriched soy preparation, demonstrating that 3 h after gavage 30% of the oral dose was absorbed and had reached tissues such as the liver, kidneys, heart, brain, lungs, and prostate, in an intact and bioactive state [102]. Although the presence of lunasin in cereals is controversial, similar reports have found bioactive lunasin in the liver, kidney, and blood of mice fed with lunasin-enriched barley [67] and rye [64], which could be explained by the presence of BBI and KTI type inhibitors in cereals, legumes, and grasses [104]. Lunasin was also found in the liver of rats fed with lunasin enriched wheat [68]. In this study, the presence of the peptide was assumed as

positive by matching the sequence KQLQGVNLTPCEKH. Interestingly this peptide contains most of the fraction RKQLQGVN, which was identified years later as involved in the paracellular transport of the peptide [60]. Lunasin found in the plasma of healthy male subjects fed with 50 g of soy protein, indicating the bioavailability of the soybean peptide in humans [105].

1.2.7.2 Bioactivity of lunasin digestion-derived peptides

Although degradation of lunasin by the gastrointestinal digestive enzymes may represent a limitation for oral delivery, evidence suggests that lunasin derived peptides may still exert a biological effect. The first hint was provided by Cruz-Huerta and coworkers, who evaluated the antiproliferative effect of the digestion product after gastric and duodenal digestion of lunasin, over the CRC cell line HT-29, showing a significant decrease of viable cells [106]. In this study, the digestion-derived peptides were identified and it was revealed that BBI can exert a protective effect even after inactivation of its protease inhibitory activity [106]. Further research reported that some of these fragments are resistant against brush-border peptidases and decrease the viability of CRC cells [60], retain free radical scavenging properties, have a protective effect over the viability and oxidative status on murine macrophage under oxidative stress, as well as reduce the production of nitric oxide in macrophages induced into a pro-inflammatory state [107]. Recent reports corroborated the anti-inflammatory and anti-cancer activity of peptides derived from *in vitro* gastrointestinal digestion of soybean proteins [108]. How the full set of bioactive properties of lunasin may be

modified after the proteolytic environment of the gastrointestinal tract is still an emerging topic of research, but the evidence reported so far supports the possibility of lunasin derived peptides exerting, at least to some extent, anti-inflammatory, antioxidant, and anti-cancer properties. Further research is required, *in vitro* and *in vivo,* to identify the potential biological activity of lunasin digestion-derived peptides.

1.2.7.3 Biological activity of lunasin in the gut

Investigation into the biological activity of lunasin has been largely focused on extraintestinal diseases, hence little is known about its properties in the gastrointestinal tract. So far, *in vitro* approaches in different CRC cell lines have shown that the peptide can induce cell cycle arrest in the G2/M phase and apoptosis by the mitochondrial pathway, downregulate the activation of extracellular matrix and adhesion genes, reduce migratory capacity, inhibit the activation of focal adhesion kinase, increase the expression of the inhibitor of kappa B alpha, and hinder the tumorsphere-forming capacity [84–86,109]. Lunasin also protects intestinal epithelial cell cultures from chemically induced oxidative damage [100]. *In vivo* models of metastatic CRC, generated by intrasplenic implantation of colon cancer cells, showed that intraperitoneal but not oral administration of lunasin, significantly abrogated metastasis to the liver, reduced histone acetylation markers and proliferating cells nuclear antigen, and potentiated the effect of the chemotherapeutic oxaliplatin by suppressing the FAK/ERK/NF-kB pathway upon binding α5β1 integrins and internalization into the

nucleus [109,110]. Although these results show a potential use of lunasin in the context of CRC, none of them address the activity of lunasin in the colon.

Recently, an *ex vivo* approach using human intestinal biopsies from healthy individuals showed that lunasin exerts an immunomodulatory effect in the intestinal mucosa [111]. Exposure of the explants to lunasin induced the mRNA expression of *IL-1β, TNF-α, IL-17A, PGE2-COX2, IL-10, and TGFβ* while downregulating *iNOS* and *p65* subunit from *NF-κB*. In contrast, lunasin significantly inhibited the expression of IL-17A and IFNγ, tended to restore *PGE2-COX2 expression and increased IL-10 and TGFβ,* when the explants were exposed to the presence of LPS as a pro-inflammatory stimulus.

1.2.7.4 Prior Work

Lunasin protease inhibitor concentrate (LPIC) is a soybean-derived combination of BBI, KTI, and lunasin that exhibited high chymotrypsin inhibitory activity and strong suppression of mRNA levels of pro-inflammatory cytokines IL-1β and IL-6 in activated human macrophages [112]. In our research group, preliminary experiments using an *in vivo* model of relapsing UC treated with LPIC indicated that the combination of compounds significantly decreased serum levels of TNF-α, colonic levels of IL-6, IL-1β, iNOS, and markers of neutrophils activity in the colon [113]. However, some adverse effects were observed such as increased body weight loss, sustained higher levels of fecal occult blood, and increased disease activity index associated with softer stool consistency and presence of visible blood, indicating that the decrease in the pro-inflammatory

markers was not reflected in the clinical manifestations of the disease. A similar outcome was obtained following the same approach to study BG-4, a 4-kDa peptide from bitter melon seeds that strongly reduced pro-inflammatory cytokines and radical oxygen species in activated THP-1 cells [114]. Indeed, treatment with BG-4 exacerbated rectal bleeding, body weight loss, neutrophils infiltration, and induced significant colon shortening while still decreasing pro-inflammatory cytokines [115]. BG-4 has been reported to exhibit trypsin inhibitory activity 8.6 times higher than KTI [116]. The fact that these two compounds exhibited similar adverse effects that seemed proportional to their protease inhibitory activity suggests that the utilization of preparations with high protease inhibitory activity in the context of IBD may not be beneficial.

1.3 Hypothesis and objectives

Over the last two decades, the investigation of the biological activity of lunasin in *in vivo* models has shown promising results. *In vitro* approaches using different cell lines have allowed researchers to gain more insights into the potential mechanisms of action triggered by the peptide. However, the investigation of lunasin has been focused on diseases occurring outside the gastrointestinal tract, with unknown results on whether dietary consumption of lunasin might be beneficial for intervention of bowel diseases. Recent efforts have been made to understand how gastrointestinal digestion might affect lunasin and its biological properties. The possible outcomes of the interaction

between the peptide and the intestinal mucosa in healthy and inflammatory states needed to be investigated.

Therefore, the overall objective of this research was to investigate the biological activity of lunasin against bowel related diseases in *in vivo* models. The main hypothesis was that lunasin can exert anti-inflammatory properties in the bowel and mitigate inflammation-related diseases. To test this hypothesis, two different scenarios of IBD were considered: i) chronic inflammation associated with genetic susceptibility, which results in the spontaneous development of the disease, and ii) chronic inflammation triggered by external factors, that progress into colitis-associated colorectal cancer. Attending to the possible variations in biological activity between the synthetic and naturally occurring lunasin, this study pursued the utilization of a purified form of the peptide, using a byproduct of the soybean industry as starting material. Three specific objectives were pursued:

The first objective was to design a process for the isolation of lunasin from tofu whey. Tofu whey prepared at laboratory scale was used to establish the isolation conditions. The combined use of ethanol, calcium, and acidification to different pH were tested to obtain a solid fraction enriched in lunasin from the whey. Preparative gel filtration chromatography was used for purification, and composition of the mobile phase, injection volume, and preparation grade of the chromatographic column were evaluated. The designed isolation process was then applied to commercially produced tofu whey. The purity and identity of the

final product were established using immunologic and chromatographic techniques, and bioactivity was assessed in murine macrophages stimulated into an inflammatory state.

The second objective was to evaluate the potential of lunasin isolated from tofu whey to reduce inflammasome-related IBD. IL-$10^{-/-}$ mice were used as *in vivo* model of spontaneous colitis. Manifestations of symptoms and pro-inflammatory markers in different sections of the small and large intestine were assessed in mice left untreated, orally administered with lunasin, or treated with a caspase inhibitor as a control for the blockade of the inflammasome. Human macrophages were used *in vitro* to identify the potential mechanism by which lunasin modulates the inflammasome.

The third objective was to evaluate the ability of lunasin to mitigate chemically induced colitis-associated carcinogenesis. Dextran sulfate sodium and azoxymethane were used to mimic relapsing cycles of inflammation and induce colitis-associated CRC. Mice receiving placebo jelly or jelly containing lunasin were monitored for clinical symptoms of the disease. Pro-inflammatory markers, tumor burden, and histopathological assessment for inflammation and dysplasia were evaluated in the colon.

The results of this study are expected to serve as evidence of the biological activity of lunasin in the bowel, and to indicate its potential utilization to lessen bowel-inflammation related diseases. In addition, further investigation

about the inclusion of lunasin as an active preventive and therapeutic agent in the context of IBD and intestinal cancers.

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Appendix

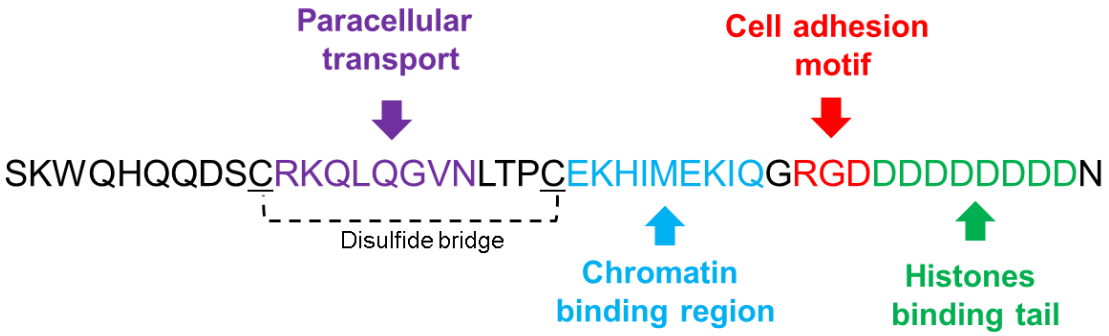


Figure 1.1 Primary sequence of lunasin indicating the functional regions.

Table 1.1 Summary of reported use of lunasin in *in vivo* models of cancer and inflammation

Source (Purity)	Disease Model	Route of administration	Dosage	Observed results	Ref.
Soybeans (>99%)	Melanoma and non-small cell lung cancer	Intraperitoneal injection	10 or 30 mg/kg bw daily	Significant reduction of tumor size over time.	[117]
Soybeans (>99%)	Melanoma	Intraperitoneal injection	30 mg/kg bw daily	Significant reduction of tumor size and weight, and delayed detection of palpable tumors	[88]
Soybeans (>95%)	Metastatic Melanoma	Intraperitoneal injection	30 mg/kg bw daily	Reduced number of macro-metastatic outgrowths, and lower area of micrometastatic lesions to the lungs	[89]
Synthetic (NR)	Skin tumorigenesis	Topic	2.5, 25 and 250 µg/week dissolved in ethanol twice a week	The highest dose reduced skin tumor incidence and delayed the appearance of tumors.	[80]
Soybeans (>95%)	Metastatic colorectal cancer	Intraperitoneal injection vs. Oral gavage	Intraperitoneal: 4 mg/kg daily. Oral: 8 and 20 mg/kg bw daily	Significant reduction of metastasis to the liver by IP but not by oral administration.	[110]
Soybeans (>95%)	Non-small cell lung cancer (NSCLC)	Intraperitoneal injection	30 mg/kg bw daily	Significant reduction of tumor size over time.	[90]

Table 1.1 Continued

Source (Purity)	Disease Model	Route of administration	Dosage	Observed results	Ref.
Soybeans (>90%)	Metastatic colorectal cancer	Intraperitoneal injection	4 mg/kg bw alone daily or with oxaliplatin twice a week	Inhibition of metastasis to the liver and potentiated effect of oxaliplatin	[109]
Aqueous extraction of soy protein concentrate (0.23% w/w)	Breast cancer	Oral via diet	0.15% or 0.23% lunasin in the diet + 0.6% BBI	Non-significant reduction in tumor incidence in high-dose diet only.	[91]
Synthetic (No Report)	Breast cancer	Intraperitoneal injection	4 and 20 mg/kw bw three times a week	Equal decreased tumor incidence with the two doses.	[102]
Synthetic (>95%)	Obesity-induced inflammation	Intraperitoneal injection	20 mg/kg bw daily	Females: reduced IL-1β, TNF-α, MCP-1, serum leptin, and malondialdehyde. Males: Inhibition of spleen enlargement and IL-6, promotion of IL-2 and INF-γ.	[97]
Synthetic (>95%)	Asthma	Intranasal	20 μg/mouse on specific days	Reduced cells infiltration, goblet cell metaplasia and IL-4 production, increased regulatory T cells	[98]

CHAPTER 2. UTILIZATION OF TOFU PROCESSING WASTEWATER AS A SOURCE OF THE BIOACTIVE PEPTIDE LUNASIN

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My primary contributions to the paper include: 1) conceptualization of the problem, 2) experimental design, 3) performance of experimental work, 4) collection, analysis, and interpretation of data, 5) writing original draft and 6) editing of the manuscript based on co-authors contributions and peer reviewer comments.

2.1 Abstract

Tofu whey is a waste byproduct generated in large quantities during tofu manufacturing. Lunasin is a naturally occurring peptide present in soybeans with biological properties of remarkable interest for human health. A simple and feasible method to isolate bioactive lunasin from tofu whey wastewater was designed. A combination of alcoholic precipitation of high-molecular weight proteins from the whey, isoelectric precipitation of lunasin enriched material, and purification via gel filtration chromatography was selected as the best approach using tofu whey prepared at the laboratory scale. This process was applied to tofu whey produced by a local tofu factory and 773 mg of 80% purity lunasin was obtained per kg of dry tofu whey. Significant reduction of nitric oxide, and pro-inflammatory cytokines TNF-α and IL-6 over lipopolysaccharide activated murine macrophages confirm its biological activity. Our three-step process is not only simpler and faster than the previously reported methods to obtain lunasin but provides a sustainable approach for the valorization of a waste product, promoting the better utilization of soybean nutrients and active compounds.

2.2 Introduction

Tofu is a cheese-like product obtained from soybeans. Lactose intolerance, celiac disease, increased health awareness and interest in vegetarian and vegan lifestyles have positioned tofu as a globally growing market [1]. Tofu production involves the grinding of hydrated soybeans to obtain soymilk,

which is then coagulated to form the tofu curd. During the process, two waste by-products are obtained in large quantities: okara and tofu whey. Tofu whey is the liquid waste fraction that remains after the curd formation and pressing [2]. About 9 kg of whey, containing up to 1% carbohydrates, 1% fats, 0.8% proteins, and 0.4% minerals, are produced per kg of soybeans used to make tofu [3]. Recovery of nutrients and bioactive compounds, transformation into functional and alcoholic beverages, and utilization as a growth media for the propagation of biomass are the most commonly studied applications of this by-product [2,3]. However, whey continues being mostly discarded into the sewage or soil, posing an environmental threat due to its high biological and chemical oxygen demand (BOD: 6000 – 8000 mg/L; COD: 7,500 – 26,000 mg/L) [3].

Lunasin is a 5 kDa soy-derived peptide that has raised interest in its potential application as a dietary supplement for the prevention of several chronic and degenerative diseases. This is due to the increasing body of evidence that supports its chemopreventive, anti-inflammatory, antioxidant, anti-hypertensive, cholesterol-regulating, neuroprotective and immunomodulatory properties [4]. The ability of lunasin to inhibit tumor growth and invasiveness of cancer cells has been demonstrated *in vitro* and *in vivo* for melanoma, breast, lung, and colorectal cancer [5], while the anti-inflammatory potential have been mostly studied *in vitro* and is associated with the reduction of pro-inflammatory molecules such as nitric oxide (NO), TNF-α and PGE_2 [6]. Most of these properties seem to be modulated at the epigenetic level by particular amino acid sequences present in the lunasin

structure: a polyaspartic acid tail that can inhibit acetylation of core histones H3 and H4, an RGD domain that modulate integrin signaling and mediates cellular internalization, and a chromatin-binding region that provides immunomodulatory functions [7].

The high cost associated with the utilization of synthetic lunasin has led to the formulation of several approaches to isolate the peptide from natural sources. Krishnan and coworkers developed a simple procedure to isolate a protein fraction from soybeans highly enriched in lunasin, Bowman Birk inhibitor and Kunitz Trypsin inhibitor via aqueous ethanol extraction and calcium precipitation [8]. The three peptides combined account for 60% of the total protein content in the final product based on densitometric analysis, or up to 80% if anion-exchange chromatography is used for further purification. Seber *et al*. designed a six-step isolation process involving PBS extraction, ionic exchange chromatography (IEC), treatment with a reducing agent, ultrafiltration, reverse phase chromatography, and ultrafiltration, using defatted soy white flakes as starting material and yielding a product with >99% purity based on densitometric analysis [9]. The purity reported for these isolation techniques indicate the relative abundance of the active peptide only as a ratio to other peptides and proteins that are revealed during the electrophoretic separation, while the presence of other non-peptide components in the final product was not considered. Dia *et al.* developed a four-steps process that involved aqueous extraction of defatted soy flour, IEC, ultrafiltration, and gel filtration

chromatography achieving 80% purity of lunasin in the final product as assayed via HPLC [10]. These methods are complicated and costly, as they deal with the complete and complex protein composition of soybeans.

Lunasin has been found in several commercially available soybean products, including soymilk and tofu [11,12]. Then it is reasonable to hypothesize that tofu by-products, may contain remaining fractions of lunasin and can serve as alternative sources of the bioactive peptide. So far, there have been no attempts to identify or isolate lunasin from soymilk or tofu waste by-products. Our objective was to design a simple and feasible method to isolate lunasin from tofu whey wastewater. In addition, the *in vitro* anti-inflammatory properties of lunasin were assessed to corroborate that the isolated lunasin maintains its bioactive properties.

2.3 Materials and Methods

2.3.1 Materials

Non-GMO soybeans (Newest Crop) and food-grade calcium sulfate tofu coagulant (Terra Alba) were obtained from Soymerica. Industrially produced tofu whey was kindly provided by a local tofu manufacturer located in Summertown, TN, USA. Lunasin polyclonal antibody was raised in rabbits using the 15 amino acid sequence corresponding to the C-terminus of lunasin and was produced by ProteinTech Group (Chicago, IL). Lunasin standard was synthesized by LifeTein LLC (Somerset, NJ), soybean Kunitz trypsin inhibitor (KTI) and Bowman-Birk

inhibitor (BBI) standards were from VWR International LLC (Atlanta, GA) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. SDS-PAGE and Western blot gels, membrane transfer kits, reagents, and goat-antirabbit secondary antibody were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemical and biological substances were purchased from VWR International, Sigma-Aldrich, and Thermo Fisher Scientific unless otherwise specified.

2.3.2 Laboratory scale preparation of tofu whey

Tofu whey was prepared at the laboratory scale and used to design the isolation method. Briefly, 100 g of soybeans were soaked overnight. Excess water was drained, and fresh water added to a final ratio of 1:9 dry beans: water. The mixture was blended for 3 min and the solid fiber (okara) was separated from the liquid fraction (soymilk) using a cheesecloth. Soymilk was boiled for 10 min, allowed to cool down to 70°C, and 3 g of calcium sulfate suspended in water were added to promote formation of tofu curd. Tofu was separated from the whey by applying a constant pressure of about 1360 Pa over the curd using a regular kitchen tofu press until dripping of the whey stopped.

2.3.3 Lunasin enrichment

Different volumes of ethanol were added to the whey to reach a final concentration of 20, 30, 40, or 50% ethanol v/v. Samples were stirred for 10 min, centrifuged at 15,800 *g* for 15 min, and precipitates and supernatants were used to identify the effect of increasing concentrations of ethanol in the protein profile and the presence of lunasin in the soluble and non-soluble fractions. Treatment

of the whey using a final concentration of 30% v/v ethanol was chosen for further analysis. To induce the precipitation of soluble proteins, the alcoholic supernatant, after centrifugation, was adjusted to pH 4.5, 5.5 or calcium chloride was added to a final concentration of 20 mM. Samples were centrifuged and the precipitates were freeze dried, pulverized using mortar and pestle, and used to identify the effect of the three precipitation techniques over the protein profile and the presence of lunasin. Bioactivity of the three final pre-enriched products was assessed by evaluating anti-inflammatory properties.

2.3.4 Adjustment of preparative gel filtration chromatography parameters

Preparative gel filtration chromatography was used for final purification of lunasin. Evaluation of the chromatographic parameters was performed using an ÄKTA pure protein purification system (GE Healthcare Life Sciences, Piscataway, NJ) equipped with sample pump and UV-detector (280 nm).The following chromatographic parameters were evaluated: i) composition of the mobile phase, 20 mM Tris-HCl buffer + 0.15 M NaCl, pH 7.6 vs. 100 mM ammonium bicarbonate (NH_4HCO_3), pH 7.8, ii) porosity of the packed bed in the chromatographic column, also referred as preparation grade (pg), pg 30 vs. pg 75, and iii) volume of sample injected, 0.5 mL vs. 3 mL. A HiLoad 16/600 Superdex column containing the indicated packed bed (30 or 75 pg) was equilibrated with 3.0 column volumes (CV) of the indicated mobile phase (Tris-HCl or NH_4HCO_3). Laboratory prepared whey was freeze dried and resuspended in the appropriate mobile phase at a concentration of 120 mg/mL. The indicated

sample volume (0.5 or 3 mL) was injected in the column. Elution was performed using 3.0 CV of the appropriate mobile phase at 1 mL/min. Ten-mL fractions were collected during the elution phase and analyzed for protein, lunasin and total carbohydrate contents, and optical density at 400 nm. Base conditions were established performing a chromatographic run using NH_4HCO_3 mobile phase, 30pg column and 0.5 mL of sample injection.

2.3.5 Isolation of lunasin from industrially produced tofu whey.

Industrially produced tofu whey was collected and frozen for up to one month, at -80 °C until use. After thawing at room temperature, suspended solids were removed using a cheese cloth. The whey was divided into three fractions: the first fraction was freeze-dried, the second was spray-dried using a Buchi-B290 Mini Spray dryer (BÜCHI Corporation, Switzerland) at 40% pump flow rate and 170°C inlet temperature, and the third fraction was treated with our designed lunasin enrichment method described in Section 2.3.3 Briefly, ethanol was added to the whey to a final concentration of 30% v/v, followed by stirring and centrifugation as described above. The supernatant was adjusted to pH 4.5 and after centrifugation, the precipitate was freeze-dried overnight. Approximately 2.0 mg ± 0.1 of dry lunasin enriched material was obtained per mL of industrial whey. Spray-dried whey (SDW), freeze-dried whey (SDW) and lunasin enriched material were used for chromatographic purification. This approach allowed the comparison of our designed lunasin enrichment method with conventional hot (spray drying) and cold (freeze-drying) drying mechanisms. To inject samples

with equivalent protein content 120 mg/mL of SDW or FW, or 60 mg/mL of lunasin enriched material were resuspended in 100 mM NH_4HCO_3 by vortexing horizontally for 15 min. After centrifugation at 30,000 g, the supernatant was filtered, and 3 mL were injected in a HiLoad 16/600 75 pg Superdex column. Elution was performed as described above using 100 mM NH_4HCO_3. Aliquots of the collected fractions were analyzed for lunasin, optical density at 400 nm, and carbohydrates content. Fractions containing a significant amount of lunasin were placed in a shaking water bath, brought to 40 °C for 5 min to remove any remaining NH_4HCO_3 and then freeze-dried.

2.3.6 Analytical methods for the characterization of lunasin

2.3.6.1 SDS-PAGE and Immunoblotting

Samples were denatured and reduced in the presence of sodium dodecyl sulfate and β-mercaptoethanol in boiling water for 5 min and electrophoretic separation was performed at 400 mA for 35 min in a Mini-PROTEAN tetra vertical electrophoresis cell (Bio-Rad). The gel was stained with Coomassie Blue G-250 for 1 h, destained with water, and imaged using a ChemiDoc Touch Imaging System (Bio-Rad). For immunoblotting, non-fixed gels after SDS-PAGE were transferred to PVDF-membranes using a Trans-Blot Turbo transfer system (Bio-Rad), blocked with 5% non-fat dry milk in 0.01% tween 20 Tris-buffered saline (TBST) for 1 h at room temperature. The membrane was incubated with 1:1000 lunasin, soybean KTI or BBI [13] primary antibodies raised in rabbits for 1.5 h at room temperature. After three 10-min washing with TBST, goat-antirabbit

secondary antibody was added and incubated for 2 h at room temperature, followed by another cycle of TBST washings. Immunoreactive bands were visualized via chemiluminescence in a C-Digit Blot scanner (LI-COR Biosciences, Lincoln, NE) using Image Studio™ software.

2.3.6.2 Enzyme-linked immunosorbent assay (ELISA)

Lunasin content in gel filtration liquid fractions and enriched precipitates were estimated via ELISA as previously described [10] with slight modifications: the plate was blocked with 5% bovine serum albumin in 0.5% Tween 20 phosphate-buffered saline, and incubation times with primary and secondary antibodies was 1 h each.

2.3.6.3 High performance liquid chromatography

Molecular weight profile via size exclusion chromatography was analyzed as previously described [14]. For determination of lunasin in purified fractions, 20 µL of the sample was injected into an Agilent (Folsom, CA) 1200 HPLC system equipped with an Eclipse Plus C18 column (4.26 x 250 mm, 5 µm), and diode array detector (215 nm). Elution was performed with acetonitrile (buffer A) and 0.1% trifluoroacetic acid in water (buffer B) as follows: step elution starting at 10% buffer A, with 10% increments up to 50%, holding each condition for 5 min, followed by equilibration of the column with 10% buffer A for 3 min. Quantification of isoflavones was performed according to previously reported protocol [15] by injecting 20 µL of the sample in the same column and equipment used for lunasin.

2.3.6.4 Total carbohydrates, protein content and trypsin inhibitory activity

Total carbohydrates were measured in gel filtration chromatography fractions via phenol-sulfuric acid method as described previously [16]; glucose (0 – 0.5 mg/ml) was used as standard. For gel filtration chromatography fractions, the relative protein content was estimated in µg/mL as A_{280nm}/ε, where ε is the mass attenuation coefficient for the whey, calculated as 0.018 by measuring the absorbance of different dilutions of tofu whey from a stock solution with known protein content. Protein content by Bradford assay and trypsin inhibitory activity using N-α-benzoyl-D, L-arginine 4-nitroanilide substrate were measured as previously described [14].

2.3.6.5 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.

The 5 kDa protein band was excised from Coomassie-stained SDS-PAGE gel for the identification of lunasin via MALDI-TOF-MS as previously described [17].

2.3.7 Biological activity of the isolated lunasin

RAW 264.7 murine macrophages (ATCC, Manassas, VA) were cultured, treated and analyzed as previously described [18]. Cells were seeded at $5x10^3$ cells/well in a 96-well plate, allowed to attach overnight, and then treated with lunasin at the indicated concentrations, in the presence of lipopolysaccharide (LPS), for 24 h. Supernatants were collected for analysis of pro-inflammatory

cytokines IL-6 and TNF-α by ELISA (BioLegend, San Diego, CA) and nitric oxide (NO) production by Griess reagent assay. MTS assay (Promega, Madison, WI) was used to test cell viability, according to the manufacturer's protocol.

2.3.8 Statistical analysis

Results are reported as mean ± standard deviation for at least three replicates. Data were analyzed for significant differences (p-value <0.05) via ANOVA followed by Tukey posthoc test, using Statgraphics Centurion ® software (Statgraphics Inc, The Plains, VA).

2.4 Results and Discussion

The yield of soymilk, tofu, okara, and whey for the laboratory-produced tofu are presented in Table 2.1 In okara, the presence of soluble proteins is derived from the fractions of soymilk retained in the solid fiber which can be considered as a potential source of the peptide. On the other hand, upon addition of calcium sulfate to soymilk, high molecular weight proteins belonging to the 11S (glycinin) and 7S (β-conglycinin) fractions aggregate forming the curd, while the low molecular weight proteins (< 16 kDa) remain soluble in the whey [19]. It could then be expected that the 5 kDa lunasin is present in the whey. Preliminary experiments using the whey and an alkaline extract from okara indicated that the amount of lunasin available in the whey is about 45 times higher than in okara (Supplementary Table 2.1), making it a better candidate for further exploration as a source of lunasin.

2.4.1 Lunasin enrichment from tofu whey

2.4.1.1 Alcoholic precipitation of high-molecular weight proteins

The purification of proteins via precipitation with alcohols has been reported and it has been shown that methanol and ethanol were capable of producing precipitates with retained enzymatic activity [20,21]. Ethanol has been successfully used to induce the aggregation of particular protein fractions from soybean preparations with bioactive properties [8,22]. We evaluated the effect of different concentrations of ethanol (20, 30, 40, and 50% v/v) on the proteins present in tofu whey. Electrophoretic separation (Figure 2.1A) indicated that upon increasing concentrations of ethanol, proteins with higher molecular weights were preferentially precipitated. At 20% v/v there was no apparent variation in the distribution of soluble proteins, while at 30% most of proteins higher than 30kDa were almost depleted. At 40 and 50%, proteins smaller than 25 kDa remained in the supernatant. Figure 2.1B presents the size exclusion chromatographic profile of the supernatants. Retention times and molecular weight of chromatographic standards are presented in Supplementary Table 2.2. As it can be observed, proteins with retention time lower than 6 min, corresponding to molecular weights higher than 6.5 kDa, were a predominant group in the whey. Variations in the profile corroborated the preferential impact of ethanol over this subset of proteins in the whey. At a concentration of 20% the height of peaks associated with high molecular weight proteins was reduced and became comparable to the height of peaks associated with peptides with retention times between 6 and 8 min,

corresponding to molecular weights between 1 and 6.5 kDa. At higher concentration of alcohol, the peptides fraction became the predominant group in the supernatant. It is known that the addition of miscible organic solvents increases intra- and intermolecular electrostatic forces that lead to variations in the folding of the protein molecules [23]. It is feasible that in complex proteins, with higher molecular weight, augmented repulsive intramolecular interactions lead to the exposure of buried ionic and polar groups. These available groups could participate in attractive protein-protein interactions with the exposed oppositely charged groups resulting in aggregation.

Western blot analysis (Figure 2.1C) revealed that fractions of lunasin progressively precipitated as the concentration of ethanol increased. Although variations in intramolecular electrostatic interactions may not have a considerable effect over the intrinsically disordered structure of lunasin [24], the dense negative charge of the polyaspartic acid tail could participate in peptide-protein interactions with the exposed positively charged side chains from the partially unfolded structures of larger proteins. This could lead to partial co-precipitation of lunasin with high molecular weight proteins. At 20%, in addition to the 5 kDa lunasin, an immunoreactive band located around 14 kDa is present. Seber and coworkers reported a 14 kDa complex originated between lunasin and a 9 kDa peptide via disulfide bridges [9]. However, the reducing nature of our electrophoretic separation, mediated by β-mercaptoethanol, and side test using dithiothreitol suggested that the complex present in the whey is not formed by

disulfide bonds. The 30% ethanol was the lower concentration at which precipitation of most high molecular weight proteins occurred, including the 14 kDa complex, with a minor effect over the solubility of lunasin. Hence, this concentration of ethanol was selected for the pre-enrichment process.

The characterization of the precipitate induced by ethanol treatment indicated that this by-product contained between three and seven times more protein, nine to fourteen times more lunasin, and comparable biological activity to some commercially available lunasin enriched dietary supplements (Supplementary Table 2.3 and Supplementary Figure 2.1), suggesting that it could be explored as a product with potential market value.

2.4.1.2. Precipitation of lunasin enriched material

Isoelectric precipitation at pH 4.5, 5.5 or precipitation mediated by calcium were tested as potential mechanisms to recover lunasin from the alcoholic whey after removal of high-molecular weight proteins. Table 2.2 presents the characterization of the obtained lunasin enriched fractions. Calcium has been previously used as a tool for the fractionated precipitation of seed storage proteins present in soybeans, peanut, beans, peas, and alfalfa [17]. In aqueous solutions, calcium forms strong complexes with the carboxylic groups of aspartic acid and glutamic acid [25]. As lunasin contains nine consecutive aspartates at the carboxylic end, and two glutamic acids present in the structure [7], we expected that addition of calcium would lead to the formation of complexes with lunasin mediated by the interaction of the divalent cation mostly with the

polyaspartic acid tail. However, the significantly lower lunasin content in calcium precipitate in contrast to the isoelectric precipitates, indicated a low efficiency of lunasin complexation. Electrophoretic profile (Figure 2.2A) shows strong bands in the calcium precipitate that could correspond to the acid and basic fractions of glycinin (32 and 20 kDa respectively) and to the β and α subunits of β-conglycinin (52 and 68 kDa respectively). It has been shown that glycinin and β-conglycinin precipitate at a minimum calcium concentration of 0.4 mM and 1.4 mM, respectively [26]; then it is possible that calcium favored the precipitation of these two fractions, over lunasin complexation. The presence of peaks at retention times later than 10 min (Figure 2.2B), suggested the presence of compounds with molecular weight smaller than 75 Da, which may correspond to organic salts probably formed with the divalent cation. In addition, the significantly lower protein content corroborated that calcium preferentially induced precipitation of non-protein compounds, present mostly in the precipitate.

Isoelectric precipitation is one of the most used mechanisms to isolate proteins. While precipitation at pH 4.5 has been shown to provide the highest yields during the production of soy protein isolates [27], two-dimensional electrophoresis of an extracted fraction, from defatted soy flakes, enriched in lunasin exhibited a 5 kDa band at pH of 5.5 [28], suggesting this particular pH as a more specific isoelectric point for the peptide. No significant differences were found for any of the parameters quantified for the two isoelectric precipitates. A stronger band at 32 kDa in the pH 4.5 precipitate indicated a higher impact over

the acid glycinin fraction, while pH 5.5 had a higher effect over the β subunit of β-conglycinin (52 kDa) (Figure 2.2A). These observations agreed with the molecular weight distribution exhibited in the chromatographic profiles. Immunoblotting revealed that not only lunasin, but the 14 kDa complex, as well as Kunitz (KTI) and Bowman-Birk (BBI) inhibitors, were present in all the precipitates (Figure 2.2C). No detectable trypsin inhibitory activity in any of the three precipitates (Table 2.2) indicated that the protease inhibitors were present as inactive molecules. The boiling of the soymilk before the curd coagulation led to the irreversible denaturation of the active sites required for the inhibitory activity.

Soybeans are known to contain several biologically active compounds. Among them, isoflavones have gained great recognition due to their ability to have a preventive effect over several diseases such as type II diabetes, osteoporosis, cardiovascular diseases, and hormone-associated cancers [29]. As they have been reported to be present in tofu whey [30], we tested for the presence of the major soy isoflavones in the precipitates. As expected, none of the aglycone forms was detectable while the more water-soluble glucosides daidzin and genistin were both present (Table 2.2). To ensure that the biological activity was conserved, the anti-inflammatory properties of the precipitates was assessed by measuring their ability to reduce the level of nitric oxide produced by activated murine macrophages. A significant dose-dependent decrease of nitric oxide released into the media was observed (Figure 2.2E), with no effect over the

proliferation of the immune cells (Figure 2.2D), suggesting that the biological activity was still present.

During storage at 23 °C and 51% relative humidity, the pH 5.5 precipitate discolored brown and became highly hygroscopic, while pH 4.5 powder maintained its original characteristics as a white homogeneous dry powder. Considering that lunasin enriched material at pH 4.5 has higher lunasin and protein content than calcium precipitate, and apparent better storage stability than the product at pH 5.5, isoelectric precipitation of the alcoholic whey at pH 4.5 was selected for the preparation of the lunasin enriched material.

2.4.2 Adjustment of preparative gel filtration chromatography parameters

Since the presence of proteins of different sizes continued to be an interference in the lunasin enriched material, a size-based separation approach can be used for lunasin purification. Gel filtration chromatography has been widely applied for the fractionation and purification of protein, peptides, enzymes and DNA based on their size and geometry while maintaining their biological activity [31,32]. To establish the most appropriate chromatographic conditions for the separation of lunasin from tofu whey, the effects of mobile phase composition, volume of sample injected, and the porosity of the packed bed (preparation grade-pg) were evaluated., The baseline was established by injecting 0.5 mL of sample, prepared as described in the experimental section, in a 30 pg column using 20 mM Tris-HCl + 0.15 M NaCl pH 7.6 as mobile phase. Modifications in the characteristic profiles were evaluated using alternative

conditions (Figure 2.3): 100mM NH_4HCO_3 pH 7.8 as mobile phase, a 75 pg column, or injecting 3 ml of the sample.

2.4.2.1 Effect of mobile phase (non-volatile vs. volatile)

During preliminary experiments using the base conditions, we observed that buffering salts in the mobile phase became the major component of the final product after freeze drying, making the content of lunasin negligible. Desalting trials using macrosep advance centrifugal device 1K MWCO allowed removing 98% of the salts present, though, the remaining salt continued to be 10 times higher than lunasin in the final dry product. So far, only one report has clearly stated the performance of concentration and buffer exchange steps, via ultrafiltration, after the final chromatographic purification [9]. To address this issue, we proposed the use of a volatile buffer as mobile phase. Upon mild heating, NH_4HCO_3 easily decomposes into the volatile compounds ammonia and carbon dioxide, [33]. Preliminary tests indicated that after heating 100 mM NH_4HCO_3 at 40°C for 5 min no solid residue remained. Then we performed the gel filtration chromatography using 100 mM NH_4HCO_3 pH 7.8 as mobile phase. Under base conditions, using Tris-HCl buffer, lunasin eluted at 60 min and the resolution of the peaks (Figure 2.3A) and carbohydrates profile (Figure 2.3B) suggested a good level of separation of the peptide from other compounds present in the whey. Figures 2.3A and 2.3C show that the chromatographic profile, as well as retention times for lunasin and carbohydrates using ammonium bicarbonate, resemble those obtained with Tris-HCl buffer. The fact that no shift

in the retention time of any peaks was observed, indicated that nonspecific ionic interactions between tofu whey components and the packed bed were negligible, and the presence of strong ionic salts in the mobile phase, such as NaCl, was not required. Then we concluded that NH_4HCO_3 was a more suitable mobile phase than Tris-HCl for the chromatographic separation and recovery of lunasin from tofu whey.

2.4.2.2 Column preparation grade (30 pg vs. 75 pg)

Preparation grade (pg) refers to the grade of fractionation or separation that the column can achieve in relation to the relative molecular mass (Mr) of the compounds present in the sample [34]. In the 30 pg column (Mr < 10,000) probably all proteins in the range of 10 to 75 kDa traveled externally to the packing beads, leaving the column as a whole group represented by a wide peak with retention times between 40 and 50 min (Figure 2.3A). In the 75 pg column (3,000 < Mr < 70,000), a better resolution of the proteins and peptides was observed, accompanied by a shift in the retention time of lunasin peak to 88 min, 28 min later than the 30 pg column. Analysis of the relative amounts of lunasin and proteins (Figure 2.3D) showed that most of the compounds absorbing at 280 nm eluted at around 108 min, 20 min later than lunasin, while lunasin containing fractions exhibited a minimum content of proteins. The better resolution of the peaks achieved by the 75 pg column made it more suitable than the 30 pg column for purification of lunasin.

2.4.2.3 Injection volume of the sample (0.5 vs. 3 mL)

Sample volume plays a decisive role in the resolution of chromatogram peaks, as larger volumes result in peak broadening; thus a sample volume no larger than 4% CV is recommended for high resolution fractionation [34]. For a 75 pg XK16 column with CV = 120 mL, 0.5 mL sample volume (0.4% of the CV) showed a good resolution for lunasin peak (Figure 2.3A); however, the amount of dry lunasin recovered per run was negligible. Figures 2.3A and 2.3E show the impact of a 6-fold increase in the sample injection volume (3 mL, equivalent to 2.5% of the CV). As expected, lunasin recovered in the fractions increased proportionally, accompanied by a remarkable widening of the peak, which now goes from 58 to 88 min. The consequent decrease in the resolution led to the overlapping of lunasin to adjacent peaks associated with larger and smaller molecules.

The four fractions exhibiting relevant content of lunasin, corresponding to retention times 58, 68, 78, and 88 min were further analyzed. Electrophoretic separation (Figure 2.3F) showed the progressive and significant reduction of high-molecular weight proteins in the fractions: eluate collected at 58 min contained proteins between 15 and about 60 kDa while eluate at 88 min was free from proteins above 15 kDa. Immunoblotting (Figure 2.3G) indicated that while the two earlier fractions contained only the 14 kDa complex, fractions at 78 and 88 min contained mostly the 5 kDa peptide. Molecular weight distribution (Figure 2.3H) revealed that the 88 min fraction contained the main peak at 7.2 min,

corresponding to 5 kDa lunasin, while for fraction at 78 min this peak was combined with a larger one at 7.0 min, probably associated with the 14 kDa complex. The difference in retention times during HPLC size exclusion chromatography profiling observed for the isolated lunasin (7.2 min) and the synthetic lunasin standard (6.6 min) could be a result of differences in the folding and geometry of the synthetic peptide in contrast to the native state. We selected 3 mL injection volume, a 75 pg column and100 mM NH_4HCO_3 pH 7.8 as operating conditions for purification of lunasin via preparative gel filtration chromatography.

2.4.3 Application of adjusted enrichment and purification processes to industrially produced tofu whey.

We used industrially produced whey to contrast our designed enrichment process using ethanol and pH 4.5 (EtOHpH4.5) with the more commonly used hot and cold drying alternatives to obtain solid fractions from liquid streams: spray drying (SDW), and freeze-drying (FDW). The three samples were purified via gel filtration chromatography as described in section 2.3.5. Chromatographic profile (Figure 2.4A) clearly showed that the ethanolic-isoelectric concentration process drastically switched the protein profile in the whey, making more prevalent the peak associated with lunasin (retention time 88 min). Peaks for lunasin (Figure 2.4B), compounds responsible for yellow coloration (Figure 2.4C), and carbohydrates (Figure 2.4D) were present in the three samples at the same retention times, suggesting that no evident conformational changes associated

with size and geometry occurred to these set of compounds by exposure to the drying temperature and conditions, or to the enrichment process. However, in comparison to the whole whey, in our lunasin enriched material the concentration of lunasin was considerably higher, while the presence of other compounds was almost negligible. This demonstrates that ethanolic – isoelectric precipitation not only removed an important quantity of undesired proteins, but other non- protein compounds present in the whey. In addition, it favors the completion of the chromatographic purification in a shorter time.

Electrophoretic and size exclusion chromatographic profile (Supplementary Figure 2.2) of purified fractions collected at 78 and 88 min (F78 and F88 respectively), from the sample treated with our enrichment method, showed that F78 still contained significant amount of the 14 kDa complex and other proteins of similar size, while F88 mostly correspond to the 5 kDa lunasin. Approximately 193 mg of F78 and 34 mg of F88 were obtained per liter of industrial tofu whey, containing 46% and 80% w/w lunasin, respectively (Supplementary Table 2.4). MALDI-TOF-MS analysis of the 5 kDa band in F78 and F88 (Table 2.3) indicated a 34% and 56% coverage of the 2S albumin precursor, respectively, and 100% coverage of lunasin sequence, corroborating the isolation of the peptide.

A six-step isolation process involving PBS extraction, ionic exchange chromatography (IEC), treatment with a reducing agent, ultrafiltration, reverse phase chromatography, and ultrafiltration, reported a yield of 442 mg lunasin/kg

defatted soy flour with >99% purity [9]. This report based the estimation of purity on the mere visualization of a unique electrophoretic band at 5 kDa in the final product, leaving behind the presence other compounds, such as carbohydrates, that are soluble in aqueous solutions and are present in the soybeans in abundant quantities but cannot be visualized via SDS-PAGE. Another four-step process that involved aqueous extraction, IEC, ultrafiltration, and gel filtration chromatography, was able to obtain 1.3 g lunasin/kg defatted soy flour, with 80% purity as assayed via HPLC [10]. Our three-step process (Figure 2.5) that involved ethanolic precipitation, isoelectric precipitation, and gel filtration chromatography yielded 772 mg/kg of dry tofu whey with at least 80% purity as analyzed via HPLC, with the remaining fraction being proteins of slightly higher molecular weight (Supplementary Figure 2.3 and Supplementary Table 2.4). This process is simpler, faster and valorizes a by-product largely produced by the tofu industry. Hence, our method can be used to obtain the naturally occurring bioactive peptide lunasin in a more economical, and environmentally friendly process leading to reduce wastage and increase utilization of tofu whey.

2.4.4 Biological activity of isolated lunasin

Oxidative stress and inflammation have been recognized as two of the principal factors contributing to the onset and development of non-communicable and infectious diseases [35]. The antioxidant and anti-inflammatory properties of lunasin have attracted attention to this peptide as a promising alternative for the prevention and treatment of some of these diseases [10,36,37]. Upon infection,

components of pathogens, including the LPS outer membrane of gram-negative bacteria, are recognized by innate immune cells such as macrophages, triggering signaling cascades that lead to the expression and release of pro-inflammatory cytokines and nitric oxide [38,39]. However, chronic inflammation derived from the inappropriate regulation of this process can produce damage to the host cells and lead to inflammation-related diseases [40].

To verify that the isolated lunasin maintained its biological activity, we tested the ability of the two purified fractions, F78 and F88, obtained from industrially produced whey treated with our designed enrichment method, to reduce pro-inflammatory response in LPS-activated murine macrophages. No detrimental effect over the viability of the immune cells (Figure 2.4E) was detected upon treatment with increasing concentrations of the two fractions. No effect was observed in most of the cases for fractions F78, while significant reduction of the pro-inflammatory markers was observed for fraction F88 (Figures 2.4 F-H), corroborating that our isolated lunasin is biologically active, and that a considerably high level of purity is required to exert the bioactive properties. The 30% reduction of TNF-α achieved by our isolated material at 2.4 mg/mL (190 μM lunasin) is comparable to the 23% reduction of TNF-α reported using 200 μM of synthetic lunasin under similar conditions of treatment of the same cell line [37].

2.5 Conclusions

A simple and feasible approach combining ethanolic-isoelectric precipitation and gel filtration chromatography was developed for the isolation of bioactive lunasin from tofu whey. In contrast to current isolation methods that waste valuable nutrients from the defatted soy flour or whole soybeans the implementation of our approach would favor the generation of three valuable products from the soybeans: tofu, an intermediate product resulting from the ethanolic treatment of the whey with potential as a protein dietary supplement, and a fraction highly enriched in lunasin. Our three-step process is competitive with current alternatives in terms of the quantity and purity of the final product, but faster and more sustainable. It promotes the valorization of a by-product largely produced by the tofu industry and at the same time facilitates a better utilization of soybean nutrients for the benefit and well-being of humans.

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Appendix

Tables and Figures

Table 2.1 Yield of products in tofu preparation at the laboratory scale.

Product	Units	Average
Dry Soybeans	g	100 ± 0
Okara	g	289 ± 11
Soy milk	mL	617 ± 29
Tofu	g	197 ± 7
Whey	mL	362 ± 20

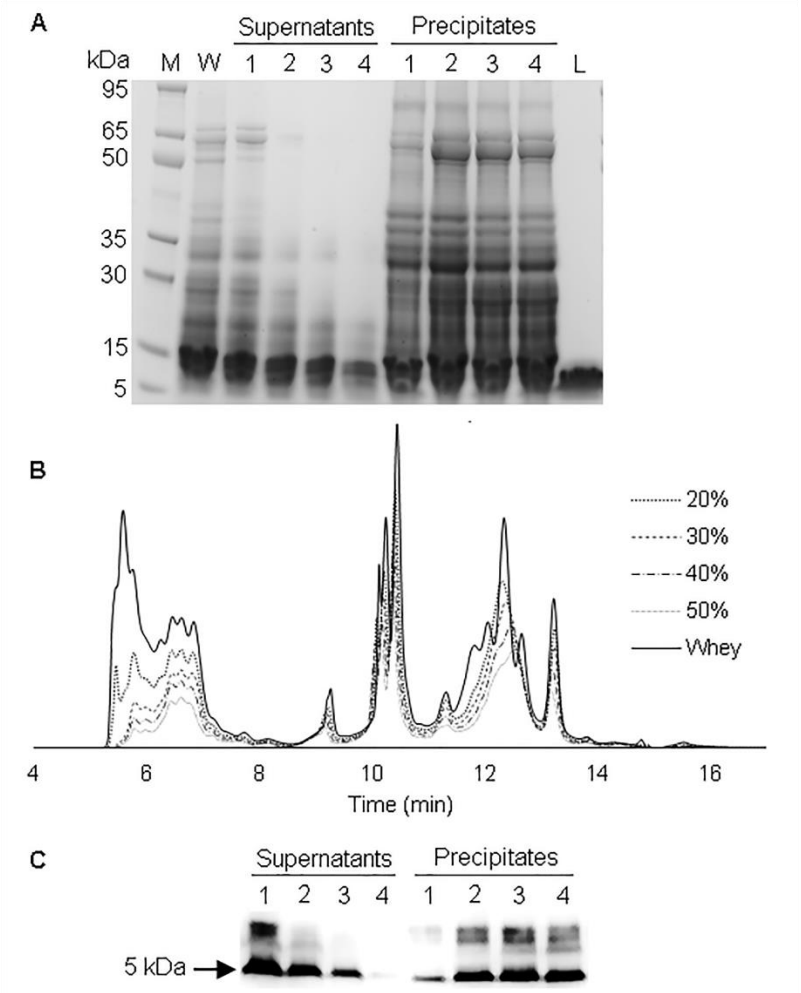


Figure 2.1 Ethanolic precipitation of high molecular weight proteins.

(A) SDS-PAGE, (B) molecular weight distribution profile, and (C) lunasin immunoblotting of supernatants (A, B, and C) and precipitates (A and C) after treatment of tofu whey with 20%, 30%, 40% or 50% v/v of ethanol (lines 1, 2, 3 and 4 respectively). Molecular weight marker (M), Non-treated tofu whey (W), and lunasin standard (L) are included as references.

Table 2.2 Characteristics of calcium and isoelectric precipitates from ethanolic whey.

Parameter	pH 4.5	pH 5.5	Ca
mg solid/mL whey	1.2 ± 0.1^{ab}	1.0 ± 0.1^{b}	1.4 ± 0.1^{a}
µg protein/mg solid	339 ± 90 ^{ab}	423 ± 55 ^{a}	247 ± 28 ^{b}
µg lunasin/mg solid*	59 ± 7^{a}	70 ± 2^{a}	39 ± 10^{b}
% lunasin in solid	5.9 ± 0.7^{a}	7.0 ± 0.2^{a}	3.9 ± 1.0^{b}
Daidzin (µg/mg)	3.7 ± 0.1^{a}	3.8 ± 0.4^{a}	2.9 ± 0.1^{b}
Genistin (ng/mg)	294 ± 3^{a}	295 ± 3^{a}	215 ± 1^{b}
Glycitin (µg/mg)	ND	ND	ND
Daidzein (µg/mg)	ND	ND	ND
Genistein (µg/mg)	ND	ND	ND
Glycitein (µg/mg)	ND	ND	ND
Trypsin Inhibitory Activity (TIA)	ND	ND	ND

ND: Non-detectable, *Estimated via ELISA. Different letters in the same row indicate significant differences among treatments ($p<0.05$)

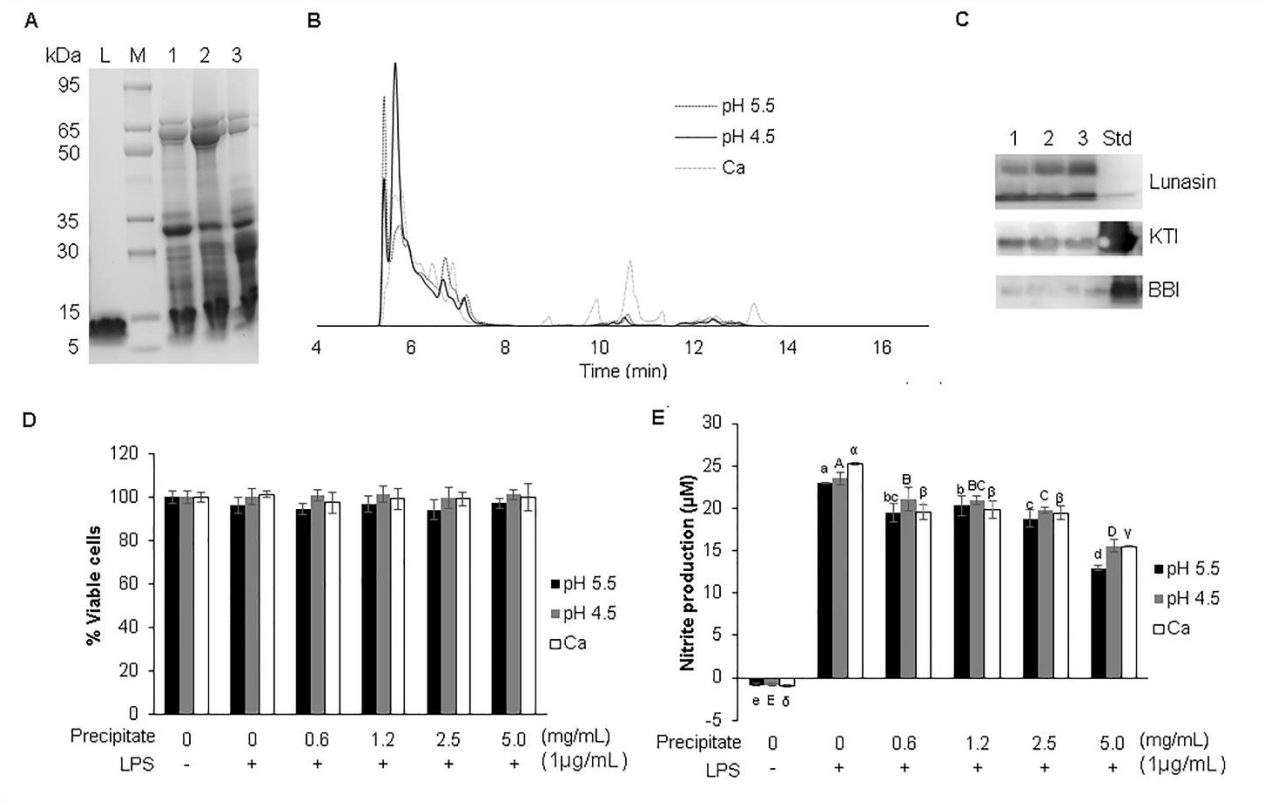


Figure 2.2 Precipitation of soluble proteins from ethanolic whey.

After treatment of the whey with 30% ethanol, lunasin enriched fractions were produced by precipitating remaining soluble proteins at pH 4.5 (line 1), pH 5.5 (line 2), or with calcium chloride (line 3). Protein precipitates were characterized via (A) SDS-PAGE, (B) size exclusion chromatography, and (C) Western blot. Lunasin (line L), Kunitz trypsin inhibitor (KTI), and Bowman-Birk inhibitor (BBI) standards (line Std) were included for reference. (D) Cell viability and (E) release of nitric oxide were measured as indicators of the biological activity of the lunasin enriched fractions. LPS-activated murine macrophages were treated with increasing concentrations of lunasin enriched fractions. Non-treated non-activated cells (Control), and non-treated LPS-activated cells (LPS) were used as

controls. Different letters indicate significant differences between the different concentrations of the same sample ($p<0.05$).

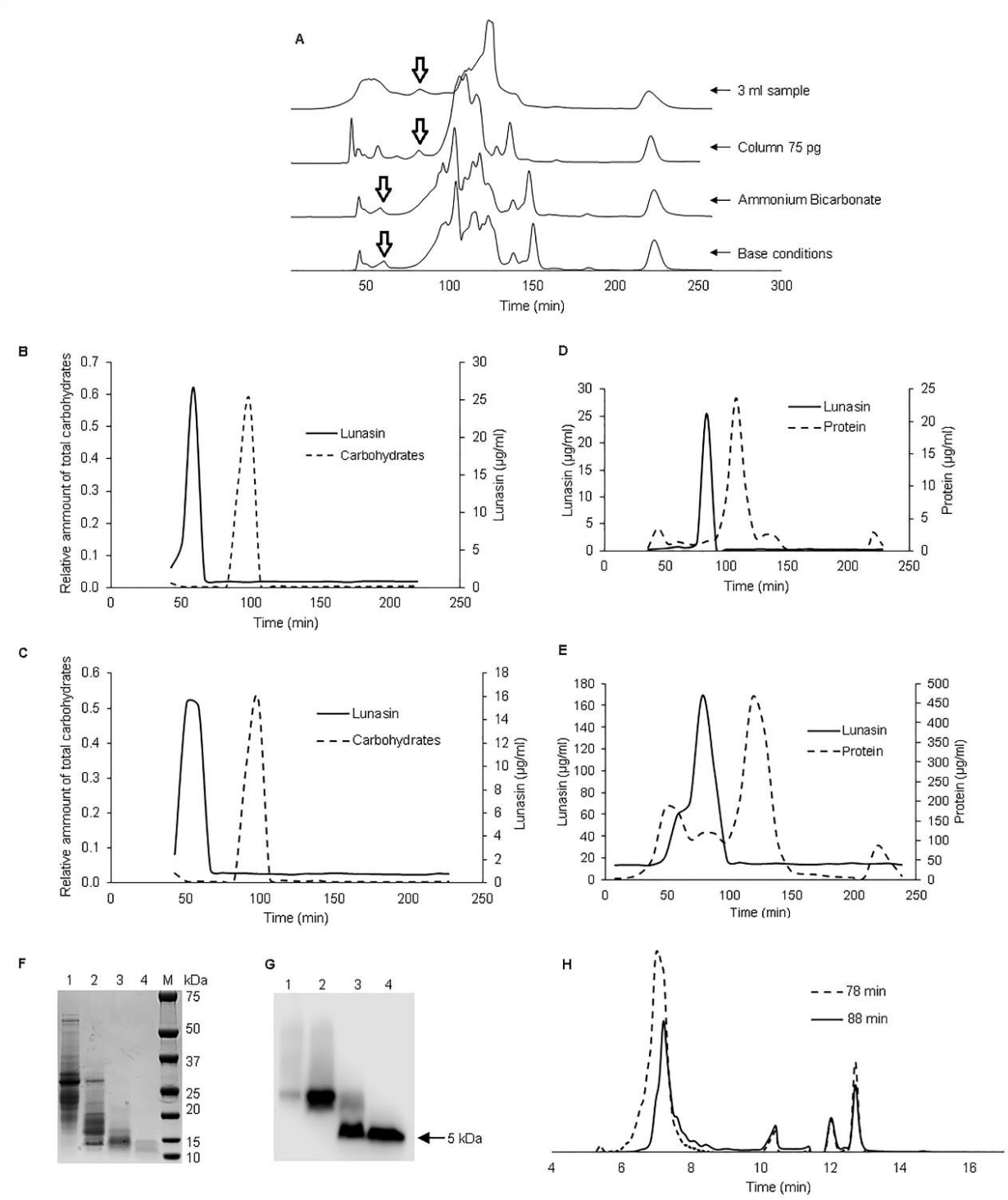


Figure 2.3 Adjustment of preparative gel filtration chromatography parameters.

The effect of the mobile phase (Tris-HCl or ammonium bicarbonate), preparation grade of the chromatographic column (30 or 75 pg) and sample volume injection (0.5 or 3 mL) were evaluated. (A) Gel filtration chromatographic profile, (B-E) lunasin, carbohydrates, and protein profiles under the different separation conditions. Base conditions: 30 pg column, Tris- HCl mobile phase and 0.5 mL injection volume (B), NH_4HCO_3 as mobile phase (C, D and E), 75 pg column (D and E), 3 mL injection volume (E). Open arrows indicate lunasin peak. As indicated in section 2.3.4, 3 mL of resuspended whey were purified using NH_4HCO_3 mobile phase and 75 pg column. Consecutive fractions of eluent were collected every 10 min. Fractions with a significant content of lunasin corresponding to average retention times of 58, 68, 78, and 88 min (lines 1 to 4 respectively) were characterized via (F) SDS-PAGE, (G) western blot against lunasin antibody and via (H) HPLC for molecular weight distribution profile.

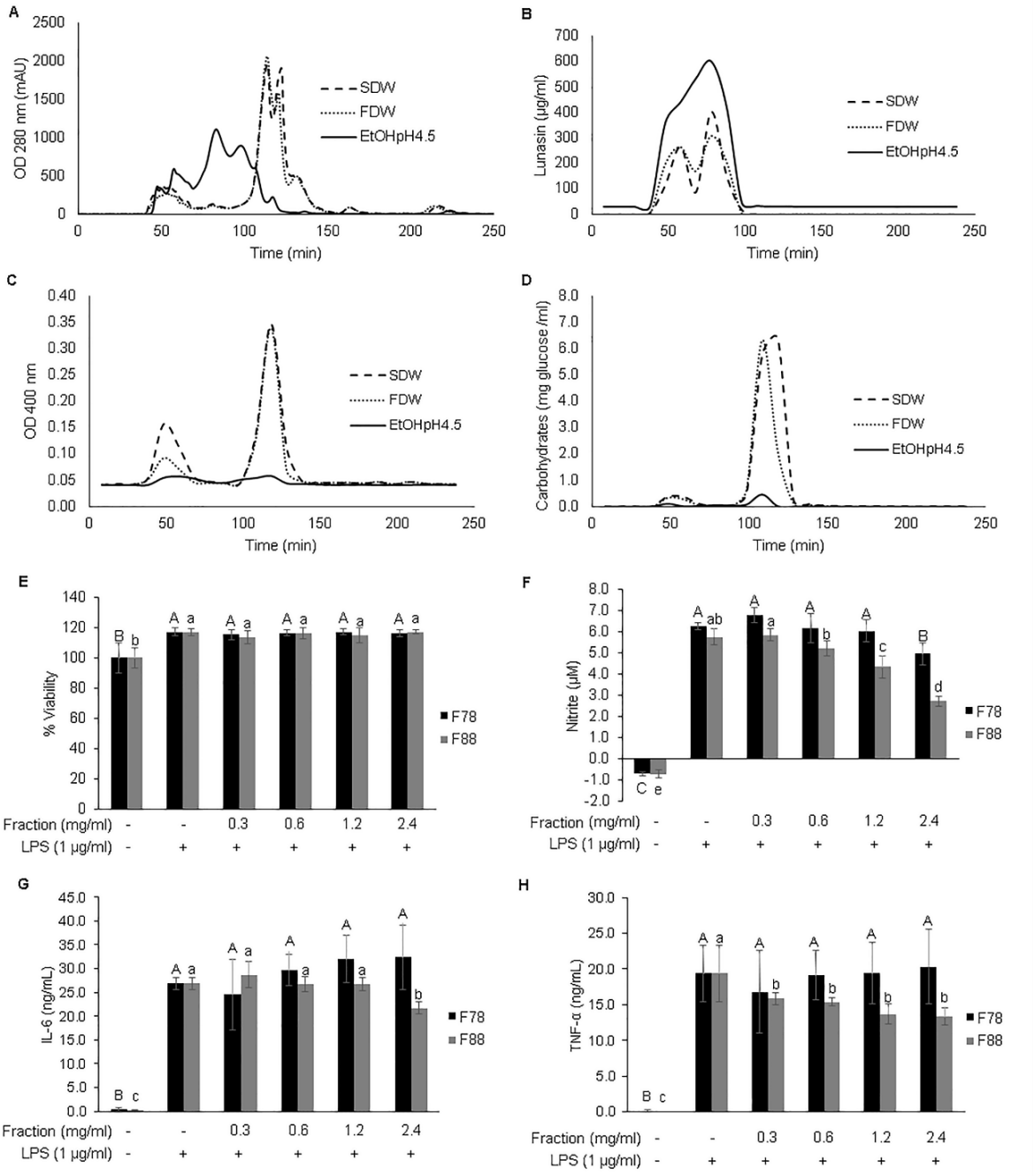


Figure 2.4 Isolation of lunasin from industrially produced tofu whey.

Industrially produced tofu whey was spray dried (SDW), freeze-dried (FDW), or concentrated using 30% ethanol and pH 4.5 (EtOHpH4.5). Each sample was

then purified via preparative gel filtration chromatography as described in section 2.3.5. Consecutive fractions of eluent were collected every 10 min and characterized for (A) protein content, (B) lunasin content, (C) yellow color intensity, and (D) total carbohydrates content. Fractions with a significant content of lunasin, corresponding to average retention times of 78min (F78) and 88 min (F88) were tested for bioactivity. (A) Viability, (B) production of nitric oxide, and release of pro-inflammatory cytokines (C) IL-6, and (D) TNF-α were evaluated in LPS-activated murine macrophages in the presence or absence of different concentrations of fractions F78 and F88.

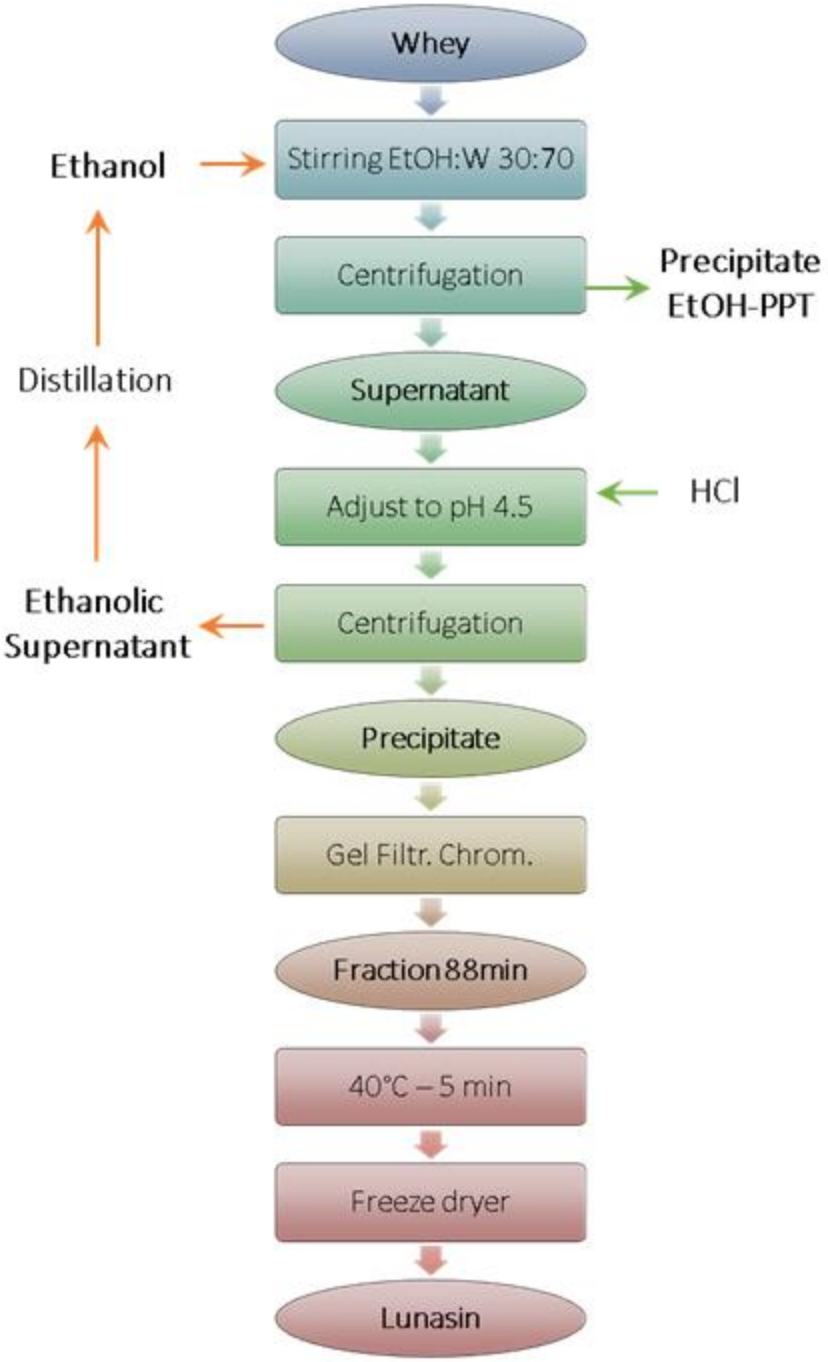


Figure 2.5 Process for the isolation of lunasin from tofu whey via ethanolic-isoelectric precipitation and gel filtration chromatography.

Table 2.3 MALDI-TOF-MS identification of the 5 kDa lunasin immunoreactive bands in fractions obtained via gel filtration chromatography of ethanolic-isoelectric enriched material from industrially produced tofu whey.

	Protein identified (NCBInr accession)	MOWSE (20 ppm)	Sequence coverage (%)	Peptides matched
Fraction F88	2S albumin precursor	462	34	HIMEKIQGR
	Gm-c1019 *Glycine max* (AW186094)			WQHQQDSCRK KWQHQQDSCR QLQGVNLTPCEK SKWQHQQDSCR FGPMIQCDLSSDD KQLQGVNLTPCEK IQGRGDDDDDDDDD IQGRGDDDDDDDDN QLQGVNLTPCEKHIMEK
	LLFCIAHTCS ASKWQHQQDS CRKQLQGVNL TPCEKHIMEK IQGRGDDDDD DDDDNHILRT MRGKINYITR NEGKDEDEEE EGHMQKCCTE MSELRSPKCQ CKALQKIMEN QSEELEEKQK KKMEKELINL ATMCRFGPMI QCDLSSDD_E VKSNVVTCTY _HMM__FM			
Fraction F78	2S albumin precursor	841	56	HIMEK
	Gm-c1055 *Glycine max* (BG042158)			CCTEMSELR ELINLATMCR WQHQQDSCR KWQHQQDSCR WQHQQDSCRK KWQHQQDSCR QLQGVNLTPCEK SKWQHQQDSCR IMENQSEELEEK FGPMIQCDLSSDD KQLQGVNLTPCEK IQGRGDDDDDDDDD IMENQSEELEEKQ IQGRGDDDDDDDDN IMENQSEELEEKQK
	GKMTKFTILL ISLLFCIAHT CSASKWQHQQ DSCRKQLQGV NLTPCEKHIM EKIQGRGDDD DDDDDDNHIL RTMRGRINYI RRNEGKDEHE EEEGHMQKCC TEMSELRSPK CQCKALHKIM ENQSEELEEK QKKKMENELI NLATMCRFGP MIQCDLSSDD			

Matched peptides are shown in red, and the underlined segment corresponds to lunasin peptide amino acid sequence (SKW…DDD).

Supplementary material

Preliminary evaluation of okara and tofu whey as potential sources of lunasin using direct calcium precipitation.

An alkaline proteinaceous extract from okara was prepared as follows: a mixture of okara: water at a mass:volume ratio 1:10 was adjusted to pH 9.0 and stirred at 80°C for 30 min to extract the soluble proteins present in the solid waste. After cooling down to room temperature, the mixture was centrifuged at 9000 *g* for 30 min. Okara extract supernatant and whey were treated with 20mM calcium chloride to induce direct precipitation of lunasin. Supernatant and precipitate of each by-product after calcium treatment were evaluated for protein and lunasin content as presented in Supplementary Table 2.1.

Supplementary Table 2.1 Characteristics of calcium precipitates from okara and tofu whey.

Parameter	Okara		Whey	
	Supernatant	**Precipitate**	**Supernatant**	**Precipitate**
mg of dry solid/g of okara or mL whey	23 ± 4	11 ± 2	24 ± 1	0.21 ± 0.05
µg protein/mg dry solid	60 ± 4	5 ± 1	91 ± 3	97 ± 14
µg lunasin/mg dry solid	0.74 ± 0.13	0.43 ± 0.02	15 ± 1	38 ± 3
% Lunasin in solid	0.07 ± 0.01	0.04 ± 0.00	1.46 ± 0.14	3.76 ± 0.41

Supplementary Table 2.2 Reference standards for HPLC size exclusion chromatography

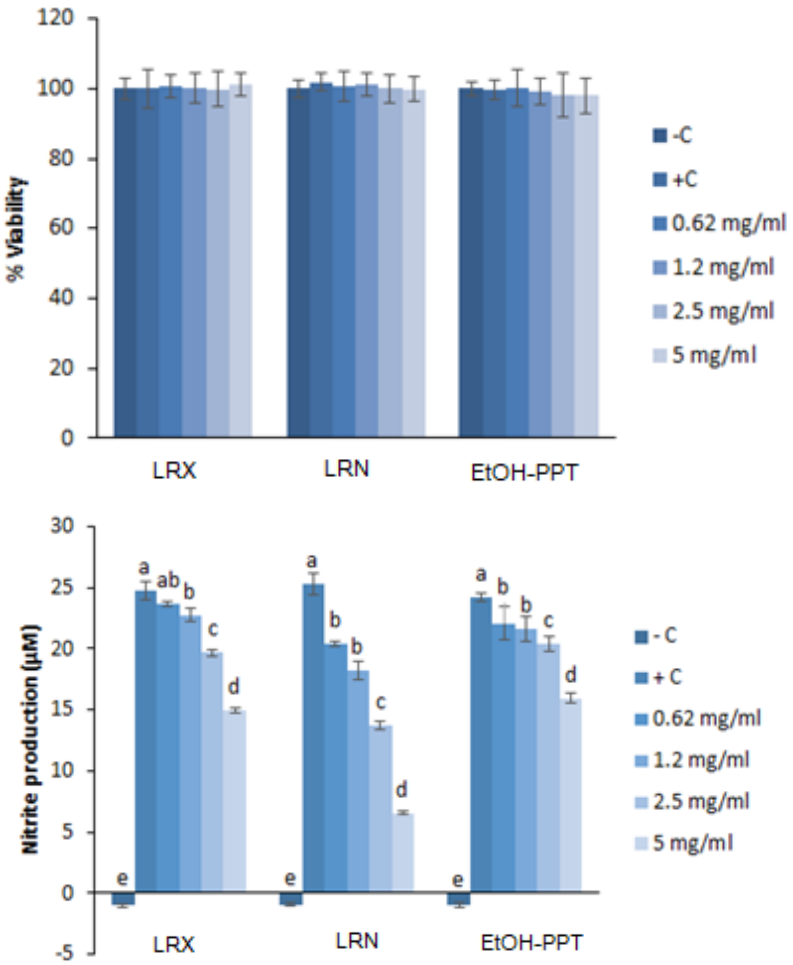
Standard	MW (Da)	RT (min)
Albumin	66000	5.281
Aprotinin	6500	6.564
Lunasin	5000	6.683
Glucagon	3842	7.351
Bradykinin Acetate	1060	7.980
Bradykinin 1-5	573	8.627
Glutathione	307	9.645
Glycine	75	9.802

Characterization of ethanolic precipitate form tofu whey

As part of the enrichment process, tofu whey is treated with 30% ethanol to induce the precipitation of high molecular weight proteins. The ethanol precipitate (EtOH-PPT) obtained during this step was characterized in contrast to commercially available lunasin dietary supplements LunaRich Now (LNR), LunaRich X (LRX), obtained from Reliv International, Inc. (Chesterfield, MO), and to defatted soy flour (DSF) from MP Biomedicals (Ohio, USA). Cell viability and release of nitric oxide were used as indicators of potential anti-inflammatory activity of the products.

Supplementary Table 2.3 Characterization of ethanolic precipitate form tofu whey and commercially available lunasin containing products.

Parameter	DSF	LRX	LRN	EtOH-PPT
µg protein/mg sample	36 ± 1	98 ± 3	41 ± 3	279 ± 33
µg lunasin/mg sample	3.4 ± 0.0	5.6 ± 0.7	3.4 ± 0.4	49.5 ± 5.3
µg lunasin/mg protein	96 ± 2	57 ± 6	83 ± 9	177 ± 4
% lunasin in solid	0.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	5.0 ± 0.5

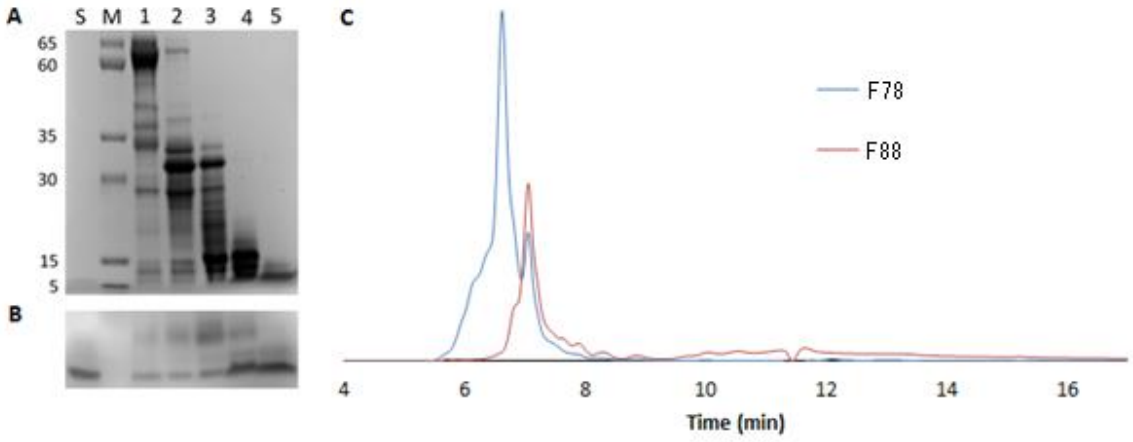


Supplementary Figure 2.1 Potential anti-inflammatory activity of ethanol precipitate and commercially available lunasin containing products.

Cell viability (top) and release of nitric oxide (bottom) were measured in murine macrophages activated with lipopolysaccharide, in the presence of different concentrations of the ethanolic precipitate or commercial products. Non-treated non-activated cells (-C), and non-treated LPS-activated cells (+C) were used as controls.

Characterization of fractions with relevant content of lunasin after enrichment and purification of industrially produced tofu whey.

Industrially produced tofu whey was treated with 30% ethanol and then adjusted to pH 4.5. The solid produced was resuspended and purified via gel filtration chromatography. Fractions of the eluate were collected every ten minutes and those with relevant content of lunasin were characterized.

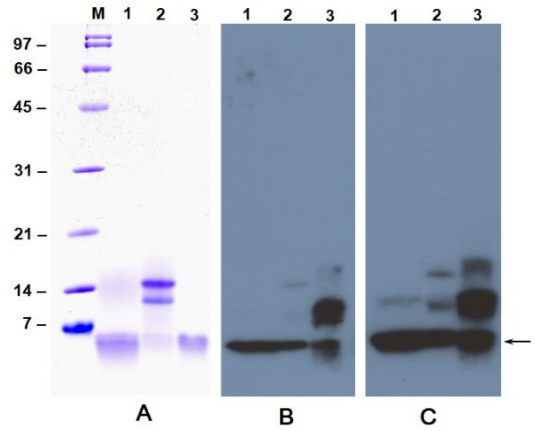


Supplementary Figure 2.2 Characterization of chromatographic fractions with a significant content of lunasin.

(A) SDS-PAGE, (B) western blot against lunasin antibody and (C) molecular weight distribution profile of fractions corresponding to retention times 48, 58, 68, 78, and 88 minutes (lines 1 to 5 respectively).

Supplementary Table 2.4 Characteristics of purified fractions obtained from industrial tofu whey via ethanolic-isoelectric precipitation and gel filtration chromatography. F78 and F 88 correspond to eluates collected at 78- and 88-min retention time.

Parameter	F78	F88
mg solid/L whey	193 ± 33	34 ± 11
µg protein/mg solid	336 ± 32	227 ± 43
µg lunasin/mg sample (HPLC)	465 ± 3	804 ± 79
% lunasin in solid	46.5 ± 0.3	80.4 ± 7.9



Supplementary Figure 2.3 Corroboration of lunasin identity using antibodies against different fractions of the peptide.

(A) SDS-PAGE, (B) Western blot using lunasin antibodies LUP 3 and (C) LUP 4 for fractions F88 (lane 1), F78 (lane 2) and synthetic lunasin peptide (lane 3).

CHAPTER 3. LUNASIN MODULATES THE ACTIVATION OF THE NLRP3 INFLAMMASOME AND REDUCES THE SUSCEPTIBILITY TO INFLAMMATORY BOWEL DISEASE IN IL-10$^{-/-}$ MICE

3.1 Abstract

Inflammatory bowel disease (IBD) is a condition of chronic inflammation that can cause severe damage to the gastrointestinal tract, compromising the health and well-being of thousands of people worldwide. Our goal was to investigate the protective effect of the soy peptide lunasin in an *in vivo* model of susceptibility to IBD and to identify *in vitro* the potential mechanism of action. In IL-10 deficient mice, oral administration of lunasin reduced the number and frequency of mice exhibiting macroscopic signs of susceptibility to inflammation and significantly decreased levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-18 by up to 95%, 90%, 90%, and 47%, respectively, in different sections of the small and large intestines. Dose-dependent decrease of caspase-1, IL-1β, and lL-18 in LPS-primed and ATP-activated THP-1 human macrophages demonstrated the ability of lunasin to modulate the NLRP3 inflammasome. The mechanism by which the inflammasome is modulated is through reduced protein levels of NLRP3. For the first time, we demonstrated that lunasin can decrease susceptibility to IBD in genetically susceptible mice by exerting anti-inflammatory properties.

3.2 Introduction

Inflammatory bowel disease (IBD) is a gastrointestinal disorder characterized by a state of chronic inflammation that results in severe damage of the bowel [1]. This results in intolerable intestinal and extra-intestinal symptoms,

and increases the risk of life-threatening conditions such as colorectal cancer [1,2]. It is estimated that more than 6.8 million people worldwide suffer from either Crohn's disease (CD) or ulcerative colitis (UC), the two major forms of IBD [3]. Immune cells and cytokines are crucial players in IBD, modulating the initiation, development, recurrence, and exacerbation of the inflammatory response and the associated clinical manifestations [4]. Hence, therapeutics frequently used to treat IBD such as steroids, aminosalicylates, and immunomodulators are molecules with strong immunosuppressive effects [5], or biologics that blockade highly specific inflammatory key targets such as tumor necrosis factor α (TNF-α), integrins, tyrosine kinases or their receptors [6]. However, serious adverse effects associated with these therapeutics include opportunistic infections, peptic ulcers, hepatotoxicity, pancreatitis, renal dysfunction, hypertension, myelosuppression, pulmonary fibrosis, and potential correlation with some malignancies [7]. Therefore, it is essential to seek effective and safe options to lessen intestinal inflammation with minimum or no adverse consequences.

Genetic susceptibility that leads to a dysregulated immune response to environmental triggers has been suggested as the potential underlying etiology of IBD [8]. Although only 26% and 19% of inheritable CD and UC cases can be explained by common genetic variants, progress has been made by identifying 163 susceptibility loci with direct implication in IBD, affecting pathways related to lymphocyte activation, innate mucosal defense, and the production of pro-

inflammatory and anti-inflammatory cytokines, particularly IFN-γ, TNF-α, IL-12 and IL-10 [8]. IL-10 and IL-10 receptor defects result in a severe early onset of the disease in humans, leading to intractable enterocolitis in infants and young children [9]. IL-10 deficient mice (IL-$10^{-/-}$) spontaneously develop colitis with transmural inflammatory infiltrates, ulcers, epithelial hyperplasia, crypt abscesses, and mucin depletion, which resemble histological characteristics of the disease in humans [10].

Activation of the inflammasome nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) has been reported in CD and UC patients with long-standing disease [11]. Also, the NLRP3 inflammasome has been found to play an important role in the development of spontaneous colitis in IL-10-/- mice [12]. Inflammasomes are multiprotein complexes that regulate the recruitment and activation of caspase-1, leading to maturation of pro-IL-18 and pro-IL-1β into active forms which are subsequently released to the extracellular environment, contributing to the inflammatory response [13]. NLRP3 inflammasome activation is a two-step process. During the priming stage, Toll-like receptors (TLR) agonists from pathogenic or commensal bacteria, act as inflammatory stimuli by inducing NF-κB mediated expression of NLRP3 and pro-IL-1β [14]. Then the activation stage is triggered by pathogen or danger-associated molecular pattern molecules (PAMPs and DAMPs, respectively) promoting the interaction and assembly of NLRP3 with the apoptosis-associated speck-like protein (ASC) containing a caspase recruitment

domain (CARD) via the pyrin domain, facilitating the interaction between the ASC and pro-caspase-1 CARD domains and mediating the cleavage of pro-caspase-1 into the active effector with proteolytic activity [14]. Blockade of the NLRP3 inflammasome by caspase-1 inhibitors has been shown to ameliorate spontaneous colitis in IL-10-/- mice [12]. Specific events, such as ion potassium efflux [15] and mitochondrial dysfunction, and particularly mROS [16], have been implicated in the activation of the NLRP3 inflammasome. Mitochondrially targeted antioxidants have been shown to suppress inflammasome-mediated cytokines and improve the outcomes of induced colitis *in vivo* [17]. Consequently, targeting the NRLP3 inflammasome has recently been proposed as a new therapeutic strategy for the treatment of IBD [18].

Lunasin is a naturally occurring soybean peptide with antioxidant, anticancer, and anti-inflammatory properties [19]. Lunasin decreases reactive oxygen species and the production of the pro-inflammatory mediators including TNF-α, IL-6, IL-1β, prostaglandin E_2, and nitric oxide through inhibition of nuclear factor – kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [20]. The ability of lunasin to regulate different inflammatory processes significantly involved in the burden of immune-related disorders has raised the scientific interest in this peptide as a potential preventive and therapeutic strategy against chronic diseases. Promising results have been found when evaluating lunasin in *in vivo* models of breast, melanoma, lung, and metastatic colorectal cancer, as well as cataracts, and cardiovascular disorders derived from lipid imbalance [20].

However, the assessment of lunasin in the context of bowel inflammation remains mostly unexplored. In this study, we aimed to explore the protective effect of lunasin on *in vivo* susceptibility to spontaneous colitis and to investigate *in vitro* the potential mechanisms involved in the modulation of the NLRP3 inflammasome.

3.3 Materials and Methods

3.3.1 Lunasin isolation

Lunasin enriched material was purified from tofu whey kindly provided by Farm Foods LLC (Summertown, TN) as previously described [21]. Briefly, ethanol was added to tofu whey to a final concentration of 30% v/v. After stirring for 10 min and centrifugation at 15800 x *g* for 15 min, the supernatant was transferred and adjusted to pH 4.5, followed by centrifugation under the same conditions. The precipitate was freeze-dried. For further purification utilizing preparative gel filtration chromatography (AKTA PURE protein purification system, GE Healthcare Life Sciences, Piscataway, NJ), the freeze-dried powder was resuspended in 100 mM ammonium bicarbonate pH 7.8 as mobile phase at 60 mg/mL, followed by filtration through a 0.45 µm nylon filter tip. Three mL of the permeate was injected in the chromatograph and separated on a HiLoad 16/600 75pg Superdex column, at room temperature and 1mL/min mobile phase flow rate. The fraction eluted between 83 and 93 min was collected, placed in a shaking water bath at 40 °C for 5 min to remove the volatile salts from the mobile

phase, and then freeze-dried. The final powder had a lunasin purity of at least 80% as assessed via HPLC.

3.3.2 *In vivo* experiments

Protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tennessee Knoxville (#2707-0619) and were conducted under the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and following ARRIVE guidelines. Eighteen IL-$10^{-/-}$ C57/BL6J 13-week-old male mice (Jackson Laboratories, Bar Harbor, ME) were housed in pairs under standard controlled conditions (12 h light/dark cycles, 30-50% relative humidity, 23 ± 3 °C) with free access to water and chow diet provided *ad libitum*. After one week of acclimatization, mice were randomly divided into three groups, control (n = 6), lunasin (n = 6), and caspase inhibitor (CI, n = 6). For 35 consecutive days, oral gavage of 100 µL of sterile water alone (control and CI groups) or containing 50 mg lunasin/kg body weight (bw) was performed. Based on the method described by Nair and Jacob [22], the human equivalent dose of lunasin supplementation is 4 mg/kg bw. The CI group received daily intraperitoneal injection of caspase-inhibitor Ac-YVAD-cmK at 1.25 mg/kg bw for 24 days before euthanasia. Due to the involvement of the inflammasome in the development of spontaneous colitis in this animal model, the CI Ac-YVAD-cmK is often used as a control treatment with positive outcomes [12]. On day 36, mice were euthanized via cervical dislocation. The small intestine was harvested and divided into duodenum,

jejunum, and ileum. Colon was dissected at the ileocecal junction and the length was recorded. Every intestinal section was gently rinsed to remove luminal contents and longitudinally dissected into two sections. One section of each tissue was fixed in formalin and paraffin-embedded for histopathological assessment, while the other half of each tissue was immediately frozen in liquid nitrogen and stored at -80 °C for further analysis.

3.3.3 Macroscopic disease activity index (DAI)

Body weight and fresh stool were monitored daily and scored from 0 to 3 according to the following parameters: stool consistency (0: normal, 1: moist and sticky, 2: soft, 3: diarrhea), hematochezia (0: normal color, 1: brown reddish stool, 2: visible blood in stool, 3: rectal bleeding), and weight loss (0: <2%, 1: 2 to 5%, 2: 5 to 10%, 3: >10% compared to the bw at the beginning of the study). The disease activity index was calculated as the sum of the scores for the three parameters, with 0 being the minimum and 9 being the maximum.

3.3.4 Analysis of fecal occult blood

Samples of stool were collected from the cages every third day, freeze-dried, and ground to analyze the presence of occult blood by quantifying hemoglobin content as previously reported [23].

3.3.5 Histopathological assessment

Hematoxylin and eosin-stained slides were used for histopathological disease scoring in a blinded fashion by board certified veterinary pathologist (MMS). Cellular infiltration, mucosal architecture, and epithelial changes were

evaluated in the three sections of the small and large intestine. Crypt abscess formation and depletion of goblet cells were also included for the evaluation of the colonic tissue. Scores were assigned following the criteria described by Erben *et al* for small intestine and colonic inflammation [24].

3.3.6 Quantification of pro-inflammatory cytokines and myeloperoxidase activity (MPO)

Tissue extracts or cell culture supernatants were used for the quantification of pro-inflammatory cytokines. Mouse TNF-α, IL-1β, and IL-6 were from BioLegend (San Diego, CA), human IL-1β and mouse IL-18 were from Invitrogen (Waltham, MA), human caspase-1 and IL-18 were from R&D Systems (Minneapolis, MN). Immunoassays were performed as described by the protocol provided by the manufacturer. Preparation of tissue extracts and evaluation of MPO activity were performed following previously published methods [23].

3.3.7 Cell culture, differentiation, and assessment of viability

THP-1 monocytes (ATCC, Manassas, VA) were cultured in RPMI-1640 basal medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 incubator. The same incubation conditions were used for all the experiments. β-mercaptoethanol at 0.05 nM was added to the complete media for regular maintenance of the culture. When required, cells were seeded at a density of 1 x 10^5 cells/well in 96-well plates, or 1 x 10^6 cells/well in 6-well plates, incubated in complete media with 100 ng/mL of phorbol 12-myristate-13-acetate (Millipore Sigma, St. Louis, MO)

for 48 h to induce differentiation into macrophages. A subsequent resting phase of 48 h in complete media alone was allowed before the initiation of the indicated experiment. After the specific treatment was performed, cell viability was assessed by the MTS assay (Promega, Madison, WI) following the manufacturer's protocol.

3.3.8 Activation of NLRP3 inflammasome via NLRP3 agonist

After the resting period, differentiated macrophages were primed for 6 h with 1 µg/mL lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, St. Louis, MO) in plain media, in the presence or absence of lunasin at the indicated concentration. Then, adenosine triphosphate (ATP) (Alfa Aesar, Tewksbury, MA) was added to each well to reach a final concentration of 5 mM and incubated for 2 h. Treatment media was collected to test for pro-inflammatory cytokines and cells were used for cell viability test or lysed and harvested for western blot analysis. Plain media without LPS or ATP were used as the negative control.

3.3.9 Western Blot

Western blot was performed following standard protocol with the following specifications: proteins in the gel were transferred to PVDF-membranes using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA), membranes were blocked with 5% non-fat dry milk in 0.01% Tween 20 Tris-buffered saline for 1 h at room temperature, primary antibodies against the indicated antigens (Proteintech, Chicago, IL) were incubated at 1:1000 dilution overnight at 4 °C, and secondary antibody at 1:2000 dilution for 1 h at room temperature.

Immunoreactive bands were visualized via chemiluminescence using a C-Digit Blot scanner (LI-COR Biosciences, Lincoln, NE) and analyzed using Image Studio™ software.

3.3.10 Mitochondrial reactive oxygen species (mROS)

Antimycin A is a powerful inhibitor of the mitochondrial respiratory chain that blocks complex III and induces superoxide production [25]. THP-1 monocytes were incubated for 1 h with plain media with or without 5 mg/mL of lunasin. Antimycin A from *Streptomyces sp.* (Sigma Aldrich, St. Louis, MO) was added to a final concentration of 40 µg/mL and incubated for 5 h. After removal of the treatment media, cells were incubated for 30 min with 3 µM MitoSOx™ (Thermo Scientific, Waltham, MA), washed with Hank's balanced salt solution (HBSS), and then analyzed using a MACSQuant10 flow cytometer (Miltenyi Biotec, Auburn, CA). For fluorescence microscopy, 1 x 10^5 cells/well were seeded in 8-well ibidi µ-slides and differentiated into macrophages as indicated in section 3.3.7. After the resting phase, cells were treated with lunasin and antimycin A as previously described. The treatment media was collected to evaluate pro-inflammatory cytokines, and the cells were incubated with MitoSOx probe. Micrographs of the cells were captured using bright-field and red fluorescence channels in an EVOS™ M7000 microscope (Invitrogen, Waltham, MA) using a 20x objective. The number of positively stained cells was manually counted in the merged image of at least six different fields taken for each replication.

3.3.11 Activation of the inflammasome via induced K^+ efflux

Differentiated macrophages were primed for 6 h with or without LPS, in the presence or absence of 5 mg/mL lunasin. K^+ conditioned media consisted of K^+ free PBS supplemented with KCl to reach the specified concentration of potassium (0, 0.5, 1.0 or 5.0 mM). Priming media was removed and K^+ conditioned media with or without 5 mg/mL of lunasin was added to the cells and incubated for the specified time to induce inflammasome activation. After treatment, K^+ conditioned media was collected and used for the quantification of pro-inflammatory cytokines. Regular growth media was used as control to compare the effect of K^+ conditioned media.

3.3.12 Statistical analysis

A Principal Component Analysis (PCA) of the data from *in vivo* experiments was performed using MATLAB (The Mathworks Inc., USA). An 18 x 127 matrix was created containing the following data: daily relative bw, stool consistency and hematochezia scores, colon length, myeloperoxidase activity, and pro-inflammatory cytokines for the duodenum, jejunum, ileum, and colon, for every mouse belonging to the three treatment groups. The technique of Wilks' lambda was carried out to select the variables with higher discriminant potential among treatments. A 18 x 42 matrix was created with the 33% of variables with the highest lambda coefficient and used to generate the PCA model with the graphical user interface for PCA developed by the Milano Chemometrics and

QSAR Research Group, Department of Earth and Environmental Sciences, University of Milano–Bicocca, Milano, Italy [26].

Results are reported as mean ± SEM for *in vivo* experiments, and as mean ± SD for *in vitro* analyses. Significant differences ($p < 0.05$) were assessed using the non-parametric Mann-Whitney test, or the parametric t-student test, accordingly, when comparing two treatments. ANOVA followed by the post hoc Tukey test, was used for the comparison of three or more treatment groups.

3.4 Results

3.4.1 Oral administration of lunasin attenuated macroscopic indicators of intestinal inflammation and reduced pro-inflammatory cytokines in the bowel of IL-10$^{-/-}$ mice.

Body weight loss, diarrhea, and the presence of blood in the stool are among the most common manifestations of IBD [1]. We applied a scoring system to keep track of noticeable changes related to these symptoms. No significant decrease in the average bw was observed for any group at any time during the experiments (Figure 3.1A). However, our records indicated that decreases of up to 7, 6, and 8% of the initial bw occurred in individual mice for the control, lunasin, and CI groups, respectively, at different time points. Also, although no significant differences were found between control and lunasin groups on any given day, the trend indicated that while lunasin administration maintained constant bw, control mice gradually gained weight over time. Interestingly, administration of the CI resulted in a

significantly lower relative bw in comparison to lunasin supplementation between days 12 and 16, and significantly lower relative bw than the control over the last 12 days of the experiment.

Upon visual inspection, no evident blood was identified in the stool or perianal region of any mice, but variation in the color of the fecal matter towards a red tone, receiving a score of one, was registered more frequently for the control group than for the lunasin and CI groups (Figure 3.1B). To account for the possible presence of occult blood in the fecal matter, hemoglobin content was measured in the stool sample. Figure 3.1C revealed two periods of time when the presence of hemoglobin, for the control group, was up to six times higher than the baseline. These results suggest that potential minor intestinal injuries led to a subtle but recurrent presence of blood in the stool of control mice, which could not be detected by simple inspection, but enough to produce a change in the color of the feces. Moreover, we found that with the lack of treatment, stool became stickier and softer (scores 1 and 2, respectively) between two and three times more often for the control group than for treated mice (Figure 3.1D). The disease activity index (Figure 3.1E) indicated that, overall, the animals exhibited mild macroscopic symptoms of bowel inflammation, with scores 1 and 2 being the more frequent for all the groups. The maximum scores observed for individual mice were 4 (1 mouse each in control and lunasin groups) and 3 (4 mice in control, 1 in lunasin, and 2 in CI groups). DAI scoring was strongly affected by the observed decrease in bw in the CI group. Taken

together, our observations indicate that the susceptibility to minor inflammatory events (frequency and severity) was reduced when oral supplementation of lunasin was provided.

To further evaluate the effect of lunasin administration on the intestinal tissues, we harvested the small and large intestines at the end of the experiment. We observed that the characteristic colon shortening in IL-10$^{-/-}$ mice (Figures 3.1F and 3.1G) was significantly alleviated by lunasin, exhibiting colons on average 13 mm longer than those from the control and CI groups. Colon shortening or stiffening is a potential outcome of the involvement of the mucosal and submucosal layers of the colon during chronic inflammation [27], hence we examined for potential microscopic morphological alterations. Interestingly, histomorphology evaluation indicated that neither the colon nor the small intestine had significant structural changes associated with inflammation. No damage in mucosal architecture, epithelial changes, depletion of goblet cells, or formation of crypt abscess was detected in any intestinal section. Low cellular infiltration with scattered individual eosinophils was seldomly observed in different sections of the small intestine in random mice but was common in the colonic mucosa of most mice in all the groups. Histological evaluation agreed with the macroscopic observations as the possible inflammation flares that led to the presence of blood in the stool occurred during the first two weeks of the experiment, but no indicators of inflammatory events were observed during the last weeks.

The potential contribution of immune cells to intestinal inflammation was investigated by quantifying MPO activity and levels of pro-inflammatory cytokines in the tissues. MPO is an enzyme that catalyzes the production of potent reactive oxygen species and has been directly correlated with immune cell trafficking during intestinal inflammation [28]. No significant differences were detected in MPO activity among the three groups for any of the intestinal sections. Neutrophils are a major source of MPO [29], hence these results corroborate histological findings and indicate that, at least by the end of the experiment, the presence and activity of granulocytes (neutrophils and eosinophils) in the intestine were not significant.

The profile of pro-inflammatory cytokines for the four intestinal sections of each mouse is outlined in Figure 3.2A. Although some sections exhibit higher levels of cytokines than others, not all the mice in the same treatment group are identically affected. The dissimilar distribution of the proinflammatory cytokines throughout the bowel for mice sharing the same environmental conditions, genetic background and feeding patterns, serves as a display of the inherent variability in predisposition to inflammation that characterizes IBD. We observed that, on average, TNF-α (Figure 3.2B) and IL-1β (Figure 3.2D) were prevalent in the distal regions of the bowel (ileum and colon), while IL-6 (Figure 3.2C) was more evenly distributed among all the sections and IL-18 (Figure 3.2E) was more strongly present in the proximal regions of the intestine (duodenum and jejunum). Reports show increased levels of TNF- α, IL-6, and IL-18, relative

to healthy controls, in biopsies of CD and UC patients in absence of mucosal markers of damage, indicating that these cytokines may exert immune activating roles without triggering immediate tissue injury [28]. Lunasin effectively reduced IL-18 in the colon by 47%, IL-6 in the jejunum by 90%, IL-1β in the duodenum and colon by 90% and 80%, respectively, and TNF-α in the colon by 95%. In contrast, the administration of caspase-1 inhibitor led to a reduction of IL-1β that was only significant in the colon but like those observed for lunasin treatment in the small intestine. Caspase inhibition led to a significant decrease of TNF-α as well, but not IL-18, in the ileum and colon.

3.4.2 Principal component analysis of characteristic in vivo parameters

Considering the high variability observed, we performed a multivariate analysis via PCA to establish the potential categorization of the mice based on the evaluated variables. We observed that the three treatment groups can be clearly differentiated (Figure 3.3A). The control group was characterized by a markedly elevated level of the pro-inflammatory cytokines TNF-α and IL-1β in the duodenum, ileum, and colon, and IL-6 in the jejunum, a noticeably softer stool consistency on days 15, 19, 27, and 30, and a perceptible change in the color of the stool on days 9, 12, and 22 that was probably due to the presence of occult blood (Figure 3.3B). The distribution of the relative bw indicate that the lunasin group had a heavier bw during the first 10 days of the experiment, while the control group weighed heavier for the last 10 days, and the CI-treated group had the lowest bw throughout the study. Collectively,

evaluation of macroscopic manifestations and intestinal tissues suggests that potential inflammatory events occurred in the intestines of mice in the control group. Administration of lunasin was effective in alleviating these events and reduced pro-inflammatory cytokines throughout the bowel.

3.4.3 Lunasin modulates the activation of the NLRP3 inflammasome.

To address the potential role of lunasin in the modulation of the inflammasome, we used LPS-primed and ATP- activated differentiated THP-1 human macrophages with or without lunasin treatment. No detrimental effect was observed over the viability of the cells upon exposure to the different stimuli (Figure 3.4A). Priming with LPS induced a significant increase in the extracellular concentration of IL-1β (Figure 3.4B) and IL-18 (Figure 3.4C). The presence of both cytokines in the medium was further increased by the addition of ATP. We observed a dose-dependent decrease in the production of these cytokines in the presence of lunasin. At 2.5 mg/mL lunasin, the release of IL-18 and IL-1β was significantly reduced by 40% and 45%, respectively. At a concentration of 5.0 mg/mL, their extracellular concentration was reduced by 63% (IL-18) and 67% (IL-1β). This dose-dependent reduction observed upon lunasin treatment suggests the involvement of a common upstream mechanism. The shared activator molecule caspase-1 (Figure 3.4D) had the same level of reduction in concentration (42% and 66% for 2.5 and 5 mg/mL treatments, respectively). To further clarify the potential role of lunasin, we analyzed the protein levels of the molecules involved in the assembly of the NLRP3

inflammasome (Figure 3.4E). Lunasin treatment led to a significant 3-fold decrease in the sensing molecule NLRP3, with no significant effect on ASC and pro-caspase-1. These data indicate that the modulatory effect of lunasin over the pro-inflammatory cytokines IL-18 and IL-1β was mediated by the abrogation of NLRP3.

3.4.4 Lunasin-induced decline of extracellular IL-1β is independent of mROS.

To assess whether the antioxidant properties of lunasin can be translated into the mitochondria, we used antimycin A to induce mROS production. Challenging the cells with antimycin A led to a 6-fold increase of IL-1β (Figure 3.5A) that was reduced by half in the presence of lunasin. Detection and quantification of mROS by the mitochondria-targeted probe MitoSOx^{TM}, via fluorescence microscopy (Figures 3.5B and 3.5C) and flow cytometry (Figures 3.5D and 3.5E) revealed that non-stimulated cells exhibit basal levels of mROS. The number of cells and the intensity of the staining, which accounts for mROS present in the cells, were significantly increased by antimycin A. No effect was observed in the presence of lunasin, demonstrating that the decreased release of IL-1β to the extracellular media was mediated by mechanism independent of mROS.

3.4.5 Lunasin-induced decline of extracellular IL-1β is not mediated by blockade of potassium efflux.

Efflux of intracellular K^+ has been demonstrated as the common and vital upstream trigger required to induce the assembly of the inflammasome [15]. Therefore, to investigate the potential involvement of lunasin in this process, we manipulated the extracellular concentration of K^+ to induce a natural efflux of the ion and the consequent activation of the NLRP3 inflammasome in the presence and absence of lunasin. First, we exposed LPS-primed cells to media with reduced K^+ levels for different time-lengths and quantified the release of IL-1β (Figure 3.6A). At 15 and 60 min of treatment, no significant differences on IL-1β were observed among the reduced concentrations of extracellular K^+. After 2 h of exposure to reduced K^+, the concentration of the pro-inflammatory cytokine in the extracellular environment was inversely proportional to the concentration of extracellular K^+ and was significantly different among all the tested conditions. This indicates that approximately 2 h is needed for the cells to elicit a response proportional to the extracellular levels of K^+. During this time, the cells sense and then react to the decreased levels of K^+, activate the inflammasome, and release mature IL-1β. Hence, 2 h was selected as treatment time for further analysis.

Next, we compared the viability and the release of IL-1β and IL-18, in non-primed (LPS -) and LPS-primed (LPS+) cells exposed to different concentrations of extracellular K^+. K^+ free medium had a detrimental effect by decreasing viable cells between 20 and 40%, while 0.5 mM only decreased viability of non-primed

cells by 8% (Figure 3.6B) when compared to cells maintained in regular media. At 1.0 and 5.0 mM, the observed significant increase in viability is probably due to an incremented metabolic activity of the cells associated with K^+ efflux. K^+ concentration lower than 1.0 mM was enough to induce activation of the inflammasome in primed and non-primed cells, leading to a significantly increased release of IL-1β (Figure 3.6C) and to a lower extent of IL-18 (Figure 6D). These results are consistent with previous observation reported by Muñoz-Planillo *et. al* in bone marrow derived macrophages [15], indicating that a low concentration of K^+ in the extracellular media is enough to activate the NLRP3 inflammasome, in absence of a priming signal or an NLRP3 agonist. In our study, the high levels of IL-1β and IL-18 concomitant to the reduced viability in K^+ free media suggests that this condition may be detrimental to the cells. Pyroptosis is a form of cell death regulated by the activation of caspases via the inflammasome [30]. Though pyroptosis may be occurring in all the tested concentrations, it seems to start becoming significant at [K^+] of 0.5 mM or lower. Based on these observations, we selected 1.0 mM K^+ as the appropriate condition to test the involvement of lunasin in the modulation of the inflammasome triggered by potassium efflux.

First, we evaluated the effect of lunasin treatment during the priming phase. We primed the differentiated macrophage with LPS (LPS+) in the presence or absence of lunasin (Lunasin+ or Lunasin- respectively). Cells treated with lunasin only (LPS- and Lunasin+) and non-stimulated cells (LPS- and

Lunasin-) were used as controls. At the end of the priming phase, the media was replaced by 1.0 mM K^+ conditioned media for 2 h to induce the activation of the inflammasome. Release of IL-1β (Figure 3.6E) and IL-18 (Figure 3.6F) was evaluated after the priming and activation phases. During the priming phase we did not detect the release of IL-1β by non-stimulated cells, but a significant release was observed in cells treated with lunasin alone. LPS-priming alone induced the release of IL-1β, but it was reduced by 60% when primed cells were simultaneously treated with lunasin during the priming phase. After activation, extracellular IL-1β increased for all samples as expected. However, LPS-primed only, lunasin-treated only, and cells primed in the presence of lunasin, all exhibited the same concentration of IL-1β after activation. In contrast, IL-18 was detected during the priming phase in all the samples, although the concentration in non-stimulated cells was significantly lower. The release of IL-18 was boosted after inflammasome activation, but surprisingly the highest levels were observed when cells had been treated with lunasin. It is possible that the presence of lunasin somehow leads to the accumulation of intracellular pro-IL-18, that upon induction of K^+ efflux was activated and released to the extracellular environment. By contrasting these results with those observed in Figures 3.4B and 3.4C we inferred that the activity of lunasin may not be limited to the priming step.

Then, in a different approach, we examined if lunasin could be engaged in the modulation of K^+ efflux during the activation phase, by evaluating the cellular

response in the presence or absence of the peptide. We postulated that if lunasin was able to inhibit K^+ efflux to some extent, extracellular concentration of both cytokines (in both LPS-primed and non-primed cells) should be lower than in conditions where lunasin was absent. This was only observed for IL-1β produced by LPS-primed cells (Figure 3.6G). As no positive effect was observed in the extracellular concentration of IL-18 (Figure 3.6H) under any condition, we deduced that lunasin was not able to inhibit potassium efflux.

3.5 Discussion

Here, for the first time, we have demonstrated that lunasin decreases markers associated with susceptibility to chronic intestinal inflammation *in vivo*. We further demonstrated the direct inhibitory role of lunasin in the maturation of the pro-inflammatory cytokines IL-18 and IL-1β by impacting the activation of the NLRP3 inflammasome via reducing the protein levels of the sensing molecule NLRP3. IL-10 acts as an anti-inflammatory cytokine that moderates the inflammatory response triggered by bacterial antigens [31]. It can suppress the release of pro-inflammatory cytokines and regulate the differentiation, proliferation, and effector functions of different subsets of innate and adaptive immune cells, acting as a key mediator of immunotolerance [31,32]. Upon the defective or absent immunoregulatory function of IL-10, intestinal homeostasis is lost, leading to a dysregulated inflammatory response characterized by the persistent activation of pro-inflammatory pathways that result in adverse consequences for the intestinal tissue [10].

Our results indicate that the exacerbated levels of pro-inflammatory signals occurring in the bowel, and the frequency of spontaneous intestinal symptoms, due to the absence of IL-10, can be modulated by the oral administration of lunasin. Upon the absence of IL-10, antigens from the natural intestinal microflora trigger an abnormal release of pro-inflammatory signals that are harmful to the intestine and lead to disease symptoms (loss of bw, diarrhea, and intestinal bleeding). When lunasin was administered, the mechanisms involved in the production of those pro-inflammatory signals, particularly TNF-α, IL-6, IL-18, and IL-1β, are hindered, counteracting the inflammatory imbalance, and therefore reducing the adverse consequences. These observed effects indicate the immunomodulatory potential of lunasin peptide as a strategy to return the balance lost when natural immunomodulatory mechanisms fail. The negative impact of the caspase inhibitor over bw can be explained by the role of caspase-1 in the regulation of metabolic functions that influence body weight, such as glucose homeostasis and lipid metabolism [33]. Treatment of obese mice for two weeks with caspase-1 inhibitors has shown a decreased gain of body weight accompanied by an altered composition of the white adipose tissue [34].

Our research group reported that pepsin and pancreatin hydrolysis can impair the ability of lunasin preparations to inhibit activation of the inflammasome [35]; however, differences in the *in vitro* and *in vivo* digestion mechanisms could result in different digestion products with distinct biological activity. In addition, it

is important to note that the lunasin enriched preparation contained considerable amounts of other proteins that were affected by the proteolytic process. It is possible that the decrease observed in the inflammasome inhibitory activity was the result of the diminished contribution of these components, rather than a direct result of lunasin digestion. This idea is supported by a recent report that indicates the antihypertensive and antioxidant activity of fragments released from the *in vitro* gastrointestinal digestion of synthetic lunasin, as well as their protective effect against oxidative damage and inflammatory stimulation of murine macrophages [36]. Our findings corroborate that upon oral administration, lunasin digestion products exert anti-inflammatory properties and can efficiently decrease levels of pro-inflammatory cytokines along the small and large intestines. Biodistribution of lunasin derived peptides along the bowel may be responsible for the variable activity observed in the cytokine's profile for each intestinal section. The substantial impact observed in the levels of IL-1β and IL-18 in lunasin treated mice suggests that the peptide could have a modulatory role over the inflammasome.

Liu *et al.* reported that the activity of NLRP3 inflammasome was elevated before colitis onset, progressively increasing as the disease worsened, and reached a maximum peak when the macroscopic indicators appeared [37]. Hence, this inflammatory mechanism has been proposed as a new therapeutic target in the pursuit of better clinical outcomes for IBD [18,38]. Here we demonstrated that lunasin can modulate the activation of the NLRP3

inflammasome. The observed trends during inflammasome activation triggered by potassium efflux indicate that the presence of the peptide during the activation phase is necessary for limiting the release of the mature IL-1β but not of IL-18. In addition, under the absence of pathogenic stimuli, lunasin can act as a priming signal to induce inflammasome activation at a lower extent and appears to promote the accumulation of pro-IL-18, but these effects are altered by the presence of inflammatory signals. These observations are in agreement with recent studies that have demonstrated immunomodulatory activity of lunasin by the upregulation of pro-inflammatory signals such as IL-6 and IL-1β in dendritic cells [39], TNF-α, IL-1β, and COX-2/ PGE_2 in *ex-vivo* explants of human mucosa [40], and interferon-γ and granzyme B in natural killer cells [41]. Our results are the first report demonstrating the upregulation of IL-18 and IL-1β in human macrophages. Further investigation is required to corroborate our observations and identify the potential mechanisms that regulate the observed effect over IL-18.

The mechanism of action of lunasin does not involve mitochondrial ROS or a direct inhibitory effect over potassium efflux, but rather the blockade of the NLRP3 inflammasome by reducing the protein levels of NLRP3. NLRP3 transcriptional regulation is dependent on the NF-κB pathway [18]. NF-κB, one of the most important regulators of proinflammatory gene expression, controls the synthesis of TNF-α, IL-6, IL-1β, and IL-8, and the expression of COX-2, among others, upon activation by pro-inflammatory signals such as PAMPs and pro-

inflammatory cytokines [42]. Treatment of RAW 264.7 LPS-activated macrophages with lunasin reduced the activation of NF-κB by inhibiting the translocation of p50 and p65 subunits of NF- κB to the nucleus, impacting its transcriptional activity [43]. Therefore, it is highly probable that inhibition of the NF-kB transcriptional activity is responsible not only for the observed decreased protein levels of NLRP3 *in vitro* but the reduced levels of TNF-α and IL-6 *in vivo*. Our observations suggest that the effect of lunasin is not limited to the downregulation of NLRP3 during the priming phase but is rather more important during the activation phase. Upon activation, the release of mature IL-1β serves as a sustained and rising priming signal that continuously triggers the NF-kB pathway, leading to the transcription of NLRP3 and pro-IL-1β, which are then activated and contribute to the progressive amplification of inflammation. The inhibitory activity of lunasin over NLRP3 appears to significantly diminish this feedback amplifying loop. It is essential to note that in contrast to NLRP3 and pro-IL-1β that are transcriptionally regulated by NF-κB, pro-IL-18 is constitutively expressed in nearly all cells in humans and animals, including endothelial cells, keratinocytes, and intestinal epithelial cells, but the active form is mostly released from macrophage and dendritic cells [44].

The NLRP3 inflammasome responds to a wide variety of intracellular stimuli such as pore-forming toxins from gram-positive and gram-negative bacteria, RNA and DNA viruses, and yeasts, indicators of damage such as ATP, monosodium urate crystals, amyloid-β aggregates, and particulate matter such

as silica, alum, and asbestos [30]. Also It has been reported that “non-canonical” activation of the NLRP3 inflammasome is mediated by the direct sensing of cytosolic LPS by caspase-11 in mice, and its human orthologs caspases 4/5 [30], which explains the increased levels of IL-1β and IL-18 observed during the priming phase without further activation stimuli. Aberrant expression and activation of the NLRP3 inflammasome due to polymorphism, gain of function mutation, and constant exposure to agonist stimuli have been implicated in the pathogenesis of multiple chronic degenerative diseases, including IBD, Alzheimer, atherosclerosis, osteoarthritis, as well as other autoimmune disorders such as lupus erythematosus and corneal inflammation-related diseases [18,45]. Therefore, the inhibitory activity of lunasin over the NLRP3 inflammasome is not only relevant in the context of IBD but can be highly beneficial in the management of other immune-related diseases. Our results not only demonstrate the modulatory properties of lunasin over the NLRP3 inflammasome but for the first time prove positive outcomes in an *in vivo* model of IBD. This indicates that lunasin supplementation is a promising strategy to control early manifestations and the potential onset of IBD.

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Appendix

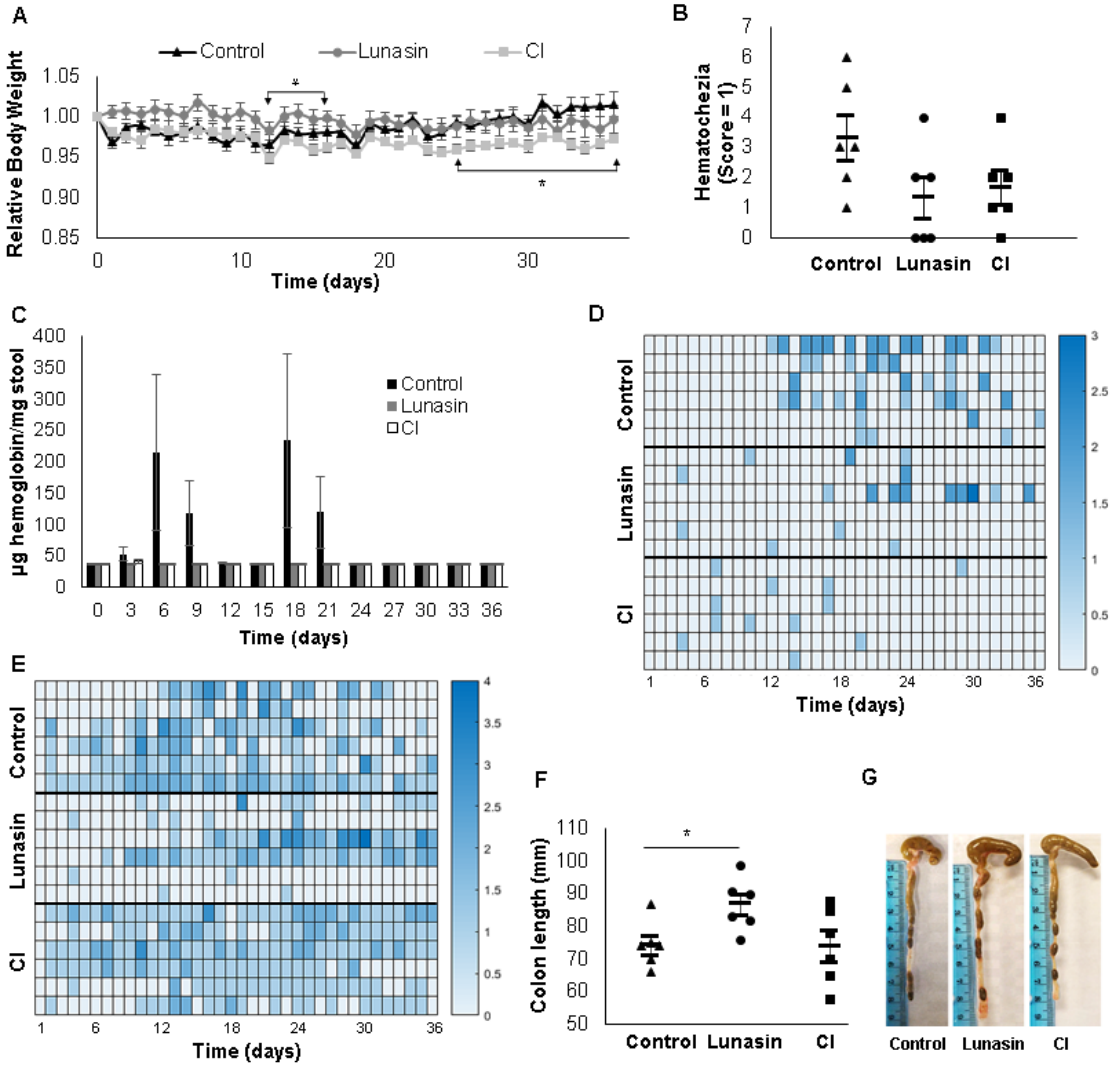


Figure 3.1 Lunasin attenuates macroscopic markers of disease.

(A) Variation of relative body weight, (B) frequency of hematochezia score, (C) average content of hemoglobin in stool as indicator of occult blood, (D) stool consistency score heatmap, (E) disease activity index (DAI) heatmap, and (F and G) colon length. Results are presented as mean ± SEM. CI: group administered with caspase inhibitor. * indicates statistically significant differences in relative

body weight between the CI and lunasin groups from day 12 to 16, and between the CI and control groups over the last 12 days of the experiment, or in colon length between the control and lunasin groups.

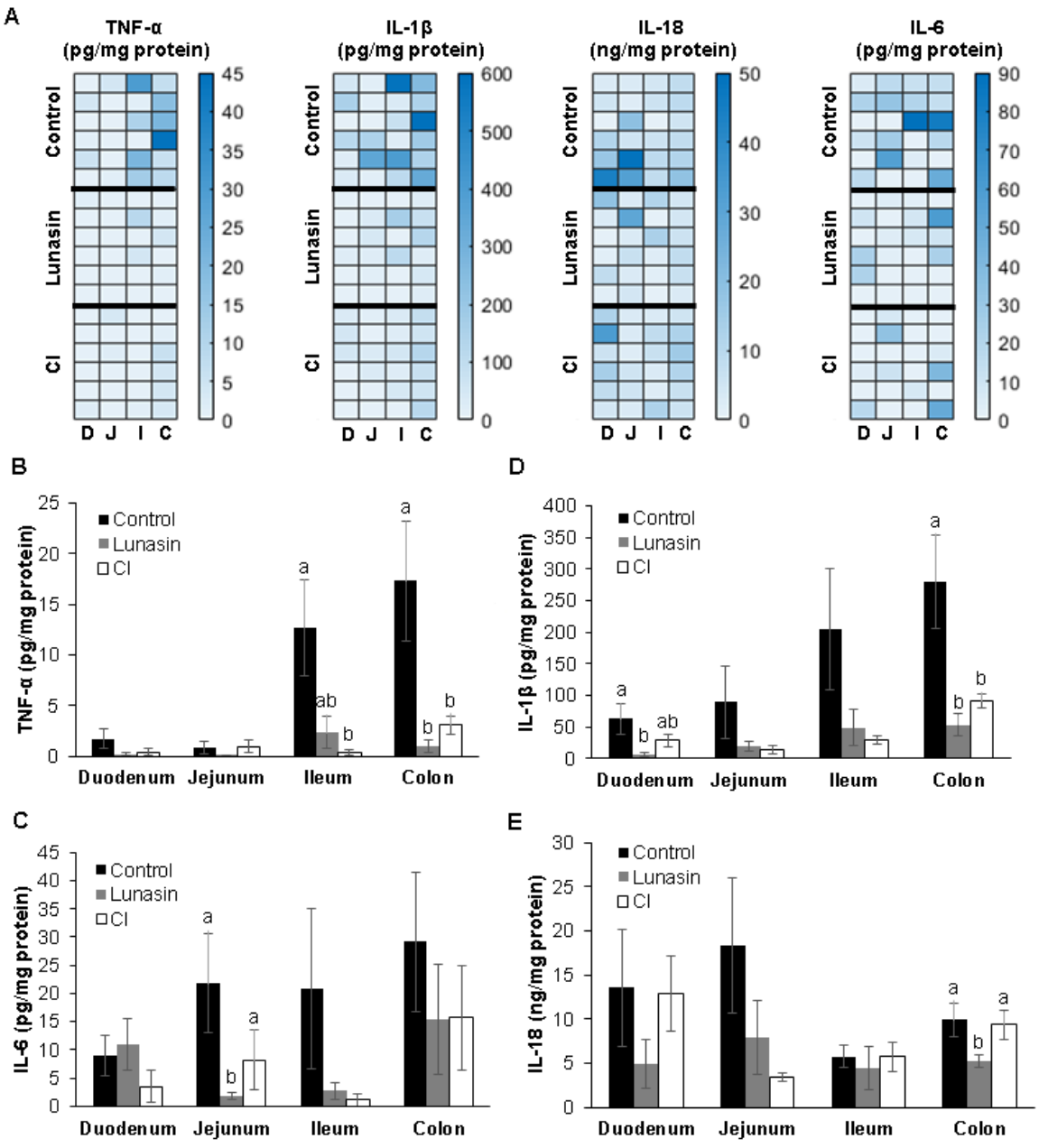


Figure 3.2 Lunasin decreases intestinal levels of proinflammatory cytokines.

(A) Heatmaps and (B-E) bar graphs of TNF-α, IL-6, IL-1β, and IL-18 in the indicated sections of the small or large intestine. In the heatmaps, D, J, I, and C indicate duodenum, jejunum, ileum, and colon, respectively. Results are

presented as mean ± SEM. Different letters indicate significant differences between the treatment groups within the same intestinal section ($p < 0.05$).

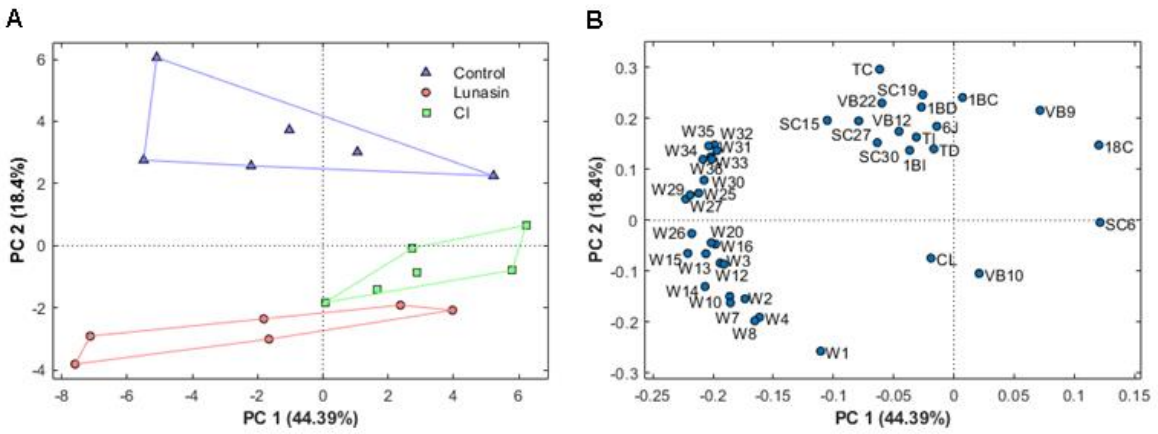


Figure 3.3 Principal component analysis showing the overall effect of lunasin over the characteristic IBD parameters evaluated in vivo.

(A) Score plot and (B) loading plot for the first two principal components (PC1 and PC2) that explain 63% of the variance of the 42 variables with higher discriminatory potential preselected using the method of Wilk's lambda. The matrix was generated using the following data: relative body weight (W), stool consistency scores (SC), and hematochezia scores (VB) from day 1 to 35, colon length (CL), MPO activity (M), IL-18 (18), IL-1β (1B), TNF-α (T), IL-6 (6) for the duodenum (D), jejunum (J), ileum (I), and colon (C).

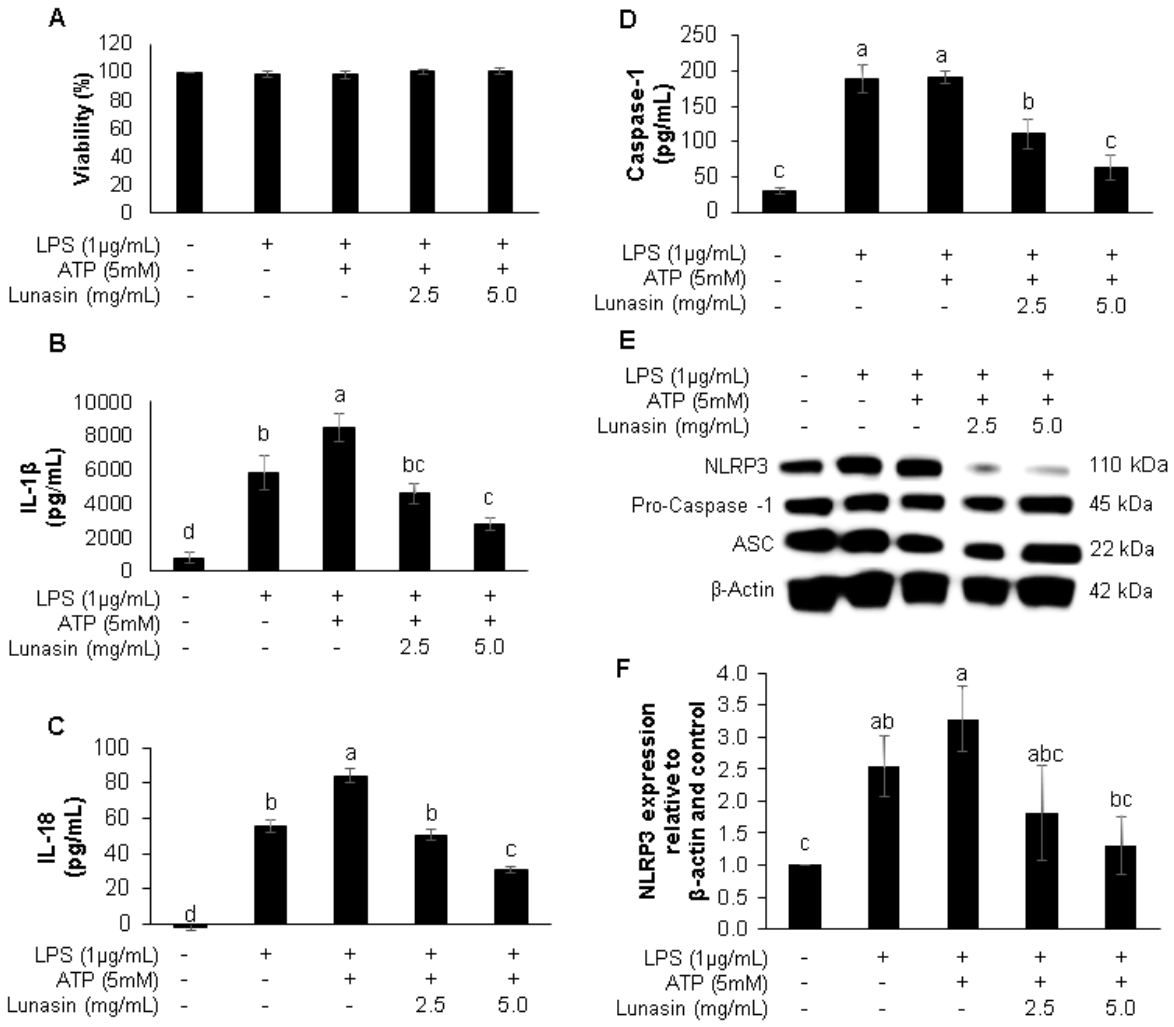


Figure 3.4 Lunasin abrogates the activation of NLRP3 inflammasome.

(A) Cell viability, (B) IL-1β, (C) IL-18, and (D) caspase-1 concentration in the extracellular media, (E) cellular protein levels of inflammasome components, and (F) quantification of the protein levels of NLRP3, upon LPS-priming and ATP-activation of the NLRP3 inflammasome in differentiated THP-1 human macrophages in the presence or absence of lunasin. Results are presented as mean ± SD. Different letters indicate significant differences between the treatments ($p < 0.05$).

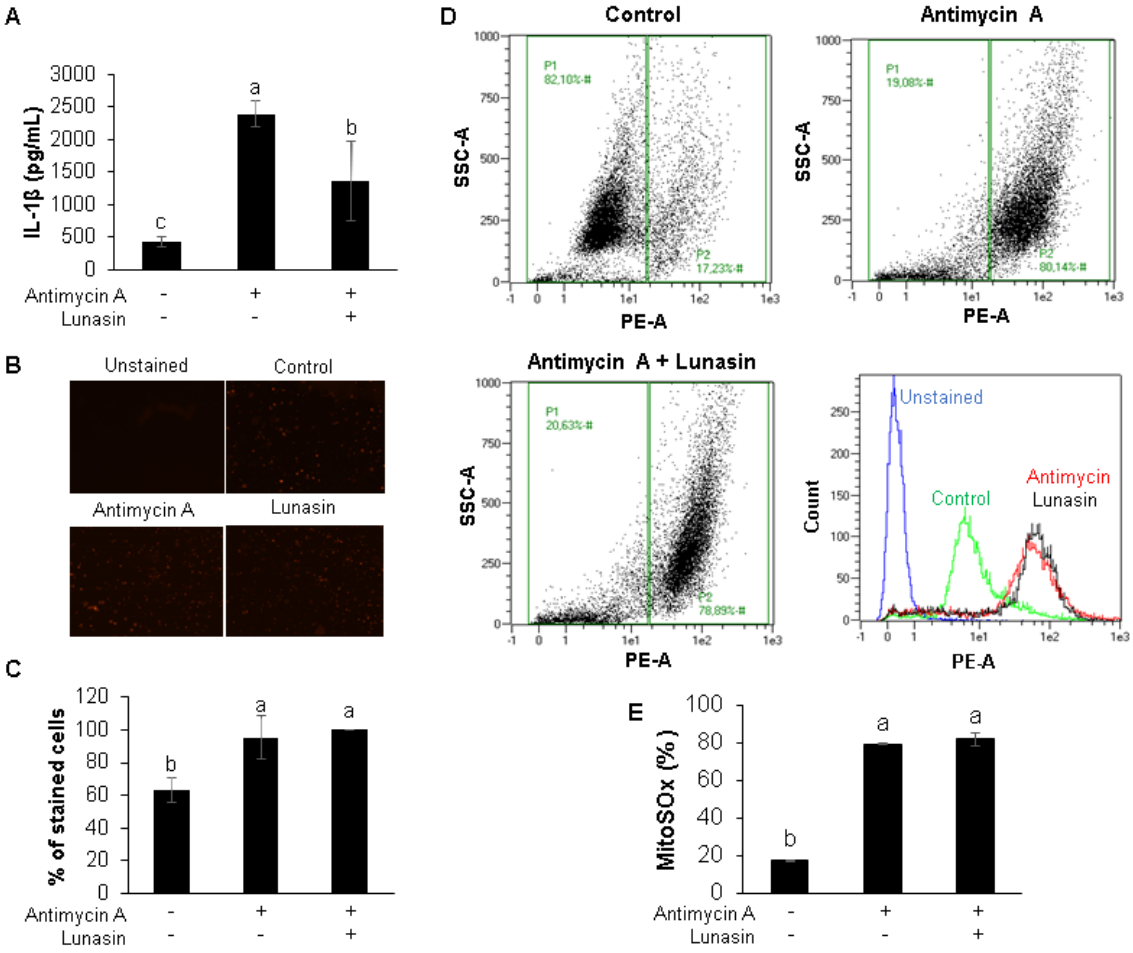


Figure 3.5 Modulation of the NLRP3 inflammasome by lunasin is independent of mitochondrial ROS.

Production of (A) IL-1β and mitochondrial ROS as detected and quantified by fluorescence microscopy (B and C) or flow cytometry (D and E) upon challenge with antimycin A in the presence or absence of lunasin. Results are presented as mean ± SD. Different letters indicate significant differences between the treatments ($p < 0.05$).

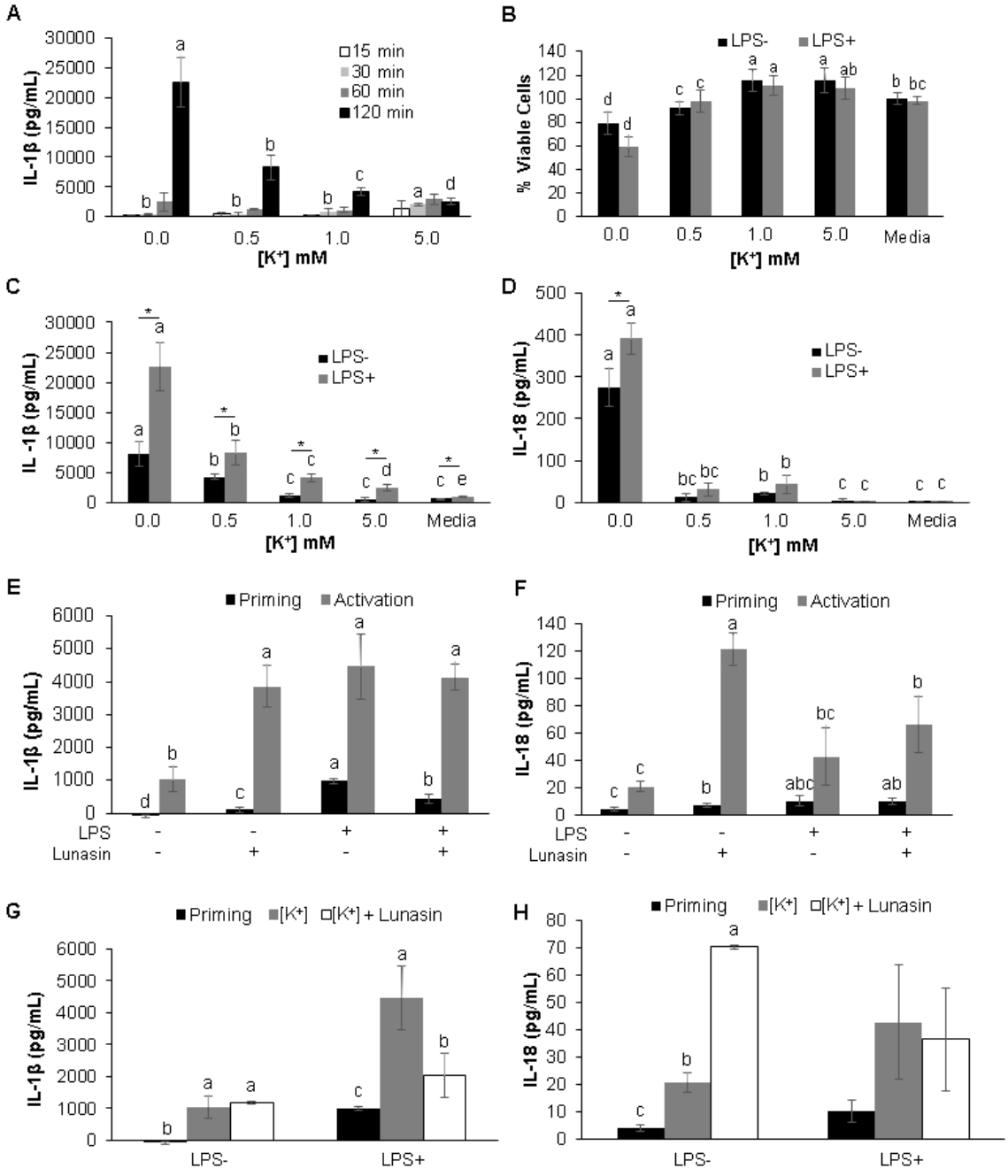


Figure 3.6 Lunasin reduces extracellular IL-1β during the activation phase but does not affect potassium efflux.

Extracellular concentration of IL-1β (A, C, E and G) or IL-18 (D, F and H), and cell viability (B) evaluated in differentiated THP-1 human macrophages exposed

to different priming and activation conditions. (A) Activation of LPS- primed cells for different times with K^+ conditioned media. (B, C and D) LPS-primed (LPS+) and non-primed (LPS-) cells were activated for 2 h with K^+ conditioned media. (E and F) Cells primed with (+) or without (-) LPS and/or lunasin, and then activated for 2 h with 1.0 mM K^+ conditioned media. (G and H) Cells primed (LPS+) or not (LPS-) with LPS, and then activated with 1.0 mM K^+ conditioned media with or without lunasin. Results are presented as mean ± SD. Different letters and * indicate significant differences between the treatments ($p< 0.05$).

CHAPTER 4. CHEMOPREVENTIVE EFFECT OF LUNASIN IN AOM/DSS-INDUCED COLITIS-ASSOCIATED COLORECTAL CANCER IN MICE

4.1 Abstract

Colitis-associated colorectal cancer (CA-CRC) accounts for the second cause of death among patients with inflammatory bowel disease (IBD). The soy-derived bioactive peptide lunasin has demonstrated *in vitro* anti-inflammatory and anti-cancer properties, as well as chemopreventive properties *in vivo*. However, the potential effect of lunasin in CA-CRC has not been addressed. In this study, we used an in *vivo* model of CA-CRC induced by azoxymethane and dextran sulfate sodium to evaluate the effect of oral administration of lunasin over the macroscopic symptoms of the disease, markers of inflammation, and tumor burden. Lunasin administration lessened the manifestation of the disease by facilitating the gain of body weight and accelerating the recovery of stool consistency during periods of mild and moderate inflammation. This effect was accompanied by a reduction in neutrophils infiltration ($p=0.024$) and activity ($p=0.062$), attenuated damage of the mucosal architecture ($p=0.68$), and the increased levels of the marker of epithelial integrity E-cadherin ($p=0.049$). Prevention of spleen enlargement ($p=0.039$) and lowering concentration of the pro-inflammatory cytokine TNF-α in the serum ($p=0.029$) suggested a potential systemic effect of lunasin. Treatment with lunasin reduced tumor incidence and tumor multiplicity ($p=0.036$) and mitigated the malignant transformation of dysplastic lesions. Our findings demonstrated for the first time the anti-inflammatory and chemopreventive properties of lunasin in CA-CRC and suggest the peptide as a promising protective agent against IBD-associated cancers.

4.2 Introduction

Colorectal cancer (CRC) is the second most deadly cancer in the United States, and the incidence of the disease is rapidly shifting toward younger populations [1]. Although the most prevalent forms of CRC are sporadic and familial-related, a higher risk of developing colitis-associated colorectal cancer (CA-CRC) is prevalent in patients affected by inflammatory bowel disease (IBD) [2]. IBD, which comprises ulcerative colitis (UC) and Crohn's Disease (CD), is a condition of persistent inflammation in the gastrointestinal (GI) tract that can cause severe damage in the small and large intestines [3]. CA-CRC is one of the most serious and life-threatening consequences of UC [4]. The risk of developing CA-CRC is estimated at 2% by 10 years, 8% by 20 years, and 18% by 30 years after UC diagnosis [5]. CA-CRC is the second most common cause of mortality among IBD patients after cardiovascular diseases, accounting for 10 to 15% of deaths [5,6]. Regular surveillance colonoscopy, polypectomy, and proctocolectomy to remove affected areas, are the standard mechanism for early detection and prevention of CA-CRC [4].

The association between chronic inflammation and cancer has been widely reported. Prolonged and extended colitis, family history of CRC, early onset of the IBD, severity of inflammation, and chronic inflammation in the bile ducts highly contribute to the increasing risk of CA-CRC [7]. Pro-inflammatory cytokines produced by the activated immune cells present in the affected tissue have been shown to contribute to tumor growth [8]. Inflammatory pathways,

particularly the nuclear factor – kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, have been recognized as key linkers between inflammation and cancer [9]. These pathways are involved in tumor initiation, promotion, and progression by upregulating the expression of anti-apoptotic genes and stimulating cell proliferation and angiogenesis via pro-inflammatory mediators [10]. TNF-α, one of the main pro-inflammatory downstream products of the NF-κB pathway, not only plays a key role in IBD [11], but also has been associated with tumor initiation via the induction of reactive oxygen species, promoting DNA damage, stimulating the expression of pro-angiogenic chemokines, and further activating the NF-κB pathway [10]. Hence, the use of anti-inflammatory therapeutics in IBD patients is also considered a chemopreventive strategy [4].

The consumption of plant-derived diets has been largely associated with health benefits. Numerous studies have demonstrated the beneficial effect of soy and soy products against cardiovascular diseases, diabetes, cancer, obesity, and other chronic and inflammation-related diseases [12]. In the context of CRC, a meta-analysis of available epidemiologic studies indicated that soy consumption was associated with approximately 21% reduced risk of CRC in women [13]. Among the different compounds with biological properties present in soybeans, the bioactive peptide lunasin has received particular attention over the last two decades as demonstrated by the large body of literature addressing its chemopreventive, anti-inflammatory, and antioxidant properties [14]. Lunasin has been demonstrated to inhibit the NF-κB pathway and downregulate the pro-

inflammatory cytokines TNF-α, IL-6, IL-1β, as well as other molecules involved in inflammation [15,16]. Mammalian cell lines and *in vivo* models of melanoma, skin, and breast cancer provide evidence of the chemopreventive and therapeutic properties of the peptide [17]. However, the biological activity of lunasin in the context of IBD and CA-CRC remains unknown. In chapter three, we demonstrated that oral administration of lunasin can reduce susceptibility to spontaneous colitis in IL-10 $^{-/-}$ mice serving as a first approach to address the promising use of the peptide in the context of IBD. In this study, we aimed to explore the effect of oral administration of lunasin over the development of colon cancer mediated by inflammation, using an *in vivo* model of chemically induced CA-CRC.

4.3 Methods

4.3.1 Isolation of lunasin and preparation of jelly for voluntary oral administration

Lunasin was isolated from tofu whey as previously described [18]. The final dry powder corresponded to isolated lunasin with at least 80% purity.

Due to the minimum impact over food intake, easy preparation and supplementation with water soluble compounds, and soft consistency that make it easily ingested during periods of stress and sickness, jelly was used as a vehicle for the dietary supplementation of lunasin. Voluntary self-administration of the jelly was selected as the mechanism for oral administration, as it allows the controlled delivery of accurate doses of lunasin, eliminates the risk of injury

associated with daily oral gavage, and reduces stress associated with restraining the animals. The jelly was prepared every 5 days using 8% gelatin and 10% sucralose (purchased from a local store) and kept at 4°C. The placebo jelly did not contain any additional ingredient, while jelly containing lunasin was incorporated with the appropriate amount of the peptide to provide a dose of 20 mg lunasin /kg of body weight (BW). Lunasin supplementation corresponds to a human equivalent dose of 1.6 mg/kg BW according to the method described by Nair and Jacob [19].

4.3.2 Animals and experimental design

Azoxymethane (AOM) and cyclic administration of dextran sulfate sodium (DSS) were used to induce CA-CRC in the mice, following previous studies in inflammation-driven tumor progression in murine models [27]. Sixteen male 8-weeks old C57/BL6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in pairs with free access to water and chow diet provided *ad libitum*. Housing facilities were controlled to maintain 23 ± 3 °C, 30-50% relative humidity, and 12 h light/dark cycles. The training was performed to overcome neophobia to the jelly and promote voluntary oral ingestion. Briefly, after three days of acclimatization, solid food was removed for eight hours during the dark cycle. At the end of the fasting period, mice were temporarily moved to individual cages and a placebo jelly was presented. After consumption of the entire piece of jelly, the mice were returned to their original cages and allowed free access to solid food. Placebo jelly was presented for the following three days, without a fasting

period, to induce the habit of jelly consumption in addition to the regular diet. At the end of the training period, mice were randomly divided into two groups AOM/DSS (n=8) and AOM/DSS+Lunasin (n=8). For the remainder of the study (11 weeks), administration of placebo jelly to the AOM/DSS group, or jelly containing lunasin to the AOM/DSS+Lunasin group was performed daily.

After one week of daily jelly supplementation, intraperitoneal injection of AOM (MP Biomedical, Santa Ana, CA) at 10mg/ kg BW was given to every mouse. One week after AOM injection, 2% DSS (MP Biomedicals, Santa Ana, CA) was administered in drinking water for seven consecutive days, followed by 14 days of regular water. The DSS administration cycle was performed two more times using 1% and 1.5% DSS for the second and third cycles, respectively. Dosage of AOM and DSS were based on previously reported study [25]. The concentration of DSS was modified for the second and third cycles due to the severe impact observed after the 2% administration cycle. Consumption of drinking water was monitored at least twice a week to ensure equal exposure to DSS. Experimentation was conducted following the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and ARRIVE guidelines. Protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of the University of Tennessee Knoxville (#2786-0920).

4.3.3 Blood and tissue collection

At the end of the third DSS cycle, blood was collected via cardiac stick under isoflurane anesthesia as a terminal procedure, immediately followed by cervical dislocation to confirm death. Blood was left to clot for 30 min at room temperature and then centrifuged at 1500 x *g* for 10 min. Serum was collected, aliquoted, and stored at -80 °C for further analysis. The spleen and the colon were harvested, and weight and length were measured. The colon was opened, and the fecal content carefully removed using tweezers. The number of macroscopic tumors was recorded, and their size (length, width, and height) was measured using a digital caliper. Tumor volume was calculated according to the formula V=0.5 (length x width x height). The colon was longitudinally dissected in two sections. One section was rolled and fixed in formalin for histological examination. The other section was snap-frozen in liquid nitrogen and stored at -80 °C for further analysis.

4.3.4 Disease activity index (DAI)

Body weight was recorded every three or four days during the first three weeks, and every day after the first cycle of DSS was initiated. Individual fresh stool samples were evaluated weekly during the first three weeks and every two or three days upon initiation of the first DSS cycle. Scores were assigned to changes in the BW and the stool, based on criteria previously described [25], with slight modifications as follows: i) loss of relative body weight: 0= no loss or gain, 1= <5%, 2= between 5 and 10%, 3= between 10 and 15%, and 4= >15%

compared to the BW at the beginning of the appropriate DSS cycle; ii) stool consistency: 0= normal stool, 1= moist and sticky, 2= loose pasty stool, 4= watery diarrhea; iii) hematochezia: 0= no visible blood in the stool, 1= brown reddish color in the stool, 2= blood present in the stool, 4= gross rectal bleeding. The DAI was calculated as the sum of the three scores for each mouse, with 0 being the minimum (no disease) and 12 the maximum score (severe disease). In addition, stool samples were collected from the cages at the indicated time points and used to evaluate the presence of hemoglobin, as a marker of fecal occult blood, using previously reported methods [20].

4.3.5 Myeloperoxidase (MPO) activity and cytokines

Extracts from colonic tissue and measurement of MPO activity, as an indicator of neutrophils infiltration, were performed according to previously described methods [20]. For assessment of cytokines, tissue extracts were prepared by homogenizing 50 mg of colonic tissue in 1 mL of T-PER^{TM} lysis buffer (Thermo Scientific, Waltham, MA) supplemented with 5 µL of protease inhibitor cocktail (VWR International, Radnor, PA), using 1.0 mm zirconium beads (OPS Diagnostics, Lebanon, NJ) for 5 min at 30 Hz. Homogenate was centrifuged and the supernatant was aliquoted and stored at -80 °C until analysis. The protein content of tissue extracts was quantified using Bradford assay. Pro-inflammatory cytokines TNF-α and IL-1β were analyzed in colonic extracts and blood serum using commercially available ELISA kits (Biolegend, San Diego, CA) according to the protocol provided by the manufacturer.

4.3.6 Western Blot

Colonic tissue extracts for cytokine analysis were used to perform western blot immunoassay. Briefly, 20 µg of protein were separated via gel electrophoresis following standard protocol. Then Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA) was used to transfer the proteins into polyvinylidene difluoride membranes. After 1 h blocking with 5% non-fat dry milk in 0.01% Tween 20 Tris-buffered saline (TBST), membranes were incubated overnight at 4 °C with Bcl-2-associated X protein (BAX), X-linked inhibitor of apoptosis protein (XIAP), Caspase-3, E-Cadherin, Claudin 3 and β-actin primary antibodies (Proteintech, Chicago, IL) at 1:1000 dilution. The next day membranes were washed three times with TBST, and then incubated with secondary antibody at 1:2000 dilution for 1 h at room temperature. Membranes were imaged using a C-Digit Blot scanner (LI-COR Biosciences, Lincoln, NE) and the optical density of the immunoreactive bands was analyzed using Image Studio™ software.

4.3.7 Histopathological assessment

Formalin-fixed colonic tissue was paraffin-embedded, cut into 4 µm sections and routinely stained with hematoxylin and eosin- (H&E). Sections of colon were assessed for inflammation, dysplasia, and neoplasia in a blinded fashion by a board-certified anatomic pathologist (MMS). Inflammation was evaluated in areas not affected by dysplasia, and the scoring was performed according to the guidelines published by Erben *et al*. [21], based on the following

criteria: i) changes in mucosal architecture: 0= none, 1= focal erosion, 2= multifocal erosion and/or evidence of focal ulceration, and 3= evidence of extended ulceration with granulation tissue; ii) goblet cell depletion: 0= minimum or no evident changes, or 1= evident decrease in the population of goblet cells; iii) density of inflammatory cellular infiltration: 0= no observed, 1= < 10% of the tissue, 2= 10 to 25%, 3= 26 to 50%, and 4= > 51%; iv) extent of infiltration: 1= limited to the mucosa, 2= mucosa and submucosa, and 3= transmural infiltration. The inflammation score was calculated as the sum of the four individual scores, with 11 being the maximum possible score. Dysplastic lesions were classified as indefinite dysplasia or hyperplasia, low-grade dysplasia with nuclei remaining in basilar location, high-grade dysplasia with nuclei which span height of cell, and invasive carcinoma [22].

4.3.8 Statistical analysis

Results are reported as mean ± SEM. The non-parametric Mann-Whitney test or the parametric Student t-test were applied accordingly to identify significant differences ($p < 0.05$). Statistical analyses were performed using the software Minitab ® 17.1.0 (Minitab Inc., USA) or Excel Microsoft 365 (Microsoft Corporation, USA).

4.4 Results

4.4.1 Lunasin facilitated the recovery of body weight and stool consistency.

Variations in the BW relative to the individual BW at the beginning of each DSS cycle are presented in Figure 4.1A. Loss of BW is common in patients with

CD due to the impairment in the digestive and absorptive functions of the intestine, but it only occurs in the most severe cases of UC [23]. This fact agrees with the lack of effect observed over the relative BW when DSS was administered at the lowest dose, in contrast to the marked and very drastic drop in BW when higher concentrations were used. Although significant differences between the two groups were found only during the last 10 days of the study, records from daily monitoring indicated that 75% and 38% of the mice in the AOM/DSS group and the lunasin treatment group, respectively, lost more than 14% of BW during the first DSS cycle. Administration of 1.5% DSS (third cycle) resulted in more than 8% loss of BW in 3 mice from the AOM/DSS group and only 1 mouse in the AOM/DSS + lunasin group.

The average DAI (Figure 4.1B) clearly shows three peaks that are proportional to each cycle of DSS. Upon inflammatory flares, decrease of the DAI to a minimum level took 15 days after the first cycle and 2 days after the second cycle for both groups, but 15 days for AOM/DSS and 5 days for AOM/DSS + Lunasin group after the third cycle. These results suggest that an extended period is required for the recovery of mild (1.5% DSS) and severe (2.0% DSS) episodes of inflammation and that lunasin significantly accelerated the recovery from inflammation when CA-CRC was already established. This faster recovery seems to be concomitant to the observed improvement in BW. Significant differences particularly on days 48, 54, and 67 were associated with favorable changes in stool consistency. Overall, when lunasin was given, a score of 0 (no

signs of disease) was 10% more frequent, while scores associated with any level of disease were less common (Figure 4.1C).

No significant differences were observed in the hemoglobin content (Figure 4.1D) between the two groups. However, as shown by hematochezia scores (Figure 4.1E), one mouse in the AOM/DSS group exhibited gross rectal bleeding, while none did in the AOM/DSS + Lunasin group. Lunasin also contributed to a faster recovery of the stool regular consistency (Figure 4.1F) as observed in the trends of the scores following the second and third inflammatory peaks. Four episodes of diarrhea were observed in each group, but only during the first cycle (Figure 4.1G). It is to be noted that watery diarrhea in the AOM/DSS + Lunasin group was observed in two mice that overall showed a low response to the peptide. Analysis of the frequency of the scores indicated that mice receiving lunasin were 5% and 8% less prone to exhibit any indicator of potential blood in the stool and change of stool consistency, respectively.

4.4.2 Lunasin treated mice had lower relative spleen and colon weight.

Spleen enlargement is a common feature in DSS-induced colitis [24] and CA-CRC [25]. In mice administered with lunasin, the relative spleen weight (Figure 4.2A) and the relative colon weight (Figure 4.2B) were significantly lower, by 0.6 mg/g and 4 mg/g, respectively. As no statistical differences were found in the length of the colon between the groups (Figure 4.2C), the increased colon weight in the non-treated mice was associated with the presence of inflammation and tumors.

4.4.3 Lunasin reduced histological markers of inflammation.

On average, AOM/DSS mice exhibited moderate cellular infiltration (Figure 4.3A) that was extended transmurally through the mucosa, submucosa, and muscularis externa (Figure 4.3B), accompanied by multifocal areas of erosion, evidence of previously extended ulcers, and granulation tissue (Figure 4.3C). Lunasin supplementation significantly reduced the density of cellular infiltration. Although transmural infiltration was found in 50% of the mice in this group, 38% had infiltration limited to the mucosa only. No evidence of extended ulcers or granulation tissue was found in this group, and 38% of the mice did not exhibit any sign of mucosal inflammatory damage. With exception of one mouse in the AOM/DSS group, no overt ulcers were found in general, indicating that the 14 days of recovery after the last cycle of DSS administration allowed for a considerable extent of mucosal healing. Also, 50% of the mice in the AOM/DSS group exhibited evident depletion of goblet cells, while only 25% in the AOM/DSS + lunasin group did ($p= 0.347$). Overall, the inflammatory score was 8 for the AOM/DSS group, and 5 for the AOM/DSS + Lunasin group (Figure 4.3D). Although the difference was not statistically significant, there was an evident favorable response in three of the mice across all the evaluated parameters, suggesting that this subset of mice was highly responsive to the therapeutic effect of the peptide. The trend observed in MPO activity (Figure 4.3E) indicated that neutrophil activity was about 50% lower in the AOM/DSS + lunasin group, which is consistent with histological evaluation of cellular infiltration. Figure 4.3F

presents representative micrographs of H&E staining of colonic tissue for each group, indicating the evaluated parameters previously described.

4.4.4 Lunasin reduced tumor burden and malignant histological features.

Pictures of colons showing the presence of tumors are presented in Figure 4.4A. The AOM/DSS group had 100% of tumor incidence with an average of four macroscopic tumors per mouse (Figure 4.4B). In contrast, the AOM/DSS + Lunasin treatment group had a 75% tumor incidence with an average of two macroscopic tumors per mouse, indicating a 50% reduction in tumor multiplicity. The maximum number of tumors detected in a single mouse in the AOM/DSS group was 10, while, for the AOM/DSS + Lunasin group, it was four. Mice bearing the highest number of tumors also corresponded to the highest relative colon and spleen weight in their respective groups. No differences were found in the average accumulative tumor volume ($p=0.273$, Figure 4.4C).

The histopathological evaluation indicated that every mouse in the AOM/DSS group developed several polypoid masses with multifocal dysplastic lesions ranging from hyperplasia to high-grade dysplasia (Table 4.1), with 25% of the mice having invasive carcinoma. Lunasin administration abrogated any kind of dysplastic lesion in 50% of the mice and inhibited the development of carcinomas, suggesting that the peptide significantly prevented abnormal cell growth. Figure 4.4D shows representative micrographs of the dysplastic regions found in both groups.

4.4.5 Lunasin decreased serum levels of TNF-α.

Assessment of pro-inflammatory cytokines (Figure 4.5) revealed that lunasin did not significantly affect the average levels of IL-1β in the colon or the serum, but there was a significant reduction of TNF-α by 70% in the blood. Although colonic levels of TNF-α between the two groups were not significantly different, the mice with the highest concentration of colonic TNF-α in each group corresponded with the highest observed level of MPO activity, the presence of overt ulcers, and high histological inflammation scores, suggesting that these mice had unresolved inflammation at the end of the study.

4.4.6 Lunasin administration resulted in higher levels of E-cadherin but not of apoptotic markers.

Protein levels of the adherent junction E-cadherin (Figure 4.6A) was significantly increased by 40% in mice treated with lunasin ($p=0.049$), while the tight junction Claudin-3 (Figure 4.6B) was increased by 20% ($p=0.133$). These results suggest a partial effect of the peptide over molecules involved in the maintenance of the epithelial barrier. Evaluation of the pro-apoptotic markers BAX (Figure 4.6C) and cleaved caspase-3 (Figure 4.6D) indicated a non-significant increase of 30% in the colon of mice supplemented with lunasin ($p=0.064$ and $p=0.213$, respectively). No significant differences were found between AOM/DSS and AOM/DSS + Lunasin groups ($p=0.382$) when comparing the protein levels of the anti-apoptotic marker XIAP in mice affected by inflammation. However, it is worth mentioning that in the subset of mice highly

responsive to lunasin, with no signs of dysplasia, mucosal damage, and low inflammation scores, a 7-fold increase in XIAP was observed in contrast to the average levels in less responsive mice from the same treatment group (p=0.022).

4.5 Discussion

In murine models, cyclic administration of DSS results in chronic colitis [26]. When combined with the genotoxic carcinogen AOM, the model recapitulates colorectal carcinogenesis driven by inflammation and resembles the key features observed in human disease [27]. The administration of AOM and DSS resulted in relapsing episodes of moderate to severe inflammation, characterized by a significant decrease of BW, diarrhea, and rectal bleeding (Figure 4.1). Daily supplementation of lunasin led to a significant improvement in the BW when CA-CRC was established, and faster recovery from diarrhea-like symptoms as indicated by an early return to regular stool consistency after episodes of mild and moderate inflammation. IBD patients face typically four episodes of diarrhea per day [23], making it one of the most predominant and debilitating symptoms of the disease [28]. Although lunasin did not fully prevent episodes of severe watery diarrhea from happening after the inflammatory peak, harder stool consistency was achieved from 2 to 10 days earlier in mice under lunasin supplementation (Figure 4.1F). These observations suggest that the peptide might facilitate or promote repair mechanisms that reduce the abnormal presence of water in the stool that causes stool softening.

In IBD, altered function, localization, or expression of the tight and adherent junctions that regulate paracellular flux result in the breakdown of the intestinal barrier function, leading to diarrhea via the backflow of absorbed solutes and water [28]. Downregulation of the tight junction molecule claudin-3 [29] and the adherent junction E-cadherin [30] has been reported in IBD patients, and in animal models of intestinal inflammation induced by DSS [31]. The higher levels of E-cadherin in the colon of mice receiving lunasin (Figure 4.6A) suggests that the peptide might contribute to the recovery of the colonic epithelial integrity. Lunasin has been shown to induce gene and protein expression of E-cadherin in murine HC11 mammary cells [32,33], and to have a slight promotor effect in human KM12L4 colon cancer cells [34]. This is the first report indicating the potential effect of lunasin on E-cadherin levels *in vivo*. Downregulation of E-cadherin in inflamed tissues has been particularly found to occur in areas of ulceration [35]. Massive infiltration of neutrophils is a hallmark of UC and is accompanied by the release of reactive oxygen species, serine proteases, MPO, and matrix metalloproteinases [36]. It has been demonstrated that E-cadherin is cleaved by the proteolytic activity of neutrophil elastase [37]. Under this scenario, the initial damage to the colon caused by DSS results in severe neutrophil infiltration in ulcerated areas, accompanied by the cleavage of E-cadherin, causing further disruption of the epithelial barrier. In consequence, the disrupted paracellular efflux allowed back flux of water to the lumen, resulting in softening of the stool and diarrhea-like symptoms. The multifocal ulceration and extensive

dense cellular infiltration observed in the intestinal wall of AOM/DSS mice was significantly alleviated by lunasin treatment (Figure 4.3A). This was accompanied by a marked decrease in MPO activity (Figure 4.3E), corroborating a reduced presence of neutrophils. It is possible that after removal of DSS, lunasin decreased the presence and activity of neutrophils in the tissue, facilitating the maintenance of E-cadherin. This contributed to the progressive recovery of the epithelial barrier and appropriate regulation of paracellular efflux, as manifested by the positive change in stool consistency (Figure 4.1F).

Inflammation plays an important role in CA-CRC by creating a favorable environment that stimulates oncogenic pathways, favors the survival of tumor-initiating cells, promotes tumor growth, and influences metastasis [38]. It is believed that inflammatory cells, via reactive oxygen species and nitrogen reactive intermediates, create a highly reactive and mutagenic environment [38]. Simultaneously, the resistance to cell death mediated by pro-inflammatory pathways increases the chance of survival of mutation-bearing cells. Thus, the wound-healing-regeneration response promotes the proliferation of mutant cells, giving place to tumor formation. Local repetitive injury and chronic inflammation, not only facilitate tumorigenesis but also contributes to tumor progression [38]. In this study, histological observations of normal epithelium overlying areas with mixed inflammation and crypt loss suggested mucosal healing of previous ulcerative injuries created during the flare (Figure 4.3C). The prolonged period of recovery after the last DSS cycle allowed for local mucosal repair and resolution

of acute inflammation by the end of the experiment, explaining the lack of significant differences between the two groups in the average colonic levels of the pro-inflammatory mediators TNF-α and IL-1β (Figure 4.5). However, it is known that during active inflammatory flares these pro-inflammatory signals are highly expressed by resident and infiltrating immune cells [11]. The effect of lunasin over pro-inflammatory signals has been studied and demonstrated *in vitro* [14]. In chapter 3 it was demonstrated that oral administration of lunasin significantly decreased TNF-α and IL-1β in the colon of an *in vivo* model of spontaneous colitis (Figure 3.2), suggesting that lunasin can exert anti-inflammatory activity in the bowel. Hence, it is reasonable to speculate that during the active flares lunasin could have attenuated to some extent the storm of pro-inflammatory cytokines in the colon, at least by reducing the presence of infiltrating immune cells, as observed in the tissues and corroborated by MPO activity trend (Figure 4.3). The high tumor multiplicity generated by the AOM/DSS treatment was significantly abrogated by lunasin (Figure 4.4B) and accompanied by a 70% decrease in the serum levels of TNF-α (Figure 4.5B). It is possible that the effect observed over tumor incidence and burden was mediated, at least partially, by the anti-inflammatory properties of lunasin. It is worth pointing out that, although no differences were found in average colonic levels of TNF-α, mice with detected carcinoma exhibited 2-fold increased concentration in comparison to their group peers, suggesting the potential involvement of this cytokine and inflammation in the carcinogenic process. Cell cycle arrest at the G2/M phase

and induction of caspase-3 -mediated apoptosis, via the upregulation of BAX and downregulation of BCL-2 *in vitro*, have been shown to reduce the proliferative potential of colorectal and mammary cancer cells treated with lunasin [15]. However, intraperitoneal injection of lunasin in a model of metastatic colorectal cancer significantly reduced metastasis to the liver with no significant effect over the pro-apoptotic markers [39], which is consistent with our observations in the protein levels of BAX and caspase-3 in the colon (Figure 4.6). In addition to its involvement in apoptosis, XIAP plays an important role in the regulation of intestinal immunity and inflammation in IBD [40]. XIAP deficiency has been reported to cause a very early onset of intractable colitis [41]. Then, it is possible that the increased levels of this protein might have contributed to the observed favorable outcomes in mice with increased response to lunasin treatment. Further mechanistic investigation is necessary to establish the potential involvement of lunasin in the upregulation of XIAP.

Enlargement of the spleen has been previously reported in DSS-induced UC [24] and CA-CRC [25] and is considered a marker of disease severity and systemic inflammation [42]. We observed a significant reduction of the relative spleen weight in mice supplemented with lunasin (Figure 4.2A). Intraperitoneal injection of lunasin has been shown to significantly decrease the enlarged spleen weight of male mice fed with a high-fat diet in a model of obesity-induced inflammation [43]. As such, the effect of lunasin over serum levels of TNF-α and the spleen weight suggests that the biological activity of the peptide, even upon

gastrointestinal digestion, is not limited to the large intestine but could directly or indirectly have an impact at the systemic level. Extraintestinal manifestations are observed in 25 to 40% of IBD patients and affect the eyes, skin, liver, joints, skeletal muscle, kidneys, lungs, as well as metabolic processes [44]. Some of these manifestations such as arthritis, erythema nodosum, pyoderma gangrenosum, iritis, and uveitis, have been demonstrated to have a pathogenic mechanism mediated by TNF-α [45]. Then, the anti-inflammatory effect of lunasin, and particularly its impact on the systemic levels of TNF-α, could be beneficial not only in the bowel but also in some of the extraintestinal manifestations of the disease.

In contrast to sporadic CRC that follows the adenoma-carcinoma sequence, CA-CRC progresses through the inflammation-dysplasia-carcinoma sequence that involves inflammation, low-grade, high-grade dysplasia, and eventually invasive carcinoma [46]. Dysplasia in CA-CRC represents a pre-malignant phase [47]. In our observations, Table 4.1, all mice in the untreated group developed some grade of dysplasia, with half of them exhibiting high-grade dysplasia and 25% invasive carcinoma. Administration of lunasin fully abrogated dysplasia in 50% of the mice and delayed the progression of the sequence in another 25%. Genomic alterations that affect genes engaged in cell proliferation, death, and differentiation are highly encountered in CRC and CA-CRC. Mutation in p53, a protein that prevents the clonal expansion of mutant cells, is an early event in CA-CRC that has been found in inflamed mucosa, near 33% of low-

grade, 63% of high-grade dysplasia, and 85% of cancerous lesions [48]. Loss of tumor suppressor genes such as *APC*, and activation of the proto-oncogenes *k-ras* and *c-src,* are also important in CA-CRC but usually occur as late events [48]. Regular surveillance colonoscopy, starting 8 to 10 years after diagnosis of UC, accompanied by random biopsy, is recommended for the early detection of dysplastic alterations, and polypectomy or proctocolectomy is usually performed when high-grade dysplasia is detected in flat mucosa [4]. Our results indicate that lunasin can potentially delay or minimize the genomic processes involved in the abnormal growth of cells that results in dysplastic lesions, and their malignant progression towards cancer. Hence, it would lessen the need for preventive removal of sections of the large intestine.

In conclusion, it was demonstrated for the first time the anti-inflammatory and chemopreventive properties of lunasin in a model of relapsing UC and CA-CRC. The oral administration of lunasin decreased the impact of inflammation over the colon, reduced tumor incidence, and multiplicity, and delayed the progression of malignant features, by favoring the recovery of the epithelial barrier integrity and reducing the susceptibility to neoplastic initiating events during the inflammatory flares. Our findings provide an alternative mechanism to facilitate the control of underlying inflammation during UC and to prevent the development of CA-CRC, which in consequence would contribute to the improvement in the life expectancy and quality of IBD patients, and particularly in those affected by refractory forms of the disease.

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Appendix

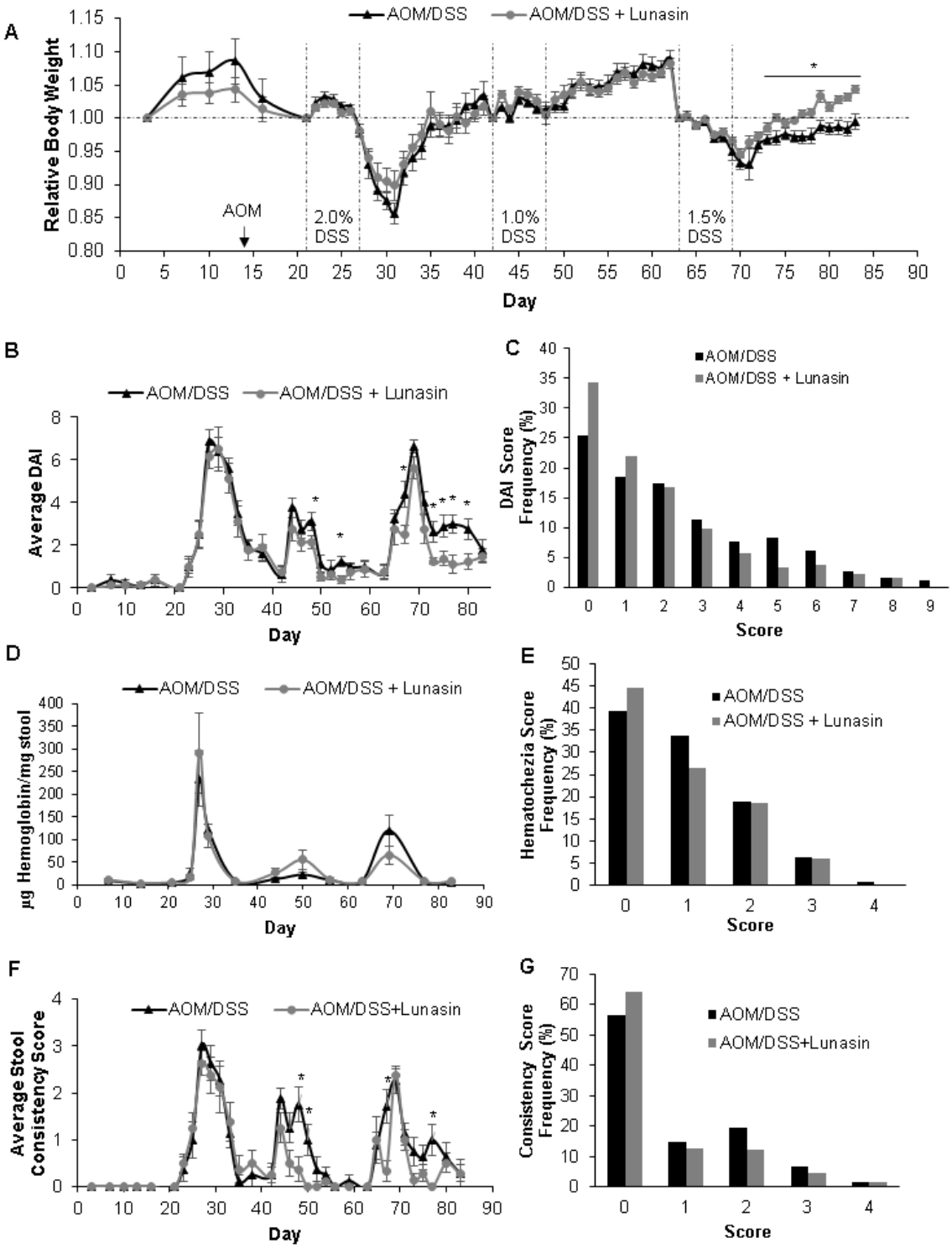

A
AOM/DSS
AOM/DSS + Lunasin
Relative Body Weight
AOM
2.0% DSS
1.0% DSS
1.5% DSS
Day
B
AOM/DSS
AOM/DSS + Lunasin
Average DAI
Day
C
AOM/DSS
AOM/DSS + Lunasin
DAI Score Frequency (%)
Score
D
AOM/DSS
AOM/DSS + Lunasin
µg Hemoglobin/mg stool
Day
E
AOM/DSS
AOM/DSS + Lunasin
Hematochezia Score Frequency (%)
Score
F
AOM/DSS
AOM/DSS+Lunasin
Average Stool Consistency Score
Day
G
AOM/DSS
AOM/DSS+Lunasin
Consistency Score Frequency (%)
Score

Figure 4.1 Macroscopic markers of the disease.

(A) Variation of the average relative body weight, (B) Average and (C) Frequency for disease activity index (DAI) scores, (D) Hemoglobin content in stool as an indicator of occult fecal blood, (E) Frequency for hematochezia score, (F) Average and (G) Frequency for stool consistency score. Results are presented as average ± SEM. * Indicate significant differences ($p<0.05$) between AOM/DSS and AOM/DSS + Lunasin groups. Over the last 10 days of the experiment, the relative body weight of the AOM/DSS + Lunasin group was significantly higher than the relative body weight for the AOM/DSS group (indicated by a horizontal bar).

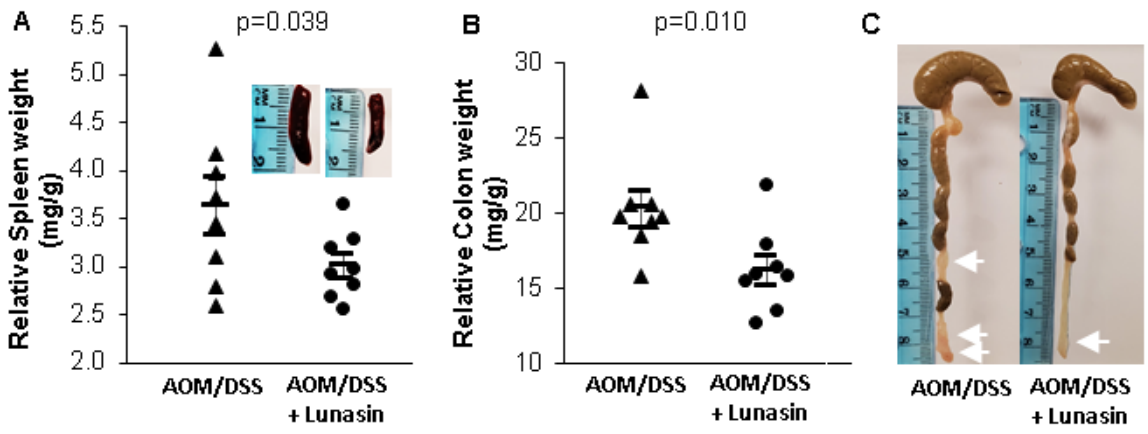


Figure 4.2 Effect of lunasin over spleen and colon.

(A) Relative spleen weight, representative pictures of the spleens as insets, (B) Relative colon weight, and (C) Representative pictures of colon length, with white arrows indicating tumors. Results are presented as average ± SEM.

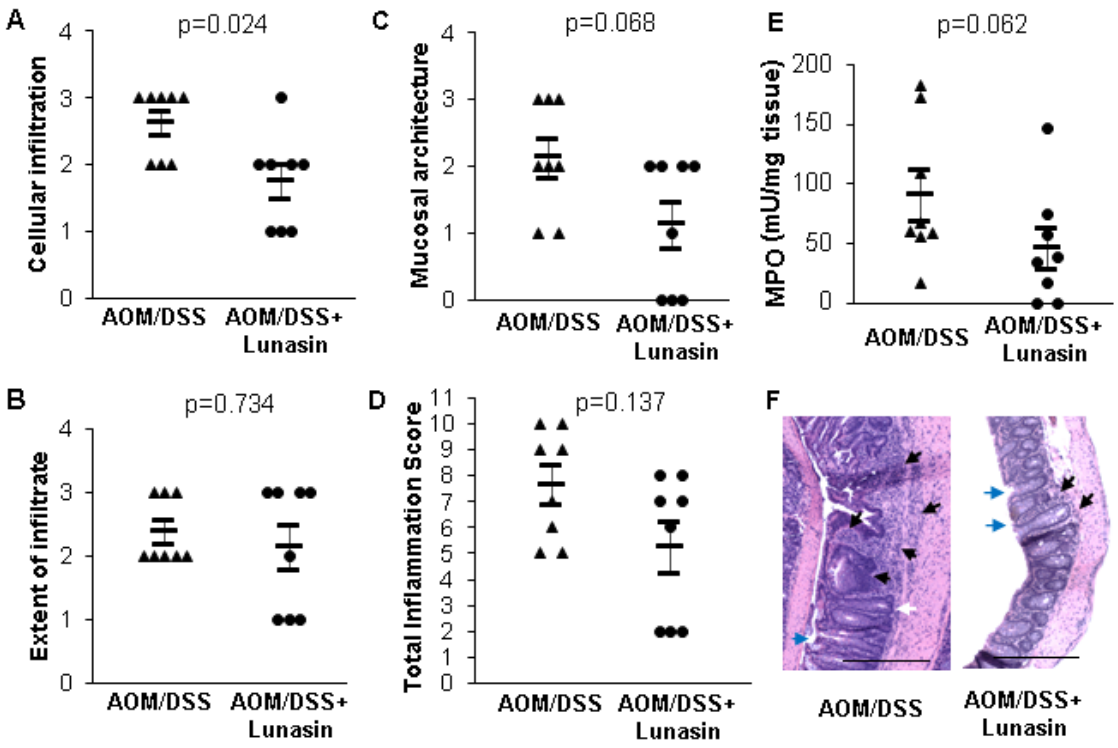


Figure 4.3 Evaluation of histopathological markers of inflammation

Scores for (A) Density of cellular infiltration, (B) Extent of infiltrate, (C) Mucosal architecture, and (D) Total inflammation. (E) Quantification of MPO activity in the colon, (F) H&E micrographs of areas affected by inflammation. Scale bars (bottom right) is 275 µm. Arrows in (F) indicate cellular infiltration (black), goblet cell depletion (white), and erosion (blue). Results are presented as average ± SEM.

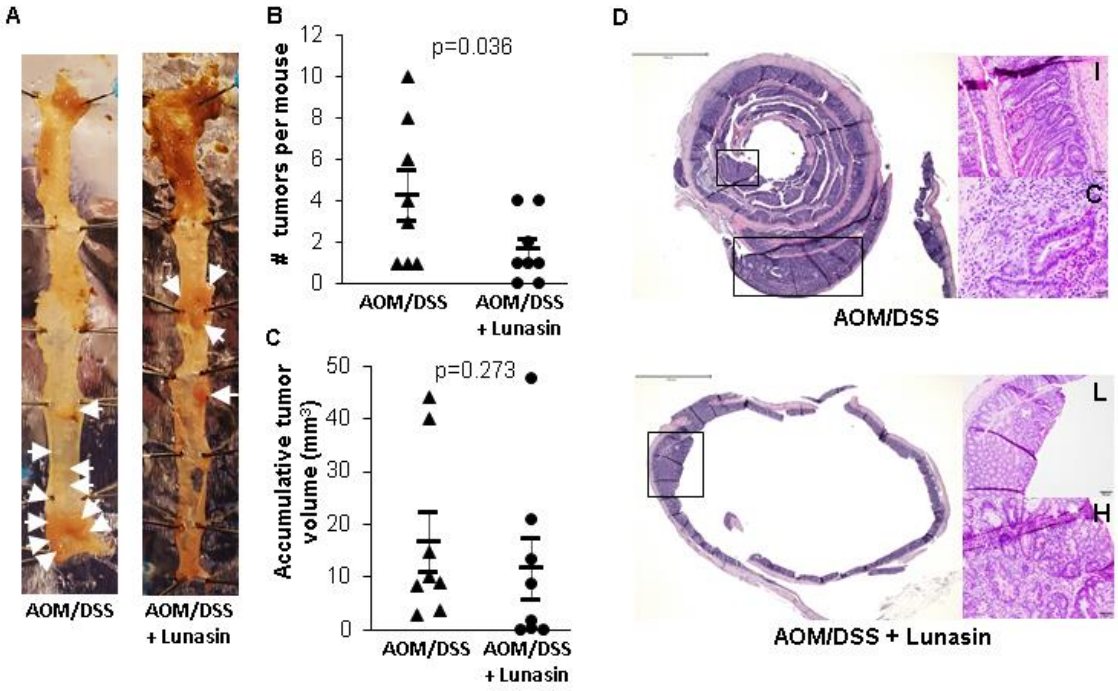


Figure 4.4 Tumor burden and dysplasia.

(A) Representative pictures of longitudinally opened colons indicating tumors (white arrows), (B) Total number of tumors per mouse, (C) Cumulative tumor volume, and (D) H&E micrographs of colon sections from each treatment group indicating polypoid dysplastic masses (black squares) and representative pictures of L: low, H: high, and I: indefinite dysplasia, and C: carcinoma. Results are presented as average ± SEM.

Table 4.1 Number of mice exhibiting the indicated grade of dysplasia.

Dysplasia	AOM/DSS	AOM/DSS + Lunasin
None	0	4
Indefinite	4	1
Low	4	3
High	4	2
Carcinoma	2	0

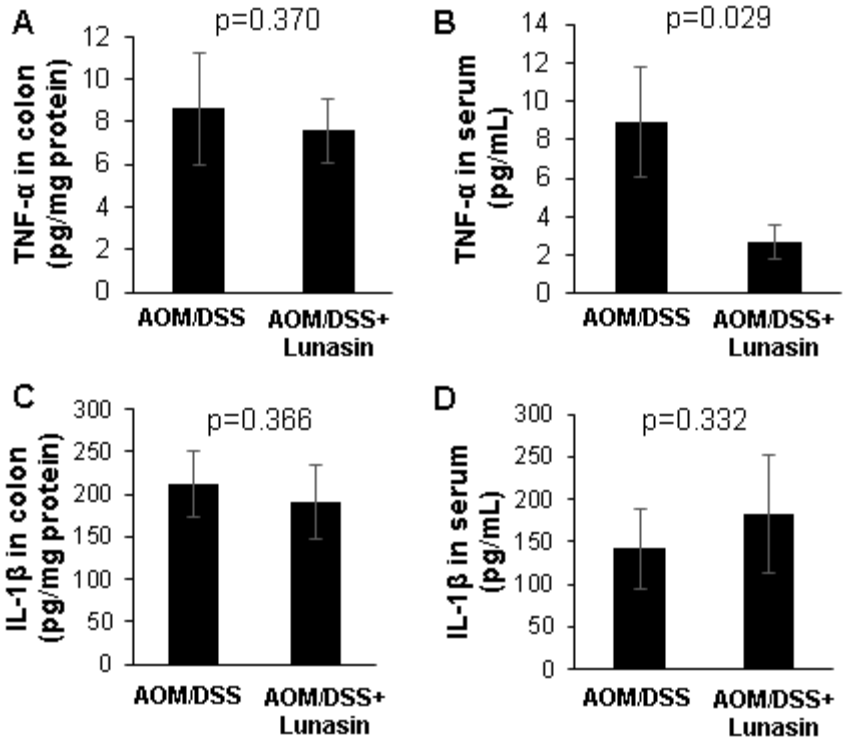


Figure 4.5 . Colonic and systemic levels of pro-inflammatory cytokines.

Levels of pro-inflammatory cytokines TNF-α (A and B) and IL-1β (C and D) in the colon (A and C), and in the blood serum (B and D). Results are presented as average ± SEM.

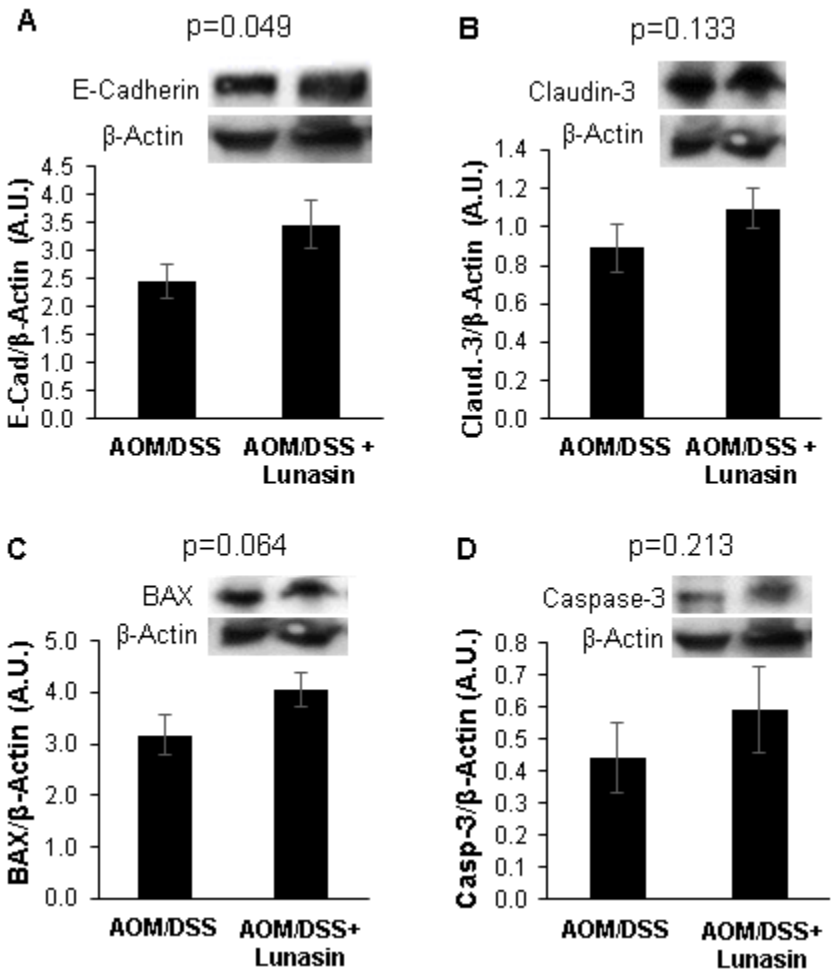


Figure 4.6 Markers of epithelial integrity and apoptosis.

Relative protein levels of (A) E-Cadherin and (B) Claudin-3 as indicators of epithelial integrity, and (C) BAX and (D) Caspase-3 as indicators of apoptosis. Results are presented as average ± SEM. A.U.: Arbitrary units.

CHAPTER 5. CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusion

In this study, a three-step process for the isolation of bioactive lunasin (>80% purity) from tofu whey was designed. The process involves ethanolic removal of high molecular weight proteins, acidification of the clarified whey to induce the separation of lunasin enriched material, and purification via preparative gel filtration chromatography. The designed process is competitive with current isolation mechanisms but faster and easier and allows the valorization of a waste product generated in large quantities by the soybean industry. Lunasin reduced the susceptibility of IL-10$^{-/-}$ mice to minor inflammatory events, which was reflected in a lower frequency of macroscopic manifestations of the disease. The decreased levels of pro-inflammatory cytokines in the small and large intestines indicated that upon oral administration, lunasin or its digestion products reached and exert anti-inflammatory activity in all the sections of the bowel. The anti-inflammatory activity of lunasin was partially mediated by the inhibition of the NLRP3 inflammasome via the downregulation of the NLRP3 protein, and independent from mitochondrial ROS and potassium efflux. Moreover, lunasin exerted chemopreventive properties in the colon by reducing tumor incidence and multiplicity, mitigating the malignant transformation of dysplastic lesions, and attenuating the damage resulting from the chemically induced inflammatory flares.

Overall, this study for the first time proposed and addressed the isolation of bioactive lunasin from a liquid byproduct of the soybean industry,

demonstrated the anti-inflammatory and chemopreventive properties of lunasin in the bowel, and showed the modulatory effect of lunasin over the NLRP3 inflammasome. Furthermore, it served to prove *in vivo* the biological activity of the products resulting from the gastrointestinal digestion of lunasin when orally consumed.

The incorporation of lunasin in the diet could be partially achieved by the consumption of soybean products. However, the low bioavailability of the peptide, and the limited amount of lunasin could post a challenge for the peptide to reach the bowel as a target site in appropriate concentrations, as it would be absorbed and passed to the systemic circulation. Generation of lunasin supplements or the incorporation of purified forms of the peptide in vehicle foods, as imitated in the last chapter, could be helpful to achieve an increased intake, and activity in the bowel. Possible interactions between the food matrix and lunasin, as well as changes in the biological activity resulting from food processing steps and storage, require further investigation.

5.2 Future directions

Further research to gain a better understanding of the potential utilization of lunasin in gastrointestinal disorders might include:

- Identification of the products resulting from the *in vivo* digestion of lunasin and their biodistribution in the different sections of the bowel, as well as the potential correlation between structure and activity.

- Variation in the composition and functionality of the intestinal microbiota because of lunasin consumption in healthy and diseased conditions, as well as the effect of lunasin and digestion-derived peptides over the crosstalk between epithelial, immune, and microbial cells.
- Potential chemopreventive activity of lunasin against sporadic and familiar forms of CRC, as well as the potential contribution of orally consumed lunasin in extraintestinal manifestations of IBD.

Research ideas inspired in the outcomes and results observed in this research, but not related to gastrointestinal health include:

- Effect of lunasin over other inflammasome structures, and the potential utilization of the peptide in diseases with a critical contribution of the inflammasomes.
- Causes underlying differential outcomes between individuals that show either high or low response to the peptide.

VITA

Andrea Nieto was born in Bogotá, Colombia. She attended Universidad Nacional de Colombia (Bogotá Colombia) where she received her bachelor's degree in Chemical Engineering and worked for four years as undergraduate research assistant in the Institute of Food Science and Technology under the guidance of Dr. Martha Quicanzan and was awarded with the grant Young Researchers and Innovators from Minciencias.

After her graduation, she continued her master's education in Advances in Food Safety at Universidad de Jaen (Jaen, Spain) supported by a scholarship from the Association of Ibero-American Universities for Postgraduate Studies – AUIP. She then participated in a short-term research program in the Universidad Politecnica de Valencia (Valencia, Spain) supported by the program Young Professors and Researchers from Santander Universities.

Andrea was awarded the Fulbright-Minciencias Scholarship to carry out her doctorate studies in the USA and moved to Knoxville to attend the University of Tennessee and pursue her PhD in Food Science. Under the direction of Dr. Vermont P. Dia she worked on the evaluation of bioactive properties of food-derived compounds using *in vitro* and *in vivo* approaches. Particularly she focused her dissertation work on the anti-inflammatory and chemopreventive activity of the soybean peptide lunasin in gastrointestinal diseases using *in vivo* models of inflammatory bowel disease and colitis-associated colorectal cancer.