TRYPSIN INHIBITORS AS DRIVERS OF DIGESTIVE INTELLIGENCE

A. Introduction: Digestive System and Trypsin

Human digestive system has been shaped over millions of years of evolutionary challenges to reach its current state. As omnivores, humans have relied on varied sources of protein and fat nutrition and evolved a specialized menu of digestive enzymes to breakdown macronutrients comprising of proteins, carbohydrates and fats into their simpler constituents. These simpler constituents comprising of amino acids, fatty acids, and sugars then construct, operate and feed the finely crafted molecular machinery in our body to sustain life. Digestive enzymes embody the intelligence driving the process of deriving sustenance from food. Of the many classes of digestive enzymes in the body, peptidases which break down proteins are of critical importance as they provide the amino acids or building blocks for all other body systems and processes. Protein nutrition is often a bottleneck in plant based diets and the various peptidases in gastroenteric system play a critical role in health of vegetarians and vegans.

Over one-third of the known peptidases in the body are classified as serine proteases as they cleave the peptide bond in proteins by utilizing the amino acid serine as an electron donor at their active site. (Hedstrom 2002) Serine proteases are involved in a multiple biological process including digestion, immune responses, and serve as essential signaling molecules in gastrointestinal physiology (Vergnolle 2016). The secretion of these proteases affects and is in turn strictly regulated by physiological conditions in intestinal cells, infiltrating immune cells and the gut microbiome(Biancheri, Di Sabatino et al. 2013). Trypsin is the leading serine protease in the body, often referred to as the master digestive enzyme, as it regulates the secretion and activity of other digestive enzymes. While, the primary role of trypsin is to continue the digestion of proteins in small intestine following the initial breakdown of proteins in stomach by pepsin, it also acts as the common activator of all other pancreatic zymogens (inactive forms of enzymes) such as trypsinogen, chymotrypsinogen, proelastase, procarboxypeptidase, and prolipase,(Jablaoui, Kriaa et al. 2020) and thus controls the expression levels of active enzymes responsible for a wide gamut of protein and fat metabolism and other systemwide catabolic and anabolic physiological activities.

A.1. Native Trypsin Inhibitors

Trypsin is stored as its inactivated precursor: trypsinogen in zymogen granules in the pancreas. (Hirota, Ohmuraya et al. 2006)Typically, activation of pancreatic digestive enzymes is strictly

controlled to prevent autodigestion of the pancreas, which clinically presents as pancreatitis. Trypsin activity is inhibited in the pancreatic acinar cells by a native trypsin inhibitor known as pancreatic secretory trypsin inhibitor (PSTI) which can inhibit up to 20% of the trypsin activity by binding to its protease catalytic site. (Hirota, Ohmuraya et al. 2006)If trypsin activation exceeds the capacity of PSTI to neutralize it, a subsequent cascade of events leads to the activation of various proteases that damage cells lining the pancreas and duodenum. The importance of this endogenous trypsin inhibitor can be appreciated by the fact that if PSTI gene is knocked out in mice, it results in the disappearance of the pancreas within a few days after birth.(Ohmuraya, Hirota et al. 2005, Ohmuraya, Hirota et al. 2006) Thus, similar to the combative relationship between a sword and a shield, the balance between trypsin and native trypsin inhibitor activity protects the GI system from autodigestion while performing its digestive role.

A.2. Trypsin Inhibitors in Legumes

Apart from native trypsin inhibitors produced in pancreas, a wide variety of compounds which can inhibit the protease activity of trypsin are found in the plant kingdom, primarily in the legume family. Legumes are important sources of proteins in human diets, but their bioavailability is lower than that of animal proteins. While food legumes are second only to cereals as a source of human and animal food, less than 4% of global legume production is consumed as human food, primarily because of concerns about low bioavailability and need for processing to remove the trypsin inhibitor content.(Wati, Theppakorn et al. 2009) Thus, enormous food science and technology effort has been spent on characterizing and neutralizing the TI content in legumes to improve protein nutritional quality, and as a strategy to fulfil the growing global demands for proteins without the cost and environmental concerns associated with animal derived protein.

Plant seeds contain several biologically active proteins that play specialized functions such as resisting pests or discouraging consumption by animals. These proteins include hydrolytic enzymes, protease inhibitors, lectin and ribosome inactivating proteins(Duranti, Barbiroli et al. 2003). Proteinase inhibitors such as trypsin inhibitors have been isolated from plant storage organs such as seeds, tubers and endosperm. Trypsin/chymotrypsin inhibitors from the legume family members such as red kidney bean, Brazilian pink bean, lima bean, and soybean are closely related with high homology. Hirano et al.(Hirano, Kagawa et al. 1992) showed that soybean seeds and other legume seeds incubated in water at 60°C release into the medium various polypeptides, including those responsible for the trypsin and other enzyme inhibitory activity. The serine protease inhibitors isolated from leguminous seeds are classified into three families: the

Bowman–Birk type inhibitors (BBIs), the Kunitz type inhibitors (KIs), and the potato inhibitor I families.(Zhang, Kouzuma et al. 2008)

Soybean trypsin inhibitors are the most well-studied. The major protein classes in soybeans and other legumes showing trypsin inhibitor activity are BBIs and KIs and these have been identified and studied since 1960s. (Clawson 1996, Duranti, Barbiroli et al. 2003) The Kunitz type inhibitors have molecular weight (MW) of approximately 20 kDa, with low cysteine content and a single reactive site. KI was first described as an alcohol-insoluble factor. (Liu 1997) They inactivate trypsin in a 1:1 stoichiometric ratio, i.e. one molecule of KI binds with one trypsin molecule. The complex that forms is analogous to an enzyme–substrate complex that, unlike the usual enzyme–substrate complex, is very unstable. Three closely related isoforms of KIs have been described.(Kim, Hara et al. 1985) The 3D structure of KI has also been determined(Song and Suh 1998). KIs have low thermal stability and have been investigated for a wide range of pharmacologic interventions, particularly due to their anti-inflammatory action. These include studies for cancer chemoprevention and for other biomedical applications,(Kennedy 1998) including stimulation of hair growth, size, and pigmentation.(Seiberg, Liu et al. 2001)

The second class of trypsin inhibitors is referred as the Bowman–Birk type (BBIs). BBIs have MW of 8–10 kDa with high cysteine content and two reactive sites for trypsin binding(Kennedy 1998) thus each BBI molecule can inactivate two trypsin molecules. BBIs can also simultaneously inhibit trypsin and chymotrypsin at independent sites. In common beans, lima beans, cow peas, and lentils, protease inhibitors are mainly the members of the Bowman–Birk family.(Matheyambath, Padmanabhan et al. 2016),(Alfonso Clemente 2014) The soybean BBI and homologous proteins have received much attention as anti-nutritional components of legume seeds used for animal feed. (Brenes, Jansman et al. 2004, Jezierny, Mosenthin et al. 2010) This is primarily because of the fact that BBIs found in majority of legumes are extremely resistant to denaturation by heat and secondly that, in common with other classes of protease inhibitors, BBI have been claimed to be the major cause of inhibition of protein idigestion and amino acid availability due to the negative feedback loop caused by their inhibition of both trypsin and chymotrypsin at multiple sites, which in turn inhibits other proteolytic enzymes in the GI system.(Lajolo, Genovese et al. 2004) This feedback enhanced inhibition can lead to over-production of digestive enzymes with possible pancreatic hypertrophy. BBI from soybean, tepary bean (Phaseolus acutifolius) and lima bean (Phaseolus lunatus) are remarkably stable in response to boiling, under either neutral or acidic conditions, (Osman, Reid et al. 2002) and are resistant to the action of proteolytic enzymes

of the upper GIT *in vitro*. (Yavelow, Finlay et al. 1983, Jae, Hyung et al. 2007) BBI are active at low pH of 1.5 in the stomach with no loss of activity.(Weder 1986) Although it has been shown that BBI can withstand many of the industrial food processes such as heating and pelleting, it is not well known if the pancreatic and associated effects documented in many small animal and poultry studies(Clarke and Wiseman 2005) apply also to larger farm animals.

A.3. Trypsin Inhibitors inactivation and extraction

TIs were earlier classified as anti-nutrients due to their inhibition of protein assimilation and numerous food processing techniques have been developed for eliminating their action or reducing of TI concentration below the threshold limits of 1–1.5 TIU/mg.(Wati, Theppakorn et al. 2009, Pedrosa, Cuadrado et al. 2012) One of the oldest studies regarding the use thermal inactivation of TIs of pulses was reported by Westfall and Hauge in 1948,(Westfall and Hauge 1948) by cooking soybean at temperatures around 108 °C for 15 to 30 minutes. Now a days, other thermal treatments are used, including the high temperature, short time extrusion cooking which is considered the fastest and most effective thermal processing for TIs inactivation (van der Poel, Stolp et al. 1992, Edwards, Becker et al. 1994, Avilés-Gaxiola, Chuck-Hernández et al. 2018).

With the emerging knowledge about beneficial aspects of TIs, extraction of TIs while maintaining their enzymatic inhibition activity has gained renewed importance. Multiple methods have been reported in literature for extraction of TIs. Especially for extracting KIs which are sensitive to thermal deactivation, typically the raw and extruded legume flours are stirred in weakly acidic suspensions at 4 degree C, followed by centrifugation at >6000g for 10 minutes. The KIs are present in the resultant clear supernatant which needs to be frozen until use. Welham and Domoney(Welham and Domoney 2000) have published small scale quantitative assays for measuring the trypsin inhibitor activity in such samples. Recently, the separation and purification of enzymes have been developed to be more efficient, economical and scalable in order to isolate target proteins(Roy and Gupta 2000, Wati, Theppakorn et al. 2009) and three-phase partitioning (TPP) has been reported for efficient extraction of BBIs from legumes. TPP methods are scalable and could be used directly with the crude suspensions of legume flours for extracting TIs.

B. TIs in human health and disease

Historically, TIs have been treated as villains of human nutrition due to their inhibition of protein digestion, however as the role of trypsin in regulating multiple proteolytic enzymes is understood in greater depth and multivalent TI actions are discovered, TIs have seen a resurgence as

compound of great interest for many lifestyle driven diseases of the modern age. These health effects rely on the serine protease deactivation of TIs, when deployed in correct stoichiometric amounts and in specific physiological condition. Preclinical and limited clinical studies on the positive role of TIs in health have been conducted in the areas of obesity mitigation, cancer prevention and treatment, and in managing inflammatory and auto-immune diseases.

B.1. TIs for obesity treatment

Obesity is a major health and economic burden in advanced countries as well as rapidly modernizing emerging nations of India, China and Latin America. Obesity is defined as concentrated or generalized fatty acid deposition in body (Carvalho, Lima et al. 2019) and often measured by a high (>30) body mass index of BMI. Obesity is caused primarily due to nutritional or caloric imbalance and often associated with genetic or endocrine metabolic disorders.(Bookout, De Groot et al. 2013) Obesity has very few pharmacological treatment options other than surgical interventions, and lifestyle change with diet and exercise if often prescribed, with poor patient compliance. The health burden of obesity increases as it is associated with an enhanced risk of developing other serious comorbidities such as type 2 diabetes mellitus, hypertension, coronary vessel disease, dyslipidemias, and certain types of cancer and other circulatory disorders. (Christodoulides, Dyson et al. 2009, Braud, Ciufolini et al. 2012) Obesity is a complex disease which once established is sustained by feedback processes from the adipose tissue. Adipose tissue is frequently infiltrated by immune cells such as activated macrophages which release inflammatory cytokines such as tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor 1 (PAI-1), interleukin-6 (IL-6), retinol-binding protein 4, macrophages chemoattractant protein 1 (MCP-1), and acute phase proteins.(De Sousa-Coelho, Marrero et al. 2012) These factors perpetuate local inflammation in the adipose tissue and induce insulin resistance and vascular and cardiac dysfunctions.(Garcia Caraballo, Comhair et al. 2017) TNF-α is produced, not only by immune cells, but also by cells of adipose tissue directly. (Goke, Stockmann et al. 1984) TNF-α has been established as a major target of pharmacologic intervention because of role in promoting insulin resistance, as a regulator of adipose tissue mass, and its increased concentrations in the hypothalamus of animals submitted to hyperlipidic and hyperglycemic diet. (Gumbmann, Dugan et al. 1989, Inagaki, Lin et al. 2008, Hill, Laeger et al. 2017) TNF-α also acts directly on the adipocytes to regulate apoptosis and lipogenesis to control accumulation of fat in adipose tissue.(Arner 1995, Petros and Peters 1996, Weisberg, McCann et al. 2003, Kanneganti and Dixit 2012) A second pathologic feature of obesity is aberrant satiety signaling comprising of hormones leptin and ghrelin which regulate satiety and hunger. In obese patients, hunger reducing hormone

leptin becomes less effective due to de novo or acquired leptin resistance and the relationship of caloric intake and feeling of satiety is broken. Thus, obesity is often resistant to lifestyle interventions based on diet or exercise.

There are very limited effective therapies for obesity. The United States Food and Drug Administration (FDA) approved weight-loss medication orlistat acts by inhibiting the intestinal digestive enzyme lipase.(Albarazanji, Jennis et al. 2019) Orlistat has low systemic exposure after oral administration and considered safe but leads to unpleasant off-target effects. Orlistat is associated with a fecal continence problem or intermittent loss of oil in obese subjects. It also decreases high-density lipoprotein cholesterol,(Khera, Pandey et al. 2018) which increases cardiovascular disease risk. An alternative safe approach for controlling obesity would be to inhibit trypsin and related serine protease inhibitors such as enteropeptidase, which can indirectly also control lipase secretion.(Albarazanji, Jennis et al. 2019) However, efforts to optimize trypsin inhibitor compounds to reduce weight are hampered by lack of detailed understanding of mechanisms underlying their efficacy and dose-response.

Nutrition researchers (McLaughlin, Peikin et al. 1983) have long noted that decreased body weight and increased pancreas weight occur in rats fed raw soybeans, and this is thought to be due to the presence of trypsin inhibitors in the raw soybeans. Trypsin is postulated to be a negative feedback signal for cholecystokinin (CCK) secretion, trypsin inhibitors can increase secretion of CCK. CCK is a putative satiety signal; thus, increased secretion of CCK could decrease food intake, and, if maintained over a period of time reduce body weight. McLaughlin et.al. (McLaughlin, Peikin et al. 1983) report the effects of a trypsin inhibitor [N,N-dimethylcarbamoyl 4-(4-guanidino-benzylyloxy)-phenyl acetate methane-sulfate (DGPM)]on feeding pattern was investigated in Zucker obese and lean rats. Administration of 25–200 mg/kg DGPM to 6-hr fasted rats decreased daily food intake by dose-dependently decreasing average meal size in both obese and lean rats, but the response was greater in obese rats. Administration of 100 mg/kg DGPM twice daily for 7 days decreased food intake and body weight in obese but not lean rats. Thus, these results suggested that soybean trypsin inhibitors can decrease body weight partly as a result of increased secretion of the putative satiety peptide CCK which reduces average meal intake.

Other researchers(Carvalho, Lima et al. 2019) have studied the impact of purified legume derived trypsin inhibitors in obesity prone rats, and noted that in addition to satiety-genic effect, TIs directly

reduce TNF-α levels to undetectable concentration in animals treated with TIs. As TNF-α plays a central role in chronic inflammation, this is an intriguing finding with relevance to a broad class of diseases including obesity. TIs were reported to reduce TNF-α and inflammatory markers to concentrations equal to those of healthy animals, regardless of weight loss. TIs were reported to reduce both the serum TNF-α and the relative expression of its gene with negative immunostaining in the visceral adipose tissue. These findings demonstrate that legume derived TIs can act as a potent anti-TNF-α blockers. Moreover, TI treated animals not only had reduced inflammation, but also reduced levels of serum cholesterol and triglycerides. Thus, TIs may also decrease cardiovascular risk in addition to lipogenic processes of obesity. This is of great relevance to the pharmaceutical industry.

While, there is no known human clinical use of TIs for obesity intervention, similar compounds such as Camostat (CS),(Albarazanji, Jennis et al. 2019) a serine protease trypsin inhibitor has been effectively used for chronic pancreatitis in Japan and has been shown to have beneficial metabolic effects. Camostat has been recently reported to be effective in protecting cells against SARS-Cov-2 novel corona virus as well, primarily due to its anti-inflammatory action.(Hoffmann, Kleine-Weber et al. 2020) In an 8-year Japanese study, CS use led to the reduction of the incidence of newly diagnosed type 2 diabetes (T2D) in pancreatitis patients from 32.7% to 24.3%.(Ito, Otsuki et al. 2007) Camostat and plant-based trypsin inhibitors also successfully reduced body weight gain by decreasing food intake in various obese rodent models(McLaughlin, Peikin et al. 1983, Muller, Goebell et al. 1988) and in several other species (Struthers and MacDonald 1983) As reported previously, this satiety-mediated effect is due to increased secretion of the peptide cholecystokinin (CCK).(McLaughlin, Peikin et al. 1983) In rats, protein digestion in the small intestine is a potent stimulus for CCK release, which acts on CCK receptor 1 (CCK1R) to delay gastric emptying and inhibit food intake.(Liou, Chavez et al. 2011) CS acts as a non-nutrient stimulant of endogenous CCK release to reduce food intake, delay gastric emptying, and increase gall bladder emptying and pancreatic zymogen secretion.(Liddle 1995, Nishi, Hara et al. 2003) Multiple other studies have demonstrated satiety-independent weight loss by trypsin inhibition in rats and mice. Prolonged CS treatment reduced weight gain and hyperglycemia in Otsuka Long-Evans Tokushima fatty (OLETF) rats. (Jia, Taguchi et al. 2005, Jia, Taguchi et al. 2005) These rats are CCK1R deficient (Miyasaka, Masuda et al. 1997) and are thus do not have CCK dependent satiety signaling. They can gain weight and do not compensate for eating larger meals by decreased meal frequency.(Bi and Moran 2016) In OLETF rats, CS administration reduced weight gain without reducing food intake. (Jia, Taguchi et al. 2005) In

similar studies in mice, novel boropeptide enteropeptidase inhibitors which stop enteropeptidase from converting trypsinogen to trypsin, also reduced weight gain without reducing food intake.(Braud, Ciufolini et al. 2012) In these studies where caloric intake was not reduced but weight was lost as a result of TI treatment, caloric excretion in feces was increased thus maintaining the energy balance.(Jia, Taguchi et al. 2005, Braud, Ciufolini et al. 2012) These studies suggests an attractive mode of weight loss for human use without caloric restriction, which might be easy to comply with as strict dietary intake control may not be necessary for weight loss with optimized TI dosing.

B.2. TIs for chemoprevention of cancer

In spite of major investments in medical research and pharmaceutical interventions, cancer remains the leading cause of mortality in United States and globally. Cancer treatments, whether they are surgery, chemotherapy, and/or radiation therapy are usually only curative in earliest stages of disease and entail severe loss in quality of life of patients due to morbid off-target effects. Chemoprevention strategies with chronic administration of safe pharmaceuticals or nutraceuticals derived from food or plant sources can either prevent the occurrence or cancer or result in benign disease. This promise of chemoprevention has spurred tremendous research effort in studying plant derived substances such as curcumin from turmeric, gingerols from ginger, and phenolics or terpenes from substances like tea, spices, and fruits for their role in inhibiting cancer. Soy and other legume derived trypsin inhibitors have a proven role in anti-inflammation in animal studies and thus have acquired substantial researcher attention for assessing their role in chemoprevention of cancer.

Legume seeds contain proteins, including lectins,(Rubio, Pedrosa et al. 2006) protease inhibitors(Clemente, Jimenez et al. 2008, Marín-Manzano, Ruiz et al. 2009) and albumin storage proteins,(Moreno and Clemente 2008) that are resistant to gut protein breakdown and remain biologically functional through their passage in the gastrointestinal tract.(Clemente, Sonnante et al. 2011) Bowman-Birk inhibitors (BBI) from legumes, such as soybean, pea, lentil and chickpea have been demonstrated to have potential anti-inflammatory and chemopreventive properties in gastrointestinal tract. BBIs protein structure is marked by extensive disulphide linkage and resistant to the harsh upper gastrointestinal tract environment. Recent reports suggest that trypin and chymotrypsin in intestines may have a role in early carcinogenesis and BBIs by virtue of inhibiting both these enzymes may exert chemopreventive action against colorectal cancer, but the precise mechanism of BBI action in intestinal tract is not understood.(Clemente, Sonnante et

al. 2011) Advances have been made in unravelling the beneficial effects of plant derived protease inhibitors on human health not only as gut, metabolic, immunological and hormonal regulators but also as naturally-occurring chemotherapeutic or radioprotective agents.(Pusztai, Gran et al. 1992, Pusztai, Bardocz et al. 2004, Gelencsér 2009) The mutual regulation of proteolytic enzymes with their cognate inhibitors plays a central role in multiple physiological and pathological processes, such as cancer and other inflammatory diseases. Serine proteases such as trypsin and chymotrypsin have been linked to tumor growth, invasion, metastasis, and angiogenesis.(Lee, Dickson et al. 2000, Darmoul, Gratio et al. 2003) The increasing understanding of the role played by these proteases in the biological processes associated with disease offers novel opportunities for therapeutic intervention by utilizing their natural inhibitors found in legumes.(Turk 2006)

A target for TI based cancer chemoprevention is colorectal cancer (CRC), which is growing in incidence due to reliance on processed and industrial foods and increasingly affects patients under 40 years of age. Like many other cancers etiology of CRC is complex and depends on genetics, lifestyle, environment and dietary composition. Dietary intervention has been suggested as a key approach to prevent and reduce the incidence of CRC.(Cummings and Bingham 1998, Reddy 2000, Macfarlane and Stover 2007) Chemoprevention via standardized dietary ingredients has major public health implications as the widespread long-term use of constituents such as legume derived TIs can be promoted in a cost effective manner, especially in economically weaker countries. Soybean BBIs and homologous proteins have been associated with a suppression of cancer development within the GIT (Kennedy, Billings et al. 2002, Clemente, Gee et al. 2005, Clemente, Moreno et al. 2010) including under conditions of direct carcinogen assault as seen in dimethylhydrazine (DMH)-treated animals when soy BBIs used at concentrations as low as 10 mg/100 g diet, reduce the incidence and frequency of colon tumors in mice(Clair, Billings et al. 1990) and rats(Kennedy, Billings et al. 2002), without any adverse or off-target effects.

In vitro studies on human colon adenocarcinoma cell line HT29 have reported dose and treatment time dependent inhibition of growth via cell-cycle arrest with soy BBI treatment. Similar anti-proliferation behavior was observed with BBIs derived from pea(Clemente, Gee et al. 2005) and lentil(Caccialupi, Ceci et al. 2010). The anti-carcinogenic properties of soybean BBI have been mechanistically linked to its chymotrypsin inhibitory domain, leading to the hypothesis that chymotrypsin-like proteases are potential targets of BBI in anti-cancer effects.(Clair, Billings et al. 1990, Kennedy, Billings et al. 2002) Yavelow et al.(Yavelow, Collins et al. 1985) have reported that an enzymatically modified soybean BBI having only chymotrypsin inhibitory activity can

suppress carcinogensis, whereas the protease inhibitor with only trypsin inhibitory activity is not effective. This suggests the importance of whole extracts of TIs from soy rather than purified extracts might be more effective.

As BBIs have been shown to exert a potent suppression of carcinogenic processes in a variety of in vitro and in vivo models,(Kennedy 1998, Clemente and Domoney 2008) soy derived BBI enriched extract was given the status of Investigational New Drug status by FDA, with multiple clinical trials in progress. Human trials investigating the role of soy BBIs have been completed in precancerous conditions such as benign prostatic hyperplasia(Bruce Malkowicz, McKenna et al. 2001), oral leukoplakia(Armstrong, Kennedy et al. 2000, Armstrong, Kennedy et al. 2000) and ulcerative colitis(Lichtenstein, Deren et al. 2008) with varying degree of positive effects. In ulcerative colitis clinical trial, 50% of patients responded and 36% showed remission of disease. No clinical toxicity or other adverse events such as allergy or gastrointestinal discomfort have been reported in these trials with long term BBI administration, thus it indicates a strong potential for assessing BBIs for chemoprevention of multiple cancers.

B.3 TIs as immunomodulators

Inflammatory and associated auto-immune disorders such as rheumatoid arthritis, Crohn's or inflammatory bowel disease, lupus, psoriasis, ankylosing spondylitis, and multiple sclerosis have seen a rapid rise in incidence in the past few decades. Most of these diseases do not have curative treatments and are managed with steroidal or immune-suppressive drugs with severe off-target effects. Collectively these diseases induce severe lack of quality of life and impose public health burden due the cost of managing these diseases in patient populations which are distributed over both working young and old age groups. Among the biological agents approved for the treatment of inflammatory diseases, a major class comprises of compounds that act as blockers or antagonists of TNF-α, (Carvalho, Lima et al. 2019) also called anti-TNF-α. (Lim, Lee et al. 2018, Melo and Magina 2018) Currently, five TNF-α blockers are FDA approved and widely available: etanercept (Enbrel®, Pfizer), infliximab (Remicade®, Cilag AG), adalimumab (Humira®, AbbVie), certolizumab-pegol (Cimzia®, Vetter Pharma-Fertigung GmbH & Co.), and golimumab (Simponi®, Baxter Pharmaceutical Solutions LLC).(Mitoma, Horiuchi et al. 2018) All these biologic drugs are known to adversely impact the serum lipid profile, by increasing triglycerides, promote early onset of type 2 diabetes and increase the risk of atherosclerotic vascular disease.(Cacciapaglia, Navarini et al. 2011) Thus, there is a strong impetus for search of effective TNF-α without these adverse effects. Other molecules with TNF-α inhibitory actions

have been investigated.(Yanovski and Yanovski 2002, Li, Maglione et al. 2005) Weight loss drugs such as orlistat which inhibits lipase action and reduces the digestion and/or absorption of nutrients,(Shah, Mehta et al. 2008) Specific serotonin reuptake inhibitors (fluoxetine),(Yanovski and Yanovski 2002) Herbal medicines, such as Potein® (Dermo manipulações, Brazil), composed of isolated trypsin inhibitors, all have been investigated as treatments of inflammatory disorders(Chen, Hira et al. 2012) however, strong efficacy and mechanistic understanding of their anti-inflammatory action is lacking. TNF-α initiates the inflammatory cascade and is responsible for the activation of immune cells, stimulates release of proteolytic enzymes from macrophages, and promotes production of other inflammatory cytokines which play a key role in the perpetuation of inflammation in diseases such as rheumatoid arthritis, ankylosing spondylitis, obesity, atherosclerosis, inflammatory bowel diseases, metabolic syndrome, and type 2 diabetes. (Lozano, Van Der Werf et al. 2016, Melo and Magina 2018, Mitoma, Horiuchi et al. 2018) Plant based and safe TNF- α blockers such as soy trypsin inhibitors can have an important role in addressing the challenges posed by these inflammatory diseases.

There is evidence for the role of serine proteases such as trypsin and chymotrypsin in inflammatory bowel disease (IBD).(Jablaoui, Kriaa et al. 2020) Serine protease activity is tightly regulated and the disequilibrium of this proteolytic activity is linked to several gastrointestinal disorders including inflammatory bowel diseases (IBD). (Kaplan 2015, Edgington-Mitchell 2016, Masaki, Kishiki et al. 2018) An increased expression level of host serine proteases has been observed in colonic and fecal samples from IBD patients.(Raithel, Winterkamp et al. 2001, Dabek, Ferrier et al. 2009, Motta, Bermúdez-Humarán et al. 2012, Hamilton, Frei et al. 2014) Such dysregulated proteolysis can elicit structural and functional changes in the mucosal barrier and participate in persistent inflammation. (Van Spaendonk, Ceuleers et al. 2017) Further in situ assays in GIT have revealed increased proteolytic activity within the inflamed epithelium.(Rolland-Fourcade, Denadai-Souza et al. 2017) Jablaoui *et.al.*(Jablaoui, Kriaa et al. 2020) demonstrated that protease activity is increased in IBD patients compared to healthy subjects and the serine protease family including trypsin, neutrophil elastase (HNE), proteinase 3 (PR3), and cathepsin G (CatG) constituted the most active protease family in GIT.

The use of anti-protease therapy via soybean BBI to diminish clinical disorders in patients with inflammatory bowel disease or ulcerative colitis has been reported.(Clarke and Wiseman 2005) The ability of soybean BBI to inhibit the activity of several serine proteases involved in bowel inflammatory disorders, such as elastase,(Ramasarma, Appu Rao et al. 1995) cathepsin G

(Ramasarma, Appu Rao et al. 1995, Osman, Reid et al. 2002) and mast cell chymase(Clemente, Marín-Manzano et al. 2013) has been demonstrated However, a clear mechanistic linkage between serine protease inhibition and anti-inflammatory properties associated with soybean BBI has not been yet demonstrated. Ionizing radiation which us used often in cancer treatment is associated with local inflammation. Soy BBIs have been demonstrated to be a potent radioprotector of normal tissue in vitro(Gariani and Leatherbarrow 1997) and in vivo(Chang, Li et al. 1990). Additionally, dietary supplementation containing 1 % BBI has been reported to inhibit unloading induced muscle weakness,(Pusztai, Bardocz et al. 2004) promote redox homeostasis in muscle fibres and blunt atrophy-induced weakness(Brauer, Kelly et al. 2002) via mechanisms reporting a novel role for BBI as antioxidants, showing the capacity of BBI to scavenge reactive oxygen species as another mode of exercising anti-inflammatory action. The role of soy BBIs has been studied for Multiple sclerosis (MS) treatment, which is an incurable inflammatory demyelinating disease of the central nervous system.(Clemente, Jimenez et al. 2008, Marín-Manzano, Ruiz et al. 2009) Soy BBIs have been demonstrated suppress the autoimmune encephalomyelitis in rodents, showing an improvement of several disease parameters (onset, severity, weight loss, inflammation, neuronal loss and demyelination) and no apparent adverse effects. BBI are effective for MS symptomatic relief even when treatment was initiated after disease onset.(Marín-Manzano, Ruiz et al. 2009) Although the mechanism of action remains unknown, BBIs can play a significant role as a therapeutic modulator in MS lesion areas.

While, the majority of studies for anti-inflammatory action of soy trypsin inhibitors have focused on BBIs, recent reports by Ribeiro et.al.(Ribeiro, Cunha et al. 2010) indicate that Kunitz type or KIs also have a strong anti-inflammatory action. In particular, these researchers have noted that purified soy KIs can efficiently inhibit human neutrophil elastase (HNE) with an IC(50) value of 8mug or 0.3nM, while having no adverse effects on organs, blood cells or liver. The imbalance between human neutrophil elastase (HNE) and endogenous serine proteinase inhibitors is considered to cause a variety of HNE-mediated inflammatory disorders such as rheumatoid arthritis, pulmonary emphysema, adult respiratory distress syndrome (ARDS), cystic fibrosis, COPD, asthma, and delayed wound healing.(Siedle, Hrenn et al. 2007) HNE is known to be present in psoriatic lesion and it induces keratinocyte hyperproliferation by signaling cascades involving TGF-a.(Rogalski, Meyer-Hoffert et al. 2002, Meyer-Hoffert, Wingertszahn et al. 2004) Soy KIs could suppress inflammation in a dose dependent manner in mice with acute lung injury and the results reported by Ribeiro *et. al.*(Ribeiro, Cunha et al. 2010) can be highly significant for developing effective plant based therapies for skin related or cosmetic inflammatory conditions.

C. Summary and Future Outlook

As illustrated in previous sections, promising scientific data and proof-of-concept studies are available in support of the role of legume and soy derived trypsin inhibitors for preventing and treating a wide variety of pathologies. There are numerous gaps in knowledge about optimum use of these compounds. In order to exert any health benefits, either local or systemic, TIs need to resist and survive the digestive process within the gastrointestinal tract (GIT) and reach the target organs. (Clemente and Domoney 2008) While, a subset of soy trypsin inhibitors composed of BBIs are structurally resistant to the challenges of proteolytic enzymes of the upper GIT in vitro(Yavelow, Finlay et al. 1983, Jae, Hyung et al. 2007) and are active at pH as low as 1.5 in the presence of pepsin with no loss of activity,(Weder 1986) only a few studies have reported the intestinal survival of BBI. Not much is known about the structural and functional stability of KI type trypsin inhibitors in physiologic conditions. Further research on effective packaging of these compounds for in vivo delivery is needed. Recent efforts have focused on nanotechnology based methods for targeted delivery of TIs in vivo, by leveraging the tunable pharmacokinetics and composition of nanoparticles.(Rekha and Sharma 2013, Chanphai and Tajmir-Riahi 2016, Liu, Cheng et al. 2016, Liu, Cheng et al. 2017) Nanoparticles can be designed with controlled size, shape and surface functionalization to achieve enhanced systemic circulation and ensure survival of TIs until reaching the organ specific action sites. Nanoparticles can also achieve controlled release of TIs to modulate their therapeutic action and further increase the safety profile. In addition to delivery concerns, as plant based compound complexes, trypsin inhibitors require the scaling, standardization and development of quality manufacturing standards for easily advancing to FDA-IND (Investigational new drug) application standards. The expense involved in these endeavors will need government and commercial investments to realize the promise of safe, effective, and environmentally friendly plant based therapies for modern ailments ranging from obesity, cancer, and auto-immune disorders.

D. Bibliography

Albarazanji, K., M. Jennis, C. R. Cavanaugh, W. Lang, B. Singh, J. C. Lanter, J. M. Lenhard and P. J. Hornby (2019). "Intestinal serine protease inhibition increases FGF21 and improves metabolism in obese mice." American Journal of Physiology-Gastrointestinal and Liver Physiology **316**(5): G653-G667.

Alfonso Clemente, M. d. C. A. (2014). "Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents." World Journal of Gastroenterology : WJG **20**(30): 10305-10315.

Armstrong, W. B., A. R. Kennedy, X. Steven Wan, T. H. Taylor, Q. A. Nguyen, J. Jensen, W. Thompson, W. Lagerberg and F. L. Meyskens, Jr. (2000). "Clinical modulation of oral leukoplakia and protease activity by Bowman-Birk inhibitor concentrate in a phase IIa chemoprevention trial." Clinical Cancer Research **6**(12): 4684-4691.
Armstrong, W. B., A. R. Kennedy, X. S. Wan, J. Atiba, C. E. McLaren and F. L. Meyskens Jr (2000). "Single-dose administration of Bowman-Birk inhibitor concentrate in patients with oral leukoplakia." Cancer Epidemiology Biomarkers and Prevention **9**(1): 43-47.
Arner, P. (1995). "Differences in lipolysis between human subcutaneous and omental adipose tissues." Annals of Medicine **27**(4): 435-438.
Avilés-Gaxiola, S., C. Chuck-Hernández and S. O. Serna Saldívar (2018). "Inactivation Methods of Trypsin Inhibitor in Legumes: A Review." Journal of Food Science **83**(1): 17-29.
Bi, S. and T. H. Moran (2016). "Obesity in the Otsuka Long Evans Tokushima Fatty Rat: Mechanisms and Discoveries." Frontiers in Nutrition **3**.
Biancheri, P., A. Di Sabatino, G. R. Corazza and T. T. MacDonald (2013). "Proteases and the gut barrier." Cell and Tissue Research **351**(2): 269-280.
Bookout, A. L., M. H. M. De Groot, B. M. Owen, S. Lee, L. Gautron, H. L. Lawrence, X. Ding, J. K. Elmquist, J. S. Takahashi, D. J. Mangelsdorf and S. A. Kliewer (2013). "FGF21 regulates metabolism and circadian behavior by acting on the nervous system." Nature Medicine **19**(9): 1147-1152.
Braud, S., M. A. Ciufolini and I. Harosh (2012). "Enteropeptidase: A Gene Associated with a Starvation Human Phenotype and a Novel Target for Obesity Treatment." PLoS ONE **7**(11).
Brauer, A. B. E., G. Kelly, S. J. Matthews and R. J. Leatherbarrow (2002). "The 1H-NMR solution structure of the antitryptic core peptide of Bowman-Birk inhibitor proteins: A minimal 'canonical loop'." Journal of Biomolecular Structure and Dynamics **20**(1): 59-70.
Brenes, A., A. J. M. Jansman and R. R. Marquardt (2004). "Recent progress on research on the effects of antinutritional factors in legume and oil seeds in monogastric animals." Recent Advances of Research in Antinutritional Factors in Legume Seeds and Oilseeds: 195-217.
Bruce Malkowicz, S., W. G. McKenna, D. J. Vaughn, X. S. Wan, K. J. Propert, K. Rockwell, S. H. F. Marks, A. J. Wein and A. R. Kennedy (2001). "Effects of Bowman-Birk inhibitor concentrate (BBIC) in patients with benign prostatic hyperplasia." Prostate **48**(1): 16-28.
Caccialupi, P., L. R. Ceci, R. A. Siciliano, D. Pignone, A. Clemente and G. Sonnante (2010). "Bowman-Birk inhibitors in lentil: Heterologous expression, functional characterisation and anti-proliferative properties in human colon cancer cells." Food Chemistry **120**(4): 1058-1066.
Cacciapaglia, F., L. Navarini, P. Menna, E. Salvatorelli, G. Minotti and A. Afeltra (2011). "Cardiovascular safety of anti-TNF-alpha therapies: Facts and unsettled issues." Autoimmunity Reviews **10**(10): 631-635.
Carvalho, F. M. C., V. C. O. Lima, I. S. Costa, A. B. S. Luz, F. V. L. Ladd, A. C. Serquiz, R. H. Bortolin, V. N. Silbiger, B. L. L. Maciel, E. A. Santos and A. H. A. Morais (2019). "Anti-TNF-α Agent Tamarind Kunitz Trypsin Inhibitor Improves Lipid Profile of Wistar Rats Presenting Dyslipidemia and Diet-induced Obesity Regardless of PPAR-γ Induction." Nutrients **11**(3): 512.
Chang, J. D., J. H. Li, P. C. Billings and A. R. Kennedy (1990). "Effects of protease inhibitors on c-myc expression in normal and transformed C3H 10T1/2 cell lines." Molecular Carcinogenesis **3**(4): 226-232.

Chanphai, P. and H. A. Tajmir-Riahi (2016). "Chitosan nanoparticles conjugate with trypsin and trypsin inhibitor." Carbohydrate Polymers **144**: 346-352.
Chen, W., T. Hira, S. Nakajima, H. Tomozawa, M. Tsubata, K. Yamaguchi and H. Hara (2012). "Suppressive effect on food intake of a potato extract (Potein ®) involving cholecystokinin release in rats." Bioscience, Biotechnology and Biochemistry **76**(6): 1104-1109.
Christodoulides, C., P. Dyson, D. Sprecher, K. Tsintzas and F. Karpe (2009). "Circulating fibroblast growth factor 21 is induced by peroxisome proliferator-activated receptor agonists but not ketosis in man." Journal of Clinical Endocrinology and Metabolism **94**(9): 3594-3601.
Clair, W. H. S., P. C. Billings, J. A. Carew, C. Keller-McGandy, P. Newberne and A. R. Kennedy (1990). "Suppression of Dimethylhydrazine-induced Carcinogenesis in Mice by Dietary Addition of the Bowman-Birk Protease Inhibitor." Cancer Research **50**(3): 580-586.
Clarke, E. and J. Wiseman (2005). "Effects of variability in trypsin inhibitor content of soya bean meals on true and apparent ileal digestibility of amino acids and pancreas size in broiler chicks." Animal Feed Science and Technology **121**(1-2): 125-138.
Clawson, G. A. (1996). "Protease inhibitors and carcinogenesis: A review." Cancer Investigation **14**(6): 597-608.
Clemente, A. and C. Domoney (2008). "Therapeutic properties of legume protease inhibitors from the Bowman-Birk family." Recent Progress in Medicinal Plants **20**: 397-417.
Clemente, A., J. M. Gee, I. T. Johnson, D. A. MacKenzie and C. Domoney (2005). "Pea (Pisum sativum L.) protease inhibitors from the Bowman - Birk class influence the growth of human colorectal adenocarcinoma HT29 cells *in vitro*." Journal of Agricultural and Food Chemistry **53**(23): 8979-8986.
Clemente, A., E. Jimenez, M. C. Marin-Manzano and L. A. Rubio (2008). "Active Bowman-Birk inhibitors survive gastrointestinal digestion at the terminal ileum of pigs fed chickpea-based diets." Journal of the Science of Food and Agriculture **88**(3): 513-521.
Clemente, A., M. d. C. Marín-Manzano, M. d. C. Arques and C. Domoney (2013). Bioactive Food Peptides in Health and Disease. B. Hernandez-Ledesma and C.-C. Hsieh, IntechOpen.
Clemente, A., F. J. Moreno, M. C. Marín-Manzano, E. Jiménez and C. Domoney (2010). "The cytotoxic effect of Bowman-Birk isoinhibitors, IBB1 and IBBD2, from soybean (Glycine max) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases." Molecular Nutrition and Food Research **54**(3): 396-405.
Clemente, A., G. Sonnante and C. Domoney (2011). "Bowman-Birk Inhibitors from Legumes and Human Gastrointestinal Health: Current Status and Perspectives." ingentaconnect.com **12**(5): 358-373.
Cummings, J. H. and S. A. Bingham (1998). "Diet and the prevention of cancer." BMJ **317**(7173): 1636-1640.
Dabek, M., L. Ferrier, R. Roka, K. Gecse, A. Annahazi, J. Moreau, J. Escourrou, C. Cartier, G. Chaumaz, M. Leveque, A. Ait-Belgnaoui, T. Wittmann, V. Theodorou and L. Bueno (2009). "Luminal cathepsin G and protease-activated§ receptor 4: A duet involved in alterations of the colonic epithelial barrier in ulcerative colitis." American Journal of Pathology **175**(1): 207-214.
Darmoul, D., V. Gratio, H. Devaud, T. Lehy and M. Laburthe (2003). "Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells." American Journal of Pathology **162**(5): 1503-1513.

De Sousa-Coelho, A. L., P. F. Marrero and D. Haro (2012). "Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation." Biochemical Journal **443**(1): 165-171.
Duranti, M., A. Barbiroli, A. Scarafoni, G. Tedeschi and P. Morazzoni (2003). "One-step purification of Kunitz soybean trypsin inhibitor." Protein Expression and Purification **30**(2): 167-170.
Edgington-Mitchell, L. E. (2016). "Pathophysiological roles of proteases in gastrointestinal disease." American Journal of Physiology - Gastrointestinal and Liver Physiology **310**(4): G234-G239.
Edwards, R. H., R. Becker, A. P. Mossman, G. M. Gray and L. C. Whitehand (1994). "Twin-screw extrusion cooking of small white beans (phaseolus vulgaris)." LWT - Food Science and Technology **27**(5): 472-481.
Garcia Caraballo, S. C., T. M. Comhair, C. H. C. Dejong, W. H. Lamers and S. E. Koehler (2017). "Dietary treatment of fatty liver: High dietary protein content has an antisteatotic and antiobesogenic effect in mice." Biochimica et Biophysica Acta - Molecular Basis of Disease **1863**(7): 1789-1804.
Gariani, T. and R. J. Leatherbarrow (1997). "Stability of protease inhibitors based on the Bowman-Birk reactive site loop to hydrolysis by proteases." Journal of Peptide Research **49**(6): 467-475.
Gelencsér, É. (2009). "Novel research strategy for safe use of legume proteins in human nutrition." Acta Alimentaria **38**: 61-70.
Goke, B., F. Stockmann and R. Muller (1984). "Effect of a specific serine protease inhibitor on the rat pancreas: Systemic administration of camostate and exocrine pancreatic secretion." Digestion **30**(3): 171-178.
Gumbmann, M. R., G. M. Dugan, W. L. Spangler, E. C. Baker and J. J. Rackis (1989). "Pancreatic response in rats and mice to trypsin inhibitors from soy and potato after short- and long-term dietary exposure." Journal of Nutrition **119**(11): 1598-1609.
Hamilton, M. J., S. M. Frei and R. L. Stevens (2014). "The multifaceted mast cell in inflammatory bowel disease." Inflammatory Bowel Diseases **20**(12): 2364-2378.
Hedstrom, L. (2002). "Serine protease mechanism and specificity." Chemical Reviews **102**(12): 4501-4523.
Hill, C. M., T. Laeger, D. C. Albarado, D. H. McDougal, H. R. Berthoud, H. Münzberg and C. D. Morrison (2017). "Low protein-induced increases in FGF21 drive UCP1-dependent metabolic but not thermoregulatory endpoints." Scientific Reports **7**(1).
Hirano, H., H. Kagawa and K. Okubo (1992). "Characterization of proteins released from legume seeds in hot water." Phytochemistry **31**(3): 731-735.
Hirota, M., M. Ohmuraya and H. Baba (2006). "The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis." Journal of Gastroenterology **41**(9): 832-836.
Hoffmann, M., H. Kleine-Weber, S. Schroeder, N. Krüger, T. Herrler, S. Erichsen, T. S. Schiergens, G. Herrler, N.-H. Wu and A. Nitsche (2020). "SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor." Cell.

Inagaki, T., V. Y. Lin, R. Goetz, M. Mohammadi, D. J. Mangelsdorf and S. A. Kliewer (2008). "Inhibition of Growth Hormone Signaling by the Fasting-Induced Hormone FGF21." Cell Metabolism **8**(1): 77-83.
Ito, T., M. Otsuki, T. Itoi, T. Shimosegawa, A. Funakoshi, K. Shiratori, S. Naruse and Y. Kuroda (2007). "Pancreatic diabetes in a follow-up survey of chronic pancreatitis in Japan." Journal of Gastroenterology **42**(4): 291-297.
Jablaoui, A., A. Kriaa, H. Mkaouar, N. Akermi, S. Soussou, M. Wysocka, D. Wołoszyn, A. Amouri, A. Gargouri, E. Maguin, A. Lesner and M. Rhimi (2020). "Fecal Serine Protease Profiling in Inflammatory Bowel Diseases." Frontiers in cellular and infection microbiology **10**: 21.
Jae, H. P., J. J. Hyung and B. O. De Lumen (2007). "In vitro digestibility of the cancer-preventive soy peptides lunasin and BBI." Journal of Agricultural and Food Chemistry **55**(26): 10703-10706.
Jezierny, D., R. Mosenthin and E. Bauer (2010). "The use of grain legumes as a protein source in pig nutrition: A review." Animal Feed Science and Technology **157**(3-4): 111-128.
Jia, D., M. Taguchi and M. Otsuki (2005). "Preventive and therapeutic effects of the protease inhibitor camostat on pancreatic fibrosis and atrophy in CCK-1 receptor-deficient rats." Pancreas **30**(1): 54-61.
Jia, D., M. Taguchi and M. Otsuki (2005). "Synthetic protease inhibitor camostat prevents and reverses dyslipidemia, insulin secretory defects, and histological abnormalities of the pancreas in genetically obese and diabetic rats." Metabolism: Clinical and Experimental **54**(5): 619-627.
Kanneganti, T. D. and V. D. Dixit (2012). "Immunological complications of obesity." Nature Immunology **13**(8): 707-712.
Kaplan, G. G. (2015). "The global burden of IBD: From 2015 to 2025." Nature Reviews Gastroenterology and Hepatology **12**(12): 720-727.
Kennedy, A. R. (1998). "Chemopreventive agents: Protease inhibitors." Pharmacology and Therapeutics **78**(3): 167-209.
Kennedy, A. R., P. C. Billings, X. S. Wan and P. M. Newberne (2002). "Effects of Bowman-Birk inhibitor on rat colon carcinogenesis." Nutrition and Cancer **43**(2): 174-186.
Khera, R., A. Pandey, A. K. Chandar, M. H. Murad, L. J. Prokop, I. J. Neeland, J. D. Berry, M. Camilleri and S. Singh (2018). "Effects of Weight-Loss Medications on Cardiometabolic Risk Profiles: A Systematic Review and Network Meta-analysis." Gastroenterology **154**(5): 1309-1319.e1307.
Kim, S. H., S. Hara, S. Hase, T. Ikenaka, H. Toda, K. Kitamura and N. Kaizuma (1985). "Comparative study on amino acid sequences of kunitz-type soybean trypsin inhibitors, Tia, Tib, and Tic." Journal of Biochemistry **98**(2): 435-448.
Lajolo, F. M., M. I. Genovese, I. F. Pryme and M. Dale (2004). "Beneficial (antiproliferative) effects of different substances." Recent Advances of Research in Antinutritional Factors in Legume Seeds and Oilseeds(110): 123-135.
Lee, S. L., R. B. Dickson and C. Y. Lin (2000). "Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease." Journal of Biological Chemistry **275**(47): 36720-36725.
Li, Z., M. Maglione, W. Tu, W. Mojica, D. Arterburn, L. R. Shugarman, L. Hilton, M. Suttorp, V. Solomon, P. G. Shekelle and S. C. Morton (2005). "Meta-analysis: Pharmacologic treatment of obesity." Annals of Internal Medicine **142**(7): 532-546.

Lichtenstein, G. R., J. J. Deren, S. Katz, J. D. Lewis, A. R. Kennedy and J. H. Ware (2008). "Bowman-Birk inhibitor concentrate: A novel therapeutic agent for patients with active ulcerative colitis." Digestive Diseases and Sciences **53**(1): 175-180.
Liddle, R. A. (1995). "Regulation of cholecystokinin secretion by intraluminal releasing factors." American Journal of Physiology - Gastrointestinal and Liver Physiology **269**(3 32-3): G319-G327.
Lim, H., S. H. Lee, H. T. Lee, J. U. Lee, J. Y. Son, W. Shin and Y. S. Heo (2018). "Structural biology of the TNFα antagonists used in the treatment of rheumatoid arthritis." International Journal of Molecular Sciences **19**(3).
Liou, A. P., D. I. Chavez, E. Espero, S. Hao, S. A. Wank and H. E. Raybould (2011). "Protein hydrolysate-induced cholecystokinin secretion from enteroendocrine cells is indirectly mediated by the intestinal oligopeptide transporter PepT1." American Journal of Physiology - Gastrointestinal and Liver Physiology **300**(5): G895-G902.
Liu, C., F. Cheng, Z. Wan, Y. Zou, J. Wang, J. Guo and X. Yang (2016). "Fabrication and delivery properties of soy Kunitz trypsin inhibitor nanoparticles." RSC Advances **6**(88): 85621-85633.
Liu, C., F. Cheng and X. Yang (2017). "Fabrication of a Soybean Bowman-Birk Inhibitor (BBI) Nanodelivery Carrier To Improve Bioavailability of Curcumin." Journal of Agricultural and Food Chemistry **65**(11): 2426-2434.
Liu, K. S. (1997). "Trypsin inhibitors." Soybeans: Chem, Technol and Utilization: 48.
Lozano, I., R. Van Der Werf, W. Bietiger, E. Seyfritz, C. Peronet, M. Pinget, N. Jeandidier, E. Maillard, E. Marchioni, S. Sigrist and S. Dal (2016). "High-fructose and high-fat diet-induced disorders in rats: Impact on diabetes risk, hepatic and vascular complications." Nutrition and Metabolism **13**(1).
Macfarlane, A. J. and P. J. Stover (2007). "Convergence of Genetic, Nutritional and Inflammatory Factors in Gastrointestinal Cancers." Nutrition Reviews **65**(SUPPL.3): S157-S166.
Marín-Manzano, M. C., R. Ruiz, E. Jiménez, L. A. Rubio and A. Clemente (2009). "Anti-carcinogenic soyabean Bowman-Birk inhibitors survive faecal fermentation in their active form and do not affect the microbiota composition in vitro." British Journal of Nutrition **101**(7): 967-971.
Masaki, T., T. Kishiki, K. Kojima, N. Asou, A. Beniya and H. Matsuoka (2018). "Recent trends (2016-2017) in the treatment of inflammatory bowel disease." Annals of Gastroenterological Surgery **2**(4): 282-288.
Matheyambath, A., P. Padmanabhan and G. Paliyath (2016). Encyclopedia of food and health, Elsevier Press, Oxford, UK.
McLaughlin, C. L., S. R. Peikin and C. A. Baile (1983). "Trypsin inhibitor effects on food intake and weight gain in zucker rats." Physiology & Behavior **31**(4): 487-491.
Melo, F. J. and S. Magina (2018). "Clinical management of Anti-TNF-alpha-induced psoriasis or psoriasiform lesions in inflammatory bowel disease patients: a systematic review." International Journal of Dermatology **57**(12): 1521-1532.
Meyer-Hoffert, U., J. Wingertszahn and O. Wiedow (2004). "Human leukocyte elastase induces keratinocyte proliferation by epidermal growth factor receptor activation." Journal of Investigative Dermatology **123**(2): 338-345.
Mitoma, H., T. Horiuchi, H. Tsukamoto and N. Ueda (2018). "Molecular mechanisms of action of anti-TNF-α agents – Comparison among therapeutic TNF-α antagonists." Cytokine **101**: 56-63.

Miyasaka, K., M. Masuda and A. Funakoshi (1997). "Regulation of cholecystokinin release and transcription in a rat without gene expression of cholecystokinin-a receptor." Digestion **58**(2): 104-110.
Moreno, F. J. and A. Clemente (2008). "2S albumin storage proteins: What makes them food allergens?" Open Biochem J **2**: 16-28.
Motta, J. P., L. G. Bermúdez-Humarán, C. Deraison, L. Martin, C. Rolland, P. Rousset, J. Boue, G. Dietrich, K. Chapman, P. Kharrat, J. P. Vinel, L. Alric, E. Mas, J. M. Sallenave, P. Langella and N. Vergnolle (2012). "Food-grade bacteria expressing elafin protect against inflammation and restore colon homeostasis." Science Translational Medicine **4**(158).
Muller, M. K., H. Goebell, R. Alfen, J. Ehlers, M. Jager and H. Plumpe (1988). "Effects of camostat, a synthetic protease inhibitor, on endocrine and exocrine pancreas of the rat." Journal of Nutrition **118**(5): 645-650.
Nishi, T., H. Hara and F. Tomita (2003). "Soybean β-conglycinin peptone suppresses food intake and gastric emptying by increasing plasma cholecystokinin levels in rats." Journal of Nutrition **133**(2): 352-357.
Ohmuraya, M., M. Hirota, K. Araki, H. Baba and K. I. Yamamura (2006). "Enhanced trypsin activity in pancreatic acinar cells deficient for serine protease inhibitor Kazal type 3 [4]." Pancreas **33**(1): 104-106.
Ohmuraya, M., M. Hirota, M. Araki, N. Mizushima, M. Matsui, T. Mizumoto, K. Haruna, S. Kume, M. Takeya, M. Ogawa, K. Araki and K. I. Yamamura (2005). "Autophagic cell death of pancreatic acinar cells in serine protease inhibitor Kazal type 3-deficient mice." Gastroenterology **129**(2): 696-705.
Osman, M. A., P. M. Reid and C. W. Weber (2002). "Thermal inactivation of tepary bean (Phaseolus acutifolius), soybean and lima bean protease inhibitors: Effect of acidic and basic pH." Food Chemistry **78**(4): 419-423.
Pedrosa, M. M., C. Cuadrado, C. Burbano, K. Allaf, J. Haddad, E. Gelencsér, K. Takács, E. Guillamón and M. Muzquiz (2012). "Effect of instant controlled pressure drop on the oligosaccharides, inositol phosphates, trypsin inhibitors and lectins contents of different legumes." Food Chemistry **131**(3): 862-868.
Petros, W. P. and W. P. Peters (1996). "Cytokines." Therapeutic Immunology: 229-279.
Pusztai, A., S. Bardocz and M. A. Martin-Cabrejas (2004). "The mode of action of ANFs on the gastrointestinal tract and its microflora." Recent Advances of Research in Antinutritional Factors in Legume Seeds and Oilseeds: 87-100.
Pusztai, A., G. Gran, D. J. Grant and C. C. Stewart (1992). "Nutritional evaluation of the trypsin {EC 3.4.21.4) inhibitor from cowpea (Vigna unguiculata Walp.)." British Journal of Nutrition **68**(3): 783-791.
Raithel, M., S. Winterkamp, A. Pacurar, P. Ulrich, J. Hochberger and E. G. Hahn (2001). "Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease." Scandinavian Journal of Gastroenterology **36**(2): 174-179.
Ramasarma, P. R., A. G. Appu Rao and D. Rajagopal Rao (1995). "Role of disulfide linkages in structure and activity of proteinase inhibitor from horsegram (Dolichos biflorus)." Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular **1248**(1): 35-42.

Reddy, B. S. (2000). "Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention." Cancer Epidemiology Biomarkers and Prevention **9**(3): 239-247.
Rekha, M. R. and C. P. Sharma (2013). "Oral delivery of therapeutic protein/peptide for diabetes-Future perspectives." International Journal of Pharmaceutics **440**(1): 48-62.
Ribeiro, J. K., D. D. Cunha, J. M. Fook and M. P. Sales (2010). "New properties of the soybean trypsin inhibitor: Inhibition of human neutrophil elastase and its effect on acute pulmonary injury." Eur J Pharmacol **644**(1-3): 238-244.
Rogalski, C., U. Meyer-Hoffert, E. Proksch and O. Wiedow (2002). "Human leukocyte elastase induces keratinocyte proliferation in vitro and *in vivo*." Journal of Investigative Dermatology **118**(1): 49-54.
Rolland-Fourcade, C., A. Denadai-Souza, C. Cirillo, C. Lopez, J. O. Jaramillo, C. Desormeaux, N. Cenac, J. P. Motta, M. Larauche, Y. Taché, P. V. Berghe, M. Neunlist, E. Coron, S. Kirzin, G. Portier, D. Bonnet, L. Alric, S. Vanner, C. Deraison and N. Vergnolle (2017). "Epithelial expression and function of trypsin-3 in irritable bowel syndrome." Gut **66**(10): 1767-1778.
Roy, I. and M. N. Gupta (2000). "Current trends in affinity-based separations of proteins/enzymes." Current Science **78**(5): 587-591.
Rubio, L. A., M. M. Pedrosa, C. Cuadrado, E. Gelencser, A. Clemente, C. Burbano and M. Muzquiz (2006). "Recovery at the terminal ileum of some legume non-nutritional factors in cannulated pigs." Journal of the Science of Food and Agriculture **86**(6): 979-987.
Seiberg, M., J. C. Liu, L. Babiarz, E. Sharlow and S. Shapiro (2001). "Soymilk reduces hair growth and hair follicle dimensions." Experimental Dermatology **10**(6): 405-413.
Shah, A., N. Mehta and M. P. Reilly (2008). "Adipose inflammation, insulin resistance, and cardiovascular disease." Journal of Parenteral and Enteral Nutrition **32**(6): 638-644.
Siedle, B., A. Hrenn and I. Merfort (2007). "Natural Compounds as Inhibitors of Human Neutrophil Elastase." Planta Medica **53**(05): 401-420.
Song, H. K. and S. W. Suh (1998). "Kunitz-type soybean trypsin inhibitor revisited: Refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from Erythrina caffra and tissue-type plasminogen activator." Journal of Molecular Biology **275**(2): 347-363.
Struthers, B. J. and J. R. MacDonald (1983). "Comparative inhibition of trypsins from several species by soybean trypsin inhibitors." Journal of Nutrition **113**(4): 800-804.
Turk, B. (2006). "Targeting proteases: Successes, failures and future prospects." Nature Reviews Drug Discovery **5**(9): 785-799.
van der Poel, A. B., W. Stolp and D. J. Van Zuilichem (1992). "Twin-screw extrusion of two pea varieties: Effects of temperature and moisture level on antinutritional factors and protein dispersibility." Journal of the Science of Food and Agriculture **58**(1): 83-87.
Van Spaendonk, H., H. Ceuleers, L. Witters, E. Patteet, J. Joossens, K. Augustyns, A. M. Lambeir, I. De Meester, J. G. De Man and B. Y. De Winter (2017). "Regulation of intestinal permeability: The role of proteases." World Journal of Gastroenterology **23**(12): 2106-2123.
Vergnolle, N. (2016). "Protease inhibition as new therapeutic strategy for GI diseases." Gut **65**(7): 1215-1224.
Wati, R. K., T. Theppakorn, S. Benjakul and S. Rawdkuen (2009). "Three-phase partitioning of trypsin inhibitor from legume seeds." Process Biochemistry **44**(12): 1307-1314.

Weder, J. K. (1986). "Inhibition of human proteinases by grain legumes." Advances in experimental medicine and biology **199**: 239-279.
Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel and A. W. Ferrante Jr (2003). "Obesity is associated with macrophage accumulation in adipose tissue." Journal of Clinical Investigation **112**(12): 1796-1808.
Welham, T. and C. Domoney (2000). "Temporal and spatial activity of a promoter from a pea enzyme inhibitor gene and its exploitation for seed quality improvement." Plant Science **159**(2): 289-299.
Westfall, R. J. and S. M. Hauge (1948). "The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures." The Journal of nutrition **35**(3): 379-389.
Yanovski, S. Z. and J. A. Yanovski (2002). "Obesity." New England Journal of Medicine **346**(8): 591-602.
Yavelow, J., M. Collins, Y. Birk, W. Troll and A. R. Kennedy (1985). "Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress x-ray-induced transformation in vitro." Proceedings of the National Academy of Sciences of the United States of America **82**(16): 5395-5399.
Yavelow, J., T. H. Finlay, A. R. Kennedy and W. Troll (1983). "Bowman-Birk soybean protease inhibitor as an anticarcinogen." Cancer Research **43**(5 Suppl.): 2454-2459.
Zhang, Y., Y. Kouzuma, T. Miyaji and M. Yonekura (2008). "Purification, characterization, and cDNA cloning of a Bowman-Birk type trypsin inhibitor from Apios americana medikus tubers." Bioscience, Biotechnology and Biochemistry **72**(1): 171-178.