PRODUCTION, CHARACTERIZATION AND EVALUATION OF ANTI-DIABETES PEPTIDES FROM PROTEINS OF IMPROVED COMMON BEAN (*Phaseolus vulgaris* L.) CULTIVARS USING BIOINFORMATIC TOOLS, ENZYMATIC SYSTEMS, AND *in vitro* AND *in vivo* MODELS

BY

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DISSERTATION

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ABSTRACT

Diabetes is an important metabolic disease expected to increase worldwide 55% by 2035; with an estimated healthcare cost of $245 billion per year in the US alone. The use of alternative plant based treatments has the advantage of no side effects and could reduce treatment cost. Common beans health benefits are attributed to bioactive compounds; including resistant starch and oligosaccharides that contribute to dietary fiber; phenolic compounds and bioactive peptides released from proteins during enzymatic digestion. Biological potential of bean peptides has been related to antihypertensive, anticancer, anti-inflammatory, antioxidant and antifungal; however, the anti-diabetes potential of common bean protein fractions and pure peptides has not been studied. In that sense, the central hypothesis of this research was that common bean protein hydrolyzed fraction contains bioactive peptides that could modulate molecular targets of type 2 diabetes. To fill the gap in the knowledge, our long-term goal was to understand the mechanism by which common bean peptides contribute to the management of type 2 diabetes. To achieve this aim, we generated anti-diabetes peptides from common bean protein and determined their efficacy using bioinformatics tools, enzymatic systems, *in vitro* and *in vivo* models.

Our research started with the selection and characterization of proteins, peptide profile, protein inhibitors, α-amylase inhibitors, phenolic compounds and anthocyanins of improved common bean cultivars from Mexico and Brazil (Aim 1). The peptides and phenolic compounds found presented outstanding anti-diabetes potential from the biochemical and *in silico* studies. Furthermore, we evaluated the peptides anti-diabetes potential after simulated gastrointestinal digestion and characterized their bioactive peptides, angiotensin converting enzyme, dipeptidyl peptidase IV, and α-glucosidase inhibition (Aim 2). Negro Otomi cultivar presented the lowest IC_{50} to inhibit DPP-IV (0.14±0.01 mg DW/ mL). The effect of processing was evaluated in Aim 3, where the goal was to determine the bioactive properties of the released peptides from commercially available precooked common beans (*Phaseolus vulgaris*). Bioactive properties and peptide profiles were evaluated in protein hydrolysates of raw and commercially precooked common beans. Hydrolysates from Navy beans were the most potent inhibitors of DPP-IV with no statistical differences between precooked and raw (IC_{50} = 0.093 and 0.095 mg protein/mL, respectively). In general, we observed that processing did not affect the bioactive properties of released peptides from precooked beans. With the objective to increase the anti-diabetes potential of common bean protein fractions, the fourth Aim was to optimize the production of

anti-diabetes protein fractions from black bean (*Phaseolus vulgaris* L.) protein isolates. The results suggested that the best combination of conditions to generate anti-diabetes peptides were with alcalase for two h and E/S of 1:20, with high inhibitions values for dipeptidyl peptidase IV (DPP-IV, 96.7%), α-amylase (53.4%) and α-glucosidase (66.1%). The protein fractions were characterized using LC-ESI-MS/MS and 33 sequences were identified. Finally, we evaluated the anti-diabetes mechanism of action of common bean protein fractions and pure peptides using *in silico, in vitro,* and *in vivo m*odels. Sequenced peptides showed outstanding potential to bind and blocking intestinal glucose transporters SGLT1 and GLUT2. Besides, using Caco-2 cells monolayer insert we observed that protein fractions reduce glucose absorption by 6.5 and 24.5 after 30 min and 24 h, respectively. It could be due to protein fractions were also able to decrease protein expression and translocation to the membrane of GLUT2 and SGLT1.

Furthermore, protein fractions were able to lower postprandial glucose by 24.5% in an oral glucose tolerance test in rats after receiving 50 mg/kg BW compared to untreated control. Also, a hyperglycemic rat model showed that protein fractions at 100 mg of PF/ Kg BW twice daily significantly reduced postprandial glucose levels by 48% compared to the non-treated diabetic group, without having consequences in body weight or other biochemical parameters. In conclusion pure peptides and protein fraction showed remarkable potential to decrease translocation of glucose transporters to the membrane and as a consequence reduction of glucose absorption.

Dedicated to my parents Margarita Contreras and Luis Mojica, my siblings Jose Roberto, Rosa Angelica, Luz Elena and Juan Carlos and all my friends, without their unconditional love and support this would not be possible.

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CHAPTER 1: INTRODUCTION

1.1 Overview

Diabetes is a metabolic disease affecting more than 24 million Americans in 2013 with an estimated healthcare cost of $245 billion per year [1]. Diabetes is a chronic noncommunicable disease and a multifactorial disorder characterized by the inability of the body to produce insulin (type 1 diabetes) or by defects in insulin secretion and action (type 2 diabetes) [1]. Type 2 diabetes accounts for 90% of the cases in adults. Impaired insulin synthesis and secretion in type-2 diabetes results from beta-cell dysfunction and reduced beta-cell mass. Uncontrolled hyperglycemia results in macrovascular and microvascular complications, including coronary artery disease and peripheral arterial disease as well as diabetic nephropathy, neuropathy, and retinopathy [1,2].

The Diabetes treatment goal is to keep glucose levels in a physiological range; treatment consists in diet changes, weight loss, exercise and the use of pharmacological drugs to improve glucose homeostasis [1]. There are eleven groups of drugs approved for the management of diabetes [1,3]. However, the use of drugs presents side effects such as hypoglycemia, weight gain, and gastrointestinal problems. On the other hand, consumption of medicinal plants, herbs, and other foods can have a positive impact on glucose regulation as a preventive mechanism [3]. For instance, soluble fiber, phenolic compounds, and peptides, from common beans and other food products have been shown to display various antihyperglycemic properties, such as inhibitory activity against digestive enzymes, insulin secretagogue effects, and activation of receptors involved in glucose metabolism [4-7].

The potential use of common beans as a nutraceutical food is well recognized in the literature [8]. Its health benefits are attributed to bioactive compounds; these compounds include

resistant starch and oligosaccharides that contribute to dietary fiber; phenolic compounds and bioactive peptides released from proteins during enzymatic digestion (Figure 1) [8-11]. Bioactive peptides from common bean have the potential to inhibit enzymes known to be molecular targets of diabetes, for instance, α-amylase, α-glucosidase and dipeptidyl peptidase IV (DPP-IV) and exert high antioxidant activity [12-14]. Peptides can block enzyme functions by interacting with amino acids in their catalytic site of certain enzymes, to inhibit their normal metabolic function. Our long-term goal was to evaluate the potential of common bean (*Phaseolus vulgaris* L.) protein fractions to modulate markers of diabetes. We hypothesized that common bean protein fractions can inhibit DPP-IV; its inhibition will increase the concentration of active glucagon like peptide-1 (GLP-1), starch degradating enzymes such as α-amylase and α-glucosidase; blocking glucose transporters, including sodium glucose linked transporter-1 (SGLT1) and glucose transporter 2 (GLUT2) to decrease glucose absorption.

The overall objective was to produce anti-diabetic peptides from common bean proteins and to determine their efficacy using bioinformatic tools, enzymatic systems and *in vitro* and *in vivo* models. Table 1 presents a summary of this research aims and outcomes.

Aim 1 was to determine the protein and peptide profile, Bowman-Birk inhibitors, Kunitz inhibitors, lectins, α-amylase inhibitors, phenolic and anthocyanin content of improved common bean cultivars from Mexico and Brazil. We produced and characterized common bean protein fractions from 15 common bean cultivars that were improved in their agronomical characteristics at research centers in Mexico and Brazil. We evaluated the potential biological function associated with the generated peptides, having anti-diabetes potential bioactivity. Results indicated that common bean from Flor de Mayo and Junio bean cultivars are an excellent source of proteins with a moderate concentration of anti-nutrient plant defense proteins. Peptides from common bean proteins presented potential biological activities related to control of type-2

diabetes and hypertension. Moreover, the phenolic compounds in common bean coats have a high antioxidant capacity and can inhibit enzymes related to starch digestion. It is important because these components in the coats also can contribute to decreasing oxidative stress and reduce hyperglycemia in a patient with diabetes by blocking starch degrading enzymes. Black beans contained delphinidin and ferulic acid.

Aim 2 of this research was to characterize bioactive peptides after simulated gastrointestinal digestion of bean proteins and evaluate the potential to inhibit angiotensin converting enzyme (ACE), DPP-IV, and α-glucosidase. Results showed that bean protein fractions and in particular peptides KKSSG, CPGNK, GGGLHK, and KTYGL have high potential to inhibit DPP-IV, ACE and α-glucosidase. The cultivar Negro Otomi and the peptide KTYGL were the ones that showed the greatest biological potential. Common bean protein isolate after simulated gastrointestinal digestion and their pure peptides demonstrated significant antioxidant, anti-diabetes and antihypertensive potential properties. Bean peptides were able to interact with amino acid residues in the catalytic site of the tested enzymes (Figure 2).

Aim 3 of this research was to determine the impact of precooking of commercially available common beans and compare the bioactive properties of the released peptides. Results demonstrated that the processing of commercially available fast cooking common beans does not affect their nutraceuticals properties negatively; on the other hand, it improved the digestibility by partial hydrolysis of proteins and increased their antioxidant capacity. This is important because processing conditions (temperature and pressure) denaturized proteins and lead to a higher degree of hydrolysis and improved protein digestibility. Processing did not affect bioactive properties of common bean protein hydrolysates such as ACE inhibition, DPP-IV inhibition, antioxidant capacity, α-amylase inhibition and α-glucosidase inhibition. The

consumption of fast cooking common beans has the advantage of reducing cooking time without affecting bioactive properties.

Aim 4 of this research was to optimize the conditions needed to generate anti-diabetic peptides from common bean proteins using commercially available enzymes and evaluate their effectiveness using biochemical and *in silico* approaches. Our most significant findings were that the common bean fractions generated with alcalase presented the highest anti-diabetes potential. From the response surface analysis output, those fractions showed the greatest inhibition potential for DPP-IV after two h of hydrolysis and E/S ratio of 1:20. In *in silico* studies of sequenced peptides showed that bean peptides have high potential anti-diabetes and antihypertensive properties. Common bean protein hydrolysates had the potential to inhibit molecular target enzymes in the management of type-2 diabetes.

The aim 5 was to optimize the extraction conditions of anthocyanins from common bean cultivar Negro Otomi to evaluate the thermal stability, color reaction kinetics, shelf life of anthocyanins under different pH and temperatures. Results showed that co-product common bean coats contain a high concentration of phenolic compounds, especially anthocyanins that can mimic commercially available colors used in beverages. These results open the possibility of using of common bean co-products generated during the protein extraction. Moreover, the anthocyanin-rich extracts exerted high antioxidant capacity as well as modulated markers of diabetes and decreased glucose uptake.

Aim 6 was to evaluate the mechanism of the anti-diabetic action of common bean bioactive peptides using *in vitro* and *in vivo* models. To test this hypothesis, we used Caco-2 colorectal cells as a model of gastrointestinal absorption, cells were first treated with different concentrations of protein fractions, as well as their pure components (synthetic peptides) and their hypoglycemic potential and other diabetic related markers were evaluated. In Caco-2 cells

we assessed DPP-IV activity, active GLP-1, and glucose absorption to have a first approach to the mechanism of action; the expression of glucose transporters GLUT2 and SGLT1 by Western blot and their localization in the cells using confocal microscopy was quantified. The hypoglycemic effect of the peptide fractions using *in vivo* oral glucose tolerance test and the anti-diabetes properties of the bean protein fractions using a streptozotocin-induced diabetic Wistar rat model were evaluated (Figure 3). Results from *in vitro* and *in vivo* models generated information needed to elucidate the role and mechanism of action of bioactive peptides on glucose homeostasis. For the *in vivo* studies, the oral glucose tolerance test demonstrated the hypoglycemic potential of common bean peptides. On the other hand, the hyperglycemic model was administered with three oral doses of bean protein fractions per day during ten days. Glucose homeostasis parameters: fasting glucose levels; insulin release related markers: DPP-IV activity, insulin and active GLP-1; lipid metabolism parameters: triglycerides, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL); liver function enzymes: alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP); and kidney function markers: urine volume and proteinuria. All these markers provided enough information to understand the anti-diabetes mechanism of action of common bean bioactive peptides. Figure 4 presents a summary of this research. The results suggested that common bean protein hydrolysates, and their peptides, have the potential to inhibit diabetes-related enzymes, decrease glucose absorption, modulate expression and translocation of glucose transporters and reduce oxidative stress. One of the principal mechanisms of action was modulating GLUT2 and SGLT1 protein expression and translocation to the cell membrane. Figure 5 presents a graphical experimental design of this research.

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1.3 Tables and figures

Table 1. Research aims summary and outcomes.

Type of Study	Aim	Publication
Characterization of bioactive components in new improved cultivars of common beans	***Aim 1***: Determine the protein and peptide profile, Bowman-Birk inhibitors, Kunitz inhibitors, lectins, α-amylase inhibitors, phenolic and anthocyanin concentration of improved common bean cultivars from Mexico and Brazil	**Mojica** L, Meyer A, Berhow MA, de Mejía EG (2015). Bean cultivars (*Phaseolus vulgaris L.)* have similar high antioxidant capacity, *in vitro* inhibition of α-amylase and α-glucosidase while diverse phenolic composition and concentration. *Food Res Int*. 69, 38–48. **Mojica L**, de Mejía EG (2015). Characterization and comparison of protein and peptide profiles and their biological activities of improved common bean cultivars (*Phaseolus vulgaris* L.) from Mexico and Brazil. *Plant Foods for Human Nutrition*. 70, 105–112.
Evaluate the effect of gastrointestinal digestion on peptides released and their anti-diabetes potential	***Aim 2:*** Characterize bioactive peptides after simulated gastrointestinal digestion of bean proteins and evaluate the potential to inhibit angiotensin converting enzyme (ACE), DPP-IV, and α-glucosidase	**Mojica L,** Luna-Vital DA, de Mejia EG (2016). Characterization of peptides from common bean protein isolates and their potential to inhibit markers of type-2 diabetes, hypertension and oxidative stress. (In Press *Journal of the Science of Food and Agriculture*-16-0187).
Evaluate the nutraceutical properties of peptides from precooked beans	***Aim 3:*** Determine the impact of precooking of commercially available common beans and compare the bioactive properties of the released peptides	**Mojica, L**., Chen, K., de Mejia, EG (2014). Impact of commercial precooking of common bean (*Phaseolus vulgaris*) on the generation of peptides, after pepsin–pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase-IV. *Journal of Food Science*. 80, 1:188-198.
Optimization of generation of anti-diabetes peptides using different proteases and computational tools	***Aim 4:*** *O*ptimize the conditions needed to generate anti-diabetes peptides from common bean proteins using commercial enzymes and evaluate their effectiveness using biochemical and *in silico* approaches	**Mojica L**, de Mejía EG (2016). Optimization of enzymatic production of anti-diabetes peptides from black bean (*Phaseolus vulgaris* L.) proteins and their characterization and biological potential. *Food & Function***,** 7**,** 713-727.
Optimization of extraction of anthocyanins from black bean coats and evaluate their stability and biological potential by biochemical and *in vitro* assays	***Aim 5***: Optimize the extraction conditions of anthocyanins from Negro Otomi and evaluate the stability in a food matrix condition, as well as the anti-diabetes potential	**Mojica** L, Tan A, Berhow MA, de Mejía EG (2016). Optimization of extraction, shelf-life and anti-diabetes potential of anthocyanin-rich extract from black bean (*Phaseolus vulgaris* L.) seed coats (*to be submitted*).
Evaluate the mechanism of the anti-diabetes action of common bean bioactive peptides using *in vitro* and *in vivo* models	***Aim 6***: Evaluate the mechanism of the anti-diabetes action of common bean bioactive peptide fractions and pure peptides using *in vitro* and *in vivo* models.	**Mojica L**, de Mejía EG., Granados-Silvestre A, Menjivar M (2016) Anti-diabetes black bean peptides reduce glucose absorption through interaction with glucose transporters: in *in silico*, *in vitro* and *in vivo* models*(to be submitted).*

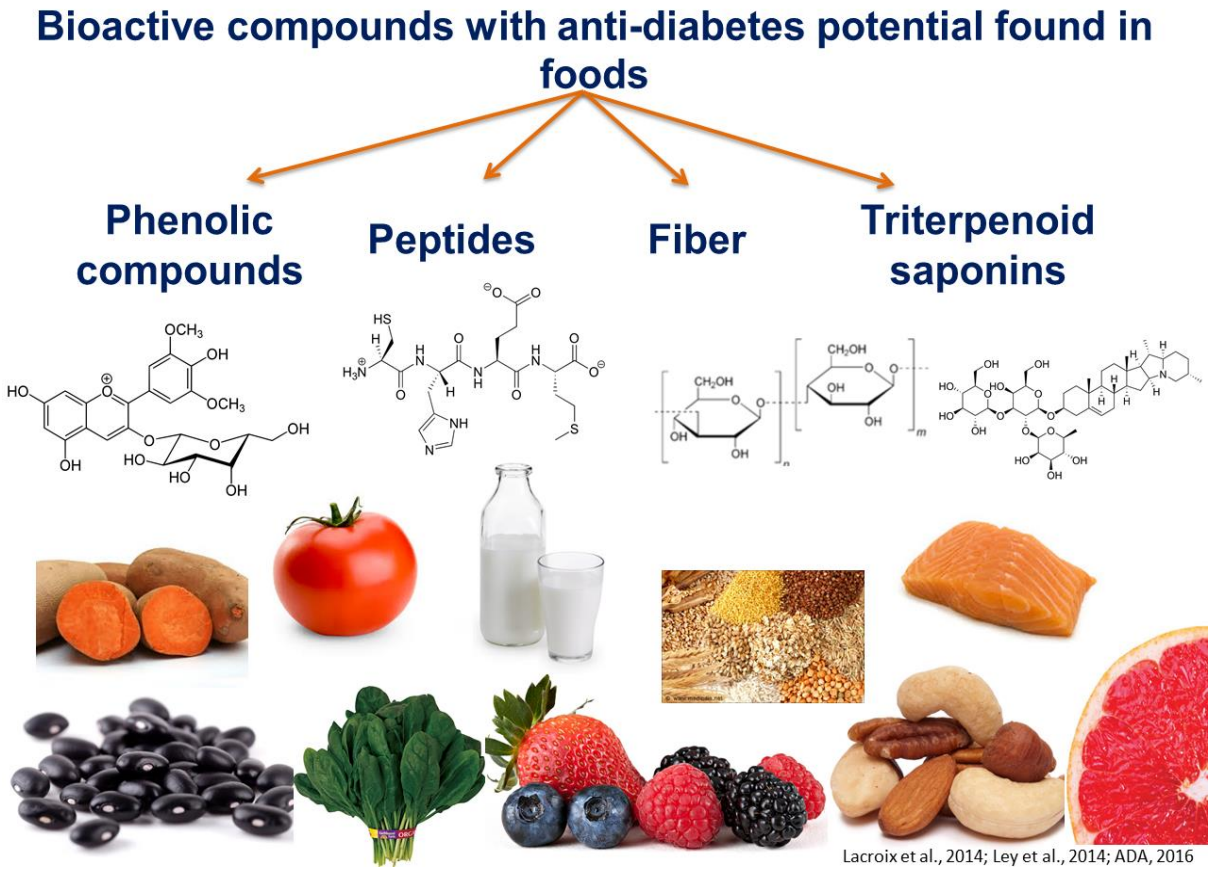

Figure 1. Dietary bioactive components with anti-diabetes potential in foods.

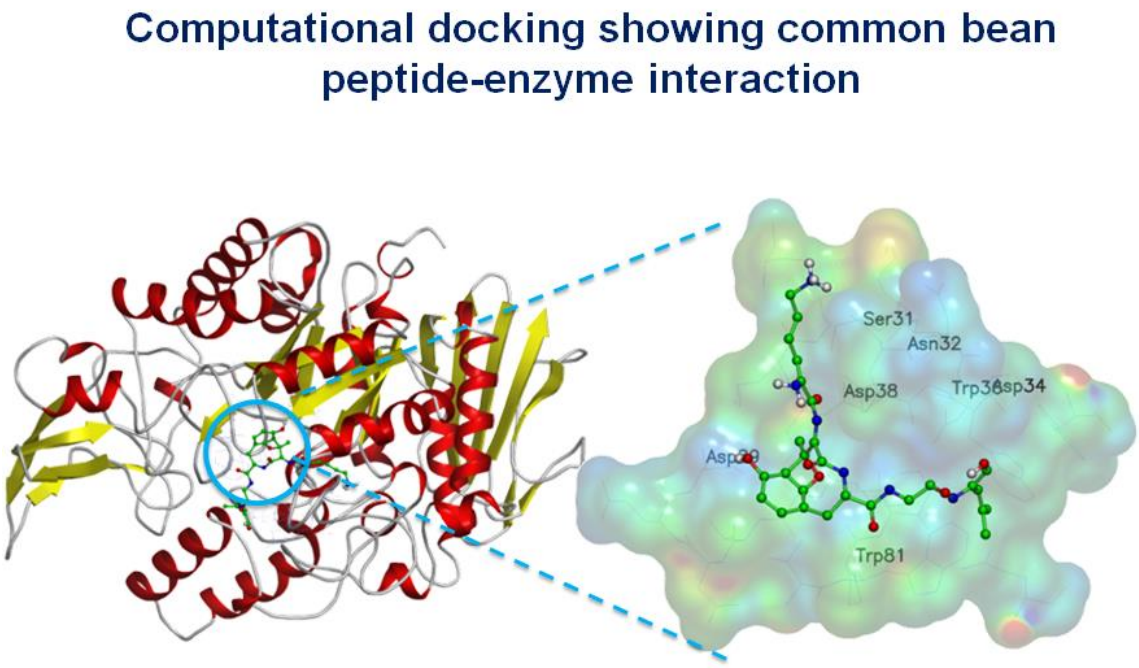

Figure 2. Enzyme inhibitor interaction using computational modeling.

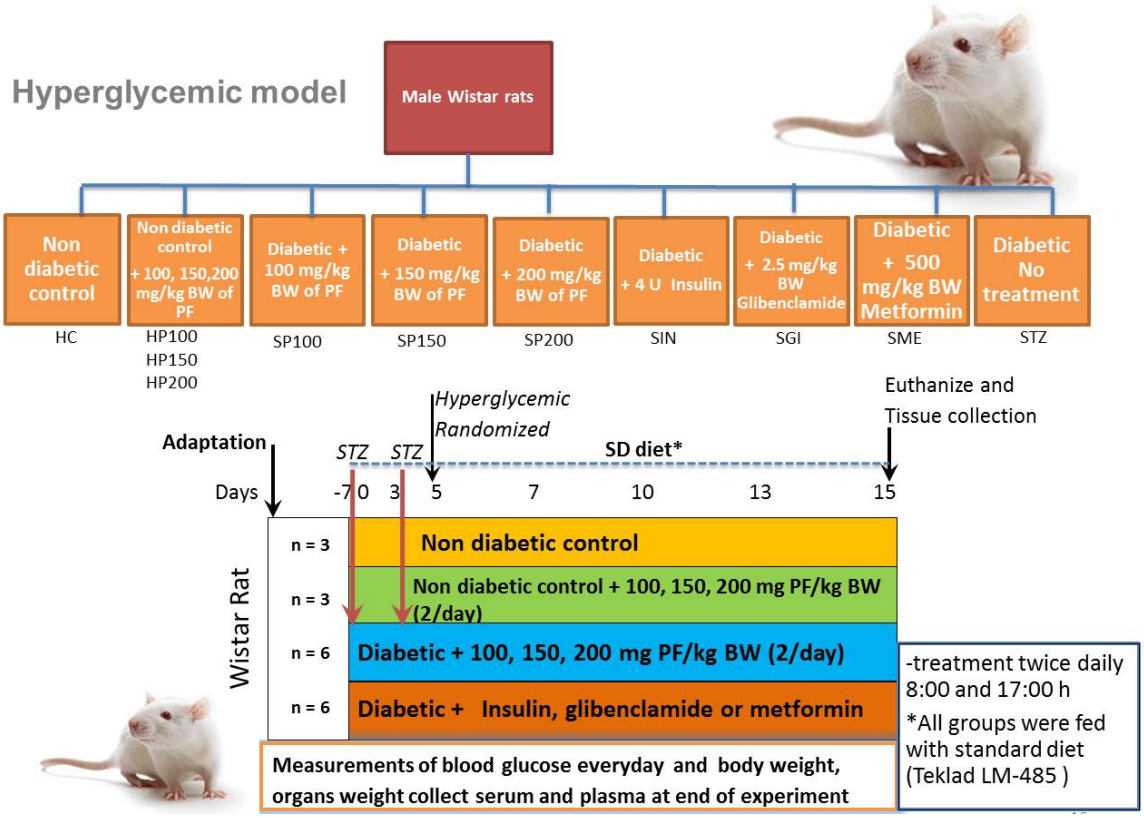

Figure 3. Experimental design to evaluate the hypoglycemic potential of black bean protein fractions.

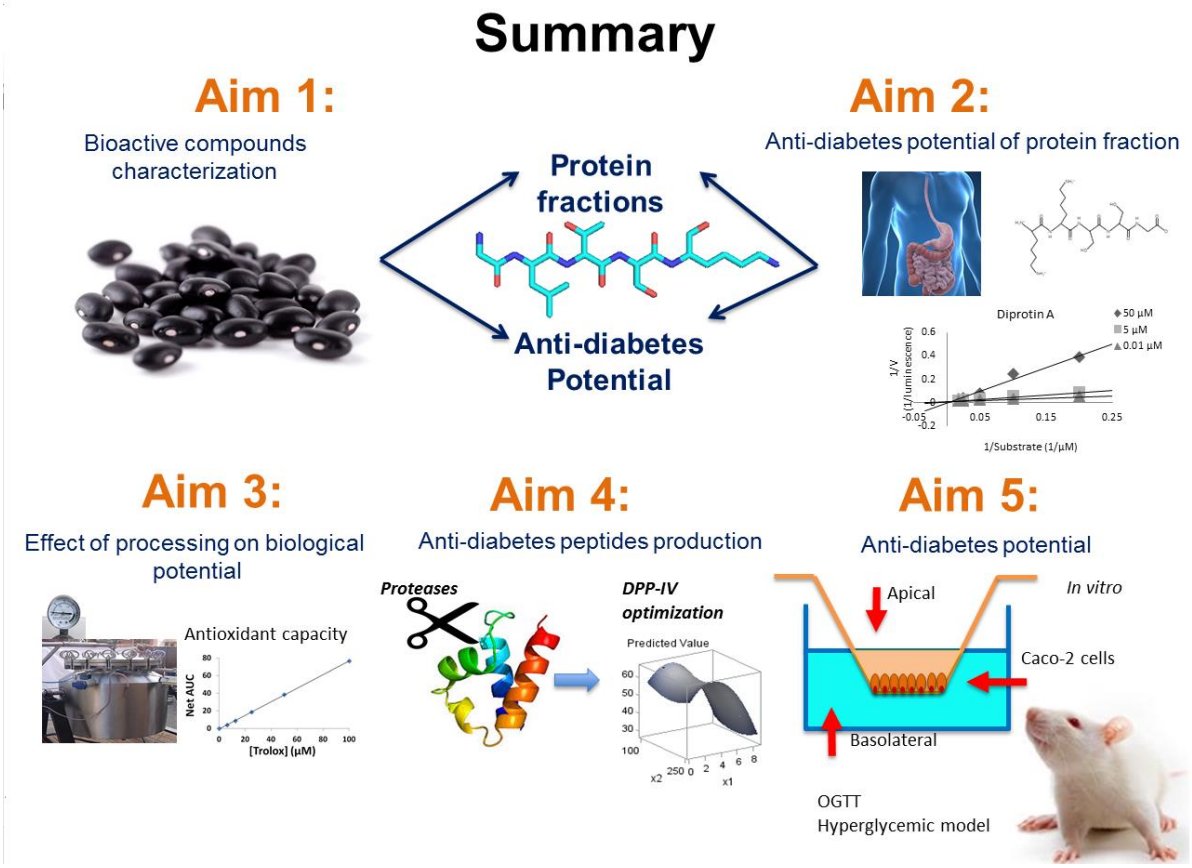

Figure 4. Research summary to produce, characterize and evaluate common bean protein fractions and synthetized peptides on type 2 diabetes markers.

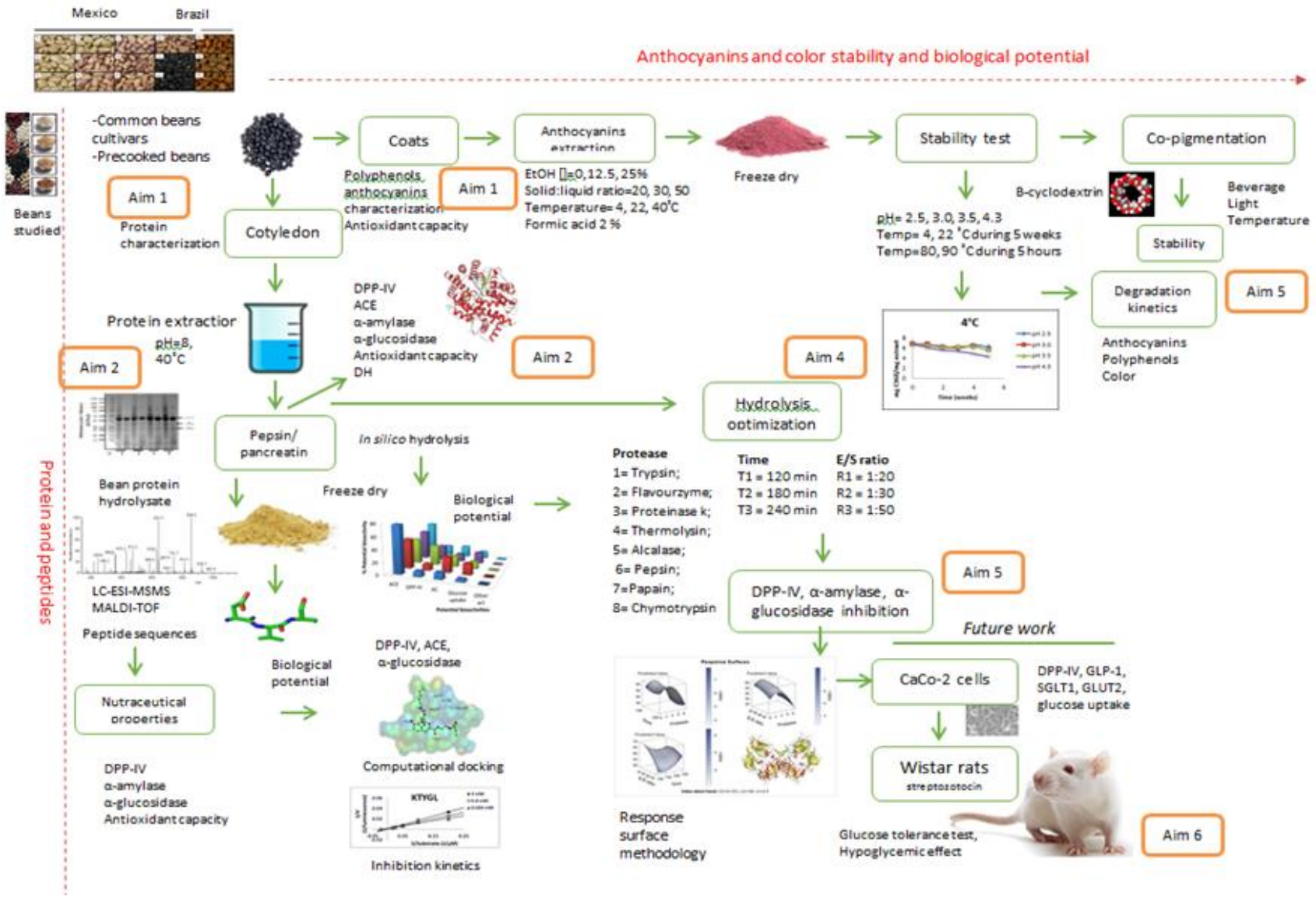

Figure 5. Summary of research experimental design.

CHAPTER 2: LITERATURE REVIEW

2.1 Diabetes and common bean peptides

2.1.1 Diabetes

Diabetes is a chronic noncommunicative disease and multifactorial disorder characterized by the inability of the body to produce insulin (type 1 diabetes) or by defects in insulin secretion and action (type 2 diabetes) [1-3]. Type 2 diabetes accounts for 90% of the cases in adults. Impaired insulin synthesis and secretion in type-2 diabetes results from β-cell dysfunction and reduction of β-cell mass. Uncontrolled hyperglycemia results in macrovascular and microvascular complications, including coronary artery disease and peripheral arterial disease as well as diabetic nephropathy, neuropathy, and retinopathy [1-3].

According to the Standard Medical Care in Diabetes (ADA, 2016) [1], the recommendations for primary care providers include healthy eating, weight control, increased physical activity, diabetes education and the use of pharmacological drugs to improve glucose homeostasis. The recommended drugs include metformin, sulfonylureas, thiazolidinediones, glucagon-like peptide (GLP)-1 receptor agonists, dipeptidyl peptidase (DPP)-IV inhibitors, sodium glucose cotransporter 2 (SGLT2) inhibitors and insulin [1]. Besides, other anti-diabetes drugs can be used, such as meglitinides, α-glucosidase inhibitors, amylin analogs, dopamine D2-receptor agonists, bile acid sequestrates, and insulin analogs [4]. However, the use of drugs presents side effects such as hypoglycemia, weight gain, and gastrointestinal problems. On the other hand, consumption of medicinal plants, herbs, and other foods can have a positive impact on glucose regulation. For instance, soluble fiber, phenolic compounds, and peptides, from common beans and other food products have shown to display various anti-hyperglycemic properties, such as inhibitory activities against digestive enzymes, insulin secretagogue effects, and activation of receptors involved in glucose metabolism [4].

2.1.2 Common bean

Common bean (*Phaseolus vulgaris* L.) is one of the most consumed legumes worldwide. It has been considered as a nutraceutical food due to its content of bioactive compounds such as polyphenols, resistant starch, oligosaccharides, non-digestible fraction and bioactive peptides [5-7]. Common bean consumption is associated with a significant reduction of the risk of non-

transmissible diseases. Common bean included as part of a healthy diet, can reduce obesity levels, to modulate cardiovascular processes, and it has also been recognized as a chemo-preventive agent mainly against colorectal cancer *in vitro* and *in vivo* [8]. Besides, common bean has been reported to ameliorate type 2 diabetes complications [9,10].

2.1.3 Common bean proteins

Common bean protein concentration ranges from 16 to 33% [8]. Proteins in beans can be classified based on their function: storage protein, carbohydrate metabolism, defense, stress response, detoxification, growth and development, protein transport and nitrogen metabolism [8]. Essential proteins in common beans are phaseolin, lectin, α-amylase inhibitor and Bowman–Birk inhibitor. Phaseolin represents 30–50% of total protein in common beans. Present secondary structure conformation consists of 10% α-helix, 9% β-turns, 48% β-sheet and 33% random coils. High amounts of β-sheet structures may limit the access of proteases. Phaseolin is mainly a glycosylated protein and consists of three polypeptide subunits α, β, and γ. Its nutritional value is limited by the low amount of methionine and cysteine and high resistance to proteolysis [11]. Lectins, α-amylase inhibitors and arcelins in plants are known to act in the defense mechanism of biotic stress and response to abiotic stress like drought, salt and wounding [12,13]. Breeding modifications explore to enhance the defense mechanism of crops to make them resistant to insect attack [13]. Protease inhibitors, Kunitz trypsin inhibitors, and Bowman–Birk inhibitors are defense related proteins commonly found in legumes. Trypsin inhibitors can inhibit the proteolytic activity of digestive enzymes. These enzymes are known to act in response to drought stress, salt stress, and wounding; its function is also to protect plants from pathogenic attacks [13]. However, lectins and protease inhibitors are thermosensitive; therefore, they are inactivated with a proper cooking process

2.1.4 Phenolic compounds in beans

Phenolic compounds have at least one aromatic ring with one or more hydroxyl groups attached and can be classified as phenolic acids, flavones, flavanones, isoflavones, flavonols, anthocyanins and condensed tannins [14]. They are products of plant secondary metabolism and are mediators of plant stress including insect and microbial defense [15]. Phenolics in common beans are found in the seed coats with lower amounts in the cotyledons. The concentration of these compounds is influenced by growing conditions and genetic factors. Tannins and anthocyanins determine the color of the seed coat; the darker the coat the higher the

concentration of these compounds [15]. Anthocyanins constitute one of the major groups of natural pigments and are responsible for many of the colors in fruits and vegetables. Anthocyanins are polyhydroxy and polymethoxy derivatives of flavilium salts are members of the flavonoid family, possessing a characteristic C3–C6–C3 carbon structure [16]. They are generally present in the plant tissues in a glycosylated form. Anthocyanins may provide anti-inflammatory and anti-diabetes benefits since they inhibit pro-inflammatory cytokines, decrease their production and prevent β-cell dysfunction leading to insulin output [17].

In general, phenolic compounds found in common beans have been reported to possess antioxidant, anticarcinogenic, antimutagenic and anti-inflammatory effects. These secondary metabolites are part of the defense response of plants, and their concentration can be affected by breeding modifications performed improving the nutritional value and agronomic characteristics of cultivars [16].

2.1.5 Bioactive peptides generation

Peptides are short-chain amino acid sequences produced from proteins they hold potential physiological functions when consumed, beyond their nutritional value [8,18]. Bioactive peptides are short sequences of approximately 2–20 amino acids that show physiological benefits when consumed. They are inactive within the protein sequence and are released during protein hydrolysis. Bioactive peptides could express their function in the intestinal tract or inside the body after being absorbed. The research on common bean peptide bioactivities not only focuses on human health benefits such as antihypertensive, antioxidant and anticancer potentials among others [8].

Proteases such as alcalase, flavourzyme, pepsin, pancreatin, chymotrypsin, papain, trypsin, and thermolysin have been used to produce bioactive peptides from common bean proteins [19]. Processing variations as the relationship of enzyme/substrate, protein pretreatment, time of hydrolysis and enzyme combination play a major role in the bioactivity of generated peptides. Several factors direct influence of peptide generation from common bean protein. Such as characteristics of the enzymes used in the production of bioactive peptides, and hydrolysis conditions. Another factor is enzyme specificity; for instance, alcalase, which is an endoprotease, presents a higher degree of hydrolysis (DH). Another model used in the generation of bioactive peptides from common bean protein is the gastrointestinal simulation using pepsin–pancreatin, which is a combination of several gastric enzymes including proteases; it has a broad

specificity on the protein sequence to generate peptides [8,19,20]. Similarly to gastrointestinal digestion, a synergistic effect has been observed with the combination of different enzymes.

Enzymes with different specificities as alcalase and flavourzyme significantly increased the DH when compared with digestion of the single enzymes. Betancur-Ancona et al. [21], suggested that the use of proteases with broad enzymatic action will release small peptides and thus increase the number of terminal amino acid residues that can be cleaved by exoproteases. Hydrolysis time also plays a major role in the generation of bioactive peptides. The use of diverse varieties of common beans with different concentration of protein has shown to influence peptide generation. The resistance to hydrolysis of phaseolin is highly variable across different cultivars [22]. Moreover, the DH of common bean proteins can be increased by a heat pre-treatment. The application of heat causes the partial denaturation, promoting the exposure of hydrophobic residues of globular structure proteins, such as phaseolin [22], and increasing their susceptibility to hydrolysis. Taking in consideration the mentioned conditions would help to improve the process by reducing operation costs and optimizing times in order to obtain high DH values that will be translated in a higher concentration of biologically efficient peptides.

2.1.6 Metabolism of peptides

Peptides are generated from dietary proteins during normal digestion or may be consumed as a processed protein fraction extract. Their bioavailability after oral administration depends on their capacity to resist further digestive hydrolysis and be absorbed to reach the target organ [23]. Small intestine epithelial cells are the primary site of absorption of nutrients such as glucose and amino acids. Most oligopeptides are extracellularly hydrolyzed by enzymes in the brush border membrane of the intestinal epithelium, generating amino acids that can be absorbed in the intestinal mucosa. However, some peptides can resist enzymatic attack due to their amino acid composition. Peptides can be transported through the intestinal epithelium by a particular carrier via paracellular transport, transcellular or transcytotic routes [24]. Smaller peptides, di- and tripeptides can be absorbed by H^+ coupled oligopeptide transporters using a transmembrane electrochemical proton gradient as a broad-specificity transport force. The proton-coupled oligopeptide transporter SLC15 family consists of four distinct isoforms: peptide transporters 1 and 2 (PEPT1/ SLC15A1 and PEPT2/SLC15A2) and peptide-histidine transporters 1 and 2 (PHT1/SLCA4 and PHT2 SLCA3). Paracellular transport for oligopeptides is an aqueous pathway involving diffusion of the peptide in the extracellular space between adjacent cells

which is restricted by tight junctions of the cells [25]. Bigger peptides are internalized primarily mediated by clathrin, caveolae and macropinocytosis endocytic pathways at the cellular membrane [23,26].

2.1.7 Glucose metabolism

An increase of blood glucose levels after food consumption activates the synthesis and release of insulin from the pancreatic β-cells. Insulin goes to systemic circulation binds to receptors in the liver, skeletal muscles, and adipose tissues. This process starts a series of reactions such as suppression of glucose production from the liver, inhibition of lipolysis, and stimulation of glucose uptake from muscle and adipose tissues, However in diabetic patients the body is unable to utilize properly carbohydrates. Leading to a hyperglycemic status, which is caused by insulin resistance that implies the incapacity of the liver, skeletal muscles, and adipose tissues to respond to insulin stimuli and impaired insulin secretion by pancreatic β-cells [27].

2.1.8 Regulation of sugar absorption during and between meals

After free glucose concentration increases, the absorption in the apical membrane occurs through SGLT1, causing activation of protein kinase CII (PKC II). Leading to activation ($t_{1/2}$ 3.5 min) of apical GLUT2, already in the membrane, and translocation of GLUT2 to the apical membrane from intracellular vesicles; this is the primary absorption channel. After glucose concentration decreases in the lumen, the process is reversed. In addition to glucose stimuli, GLUT2 translocation is also regulated by local and endocrine hormones, cellular energy status, stress, and diabetes; regulation occurs through a network of intracellular signaling pathways [28-30]. Cytosolic stores of GLUT2 have been shown to transport reversibly from cytosolic vesicles to the apical membrane to increase the glucose absorptive capacity of the enterocyte. SGLT1 transports simultaneously glucose and Na^+ into the enterocyte in a ratio of 1:2. This co-transport of Na+ into the cell causes depolarization which activates the voltage-gated, L-type calcium channel Cav1.3 promulgating an influx of Ca^{2+}. This increase in intracellular Ca2+ results in remodeling of the terminal web and cytoskeletal structures necessary for the trafficking of GLUT2 to the apical membrane [29-31]. The increased protein kinase C (PKC) activity also seems to affect GLUT2 translocation. In addition, a heterodimer protein composed of the heterodimeric sweet receptor T1R2+T1R3 proteins coupled to gustducin, a heterotrimeric G-protein consisting of a, b, and g subunits known as taste receptors. The taste receptors are located on apical section of enterocytes and enteroendocrine cells and respond to high glucose

concentrations. The adaptive response the enterocyte goes beyond GLUT2 and SGLT1 glucose transporters. Also, the PepT1 is translocated to the apical membrane similarly to GLUT2; however, PepT1 levels in the apical membrane appear to decrease when GLUT2 is increased and to increase when GLUT2 is reduced [31]. Plant materials such as polyphenols have shown attenuation of intestinal glucose absorption in *vitro* by inhibition of active uptake through SGLT1 and facilitated transport by GLUT2. Those intracellular storage vesicles containing SGLT1 are associated with microtubules involved in intracellular vesicular trafficking. The intracellular SGLT1 pool is mobile and part of a regulatory mechanism [32].

2.1.9 Glucagon-like peptide 1

Food stimulates GLP-1 secretion from enteroendocrine L cells in the gut. GLP-1 first release occurs after 10–15 min after oral ingestion mediated by neurotransmitters and the vagus nerve and potentially L cells in the proximal intestine and the second release occurs after 30–60 min by the stimulation of L-cells located in the distal region of the small intestine. GLP-1 secretion involves a glucose-sensing signal including glucokinase, ATP-dependent potassium channels, and sodium glucose cotransporter-1. Also, the sweet taste receptor is known to participate in GLP-1 secretion [33]. GLP-1 has wide-ranging effects on glucose metabolism, including the stimulation of insulin, inhibition of glucagon secretion, inhibition of gastrointestinal motility, and augmentation of satiety [34]. Enzyme DPP-IV cleavage and renal and hepatic clearance are elimination ways of circulating GLP-1, decreasing its half-life of around 2 min to increase plasma levels of GLP-, DPP-IV inhibitors such as sitagliptin are medically used. GLP-1 binds to a specific receptor, GLP-1R, which is a 7- transmembrane guanine nucleotide-binding protein-coupled receptor in β-cells, to stimulate the insulin release pathway. Since GLP-1 half-life is short, either GLP-1R agonists or DPP-IV inhibitors are used to stimulate insulin production in diabetes therapy by weekly injections [35].

2.1.10 Dipeptidyl peptidase IV

Dipeptidyl peptidase IV (DPP-IV) belongs to the prolyl peptidases family of enzymes that can cleave peptide bonds after a proline residue. This enzyme specifically removes N-terminal dipeptides from substrates containing proline, and to some extent alanine, at the penultimate position. The typical active substrate of DPP-IV is the incretin GLP-1. DPP-IV degrades GLP-1and decreases its levels in plasma. GLP-1 is secreted in the stomach producing a stimulating effect on insulin secretion after meal consumption [34]. Human DPP-IV is a 766

amino acid transmembrane glycoprotein consisting of a cytoplasmic tail (residues 1-6) a transmembrane region (residues 7-28), and an extracellular part (29-766). The protein extracellular region can be further subdivided into two domains (1) the catalytic domain (residues 508-766) which shows an a/b hydrolase fold and contains the catalytic triad Ser630 – Asp708 – His740; and (2) an eight-bladed b propeller domain (residues 56-497) which also contributes to the inhibitor binding site [36,37]. Synthetic inhibitors of the enzyme DPP-IV, are currently used for the management of diabetes type 2 [23]; however, several DPP-IV inhibitors can also be found in plant foods or be produced or release during food processing.

2.1.11 Reactive oxygen species

Reactive oxygen species (ROS), such as free radicals and peroxides are formed in the body as a consequence of metabolic processes or can reach the organism due to environmental exposure. ROS imbalance can contribute to aging and increase the risk of cancer, cardiovascular diseases, atherosclerosis, diabetes and their complications among others [38]. ROS has been suggested to impair critical signaling pathways involved in glycemic regulation, reducing insulin sensitivity and pancreatic β-cell failure [38]. Moreover, many studies have suggested that oxidative stress and chronic inflammation are interrelated factors involved in the development of type 2 diabetes and other chronic diseases. Phytochemical compounds, including flavonoids, could activate the nuclear-factor like 2-mediated antioxidant response element and promote the gene expression of antioxidant species such as superoxide dismutase-1, glutathione peroxidase 1, glutathione S-transferase, and thioredoxin, which in turn can remove ROS [4]. The amino acid sequences in the peptides contribute to their bioactivity. Hydrophobic amino acids and polar/charged amino acids contribute to the peptides antioxidant activity [38,39]. On the other hand, aspartic acid and glutamic acid may be essential for inhibition of metal-mediated oxidation process and metal chelating properties due to their ability to bind metals by their charged residues. The alkaline amino acids histidine and arginine contribute to the antioxidant effects though the capability of these amino acids to act as donors or acceptors of protons through their imidazole and guanidine groups respectively [38,40].

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CHAPTER 3: HYPOTHESIS AND OBJECTIVE

3.1 Long term goal

To provide an understanding of the role of common bean protein fractions in preventing and controlling diabetes mellitus.

3.2 Central hypothesis

Common bean protein fractions can prevent and control diabetes by DPP-IV inhibition, glucose degradation enzymes inhibition (α-amylase and α-glucosidase), block glucose transporters SGLT1 and GLUT2 to decrease glucose absorption and increase the production of GLP-1.

3.3 Overall objective

To produce anti-diabetes peptides from common bean proteins and to determine their efficacy using bioinformatics tools, enzymatic systems, *in vitro* and in *vivo* models.

3.4 Specific aims

The hypothesis of this work was evaluated through seven aims; in the first six we characterized the common bean proteins and peptides. We also characterized the phenolic compounds and evaluated their stability on a food system. Moreover, we measured the common bean peptides nutraceutical properties and the effect of processing on common bean biological potential, and we optimized the process to obtain anti-diabetes peptides. For the final aim, we used *in vitro* and *in vivo* models to understand the mechanism of action of protein fractions in managing diabetes.

Aim 1: To determine the protein and peptide profile, Bowman-Birk inhibitors, Kunitz inhibitors, lectins, α-amylase inhibitors and phenolic compounds and anthocyanins of improved common bean cultivars from Mexico and Brazil.

Aim 2: To determine the impact of precooking of commercially available common beans and compare the bioactive properties of the released peptides.

Aim 3: To characterize bioactive peptides after simulated gastrointestinal digestion of bean proteins and evaluate the potential to inhibit angiotensin converting enzyme (ACE), DPP-IV, and α-glucosidase.

Aim 4: To optimize the conditions needed to generate anti-diabetic peptides from common bean proteins using commercial enzymes and evaluate their effectiveness using biochemical and *in silico* approaches.

Aim 5: To optimize the extraction conditions of anthocyanins from common bean cultivar Negro Otomi, and to assess their stability at different pH and temperatures, and evaluate the biological potential related to type 2 diabetes.

Aim 6: To evaluate the mechanism of the anti-diabetes action of common bean bioactive peptides using *in vitro* and *in vivo* streptozotocin diabetic induced rat model.

CHAPTER 4: CHARACTERIZATION AND COMPARISON OF PROTEIN AND PEPTIDE PROFILES AND THEIR BIOLOGICAL ACTIVITIES OF IMPROVED COMMON BEAN CULTIVARS (*Phaseolus vulgaris* L.) FROM MEXICO AND BRAZIL

4.1 Abstract

Common bean (*Phaseolus vulgaris* L.) is a good source of protein, vitamins, minerals and complex carbohydrates. The objective was to compare protein profile, including anti-nutrient proteins, and potential bioactive peptides of improved common bean cultivars grown in Mexico and Brazil. Bean protein isolates (BPI) were prepared from 15 common bean cultivars and hydrolyzed using pepsin/pancreatin. Thirteen proteins were identified by SDS-PAGE and protein in-gel tryptic-digestion-LC/MS. Protein profile was similar among common bean cultivars with high concentrations of defense-related proteins. Major identified proteins were phaseolin, lectin, protease and α-amylase inhibitors. Lectin (159.2 to 357.9 mg lectin/g BPI), Kunitz trypsin inhibitor (inh) (4.3 to 75.5 mg trypsin inh/g BPI), Bowman-Birk inhibitor (5.4 to 14.3 μg trypsin-chymotrypsin inh/g BPI) and α-amylase inhibitor activity (2.5 to 14.9 % inhibition relative to acarbose/mg BPI) were higher in Mexican beans compared to Brazilian beans. Abundant peptides were identified by HPLCMS/MS with molecular masses ranging from 300 to 1500 Da and significant sequences were SGAM, DSSG, LLAH, YVAT, EPTE and KPKL. Potential bioactivities of sequenced peptides were angiotensin converting enzyme inhibitor (ACE), dipeptidyl peptidase IV inhibitor (DPP-IV) and antioxidant capacity. Peptides from common bean proteins presented potential biological activities related to control of hypertension and type-2 diabetes.

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4.2 Introduction

Common bean (*Phaseolus vulgaris* L.) is a significant source of proteins (16–33 %), some vitamins, minerals, phenolic compounds and complex carbohydrates [1–4]. Nutraceutical properties of common beans have been studied, for instance their anti-diabetes, anticancer, anti-inflammatory, antihypertensive and antioxidant properties [5–9].

Peptides are short chain amino acid sequences belonging to proteins, which hold potential physiological functions beyond their nutritional input [10]. Nowadays, there is important research on peptides from different food proteins, focusing on sources, agronomic production, physicochemical properties and biological potential [11]. The biological potential of peptides is associated with their ability to interact with amino acids of enzymes related with important diseases, and inhibit their action [9]. On the other hand, peptides can also exert antioxidant properties due to the functional groups of the amino acids present within the sequence. For instance, hydrophilic, aromatic, and basic amino acids contain phenolic, indole and imidazole groups that act as proton donors to stabilize free radicals [12]. Lectins, trypsin inhibitors, α-amylase inhibitors and arcelins in plants are known to act in defense-related mechanism of biotic stress and response to abiotic stress like drought, salt and wounding [13–15]. Mechanisms of crop resistance to pests and weather, as well as nutritional value, can be improved by breeding [15, 16]. Thermal denaturation during cooking may completely inactivate anti-nutrient proteins and they may even contribute to add nutritional value to the digested protein due to their high sulfur amino acid content, for instance lectins [13, 17].

The objective of this study was to characterize and compare the protein profile, including anti-nutrient proteins, and to characterize peptides with biological potential from improved common bean cultivars grown in Mexico and Brazil.

4.3 Materials and methods

Materials and Reagents Twelve common bean cultivars (*Phaseolus vulgaris* L.) grown in 2012 were provided by the Experimental Station of the National Research Institute for Forestry Agriculture and Livestock (INIFAP), Zacatecas, Mexico; Pintos [1: Pinto-Bayacora; 2: Pinto-Bravo; 3: Pinto-Centenario; 4: Pinto-Saltillo]; Flores de Mayo and Junio [5:FJ-Leon; 6: FJ Marcela; 7: FM-Eugenia; 8: FM-67; 9: FM-199; 10: FM −202]; Negros [11: Negro-Frijozac; 12: Negro-Otomi]. Three common bean cultivars grown in 2012 were provided by the Brazilian Agricultural Research Center (EMBRAPA), Brazil; Carioca [13: BRS-Horizonte; 14:BRS-

Pontal; 15: Perola] (Figure 6a). Seeds were harvested after complete ripening and dried in the plant seed case at environmental conditions. The dry seeds were stored at 4 °C until use. Porcine pepsin (420 U/mg solid, EC 3.4.23.1), pancreatin (8xUSP, a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9) and α-amylase from porcine pancreas (≥10 U/mg solid, type VI-BEC 3.2.1.1) were purchased from Sigma–Aldrich (St. Louis, MO). Primary antibody for lectin was rabbit monoclonal antibody, for *Phaseolus vulgaris* erythroagglutinin and leucoagglutinin (Vector Laboratories Inc, Burlingame, CA); for Kunitz trypsin inhibitor (KTI) was trypsin inhibitor rabbit polyclonal antibody, epitope: full length protein (Abcam, Cambridge, MA), for Bowman-Birk inhibitor (BBI) primary antibody, epitope: 238 I mouse monoclonal antibody (Agdia, Inc., Elkhart, IN). The secondary antibody for both lectin and KTI was antirabbit IgG alkaline phosphatase conjugate. Antimouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) was used as the secondary antibody for BBI.

4.3.1 Near Infrared Analysis of Total Protein

The total protein concentration of common bean cultivars was determined by Near Infrared Grain Analyzer (Infratec 1229, Foss Tecator) following manufacturer instructions. Briefly, one pound of sample was located in the upper dispenser of the equipment and separated in six portions, read and averaged. Results were expressed as grams of protein per 100 g of seeds.

4.3.2 Soluble Protein Determination

Quantification of soluble protein was performed using the method reported by Silva-Sanchez et al. [18]. Concentration of soluble protein was calculated using a bovine serum albumin standard curve (y=0.0002x-0.012, R2=0.99).

4.3.3 Bean Protein Isolates

Common bean cultivars were soaked in water for 16 h. After soaking the hull was manually removed and cotyledons were ground in a commercial blender in a 1:10 bean/water ratio. Protein isolates were prepared using the method reported by Oseguera-Toledo et al. [5]. Dried common bean protein isolates were stored at −20 °C for further analysis.

4.3.4 Quantification of Lectins, Kunitz Trypsin Inhibitor and Bowman-Birk Inhibitor in Common Beans

One milligram of the BPIs were solubilized in 0.05 M NaOH, and further dilutions were made in TBS. Determinations of lectins, KTI and BBI inhibitors were performed using the

methodology reported by Dia et al. [19]. Standard curves were determined using pure lectin (>80% purity), y=0.001x+0.0455, R^2=97; pure KTI (>98 % purity), y=0.0034x+0.1012, R2=98 and BBI (70−90 % purity), y=0.7348x+0.006, R^2=99. Each sample was analyzed in triplicate.

4.3.5 Quantification of α-amylase Inhibitor Activity

The α-amylase inhibitor activity was performed using the method reported by Mojica et al. [9]. Results are presented as percent inhibition relative to the positive control acarbose (AC) having 100 % inhibition.

4.3.6 Simulated Gastrointestinal (GI) Digestion

In vitro digestion of bean protein isolate was performed using the method reported by Megías et al. [20]. Common bean hydrolysates were stored at −20 °C for further analysis.

4.3.7 Gel Electrophoresis Analysis

SDS-PAGE for the protein profile of common bean cultivars and their hydrolysates were performed following manufacturer's instructions. Gels were visualized using GL 4000 Pro Imaging system (Carestream Health Inc., Rochester, NY).

4.3.8 In-gel Tryptic Digestion Protein Analysis

Protein in-gel tryptic digestion and LC/MS analysis were performed separating the electrophoresis gel in five sections. Gel slices were dehydrated and destained in 50 % acetonitrile plus 25 mM ammonium bicarbonate. The gel was dried using Speedvac (Thermo Scientific) before hydrolysis with trypsin (mass spectrometry grade, G-Biosciences at 1:50 w/w) in 25 mM ammonium bicarbonate. Hydrolysis was performed using a Discover Microwave Digester from CEM Corporation (Mathews, NC) for 15 min at 75 W, 55°C. The digested peptides were extracted using 50 % acetonitrile plus 5 % formic acid, lyophilized to dryness, and reconstituted in 10μL 5 % acetonitrile plus 0.1 % formic acid, 6μL was used for LC/MS analysis. LC/MS was performed using a Thermo Dionex Ultimate RSLC3000 operating in nano mode at 300μL/min with a gradient from 0.1 % formic acid to 60% acetonitrile plus 0.1% formic acid for 60 min. The trap column used was a Thermo AcclaimPepMap 100 (75 μm×2 cm) and the analytical column was a Thermo Acclaim PepMap RSLC (75μm×15 cm). Protein in-gel tryptic digestion analysis Xcalibur raw file was converted by Mascot Distiller into peak lists that were submitted to Mascot and searched against specific protein databases (Uniprot Database).

Peptide Sequence Identification Peptides obtained after simulated GI digestion were analyzed using the method reported by de Souza-Rocha et al. [21]. Confirmation of peptide sequences in

common bean proteins (*Phaseolus vulgaris* L.) was performed using BLAST® tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 27/05/2014). The potential biological activity of the peptides was predicted by using BIOPEP database (http://www.uwm.edu.pl/biochemia, accessed on 27/05/2014).

4.3.9 Statistical Analysis

The experiments were repeated at least three times with consistent results. Data are expressed as the mean ± standard deviation. The data obtained were analyzed using one-way ANOVA to compare experimental to control values (StatGraphics plus 5.0). Comparisons between groups were performed using LSD test, and differences were considered significant at $p<0.05$. Principal component analysis (PCA) and clustering analysis were performed using SPSS Statistics Premium GradPack 22 (Somers, NY).

4.4 Results and discussion

4.4.1 Characteristics of Common Bean Cultivars

Studied common bean cultivars: Pintos, Negros, Flor de Mayo and Junio from Mexico and Cariocas from Brazil are shown in Figure 6a. These cultivars were developed by breeding in specialized research centers to improve agronomic or nutritional aspects, or both (http://www.inifap.gob.mx/; https://www.embrapa.br/). INIFAP and EMBRAPA are currently working on these cultivars due to their potential agronomic value; most prevalent improvements in common beans were disease resistance such as anthracnose, rust and common bright. On the other hand, other cultivars had nutritional improvements as high protein, iron and zinc; also, high yield and fast growth.

4.4.2 Protein Quantification and Profiles from Common Bean Cultivars

The total protein concentration ranged from 19.0 to 27.2 g/100 g seeds in Negro Otomi and FM-202 cultivars, respectively; while soluble protein ranged from 14.4 to 22.7 g/100 g seeds in BRS-Pontal and FM-202, respectively (Table 2). With respect to protein profiles, 13 bands were separated by their molecular mass in polyacrylamide gels (Figure 6b). Protein identification was confirmed using LC/MS tryptic digestion. Protein profile showed a similar pattern among Mexican and Brazilian cultivars; nevertheless, the lower molecular mass bands, less than 10 kDa were present in Mexican lines 1 to 12, and apparently absent in the Brazilian lines 13 to 15. However, we only used SDS-PAGE to separate the proteins in this study; other methodologies to separate each protein band would be needed. Phaseolin, lectin, α-amylase inhibitor and BBI were

the most important identified proteins [13,14,22–24]. Luna-Vital et al. [11] listed 160 proteins from *Phaseolus vulgaris* reported on UniProtKB Knowledgebase, including phaseolin, lectin, α-amylase inhibitor and Bowman-Birk inhibitor, as well as several other proteins related with plant metabolic functions. Storage protein phaseolin (bands D and E) ranged from 30.2 to 53.5 % of the total protein, being the most abundant protein in common beans (Figure 6b). Similar to our results, phaseolin has been reported to present 30–50 % of the total protein in common beans [17, 23]. α-Amylase inhibitors were identified in bands J, K and L, with molecular masses of 18, 16 and 14 kDa, respectively. Lectin with MM of 33 and 25 kDa (bands F and H), trypsin inhibitor was detected at 20 kDa (band I), and BBI at less than 10 kDa (band M). Arcelin was identified in band G with a MM of 29 kDa.

4.4.3 Sequences of Peptides in Common Bean Protein Hydrolysates

The effect of GI digestion on protein profile is presented in Figure 6C. Most protein bands were digested after 4 h of hydrolysis with pepsin/pancreatin; however, phaseolin remained unhydrolyzed. This could be due to its compact structure and stability provided by its three dimensional structure and carbohydrate moiety [22–24]. Tang et al. [25] reported digestibility of red, kidney and mug beans in the range of 53–64%after 4 h of hydrolysis with pepsin/trypsin. Among cultivars, the molecular mass of the identified peptides ranged from 300 to 1500 Da. It was possible to identify 137 peptide sequences among the 15 cultivars. The highest number of peptides identified in protein hydrolysates (31 sequences) had a molecular mass in the range of 901 to 1000 Da; followed by 21 different sequences with MM of 401 to 500 Da. Low molecular mass peptides (300 to 500 and 501 to 600 Da) presented similar sequences among all cultivars (SGAM, DSSG and LLAH, YVAT, EPTE, KPKL). Figure 6d shows the structure, physical properties and biological potential of abundant peptides. Sequences of identified peptides from common bean hydrolysates are shown in Table 3. Depending on the amino acid sequences, some peptides had more than one potential biological activity; however, ACE inhibition was the most prevalent biological function of the peptides (87.5 %). ACE is an essential enzyme related to the control of high blood pressure. On the other hand, DPP-IV inhibition was present in more than half of sequences (74 peptides); this enzyme is a molecular target in the treatment of type-2 diabetes [9]. Similarly, Luna-Vital et al. [11] reviewed several reports of the potential of common bean hydrolysates to inhibit ACE and DPP-IV, and with antioxidant capacity. Another important potential bioactivity of peptides (36 sequences) was antioxidant capacity. Lectin,

Protease and α-amylase Inhibitors in Common Beans Concentration of lectins, KTI, and BBI, and the activity of α-amylase inhibitor are shown in Table 2. Common bean cultivars from Mexico showed higher concentrations of anti-nutrient proteins than Brazilian beans. Cultivars Pinto-Bayacora and BRS-Pontal presented the highest and lowest concentrations of lectins (357.9 and 159.2 mg lectin/g BPI), respectively. Regarding Kunitz trypsin inhibitors FM-199 contained the lowest concentration (4.3 mg trypsin inh/g BPI) while Pinto-Bravo presented the highest (75.5 mg trypsin inh/g BPI). Bowman-Birk inhibitor concentrations ranged from 5.4 to 14.3 μg trypsin-chymotrypsin inh/g BPI in FM-199 and Perola, respectively. Pinto-Bayacora showed the highest α-amylase inhibitor activity relative to acarbose (14.9 % inhibition relative AC/mg BPI) and Negro-Frijozac showed the lowest (2.5 % inhibition relative AC/mg BPI). Studied cultivars present higher concentrations of the lectin family proteins (12 to 26 % of total protein recovered in SPI) when compared with reported data (6 to 12 % of total protein) [1].

4.4.4 Principal Component Analysis and Cluster Analysis

Formed clusters can be observed in Table 2; groups of FM and FJ bean clusters contained large scores in PC1, related with high protein concentration. On the other hand, the cluster containing Negro-Otomi, Pinto-Bravo and Pinto-Bayacora presented large score on PC2 which represent high concentration of defense-related proteins. However, there were three clusters that have small scores in both PCs (Pinto-Centenario, Pinto-Saltillo, FM-Eugenia, Negro-Frijozac, Brazilian-Pontal, Brazilian-Horizonte and Perola); these cultivars showed balanced concentration of defense-related proteins and total protein.

4.5 Conclusions

Protein profiles among common bean cultivars from Mexico and Brazil were similar and presented high concentrations of defense-related proteins. Flor de Mayo and Junio cultivars are a good source of proteins with a moderate concentration of anti-nutrient proteins. On the other hand, Carioca beans, Negro Frijozac, Pinto-Centenario, Pinto-Saltillo and FM Eugenia present a good balance between agronomical related defense proteins and total protein to be considered as a good option for producers and consumers. New improved common bean cultivars fulfill the objective for which they were developed; they present good agronomic characteristics without losing nutritional traits. An 87.5 % of sequenced peptides presented potential to inhibit ACE, followed by inhibition of DPP-IV and antioxidant capacity. Tetra-peptides SGAM, DSSG, LLAH, YVAT, EPTE and KPKL were the most commonly found among cultivars with high

biological potential. Common beans are a good source of proteins and peptides with potential biological activities related to control of hypertension and type-2 diabetes.

4.6 References

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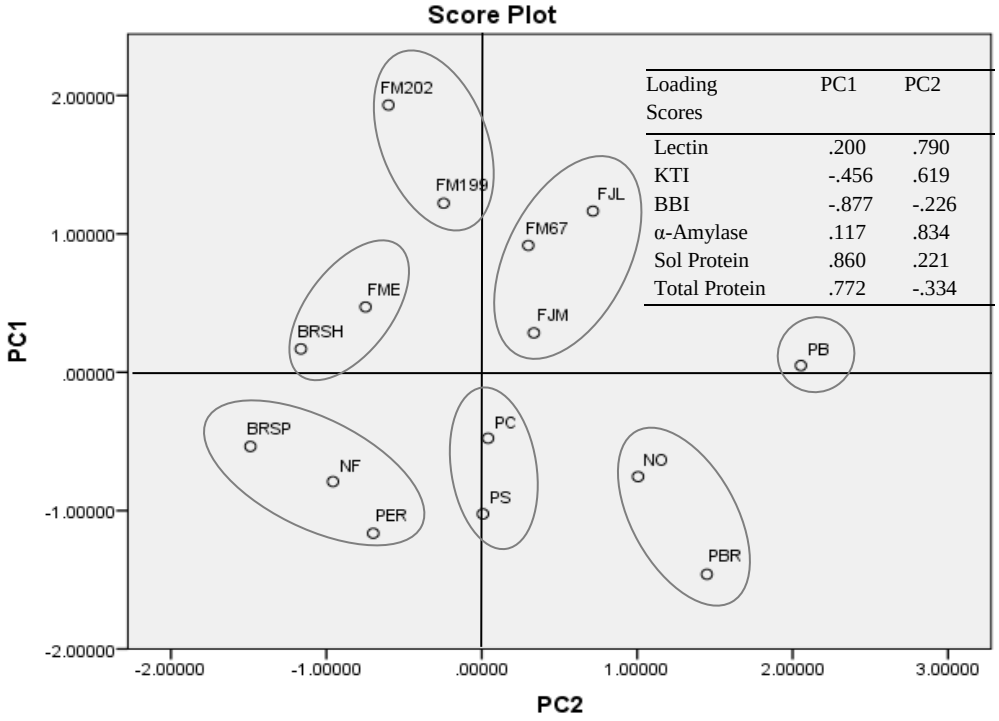
4.7 Tables and figures

Table 2. Concentration of protein, lectins, Kunitz trypsin inhibitors, Bowman-Birk inhibitors, and α-amylase inhibitor activity

Cultivar	Total Protein	Soluble protein	Lectins	Kunitz inhibitor	BBI inhibitor	α-Amylase inhibitor
	g /100 g seeds	g /100 g seeds	mg Lectin/g BPI	mg Kunitz inh/g BPI	µg BBI inh/g BPI	% inh rel AC/mg BPI
Pinto-Bayacora, PB	23.5± 1.1de	18.2± 0.0h	357.9±30.1a	44.6±13.7a	9.4±3.4de	14.9±1.7a
Pinto- Bravo, PBR	20.1± 0.2ab	15.4± 0.3bc	263.5±1.0bc	75.5±2.6a	10.0±0.8def	9.0±1.1d
Pinto-Centenario,PC	22.2± 0.6cd	16.1±0.2cde	258.9±47.1bc	26.3±7.2bcde	9.9±0.8def	6.7±1.7ef
Pinto-Saltillo, PS	19.6± 0.4[a]	15.6±0.1bcd	225.3±74.2cd	22.8±11.7bcd	10.9±2.1ef	8.2±0.0d
FJunio-Leon, FJL	24.4±0.3ef	20.5±0.4j	255.2±19.2bc	27.1±9.6bc	5.5±1.4ab	11.4±0.1bc
FJ-Marcela, FJM	23.6± 0.0def	17.8±0.3gh	240.8±17.6bc	33.7±0.5bc	7.1±0.9abc	8.7±0.1d
FM- Eugenia, FME	24.0±0.6ef	19.3±0.4i	196.0±74.8cd	15.3±6.4ed	8.8±0.3cde	5.6±0.1de
FMayo- 67, FM67	24.9±1.0fg	20.1±0.6ij	198.1±62.3cd	17.1±6.6cde	7.8±0.0bcd	14.9±0.4c
FMayo-199, FM199	26.8±0.2h	16.6±0.7ef	313.8±65.3ab	4.3±0.6e	5.4±0.6a	5.67±0.2gh
FMayo-202, FM202	27.2±0.2h	22.7±0.4k	245.7±24.7bc	14.3±2.6ed	5.7±0.1ab	3.4±0.5hi
Negro-Frijozac, NF	21.2±0.0bc	14.7±0.8ab	203.1±69.3cd	19.0±4.5bcde	9.7±0.7def	2.5±0.1i
Negro-Otomi, NO	19.0±2.5[a]	16.5±0.8def	267.7±20.2bc	24.5±8.5bcde	9.9±1.9def	13.1±0.1b
BRSHorizote, BRSH	24.8±0.3efg	17.2±1.1fg	161.5±4.4d	21.2±1.3bcde	8.5±0.7cd	4.3±0.3gh
BRS-Pontal, BRSP	26.1± 0.2gh	14.4±0.1a	159.2±11.7d	19.3±0.8bcde	11.7±0.4f	5.0±0.1efg
Perola, PER	22.5±0.1cd	16.0± 0.8cde	221.6±43.1cd	25.8±0.1bcde	14.3±0.8g	4.5±0.1fgh

Different letters among samples indicate significant differences ($p < 0.05$). At least triplicate analyses of each sample were performed. BBI: Bowman-Birk inhibitor, BPI: Bean protein isolate; AC: acarbose; inh: inhibitor, rel: relative



Principal component analysis score plot and clusters of fifteen common bean cultivars from Mexico and Brazil. Score plots for the first two principal components PC1 (39.53%) and PC2 (31.49%).

Table 3. Peptide sequences from common bean protein isolate hydrolysate and their potential biological activities (angiotensin converting enzyme, dipeptidyl peptidase IV and antioxidative.

MW / Cultivars	300-400	401-500	501-600	601-700	701-900	901-1000	1001-1200	1201-1300	1301-1400	1401-1500
Pinto-Bayacora	AGAH[1,3]	SQGY[1] GRGW[1]					LEGPKRAGW[1,3]	FLEMLLLDFL[2] RLSQERMRAQ[1]	KRLKQL-YRQQ[1,3]	
Pinto-Bravo		LLAH[1,2,3]	VRFV[1] LVRF[1] PLEAL[1]		RPESGAL[1]	REAKLYQ[1,3] SAKGPPTSAQ[1,3]		YYLRRLLLQ[1,3] PLLELELVEAAG[1,2,3] QADLQLQLLLV[1,3]		YLAGNPFAPP-HGGK[1,3]
Pinto-Centenario		YVAT[2] MPPM[1,3]		KVDNFG[1] SGPTLSK[1,3]		QLEYRAQ[1] GPKVGWAVSG[1,3] SGKKPTRW[1,3] YLRALSGW[1]		RLLFNLMLAF[1,3] FFLLEQLAATT[1,3]		
Pinto-Saltillo	CGGE[1,3]	LLAH[1,2,3] LPPAG[1,3] TTIF[1]	EKPF[1,3]	LLLLHA[2,3]	RPEGSAL[1]	SAKTPPAGSK[1,3] SASPPPFAAL[1,3] ASKPGASSAAL[1,3]				
FJ-Leon	DSSG[1]	SGAM[1] FPLV[1,3] DDLL[2]		LLLLHA[2,3]		ELEALETT[2,3]			LQLELELE-EEL[2,3]	
FJ-Marcela	SGAM[1] GTAK[1]	HALL[1,3] KPKL[2] SRVK[1] KEGY[1,3]	DLAAQ[1,3] LSGVF[2]	HAKVPK[1,3]	VYPPPAL[1,2,3] LLFFLE[1]	GSKAPEDKK[1,3]	FIFFNLET[2] LLRLLLLLSG[1,3]		RNRLEN-MLLAQ[1,3]	PNLLGLSLEL-LLLL[1,2,3]
FJ-202	SGAM[1]	FPVL[1,3] GRGW[1]			YGLVAGK[1,3]	DLLDDEVS[1,3] DWKLLRGS[1,3]	LLLLLLHAL[2] QMYLLLVR[1,2,3]	LEFLLLMLDF[2]	RRKAL-MRGQNK[1,3]	
FM-Eugenia		HALL[1,3] KPKL[2] SRVK[1] KEGY[1,3]	PKKPK[1,3]		PLTALFV[1]	KRTAGTARP[1] SGKAPPTSGGT[1,3] GSLSAKSKGK[1,3]				
FM-67	DSGS[1]	LLHA[2,3] KPQL[1,3]	KELE[1,3]		FAFQFT[1,3]	LEELYLQ[1,3] SAKGPPMGAK[1,3]		LMMLMYLLLL[1,2,3]	QFLLQM-LALRK[1,3]	
FM-199		EPTE[1]	VLRF[1]	QLKLLS[1,2,3] RASVVAL[2] FFAQFT[2]		LLLYLLTG[1,2,3]	SAWELDDKGS[2,3]			YLKNPDLEHP-SL[2,3]
Negro-Frijozac		GPALP[1,3] GLASK[1,3] KGAVT[1]	LGVHK[1] KPQKP[1,3]	FFAQFT[2]		YYTPKSKG[1,3]		TELELLFLEF[1,2,3]	RRMSLL-GKLRQ[1,3]	
Negro-Otomi	DSSG[1]	LLAH[1,2,3]		SVLLNR[1,3]		ELLELYQ[1,2,3] SANPATPTGK[1,3] SGPAKWKW[1,3]		RYAFLELLTQ[1,2,3] KRKRYEKLLLLRE-EGRSVFQ[1,3]		
BRS-Horizonte		YVAT[1]	GSVHVV[1,3]	KQLLLS[2]	RQRLPL[1,3] RLNDLLQ[1,3]	KTPGTLAKK[1,3] GKASLTDGVL[1,3] TAKGAAAAGAGK[1] TPGAPAALPGQ[1,3]		EFLDLMLLLF[1,3] RFKLRMQRK[1]		
BRS-Pontal		KPKL[1,3]	PKKPK[1,3]	ERGLAGS[1,3]	DNLVCGS[1] EQDKKL[1]	RVSGTPSKK[1] TAKGGVGAAKN[1]				
Perola	FAAG[1,3]	EPTE[1] YAAAT[1]	KEEE[1]	EGRALGS[1]	LKSLYL[1,3] KGAGGAAAH[1,3]	ASKGGGVAGKK[1,3] SKAGGVGGLSK[1,3]				DLLLLLMGES-LLF[1,3]

Peptides obtained from the HPLC elution profile with intensity of at least 50%; Sequences belonging to *Phaseolus vulgaris* protein was confirmed by BLAST tool. Potential bioactivities were obtained from the BIOPEP database. 1: Angiotensin converting enzyme inhibition; 2: Dipeptidyl peptidase-IV inhibition; 3: Antioxidant capacity. HPLC-MS-MS: high performance liquid chromatography-mass spectrometry-mass spectrometry; MW: molecular weight; Da: Daltons; FJ: flor de junio; FM: flor de mayo; BRS: Brazilian; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

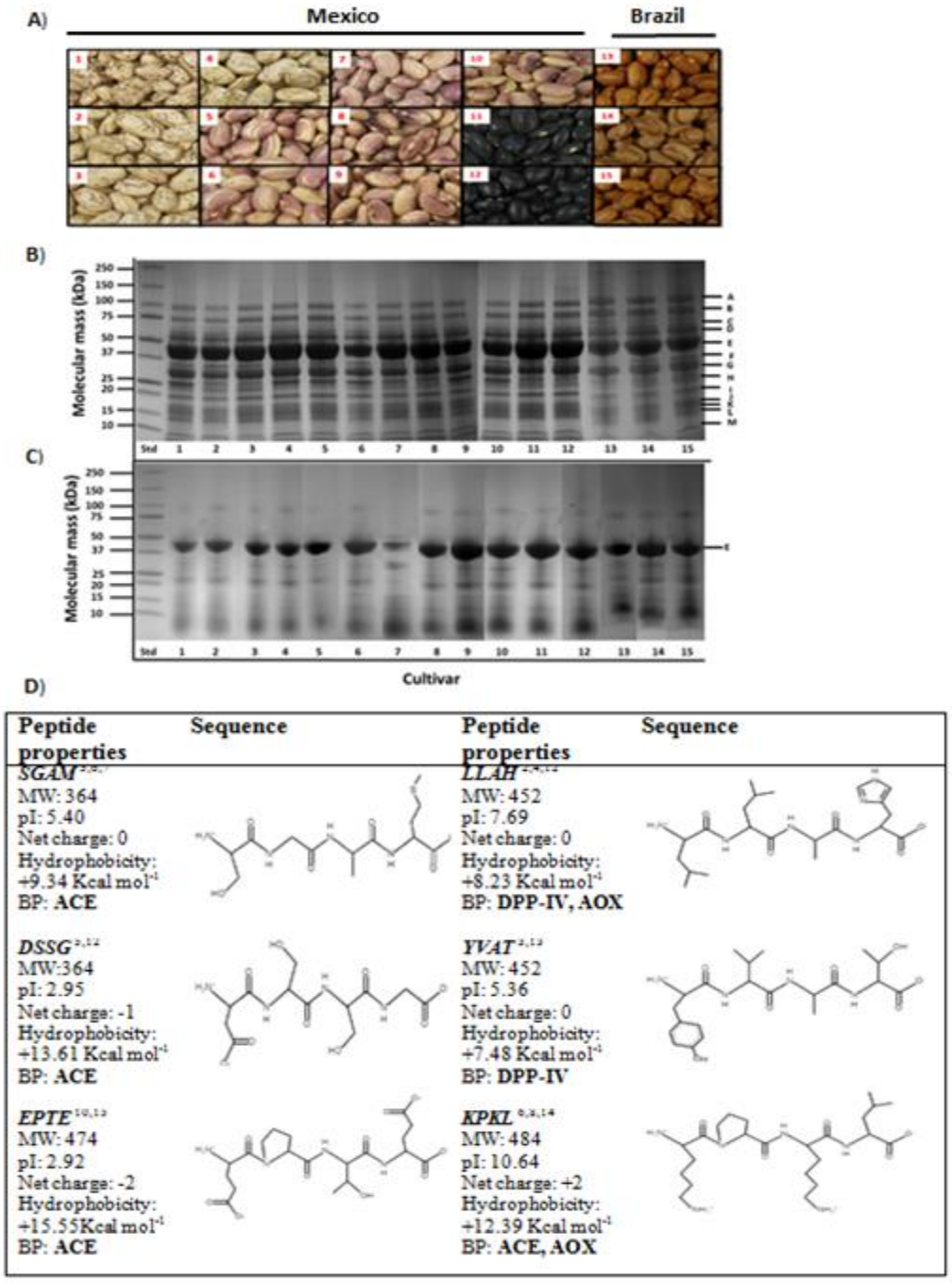


Figure 6. A) Common bean cultivars (*Phaseolus vulgaris* L.) from Mexico (1: Pinto-Bayacora; 2: Pinto-Bravo; 3: Pinto-Centenario; 4: Pinto-Saltillo; 5: FJ-Leon; 6: FJ- Marcela; 7: FM-Eugenia; 8: FM -67; 9: FM -199; 10: FM -202; 11: Negro-Frijozac; 12: Negro- Otomi), and cultivars from Brazil, 13: BRS-Horizonte; 14: BRS-Pontal; 15: Perola); B) SDS-PAGE protein profile of common bean cultivars, identification based on SDS-PAGE[a] and tryptic gel LC/MS[b] Database UniprotKB20130614 and references from the literature [5,8,11,23,24], Capital letters indicate protein bands in gels. A: linoleate 9S Lipoxygenase 1[a]; B: Legumin[a]; C: not identified[a]; D: phaseolin[ab]; E: phaseolin[ab]; F: α-amylase inhibitor[a]; G: arcelin-4[a,b]; H: phytohemagglutinin[a,b]; I: trypsin inhibitor[b]; J: Lectin[a]; K: α-amylase inhibitor-β[a], L: α-amylase inhibitor-β[ab]; M: Bowman-Birk type proteinase inhibitor[b]; C) SDS-PAGE protein profile of common bean protein isolates after simulated gastrointestinal enzymatic digestion; D) Physical properties, potential biological activities and structure of peptides most predominant in common beans, structures obtained by PepDraw tool; pI: isoelectric point; MW: molecular weight; BP: bioactivity potential; ACE: angiotensin converting enzyme inhibitory activity; DPP-IV: Dipeptidyl peptidase IV inhibitory activity; AOX: antioxidative. Small numbers indicate cultivar source.

CHAPTER 5: CHARACTERIZATION OF PEPTIDES FROM COMMON BEAN PROTEIN ISOLATES AND THEIR POTENTIAL TO INHIBIT MARKERS OF TYPE-2 DIABETES, HYPERTENSION AND OXIDATIVE STRESS

5.1 Abstract

Diabetes and hypertension are diseases affecting a high proportion of the world population; the use of food-based products such as common bean peptides may contribute to reduce the risk of complications associated to chronic diseases. The aim was to produce and characterize peptides from common bean protein isolates and evaluate their potential to inhibit markers of type-2 diabetes, hypertension and oxidative stress. Mexican black and Brazilian Carioca bean isolated proteins were characterized after pepsin/pancreatin digestion. Also, four synthesized pure peptides, originally found in these beans, were evaluated. Bean protein digests and pure peptides exerted dipeptidyl peptidase-IV (DPP-IV) inhibition, IC50= 0.03 to 0.87 mg dry weight, DW, mL-1. Lineweaver-Burk plots and computational modeling showed competitive inhibition of DPP-IV. Angiotensin converting enzyme inhibition (ACE) ranged from IC50= 0.09 to 0.99 mg DW mL-1, and α-glucosidase inhibition ranged from 36.3 to 50.1% mg-1 DW. Carioca Perola bean digested proteins presented the highest antioxidant capacity (269.3 mM Trolox equivalent, TE g-1 DW) as the peptide KTYGL ($p > 0.05$) with the most potent DPP-IV and ACE inhibition. Peptides from common bean have anti-diabetes and antihypertensive potential regardless of their antioxidant capacity

This chapter is part of the publication: Mojica L, Luna-Vital DA, de Mejía EG (2016) Characterization of peptides from common bean protein isolates and their potential to inhibit markers of type-2 diabetes, hypertension and oxidative stress. J Sci Food Agric. JSFA-16-0187. In Press.

5.2 Introduction

According to the American Diabetes Association [1], approximately 29.1 million Americans, nearly 9.3% of the population have type-2 diabetes. A strategy for the management of type-2 diabetes involves prolonging the activity of the incretin hormones glucose dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). These incretins enhance insulin secretion from pancreatic beta cells in response to nutrients [2]. However, incretins are rapidly degraded by the exopeptidase dipeptidyl peptidase IV (DPP-IV), resulting in a decreased insulin response [3]. Therefore, the inhibition of DPP-IV can be used to manage type-2 diabetes. Additionally, α-glucosidase inhibitors have also been used for the treatment of type-2 diabetes. This enzyme breaks down starch and releases free glucose; its inhibition decreases glucose intestinal absorption [4].

Hypertension is a serious public health problem due to its correlation with the incidence of cardiovascular disease (CVD). About 600,000 people die of CVD in the United States every year, representing one in every four deaths [5]. Hypertension is related to the incidence of coronary heart disease, peripheral artery disease and stroke, and kidney disease [6]. The control of hypertension may be regulated by changes in lifestyle for instance physical activity, smoking cessation, reducing sodium intake, improving diet and reducing stress [7]. One of the main mechanisms related to hypertension is the function of the renin-angiotensin aldosterone system, and specifically, angiotensin converting enzyme (ACE). ACE removes two C-terminal residues from angiotensin I and transforms it into angiotensin II, a potent vasoconstrictor, narrowing arteries and blood vessels [8]. Pharmaceutical ACE inhibitors such as captopril, enalapril and lisinopril decrease the enzymatic activity of ACE and indirectly reduce the level of angiotensin II, providing a vasorelaxing effect on blood vessels [9]. However, the use of ACE inhibitors may trigger undesirable side effects including allergic reaction, skin conditions, bone marrow suppression, and nephrotic syndrome, among others [10]. About 73% of adults with type-2 diabetes have hypertension [5]. Although it is not clearly understood why these diseases commonly occur together, oxidative stress seems to play a pivotal role in the development of both microvascular and cardiovascular diabetes complications [11]. Peptides with antioxidant capacity (AC) have been reported from peas [12], chickpeas [13], lentils [14], common beans [15], and soybean [16]. AC from peptides can be achieved due to the presence of hydrophilic, aromatic, and basic amino acids in the sequence of the peptides formed after hydrolysis.

Phenolic, indole and imidazole groups present in amino acid residues act as proton donors to the deficient radicals to maintain molecular stability [17].

Peptides from common bean proteins have been reported to have potential anti-inflammatory, anti-hypertensive, antioxidant, metal chelating and anti-fungal activities [15]. DPP-IV, α-glucosidase and ACE are important enzymes related with chronic diseases such as type-2 diabetes and hypertension [18], and their inhibition can be a potential therapeutic strategy. There is a need to characterize peptides with potential biological activity from common bean cultivars. Therefore, the objective was to characterize peptides from improved common bean cultivars and to compare the effect of their digested isolated proteins, and pure peptides on DPP-IV, α-glucosidase and ACE inhibition as well as to determine their AC. Also, the potential mechanisms of interaction of peptides with DPP-IV, α-glucosidase and ACE enzymes were proposed using molecular docking; the inhibition kinetics of pure peptides and DPP-IV was determined using Lineweaver-Burk plots.

5.3 Materials and methods

5.3.1 Materials and reagents

Four agronomically improved *Phaseolus vulgaris* L. common bean varieties (Fig. 1A) were studied: black-Otomi from Mexico (provided by the Experimental Station of the National Research Institute for Forestry Agriculture and Livestock, INIFAP, Zacatecas, Mexico), and BRS-Horizonte, BRS-Pontal and Perola (provided by the Brazilian Agricultural Research Center, EMBRAPA, Santo Antônio de Goiás, GO). The dry grains were stored at 4°C until use. Porcine pepsin (EC 3.4.23.1), pancreatin (8xUSP, a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9), human dipeptidyl peptidase IV (D4943, EC 3.4.14.5), α-glucosidase from Saccharomyces cerevisiae (EC 3.2.1.20) and DPP-IV inhibitor, diprotin A (Ile-Pro-Ile) were purchased from Sigma Aldrich (St. Louis, MO). SDS-PAGE was purchased from Bio-Rad Laboratories, Inc. (Hercules, California, USA). DPPIV-GLO® protease assay kit was purchased from Promega (Madison, WI). Angiotensin converting enzyme (EC 3.4.17.23) assay kit (ACE KIT-WST) was purchased from Dojindo (Rockville, MD). Pure peptides KKSSG, KTYGL, GGGLHK and CPGNK (purity ≥ 98%) were produced by GenScript (Piscataway, NJ). The best pure peptides candidates were selected for synthesis and further analysis using as criteria the biological potential results, the predicted interaction values, and at least one peptide was selected from each cultivar.

5.3.2 Extraction of bean protein isolate

Common bean cultivars were soaked in water for 16 h. After soaking, the hull was manually removed and cotyledons were ground in a commercial blender in a 1:10 bean/water ratio. Bean protein isolates (BPI) were prepared using the method reported by Oseguera-Toledo et al. [19]. Dried common bean protein isolates were stored at −20 °C for further analysis.

5.3.3 Simulated gastrointestinal digestion of bean protein isolates

In vitro digestion of bean protein isolate was performed using the method reported by Megías et al. [20]. Common bean digests were stored at −20 °C for further analysis.

5.3.4 Gel electrophoresis analysis (SDS-PAGE)

SDS-PAGE was performed for the visualization of the protein profile of common bean cultivars and their hydrolysates following manufacturer's instructions. Gels were visualized using GL 4000 Pro Imaging system (Carestream Health Inc., Rochester, NY).

5.3.5 Molecular mass protein profile and peptide sequence identification from common bean

Bean protein isolate digests were analyzed by matrix-laser desorption ionization–time-of-flight mass spectroscopy (MALDI-TOF-MS/MS) according to the method reported by Luna-Vital et al. [21]. Briefly, 1 mg of protein hydrolysates from Black-Otomi BRS-Horizonte, BRS-Pontal and Perola were dissolved in 1 ml of deionized water and analyzed by using an UltrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.4 software package (Bruker Daltonics). MS/MS analysis of each ion of interest was performed at 500 Hz in LIFT mode using a randomized raster, summed, and saved for analysis. Data processing was performed using the FlexAnalysis 3.4 software package (Bruker Daltonics, Bremen, Germany) and Biotools 3.2 (Bruker Daltonics, Bremen, Germany). The presence of peptides in common bean protein sequences were confirmed using BLAST® tool; (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [18 January 2016]. Potential biological activities of the peptides were obtained using BioPep tool (http://www.uwm.edu.pl/biochemia/ index.php/pl/biopep) [18 January 2016]. Physicochemical properties were predicted using PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/index.html) [18 January 2016].

5.3.6 Oxygen radical absorbance capacity from BPI digests and peptides

AC was measured by the oxygen radical absorbance capacity assay as described by Prior et al [22] using 20 μL trolox standard, 1 mg mL^{-1} of pure peptide, or 1 mg mL^{-1} protein isolate

digests, or blank (75 mmol L-1 phosphate buffer, pH 7.4), 120 µL of 116.9 nmol L^{-1} fluorescein (final concentration 70 nmol L^{-1}/well), 60 µL of 40 mmol L^{-1} α,α′-azodiisobutyramidine dihydrochloride (AAPH) per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multiwell plate reader (Biotek Instruments, Winooski, VT). Results were expressed as millimols of Trolox equivalents per g of protein (mmol TE g^{-1}).

5.3.7 Dipeptidyl peptidase (DPP-IV) inhibition biochemical assay

DPP-IV inhibition was measured using the DPP-IVGLO® Protease Assay (G8351, Promega, Madison, WI). A 50 μL of DPP-IVGLO® reagent was added to a white-walled 96-well plate containing either 50 μL of blank, 40 μL enzyme control or 40 μL BPI digests, or peptides. Bean digests and peptides were prepared in buffer (100 mmol L^{-1} Tris, pH 8.0, 200 mM NaCl, 1 m mol L^{-1} EDTA) at different concentrations (0.005 to 1 mg DW mL^{-1}). The blank contained only buffer and DPP-IVGLO® reagent, while the enzyme control and the samples contained buffer, DPP-IVGLO® reagent and 10 μL purified DPP-IV human enzyme (10 ng mL^{-1}). Luminescence was measured after mixing and incubation at 26°C for 30 min using a Synergy2 multiwell plate reader (Biotek Instruments, Winooski, VT). Percent inhibition was calculated from the blank and enzyme control for each sample. IC_{50} values were calculated using Graph Pad Prism 4.0.

5.3.8 Inhibitory kinetics study

Recombinant human DPP-IV inhibition kinetics was measured at various concentrations of four pure peptides using different concentrations (0.004, 0.4 and 4 mmol L^{-1} for each peptide KTYGL, KKSSG, CPGNK and GGGLHK; and 0.01, 5 and 50 μmol L^{-1} for diprotin A). Each concentration was evaluated in the presence of various concentrations of Gly-Pro-amino methyl coumarin (AMC) substrate (0–60 μmol L^{-1}) that generated a luminescent signal by luciferase reaction, with the amount of DPP-IV enzyme available to bind Gly-Pro-AMC proportional to relative light units (RLU) produced. DPP-IV activity was measured using the DPP-IVGLO® Protease Assay. The inhibition pattern was determined using the Lineweaver-Burk kinetics model. A double-reciprocal plot was generated from luminescence data and secondary plots were used to calculate the Ki for each peptide.

5.3.9 Angiotensin converting enzyme inhibition activity

ACE inhibition activity was measured using the ACE Kit–WST assay kit (Dojindo, Laboratories). Enzymes and indicator solutions were prepared according to manufacturer instructions. Either BPI digests, or peptides were dissolved and diluted in deionized water (0.01 to 1 mg DW mL^{-1}). A 20 μL of diluted digests and peptides (0.01 to 1 mg DW mL^{-1}), or blanks, were added to a 96 well microplate. A 20 μL of substrate buffer, deionized water, and enzyme working solution was subsequently added to each well and incubated at 37°C for 1 h. A 200 μL indicator solution was then added and the plate incubated for 10 min at room temperature before being read in a microplate reader at 450 nm. IC_{50} values were calculated using Graph Pad Prism 4.0.

5.3.10 α-Glucosidase inhibition

For the α-glucosidase assay, in a 96-well plate, 50 μL of protein digest, peptides (1 mg DW mL^{-1}), or positive control (1 mmol L^{-1} acarbose) were added to 100 µL of 1 U mL^{-1} α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 μL aliquot of a 5 mM p-nitrophenyl-α-D-glucopyranoside solution (in 0.1 mol L^{-1} sodium phosphate buffer pH 6.9) was added to each well and incubated at 25°C for 5 min before the absorbance was read at 405 nm. Results are presented as percent of inhibition per mg of dry weight (% inh mg^{-1} DW).

5.3.11 Computational docking

Docking calculations were carried out through molecular docking according to a methodology described previously [23] using on dipeptidyl peptidase IV (1RWQ), angiotensin converting enzyme (1UZE), and α-glucosidase (3AJ7) protein crystal structures. Codes in brackets refer to protein data bank entries.

5.3.12Statistical analysis

The experiments were repeated at least three times with consistent results. Data were expressed as the mean ± standard deviation. The data obtained were analyzed using one-way ANOVA to compare experimental to control values (StatGraphics plus 5.0). Comparisons between groups were performed using LSD test, and differences were considered significant at $p < 0.05$. Pearson's r correlation coefficients were determined using GraphPad® Prism version 6.

5.4 Results and discussion

5.4.1 Physicochemical characteristics of identified peptides from proteins isolate of bean cultivars

BPIs and their protein digests from the four common bean cultivars (Figure 7A) showed a characteristic protein profile. Main proteins identified in the protein isolates were phaseolin (40 kDa), lectin (29 kDa) and α-amylase inhibitor (25 kDa) (Figure 7B). After digestion with pepsin/pancreatin, phaseolin remained almost unhydrolyzed. Phaseolin has been reported to have high resistance to proteolysis due to its globular and glycosylated structure [24]. Figure 7C presents the sequence of 22 peptides from black-Otomi, BRS-Pontal, BRS-Horizonte and Perola bean cultivars, and their biological potential to inhibit enzymes related to chronic diseases. From the results obtained on the identity of the peptides and their predicted biological activities, four peptides (KKSSG, KTYGL, GGGLHK and CPGNK) with the highest potential to inhibit DPP-IV, ACE and α-glucosidase enzymes were selected to be synthesized and evaluated as pure peptides (Figure 8). From the peptide database, the biological activities were attributed to small amino acid sequences within each peptide. It was discovered that the main reported bioactivities were as DPP-IV inhibitors, ACE inhibitors, followed by AC. Table 4 presents the peptide sequences, identified in the different cultivars by MALDI-TOF-MS/MS, of less than 1 kDa peaks; peptides sequences scored ≥ 75 in the software library database (BioTools Flex analysis) and only from bean protein sources identified using the NCBI blast database. The numbers 1-4 indicate cultivar source of that specific peptide; for instance: 1, Black Otomi; 2, BRS-Perola; 3, BRS-Horizonte; 4, Pontal. Table 4 also presents the *in silico* results, based on the interactions of bean peptides with enzymes DPP-IV, ACE, and α-glucosidase, their binding inhibition constants (Ki) and their physicochemical properties. Similar physical properties were found among peptides; for instance, CPGNK, pI = 8.76, net charge = +1 and hydrophobicity of 12.82 kcal mol^{-1}; KTYGL, pI= 9.78, net charge = +1 and hydrophobicity = 10.14 kcal mol^{-1}; KKSSG, pI = 10.65, net charge = +2 and hydrophobicity of 15.57 kcal mol^{-1} and GGGLHK, pI = 8.9, net charge = +1 and hydrophobicity of 15.23 kcal mol^{-1}. According to the research conducted in this field, peptides with hydrophobicity ≤ 20 kcal mol^{-1} are candidates for penetrating the cell membrane and exerting their biological function [25].

5.4.2 Antioxidant capacity of bean proteins isolate digests and pure peptides

The AC of protein digests and pure peptides can be observed in Table 5. Bean protein isolate digests and bean pure peptides, except for the peptide KKSSG, exerted AC. The results in the present study were lower than previous reports of common bean protein hydrolysates obtained with diverse enzymes [26]. Comparing the bean digests values to a gallic acid control,

its AC value (220.30 mmol L^{-1} TE g^{-1} DW) was lower than common bean digests (245.26 - 273.85 mM TE g^{-1} DW) and peptide KTYGL (249.39 mmol L^{-1} TE g^{-1} DW). Pure peptides had lower AC than the complete BPI digests. The peptide KKSSG did not present AC; this was expected since there are several reports indicating that most of the antiradical properties of peptides rely on the presence of hydrophobic amino acids, which were not present in this peptide. AC measurements were performed, using 1 mg of pure peptide or 1 mg of bean protein isolate digest on a dry weight bases. The concentration of the sequenced pure peptides represented less than 10% of total peptides in the whole hydrolysate. The comparison between pure peptides or protein hydrolysate was done using the same amount of material during the experiments. According to the literature, an increase in the concentration of small peptides also increases the probability of finding amino acid residues that enhanced the antioxidant capacity of the hydrolysates [15].

The consumption of compounds with AC directly correlates with a reduction in the concentration of reactive oxygen species (ROS), which in turns is related to inflammation inhibition. Also, there is a relationship among the molecular signaling of inflammation with hypertension and diabetes [27]. Therefore, in addition to the DPP-IV and ACE inhibiting properties of the BPI digests and peptides, they might be able to improve the balance of ROS and potentially contribute to the management of both diseases. Furthermore, antioxidants are able to ameliorate diabetes complications through different mechanisms such as reduction of oxidized low density lipoproteins [28], known also to trigger hypertension [29].

5.4.3 Dipeptidyl peptidase IV inhibition by bean protein isolate digests and pure peptides: biochemical and molecular docking

The results for DPP-IV inhibitory activity of bean digests and pure peptides are presented in Table 5. Black Otomi bean digest was the most effective at inhibiting DPP-IV with IC_{50} = 0.14 mg DW mL^{-1}, whereas BRS-Horizonte, BRS-Pontal and Perola had IC_{50} = 0.19, 0.30 and 0.33 mg DW mL^{-1}, respectively. These values were lower, therefore more potent, than those reported by Velarde-Salcedo et al. [30] using hydrolysates from black bean protein, and higher than the IC_{50} reported by Mojica et al. [31] in raw Navy beans (0.095 mg DW mL^{-1}). Higher inhibition potential, in the latter study, could be due to the presence of phenolic compounds in the digests since bean coats were not removed before protein extraction. The pure peptides presented IC_{50} values ranging from 0.03 to 0.87 mg DW mL^{-1}. Diprotin A (a known DPP-IV

inhibitor used as a positive control) is a tripeptide with proline in N-2 position and it was a more efficient inhibitor (IC_{50}= 0.02 mg DW mL^{-1}) than the BPI digests and pure peptides; however, it was not statistically different ($p > 0.05$) than peptide KTYGL, IC_{50} (0.03 mg DW mL^{-1}). These values are similar to data obtained previously, 0.06 µmol L^{-1} (~0.024 mg/mL) using sitagliptin, a commercial antihyperglycemic agent [32]. To the best of our knowledge, this is the first report of pure peptides from common bean inhibiting DPP-IV; the results obtained suggested that common bean protein is a good source of peptides with anti-diabetes potential.

The molecular docking analysis showed that bean peptides were able to interact with several amino acid residues of the DPP-IV binding site. All of the peptides studied had predicted energy binding values ranging from -9.7 to -3.1 kcal mol^{-1}. DPP-IV active structure exists as a dimer with two domains forming a β-propeller domain with a 30–45 Å cavity between each monomer where the inhibitors bind next to the catalytic site [33], therefore, it is more likely for smaller peptides to access the binding site. The stereochemistry appears to play a role in the biological activity of the peptides [34]. The pure peptides, originally identified in common bean BPI digests, were able to interact with several binding site residues of the enzyme; therefore, they may be able to pose in the large cavity, blocking access to the substrate. The interactions between DPP-IV and common bean peptides were mainly H-bond, hydrophobic, polar and cation π bonds, as observed in Figure 9A. Figure 10A-E presents the inhibition kinetics (Lineweaver-Burk plots) and secondary plots (used to calculate Ki) [35,36] for pure peptides KTYGL, KKSSG, CPGNK, GGGHLK and Diprotein A. The peptide GGGHLK presented the lowest experimental Ki value (3.07 mmol L^{-1}); the other evaluated peptides had the following values: 3.55 mmol L^{-1} for CPGNK, 6.00 mmol L^{-1} for KKSSG, and 7.86 mmol L^{-1} for KTYGL.

It is considered that the lower the Ki value, the more potent the compound is as an inhibitor. It has been found in *in vivo* studies that if the inhibitor is found in plasma at a concentration greater than the Ki, then the interaction is likely to occur [37]. From the Lineweaver-Burk plots of DPP-IV inhibition kinetics, bean peptides inhibited the enzyme in a competitive manner by interacting in the catalytic site of the enzyme [38]. These results agree with our molecular docking findings, which showed how the pure peptides interacted with amino acid residues within the catalytic site of the enzyme.

5.4.4 Angiotensin converting enzyme inhibition by bean proteins isolate digests and pure peptides: biochemical and molecular docking

Among the cultivars tested, black Otomi had the highest inhibitory activity on ACE with IC_{50} value of 0.29 mg DW mL^{-1}, compared to Perola, BRS-Horizonte and BRS-Pontal, having IC_{50} value of 0.37, 0.43 and 0.45 mg DW mL^{-1}, respectively (Table 5). These results are in accordance with those previously reported [39] for common bean protein digests generated with pepsin-pancreatin, where IC_{50} values ranged from 0.15 to 0.77 mg DW mL^{-1} and were lower than those reported for hydrolysates obtained from hard-to-cook common beans with IC_{50} values of 6.5 mg DW mL^{-1} [26]. Regarding the inhibitory effects of pure peptides, IC_{50} values ranged from 0.09 to 0.99 mg DW mL^{-1}. Peptide Diprotin A was used as a positive control for ACE inhibition (IC_{50} = 0.05 mg DW mL^{-1}). Lam et al. [40] reported IC_{50} values of 6.87 nM (~1.42 ng mL^{-1}) for captopril using the same method.

Most of the peptides studied had free energy values for ACE ranging from -10 to -7 kcal mol^{-1}, except peptides with more than six amino acids (Table 2). In a study using Jamapa black bean hydrolysates, peptide fractions with molecular mass lower than 1 kDa, produced by ultrafiltration, presented the lowest IC_{50} values compared to higher molecular mass fractions [41]. The active sites of ACE are protected by an N-terminal lid that blocks the access of large polypeptides [42]; therefore, small peptides are more effective in inhibiting ACE activity. The most stabilized pose of the peptide bonds with ACE were obtained from computational docking, and an example can be observed in Figure 9B. This indicates that the peptides were able to inhibit ACE activity mainly through interaction forces involving hydrogen bonds, hydrophobic, polar and cation π bonds. The main interactions of the peptides were with the catalytic cavity with 8 to 12 amino acid residues depending on the peptide, out of the total 38 amino acids comprising the catalytic cavity, and particularly with His353, known to play an important role in ACE activity. Zinc is essential for the activity of ACE, which binding motifs are His383, Glu384, His387 and Glu411 [43]. Molecular docking showed that pure peptides interacted with His383 and Glu384, amino acids involved with zinc binding, thus, this interaction potentially restrains ACE activity.

5.4.5 α-Glucosidase inhibition by bean protein isolate digests and pure peptides: biochemical and molecular docking

α-Glucosidase is an enzyme involved in starch digestion, so inhibition of this enzyme decreases available glucose for intestinal absorption. The inhibition of α-glucosidase is used as a molecular target in the treatment of type-2 diabetes. No statistical differences were found among cultivars on the potential to inhibit α-glucosidase (Table 5). In general, pure peptide KKSSG (49.3% inh mg^{-1} DW) showed higher inhibition than KTYGL (36.3% inh mg^{-1} DW), $p < 0.05$. Inhibition values were lower than the ones reported by Mojica et al. [31] in protein hydrolysates from raw samples (60% - 70% inhibition); they used whole samples and some remnant phenolic compounds from bean coats could have contributed to the higher enzyme inhibition.

Molecular docking demonstrated that all the pure peptides studied docked into the binding pocket of α-glucosidase (Fig. 3C). It was observed that peptides were able to inhibit the enzyme mainly through polar interactions (Asn32, Asp34, Asp38 and Asp 89), hydrophobic interactions (Trp36 and Trp81) and hydrogen bonds (Ser31) with the residues from the binding pocket of the enzyme. There was a positive correlation of the docking analysis and the biochemical assays (r= 0.77 and 0.93) for both ACE and DPP-IV, respectively, confirming that molecular docking is an important tool that can save time and money in the screening of the biological potential of compounds. Regarding α-glucosidase inhibition using docking analysis, a negative correlation with DPP-IV inhibition using docking and biochemical assays (r=-0.82 and -0.97, respectively) was found; this means that higher α-glucosidase inhibition correlated with the potent (lower IC_{50}) inhibitors of DPP-IV (Table 5).

Based on our previous results [44,45,46] on the biological potential of peptides from improved common bean cultivars, we selected four cultivars to further the investigation using as important criteria their protein and peptide profiles. Black Otomi, BRS-Horizonte, BRS-Pontal and Perola presented good balance between agronomical related defense proteins and total proteins. Moreover, carioca bean and black bean are widely consumed in Brazil and Mexico, respectively. These common bean cultivars present potential to inhibit enzymes related to hypertension and type-2 diabetes.

5.5 Conclusions

The properties of protein isolate digests and peptides evaluated in this study support the hypothesis of the potential benefits of common bean in health; the peptides present in common bean proteins could help to promote antioxidant/oxidant homeostasis. Besides, they can also inhibit enzymes which increased activity is related with non-communicable diseases and in

general, with metabolic disorders. An improvement in redox homeostasis, combined with the diabetes-ameliorating effects of DPP-IV, the antihypertensive effect of ACE inhibition, and the α-glucosidase inhibition, can potentially improve the lifestyle of the type 2-diabetic patient. Therefore, consumption of common bean protein could represent an option of dietary supplement in the management of diabetes. To the best of our knowledge, this is the first report of the potential of pure peptides from common bean protein isolates to inhibit the diabetes-related enzymes DPP-IV and α-glucosidase.

This study demonstrated significant antioxidant, anti-diabetes and antihypertensive potential properties from common bean protein isolate after simulated gastrointestinal digestion, and their pure peptides. Bean peptides were able to interact with amino acid residues in the catalytic site of the tested enzymes. Future *in vivo* studies in our laboratory will contribute to determining the potential of common bean peptides in the management of type-2 diabetes and other health complications derived from redox homeostasis such as hypertension.

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5.7 Tables and figures

Table 4. ***In silico*** **predicted interaction values of peptides from common bean with ACE, DPP-IV and α-glucosidase enzymes and their physical properties.**

Peptide sequence	ACE predicted binding energy (kcalmol^{-1})	ACE predicted Ki (mM)	DPP-IV predicted binding energy (kcalmol^{-1})	DPP-IV predicted Ki (mM)	α-Glu predicted binding energy (kcalmol^{-1})	α-Glu predicted Ki (mM)	Amino acid Sequence Length	MM	pI	Net Charge	Hydrophobicity kcalmol $^{-1}$
SGSYS[3]	-7.98	1.42	-7.13	5.91	-6.47	0.018	5	499.2	5.37	0	9.72
GGNEGA[2]	-8.08	1.20	-6.53	16.4	-4.89	0.259	6	503.2	3.21	-1	16.33
GGDEAG[3]	-7.49	3.24	-6.32	23.53	-5.25	0.141	6	504.2	2.99	-2	19.12
GHVPP[4]	-9.25	0.16	-8.00	1.36	-6.86	0.009	5	505.3	8.31	0	11.2
KKSSG[1]	**-9.63**	**0.08**	**-9.58**	**0.09**	**-6.76**	**0.011**	**5**	**505.3**	**10.65**	**2**	**15.57**
CPGNK[2,3]	**-9.53**	**0.10**	**-8.41**	**0.68**	**-7.22**	**0.005**	**5**	**517.2**	**8.67**	**1**	**12.82**
VGTNK[3]	-7.09	6.34	-5.54	86.57	-4.63	0.404	5	517.3	9.80	1	12.49
LSGVF[3]	-9.63	3.97	-4.38	0.61	-4.62	0.409	5	521.3	5.52	0	6.09
NPYM[4]	-8.2	0.97	-6.02	38.87	-5.68	0.069	4	523.2	5.34	0	7.51
NPSLP[2]	-7.72	2.19	-6.21	27.87	-5.66	0.071	5	526.3	5.44	0	8.24
TACKD[1]	-8.33	0.78	-6.08	34.95	-5.41	0.109	5	536.2	6.08	0	15.07
CGPHGA[4]	-8.07	1.21	-6.16	30.55	-7.96	0.001	6	540.2	7.17	0	13.15
GGGLHK[4]	**-8.56**	**0.52**	**-9.22**	**0.17**	**-6.54**	**0.016**	**6**	**567.3**	**9.80**	**1**	**15.23**
KTYGL[3]	**-10.28**	**0.02**	**-9.73**	**0.07**	**-5.87**	**0.049**	**5**	**580.3**	**9.78**	**1**	**10.14**
LSFNT[1]	-6.66	13.02	-6.75	11.29	-6.94	0.008	5	580.3	5.47	0	6.50
KGPASK[2]	-7.55	2.93	-5.78	58.11	-5.96	0.043	6	586.3	10.57	2	15.75
RTLNL[3]	-7.36	4.02	-6.43	19.34	-5.89	0.048	5	615.4	11.11	1	8.31
MPHLK[3]	-9.24	0.16	-7.83	1.82	-7.82	0.002	5	624.3	9.80	1	11.25
VKFMT[1]	-9.76	0.06	-7.63	2.53	-7.66	0.002	5	624.3	9.82	1	8.11
KYMKS[4]	-7.94	1.51	-6.14	31.31	-6.77	0.011	5	655.3	10.14	2	12.58
KMARPV[1]	-9.18	0.18	-8.50	0.58	-6.93	0.008	6	700.4	11.50	2	12.02

Numbers[1-4] indicate cultivar source: [1], Black Otomi; [2], BRS-Perola; [3], BRS-Horizonte; [4], Pontal; Docking calculations were carried out using Docking Server tool. Physical properties were determined using Pepdraw tool; ACE, Angiotensin converting enzyme; Ki, Constant of inhibition; DPP-IV, Dipeptidyl peptidase-IV; MM, molecular mass; mM, mmol per liter, Da, Dalton; pI, isoelectric point; Net C, Net charge; α-Glu, α-glucosidase; Amino acid nomenclature: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

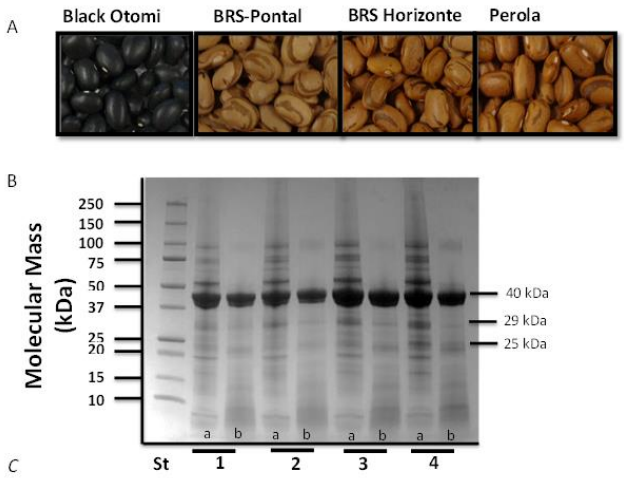
Table 5. Antioxidant capacity, inhibition of DPP-IV, ACE and α-glucosidase from common bean hydrolysates and pure peptides.

Hydrolysate/ peptides	ORAC mM TE g^{-1} DW	IC_{50} (mg DW mL^{-1})		α-Glucosidase % inh mg^{-1} DW
		DPP-IV	ACE	
Bean protein hydrolysates				
Black-Otomi	246.74±5.17c	0.14±0.01bc	0.29±0.05c	46.90±7.10ab
BRS-Horizonte	245.26±3.79c	0.19±0.03c	0.43±0.07de	50.10±5.30b
Perola	250.67±1.70cd	0.33±0.03d	0.37±0.03d	49.50±8.40b
BRS-Pontal	273.85±0.76d	0.30±0.01d	0.45±0.05e	48.00±7.60ab
Bean peptides				
KTYGL	249.39±0.84cd	0.03±0.00a	0.09±0.01a	36.30±8.80a
KKSSG	No AC	0.64±0.16e	0.20±0.02b	49.34±6.50b
CPGNK	131.31±17.06a	0.87±0.02f	0.18±0.06b	37.60±6.80ab
GGGLHK	115.83±20.92a	0.61±0.10e	0.99±0.01f	46.10±8.30ab
Diprotin A	No AC	0.02±0.00a	0.05±0.00a	-
Gallic acid	220.30±2.70b	-	-	-

	DPP-IV docking	DPP-IV biochemical	ACE docking	ACE biochemical	α-glucosidase docking
DPP-IV docking	1	**0.77**	0.33	0.067	**-0.82**
DPP-IV biochemical		1	0.57	0.23	**-0.97**
ACE docking			1	**0.93**	-0.39
ACE biochemical				1	-0.03
α-glu docking					1
α-glu biochemical					

Values represent means (n = 3), and different letters within a column represent significant differences ($p < 0.05$). IC_{50}: Amount needed to decrease the activity of the enzyme by 50% of its original activity. ACE, Angiotensin converting enzyme; DPP-IV, Dipeptidyl peptidase-IV; TE, trolox equivalents; DW, dry weight; inh, inhibition. -, experiments were not performed.

Bold numbers indicate Pearson's r correlation coefficients statistically different ($p<0.05$) between parameters for all bean cultivars.



cultivar	Sequence [1]	Biological potential	Bioactive sequence	Protein source in beans
Black Otomi	KKSSG	DPP-IV and ACE inh	SG,KK,KS	Bowman-Birk inhibitor
	TACKD	Antioxidant, DPP-IV inh	KD,TA	Vacuolar-sorting receptor
	LSFNT	DPP-IV and ACE inh	SF,FN,NT	Arcelin
	VKFMT	ACE inh, CaMPDE inh, Renin inh, DPP-IV inh	VK,KF	Zinc finger CHC2-family protein
	KMARPV	DPP-IV and ACE inh	RP,AR,MA,PV	Cyclophilin
BRS-Pontal	GHVPP	DPP-IV and ACE inh	PP,VP,VPP,GH,HV	Peroxiredoxin
	NPYM	DPP-IV inh	NP,PY,YM	60S ribosomal protein L17
	CGPHGA	DPP-IV and ACE inh, antitoxidant, antithrombotic	GP,GA,GG,HG, PH,PHG	Auxin-responsive protein
	GGGLHK	Antioxidant, DPP-IV and ACE inh	LH,LHK,GL,GG, HK	Homoglutathione synthetase
	KYMKS	DPP-IV and ACE inh	KY,KS,MK,YM	Cytochrome P450
BRS-Horizonte	SGSYS	DPP-IV and ACE inh	GS,SY	Dehydration-induced-like protein
	GGDEAG	DPP-IV and ACE inh	AG,GG,GD,EA	Peptide chain release factor
	VGTNK	DPP-IV and ACE inh	VG,GT,NK,TN	NADP-dependent malic enzyme
	CPGNK	DPP-IV and ACE inh, antiamnestic, antithrombotic	PG,NK	Arcelin
	LSGVF	DPP-IV and ACE inh	GV,NK,VF,SG	Early-responsive to dehydration
	KTYGL	DPP-IV and ACE inh, antioxidant	YG,YGL,GL,TY, KT,	Putative resistance protein TIR 34
	RTLNL	DPP-IV and ACE inh	LN,NL,TL	Polygalacturonase inhibiting protein
	MPHLK	ACE and DPP-IV inh, antioxidant	HL,PH,MP,PH, PHL,LK,	Phenylalanine ammonia-lyase class III
Perola	GGNEGA	DPP-IV and ACE inh	GA,GG,EG,NE	Leucine-rich repeat receptor-like protein
	CPGNK	DPP-IV and ACE inh, antiamnestic, antithrombotic	PG,NK,	Arcelin
	NPSLP	DPP-IV inh	LP,NP,SL,PS	Phosphatase
	KGPASK	DPP-IV and ACE inh, antiamnestic, antithrombotic	GP,PA,GPA,KG, SK,AS,	Granule bound starch synthase

Figure 7. A. Common bean cultivars Black-Otomi from Mexico, and BRS Pontal, BRS Horizonte and Perola from Brazil. B. SDS-PAGE of common bean protein profiles and their protein hydrolysate profile after simulated gastrointestinal digestion for each cultivar. a, protein before hydrolysis; b, protein after hydrolysis; 1, Black-Otomi; 2, BRS-Perola; 3, BRS-Horizonte; 4, Pontal; Std, molecular mass standard; protein identification and molecular mass from literature: Montoya et al. [20]; Rui et al. [43]; Carrasco-Castilla et al. [44]. C. Sequenced peptides from common bean hydrolysates and their potential biological function. [1]Peptides sequences obtained from MALDI-TOF-MS/MS of less than 1 kDa peaks; peptides sequences scored ≥ 75 software library database (BioTools Flex analysis). Potential biological activity was identified in BIOPEP database [18 January 2016]. Protein source of peptides was identified using the NCBI blast database [18 January 2016]; bioactive sequence, amino acids within the peptide sequence that have been reported with biological potential; ACE, angiotensin converting enzyme; DPP-IV, dipeptidyl peptidase IV; inh: inhibitor; BRS, Brazilian; amino acid nomenclature: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

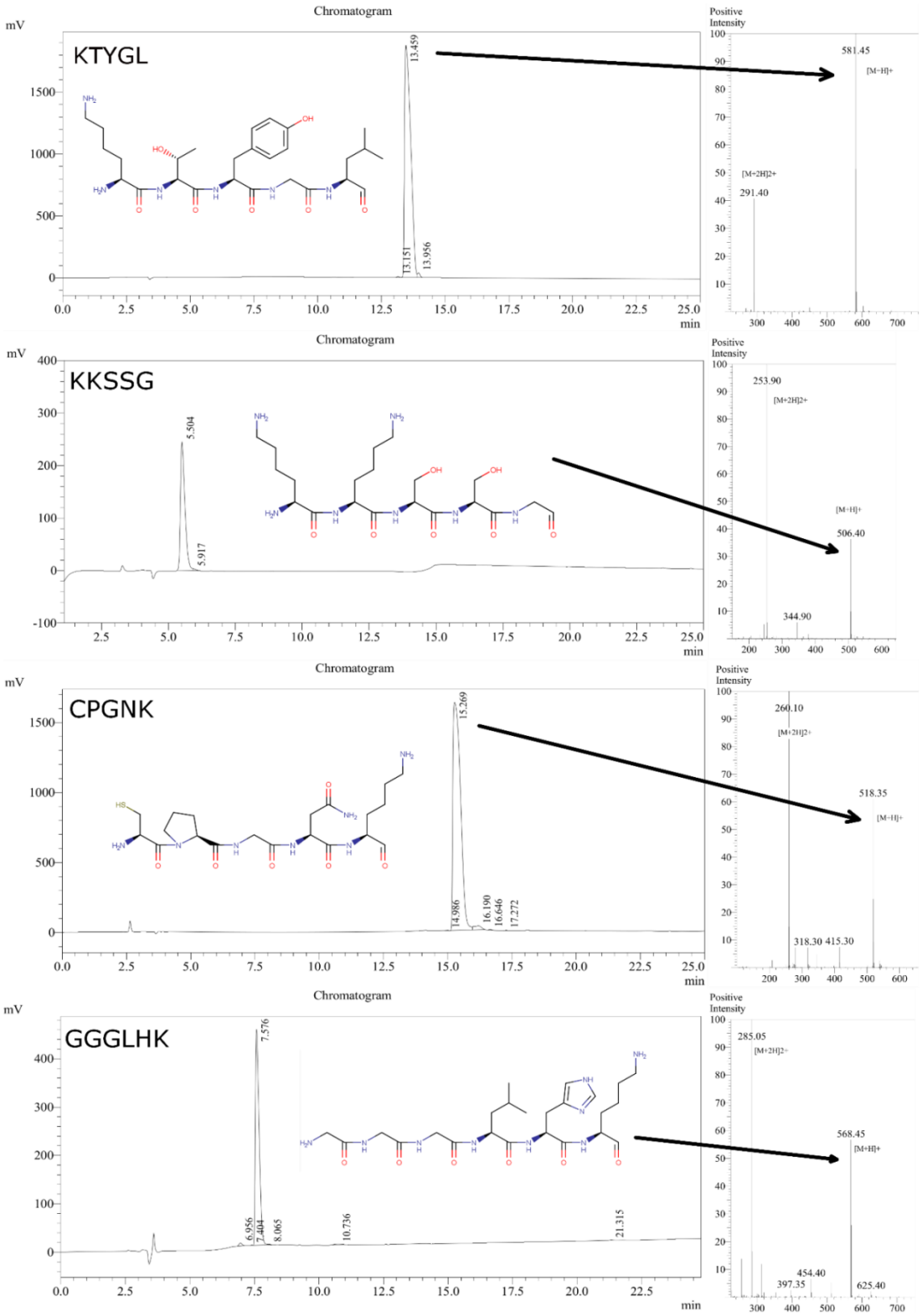


Figure 8. Representative chromatograms of high pressure liquid chromatography and MS/MS ions of the sequenced pure peptides, respectively. A, *A'*. KKSGG. B, *B'*. CPGNK. C, *C'*. GGGLHK. D, *D'*. KTYGL.

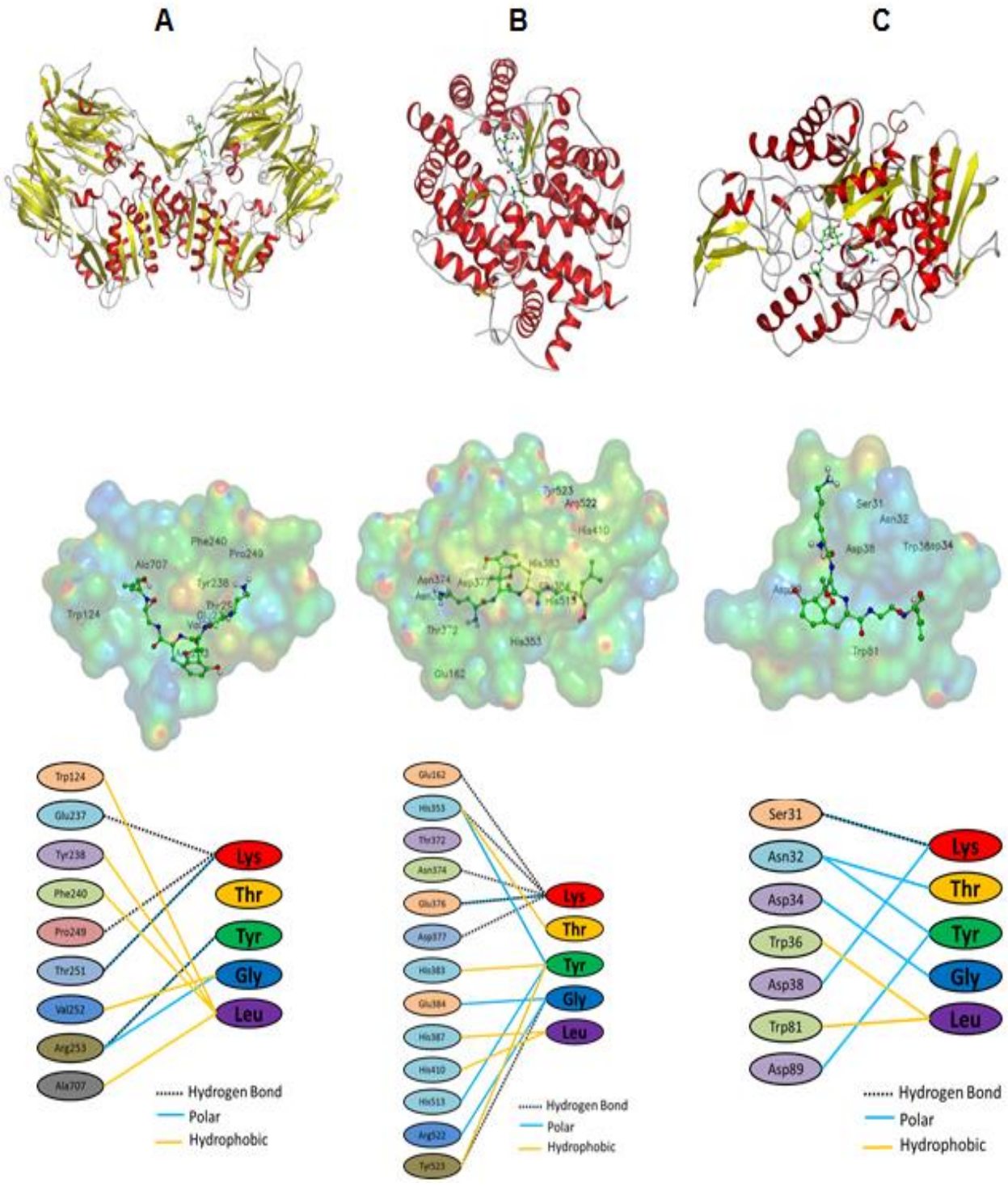


Figure 9. Molecular docking diagrams: example of peptide KTYGL interacting in the catalytic site of targered enzymes. A. Dipeptidl peptidase IV. B. Angiotensin converting enzyme. C. α-Glucosidase.

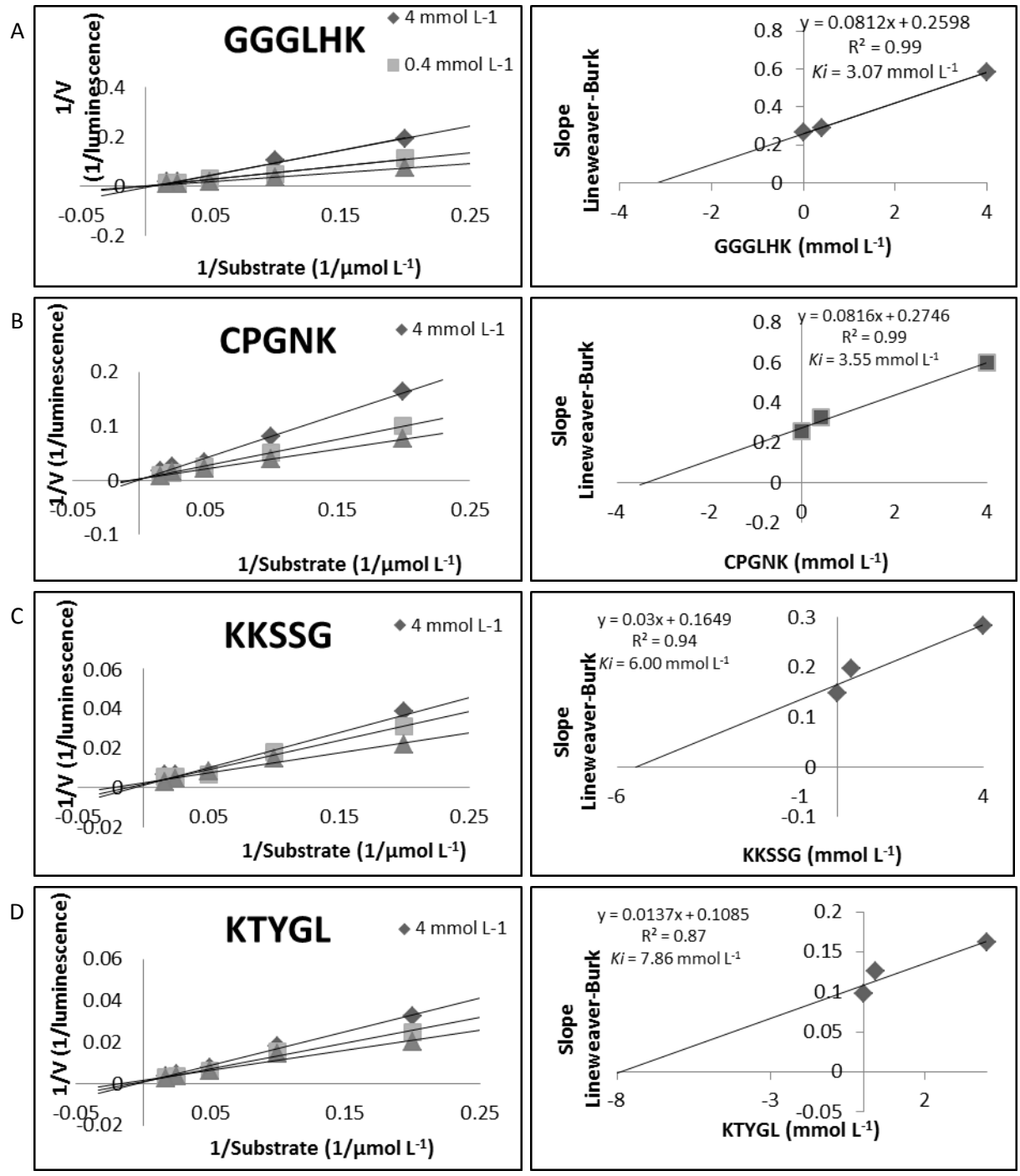


Figure 10. Enzyme inhibition kinetics for DPP-IV showing Lineweaver-Burk and secondary plots to calculate inhibition constants (Ki) of each peptide. A. GGGLHK; B. CPGNK; C. KKSSG; D. KTYGL.

CHAPTER 6: IMPACT OF COMMERCIAL PRECOOKING OF COMMON BEAN (*Phaseolus vulgaris L*) ON THE GENERATION OF PEPTIDES, AFTER PEPSIN–PANCREATIN HYDROLYSIS, CAPABLE TO INHIBIT DIPEPTIDYL PEPTIDASE-IV

6.1 Abstract

The objective of this research was to determine the bioactive properties of the released peptides from commercially available precook common beans (*Phaseolus vulgaris*). Bioactive properties and peptide profiles were evaluated in protein hydrolysates of raw and commercially precooked common beans. Five varieties (Black, Pinto, Red, Navy, and Great Northern) were selected for protein extraction, protein and peptide molecular mass profiles, and peptide sequences. Potential bioactivities of hydrolysates, including antioxidant capacity and inhibition of α-amylase, α-glucosidase, dipeptidyl peptidase-IV (DPP-IV), and angiotensin converting enzyme I (ACE) were analyzed after digestion with pepsin/pancreatin. Hydrolysates from Navy beans were the most potent inhibitors of DPP-IV with no statistical differences between precooked and raw (IC_{50} = 0.093 and 0.095 mg protein/mL, respectively). α-Amylase inhibition was higher for raw Red, Navy and Great Northern beans (36%, 31% and 27% relative to acarbose (relac)/mg protein, respectively). α-Glucosidase inhibition among all bean hydrolysates did not show significant differences; however, inhibition values were above 40% rel ac/mg protein. IC_{50} values for ACE were not significantly different among all bean hydrolysates (range 0.20 to 0.34 mg protein/mL), except for Red bean that presented higher IC_{50} values. Peptide molecular mass profile ranged from 500 to 3000 Da. A total of 11 and 17 biologically active peptide sequences were identified in raw and precooked beans, respectively. Peptide sequences YAGGS and YAAGS from raw Great Northern and precooked Pinto showed similar amino acid sequences and same potential ACE inhibition activity. Processing did not affect the bioactive properties of released peptides from precooked beans. Commercially precooked beans could contribute to the intake of bioactive peptides and promote health.

This chapter is part of the publication: Mojica L, Chen K, de Mejia EG (2014) Impact of Commercial Precooking of Common Bean (*Phaseolus vulgaris*) on the Generation of Peptides, After Pepsin-Pancreatin Hydrolysis, Capable to Inhibit Dipeptidyl Peptidase-IV. J Food Sci 80:(1) H188-H198. License Number: 3785950887461. Permission granted by John Wiley and Sons CCC.

6.2 Introduction

Common bean (*Phaseolus vulgaris*) is one of the most agronomical adaptable, economical and available legume worldwide [1]. It is especially important as a staple food in developing countries where availability of animal protein is low. Common beans provide adequate nutrition with its relatively high-quality carbohydrates [2] and proteins [3]. *P. vulgaris* cultivars have also been linked with several health benefits [4,5] such as reduction of risk of diabetes and cardiovascular disease attributed to the presence of polyphenols and bioactive peptides [6,7].

As consumer demands for precooked products increase, common beans are often subjected to processing aimed to increase stability during distribution and storage and for convenience processing methods such as extrusion and ultrasound could improve digestibility of common beans by decreasing antinutritional components and inducing protein denaturation [7]. Thermal processing promotes protein denaturation on common beans and increases exposed cleavage sites to enzymes [8]. This also improves digestibility by allowing gastrointestinal enzymes such as pepsin and pancreatin to work more efficiently by increased interaction between enzyme and denatured proteins [9]. Increases in the degree of protein hydrolysis may directly influence availability of bioactive peptides generated after digestion. Bioactive peptides may have antioxidant capacity when released from the native protein [10]. The presence of bioactive peptides can decrease the rate of oxidation and increase shelf life and stability, contributing to the overall quality of the product [11].

Angiotensin converting enzyme I (ACE) is largely responsible for regulating blood pressure by converting angiotensin I to angiotensin II, a vasoconstrictor [12]. Dipeptidyl peptidase-IV (DPP-IV), α-amylase, and α-glucosidase are enzymes related with glucose metabolism; DPP-IV inhibitors have been used as treatment for type-2 diabetes because its inhibition aids in the control of glucose homeostasis [13,14]. α-Amylase and α-glucosidase digest starches releasing free available sugars, thus, inhibition of these enzymes decreases available glucose for intestinal absorption and is therefore used in type-2 diabetes treatment [15,16].

Research on common bean proteins have been focused on the antioxidant capacity of hydrolysates and their potential for ACE inhibition, metal chelation, and NF-κB suppressor activity [17-20], antitumor activity [21,4,5], antihypertensive properties [20,22,23], antioxidant

capacity [7,19,24]; and antifungal activity [25-28]. However, the novelty of this scientific research was to discover, for the 1st time, the biological potential of peptides from commercially precooked common bean hydrolysates on enzymes related to type-2 diabetes as DPP-IV as well as provide information about functional properties of commercially available processed products.

Therefore, the objective of this study was to determine the impact of precooking of commercially available common beans and compare the bioactive properties of the released peptides, after simulated gastrointestinal digestion, from 5 varieties (Black, Pinto, Red, Navy, and Great Northern). Bioactive properties and peptide profiles were evaluated in protein hydrolysates of raw and commercially precooked common beans. Selected bean hydrolysates were compared based on their protein concentration, protein and peptide molecular mass profiles; also, peptide sequences were discovered that presented antioxidant capacity and inhibition of *α*-amylase, *α*-glucosidase, DPP-IV, and ACE. This manuscript provides, for the 1st time, the discovery of peptides from precooked commercial products and the biological activity, of their pepsin–pancreatin hydrolysates, based on markers related to type-2 diabetes.

6.3 Materials and methods

6.3.1 Materials

Raw and precooked varieties of *P. vulgaris L.* were studied including Black, Pinto, Red, Navy, and Great Northern (Figure 11A). These raw and processed common bean samples were provided by the Archer Daniels Midland Co. (ADM, Decatur, Ill., U.S.A.). In this particular study, the term "precooked" refers to the bean samples that were processed including blanching, high pressure and thermal cooking, and finally oven dry. This precooking process decreased time required for home preparation of beans; requiring only boiling the beans during 10 to 15 min. Precooked samples were prepared by boiling them for 15 min as stated on the instructions, before protein extraction. Porcine pepsin (EC 3.4.23.1), pancreatin (8xUSP, a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas, EC 232-468-9), porcine DPP-IV (D7052, EC 3.4.14.5), *α*-amylase (EC 3.2.1.1), and *α*-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). DPPIV-Glo protease assay kit was purchased from Promega (Madison, Wis., U.S.A.). Angiotensin converting enzyme assay (ACE KIT-WST) was purchased from Dojindo (Rockville, Md., U.S.A.). Precast (4% to 20%) gradient polyacrylamide Tris–HCl gels were from Bio-Rad Laboratories (Hercules, Calif., U.S.A.). DC protein assay from Bio-Rad Laboratories.

Molecular mass protein standards (10 to 250 kDa) and SimplyBlue Safe Stain were from Amersham Pharmacia Biotech (Carlsbad, Calif., U.S.A.).

6.3.2 Protein isolates extraction

Bean samples were ground in a commercial blender in a 1:10 bean/water ratio. The pH was adjusted to 8.0 with 0.1 M NaOH and the protein extraction was carried out at 35 °C with stirring for 1 h. The mixture was centrifuged at 5000 *g* for 15 min at 4 °C. The precipitate was re-extracted under identical conditions to maximize yield and both extracts were combined. Then, the pH was adjusted to 4.3 with HCl to precipitate proteins, followed by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant was discarded and the pellet was freeze-dried in a Lab Conco Freeze Dryer 4.5 (Kansas, Mo., and U.S.A.). Dried common bean protein isolates (BPI) were stored at −20 °C until further analysis.

6.3.3 Simulated gastrointestinal digestion

In vitro simulated gastrointestinal digestion of BPI was performed following the procedure by Megıas *et al.* [29] with modifications. Briefly, BPI was suspended in water (1:20 w/v) and a sequential enzyme digestion was carried out with pepsin/substrate 1:20 (w/w) (pH 2.0) followed by pancreatin /substrate 1:20 (w/w); pH 7.5 at 37 °C for 2 h each. The hydrolysis was stopped by heating at 75 °C for 20 min, and the resulting BPI hydrolysates (BPIH) were centrifuged at 20000 *g* for 15 min at 4 °C. Bean protein hydrolysates were dialyzed to eliminate salts using a 500-Da molecular weight cutoff membrane and then freeze-dried in a Lab Conco FreeZone Freeze dry system. Hydrolysates were stored at −20 °C until analysis.

6.3.4 Gel electrophoresis analysis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis [SDS–PAGE])

The freeze-dried BPI of the 5 bean types tested and their BPIH were analyzed by SDS–PAGE, which was carried out under reducing conditions (1:20 β-mercaptoethanol, β-ME). Precast (4% to 20%) gradient polyacrylamide Tris–HCl gels were used with a Bio-Rad Criterion Cell under a constant voltage of 200 V for 35 min. Standards (10 to 250 kDa) were used to calculate molecular mass. After staining with Simply Blue Safe Stain overnight, and destaining with water, the gel was visualized using a GL 4000 Pro Imaging system (Carestream Health Inc., Rochester, N.Y., U.S.A.).

6.3.5 Degree of hydrolysis (DH)

DH was performed as described by Cabra et al. [30] with some modifications. DH is expressed as percentage of the dissolved protein after precipitation with 0.2Ntrichloroacetic acid (TCA), compared to the total dissolved protein (100%) obtained after complete hydrolysis with 2N sulfuric acid at 100 °C for 4 h.

6.3.6 Molecular mass peptide profile

Common bean BPIH were analyzed by matrix-laser desorption ionization–time-of-flight (MALDI–TOF). Samples were dissolved in water and mixed with 50 mg/mL 2,5-dihydroxybenzoic acid (DHB) matrix (Sigma, St. Louis, Mo., U.S.A.) in 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid in a 1:1 ratio and deposited on a standard stainless steel target. Mass spectra were collected in positive ion mode on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.4 software package (Bruker Daltonics). Following external calibration, 2000 full scan spectra were acquired from 500 to 3000 m/z. Results are shown in Table 6 as percentage of the total area of peaks within a range.

6.3.7 Characterization of the peptides and sequence identification

The peptides obtained after simulated gastrointestinal digestions (BPIH) were analyzed by high-performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–ESI–MS) using a Q-tof Ultima mass spectrometer (Waters, Milford, U.S.A.), equipped with an Alliance 2795 HPLC system. The separation of the components was performed by using a mobile phase of Solvent A (95% H2O, 5% ACN, and 0.1% formic acid) and Solvent B (95% ACN, 5% H2O, and 0.1% formic acid) in a flow rate of 200 μL/min. The elution was in a linear gradient (0 min, 90% A; 2 min, 90% A; 40 min, 65% A; 60 min, 10% A; 65 min, 10% A; 66 min, 90% A; 80 min, 90% A). The temperature was kept in 20 °C during the whole procedure. A splitter with a split ratio of 1:10 was used, where 1 part went to the mass spectrometer and 10 parts went to the waste. The Q-tof Ultima mass spectrometer was equipped with a Z-spray ion source. Using a positive ion electrospray ionization mode (+ESI), the analysis on the Q-tof was carried out in V-mode with an instrument resolution between 9000 and 10000 based on full width at half maximum, with a flow rate of 20 μL/min. The source temperature was set at 80 °C and desolvation temperatures were set at 250 °C, respectively. The Q-tof was operated at capillary voltage of 3.5 kV and a cone voltage of 35 V. The final detector was a microchannel plate with high sensitivity. The MassLynx 4.1V software (Waters, Milford, Mass., U.S.A.) was

used to control the instruments and to process the data in order to get the highest probability of the peptides sequences. Confirmation of peptides sequence in common bean proteins (*P. vulgaris L.*) was performed using BLASTR _ tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on May 12, 2014). The potential biological activity of the peptides was predicted by using BIOPEP database (http://www.uwm.edu.pl/biochemia, accessed on May 12, 2014). Peptide structures were predicted using PepDraw tool (http://www.tulane.edu/_biochem/WW/PepDraw/, accessed on May 13, 2014).

6.3.8 Antioxidant capacity (AC)

AC was measured by the oxygen radical absorbance capacity assay as described by Prior et al. [31], using 20 μL trolox standard, BPIH raw and precooked samples, or blank (75 mM phosphate buffer, pH 7.4), 120 μL of 116.9 nM fluorescein (final concentration 70 nm/well), 60 μL of 40 mM α,α'-azodiisobutyramidine dihydrochloride (AAPH) per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multiwell plate reader (Biotek Instruments, Winooski, Vt., U.S.A.). Results were expressed as millimoles Trolox equivalents per g of protein (mmol TE/g) (Johnson et al. [32]).

6.3.9 α-Amylase inhibition

For the α-amylase assay, 500 μL of BPIH raw and precooked samples, or positive control (1mMacarbose) were added to 500 μL of 13 U/mL α-amylase solution (type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 μL of 1% soluble starch solution (previously dissolved in sodium phosphate buffer and boiled for 15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color reagent was added and the tubes were placed in 100 °C water bath for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100%

6.3.10 α-Glucosidase inhibition

For the α-glucosidase assay, in a 96-well plate, 50 μL of BPIH raw and precooked samples, or positive control (1 mM acarbose) were added to 100 μL of a 1-U/mL α-glucosidase solution (in 0.1M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 μL aliquot of a 5-mM p-nitrophenyl-α-D-glucopyranoside solution (in 0.1 M sodium phosphate buffer pH 6.9) was added briefly to each well and incubated at 25 °C for 5 min before the absorbance was read

at 405 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100% inhibition.

6.3.11 ACE inhibition

Angiotensin converting enzyme inhibition activity was measured using the ACE Kit–WST (Dojindo). Enzymes and indicator solutions were prepared according to the manual and BPIH raw and precooked samples were dissolved in deionized water and diluted to a factor of 5 × 106. A 20 μL of diluted BPIH and blanks were added to a 96-well microplate. A total of 20 μL of substrate buffer, deionized water, and enzyme working solution was subsequently added to each well and incubated at 37 °C for 1 h. A 200 μL of indicator solution was then added and the plate was incubated for 10 min at room temperature before being read in a microplate reader at 450 nm. IC_{50} values were calculated using Graph Pad Prism 4.0.

6.3.12 DPP-IV inhibition

DPP-IV inhibition was measured using the DPP-IV Glo Protease Assay (G8351, Promega). A total of 50 μL of DPP-IV Glo reagent was added to a white-walled 96-well plate containing either 50 μL of blank, 40 μL enzyme control, or 40 μL BPIH samples. Samples were prepared in an assay buffer (100 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA) at different concentrations. The blank contained only assay buffer and DPP-IV Glo reagent, while the enzyme control and the samples contained assay buffer, DPP-IV Glo reagent and 10 μL purified DPP-IV human enzyme (100ng/mL). Luminescence was then measured after gentle mixing and incubation at 26°C for 30 min using a Synergy2 multiwell plate reader (Biotek Instruments). Percent inhibition was calculated from the blank and enzyme control for each sample. IC50, defined as the concentration needed to inhibit 50% of the activity of the enzyme, was calculated using Graph Pad Prism 4.0.

6.3.13 Statistical analysis

The experiments were repeated at least 3 times with consistent results. Data are expressed as the mean ± standard deviation. The data obtained were analyzed using one-way analysis of variance (ANOVA) to compare raw to precooked values (StatGraphics plus 5.0) and across samples. Comparisons between groups were performed using least significant difference (LSD) test, and differences were considered significant at $P < 0.05$.

5.4 Results and discussion

6.4.1 Protein profile for raw and precooked beans by gel electrophoresis analysis

Figure 11(A) presents the image of raw and precooked common beans selected for this study. Figure 11(B) and (C) present the protein profile for raw and precooked beans, respectively. There are 2 columns for each bean; the 1st column represents the BPI before hydrolysis and the 2nd column contains the BPI after hydrolysis (BPIH). The precooked BPIs presented bands at lower molecular mass, not well defined, compared to those of the raw beans. This could be due to the effect of the processing condition on the protein integrity [33]. On the other hand, the raw BPI presented a characteristic band at around at 40 kDa across all samples. This band corresponds to phaseolin; main storage protein in common bean [34]. In raw samples, phaseolin was not completely hydrolyzed. Processing effects, like rigorous heat and pressure, have both been shown to denature phaseolin and other related proteins in beans [35,36]. Denaturation process opened up the tertiary structure of proteins allowing greater hydrolysis resulting in an overall more complete digestion [37,33]. Based on the electrophoresis results, precooked beans also had a greater percentage of peptides with low molecular masses (500 to 700 Da) than raw counterparts.

Thermal treatment has both positive and negative effects on proteins. First, it could inactivate nonnutritional factors as lectins [38], α-amylases, and trypsin/chymotrypsin inhibitors [39,40]. It could denature proteins increasing the exposure of their hydrophobic residues opening up the protein structure; this structural modification allows gastrointestinal enzymes to reach protein sites that in native state the enzymes cannot access [33,12].On the other hand, heat treatment may promote the formation of protein aggregates resistant to hydrolysis. Tang *et al.* [41] concluded that the influence of heating on the digestibility of kidney bean proteins was related to the extent of heating to induce aggregation. Protein/polyphenols interaction also interferes with protein digestibility, decreasing the hydrolysis of phaseolin significantly [42,43]. From the biochemical point of view, higher degree of enzymatic hydrolysis represents higher production of smaller peptides that reach the catalytic site of enzymes; therefore, increasing the biological beneficial potential of protein hydrolysates [44,45].

6.4.2 Degree of Hydrolysis

Figure 12(A) depicts the comparison of the degree of hydrolysis between the BPIH from raw and precooked beans. The degree of hydrolysis showed only significant difference for processed Black and Pinto beans. The precooked beans had a greater degree of hydrolysis, over 30%; while raw varieties had degrees of hydrolysis over 20% but no greater than 40% (Figure

12A). In general, across all 5 varieties, precooked beans presented higher values for degree of hydrolysis than raw beans. Thermal processing increased the extent of hydrolysis by pepsin and pancreatin. High temperatures caused unfolding of the protein molecules [46], which could be due to an increase in kinetic energy that disrupts noncovalent and covalent interactions. Protein unfolding results in increased accessibility of the enzyme to the substrate, resulting in a higher cleavage rate [47]. Therefore, the processed bean proteins are more susceptible to enzymatic action thereby increasing their digestibility.

6.4.3 Molecular mass peptide profile from raw and precooked bean hydrolysates

The molecular mass peptide profile shown in Table 6 highly corresponds with the electrophoresis results in Figure 11(B) and (C). Low molecular mass bands were abundant in BPIH from precooked beans. The BPIH peptide profiles from precooked beans showed larger percentages of the peptides in the range 500 to 700Da in all 5 bean varieties (44% to 73%). In BPIH from raw beans, the peptides formed were more evenly distributed across molecular masses; whereas in the precooked beans the higher percentage of the peptides had molecular masses lower than 700Da or higher than 2000 kDa. This suggests that some of the proteins were digested into small peptides but a portion remained intact, as can be seen in Figure 11(B). The BPIH from raw bean proteins did not experience thermal treatment due to precooking. As a result, the structure was less reachable by enzymes and fewer cleavages occurred.

6.4.4 Peptide sequence and predicted bioactivity

Sequences of the more abundant peptides identified in BPIH from raw and precooked beans are shown in Table 7. It was possible to identify 11 peptide sequences from raw and 17 from precooked samples. *α*-Amylase and *α*-glucosidase are enzymes that can be inhibited by the peptides produced in the stomach and intestine. However, ACE and DPP-IV inhibitory peptides need to be absorbed into the enterocyte. Di- and tripeptides can be transported into the enterocyte by H+ -peptide transporter (PEPT1) [48]. The mechanism of absorption and bioavailability of bigger peptides are still under investigation. However, Dia *et al.* [49] suggest that larger bioactive peptides from legumes can be absorbed by the human gastrointestinal system. De Mejia *et al.* [50] concluded that there is evidence of how food bioactive peptides are bioavailable and can be absorbed into the body, and proposed the mechanisms involved as the transcellular movements of cell penetrating peptides (CPPs), the paracellular pathway, and specific transporters (PEPT1, PEPT2). The potential biological function of sequenced peptides was

evaluated *in silico* (Table 7) using the BIOPEP database. Bioactive sequences within the peptide are more likely to interact and bind with amino acids of target enzymes. *In silico* studies are used to support the potential inhibition of peptides, in the protein hydrolysate, of enzymes related to chronic disease, such as type-2 diabetes.

Figure 13 shows an example of MS and MS/MS profiles during peptide sequence identification for Black and Navy beans. Identified peptides presented potential ACE inhibition, DPP-IV inhibition, and antioxidant capacity properties. Peptide structures with higher potential of inhibition of these enzymes are presented in Table 8. Amino acid type and sequence are key factors in the potential of interaction between the functional groups in the peptides with the amino acids in the catalytic site of the enzymes. α-Amylase, α-glucosidase, DPP-IV, and ACE are enzymes related to chronic degenerative diseases such as type-2 diabetes and hypertension [51-53]. Competitive inhibition of these enzymes occurs when compounds can be positioned to interact with the amino acids in the catalytic site. Interactions depend on distances and functional groups in side chains. Interactions are predicted to be caused by hydrogen bonding, polar linkages, and hydrophobic interactions, among others. Differences in amino acids in the catalytic site make enzymes specific for a substrate, as well as determine the affinity for an inhibitor.

6.4.5 Antioxidant capacity

BPIH from Black bean showed a significant higher AC in the precooked sample than the raw 610.5 and 329.7 mmol TE/g protein), respectively (Figure 12B). Pinto bean also presented significant differences between precooked and raw samples 467.5 and 297.5 mmol TE/g protein, respectively. However, the rest of the BPIH from raw and precooked common beans showed antioxidant activities from 300 to 500 mmol TE/g proteins. Higher AC values for precooked Black bean BPIH could be due to the effect of processing in the released antioxidant peptides. Black and Pinto cultivars also presented higher significant difference in the degree of hydrolysis. Oseguera-Toledo *et al.* [17] reported higher AC values in Pinto and Black beans (40 mmol TE/mg protein) after hydrolysis with pepsin/pancreatin. Tendency for antioxidant capacities for all beans was higher when compared BPIH from precooked samples compared with the raw counterparts. However, Red, Navy, and Great Northern beans were not significantly different. Peptides as antioxidants can reduce the risk of incidence in disease development caused by oxidative stress [19]. Moreover, peptides can act synergistically with nonpeptide antioxidants enhancing their protective effect [54]. Xu and Chang [55] reported that pressure boiling

treatments retained more efficiently the antioxidant capacity of phenolic compounds from common beans.

6.4.6 α-Amylase and α-glucosidase inhibition

Percent inhibition of *α*-amylase was calculated in relation to acarbose; a known pharmaceutical inhibitor of this enzyme (Figure 14A). Across all samples, percent inhibition of *α*-amylase was higher in the BPIH from raw beans than the processed ones. However, Pinto bean did not show significant differences on *α*-amylase inhibitory capacity. Lower inhibition of precooked samples could be due to the effect of processing in the degradation of naturally occurring *α*-amylase inhibitors. Common beans contain *α*-amylase inhibitors that are part of the defense response mechanism of the plant against insects [56]. During processing, thermal treatment induced protein denaturation and inactivation of nonnutritional proteins as lectins, trypsin/chymotrypsin, and *α*-amylase inhibitors. Based on this, the capacity of inhibition of *α*-amylase is higher for unprocessed beans. *α*-Glucosidase inhibition from BPIH raw and precooked beans are observed in Figure 14B. Most raw samples fall between 60% and 70% inhibition, and precooked samples ranged between 40% and 60% inhibition. *α*-Glucosidase inhibition differences between BPIH from raw and processed protein were not statistically significant except for Black beans. Common bean protein hydrolysates with potential to inhibit *α*-amylase and *α*-glucosidase have not been reported previously. However, *in vivo* studies suggest that consumption of cooked common beans could improve the glycemic status in controlling diabetes [57]. This is done by decreasing the digestion of polysaccharides thereby reducing the amount of glucose available for absorption.

6.4.7 ACE inhibition activity

Figure 15(A) presents the results of ACE inhibitory activity by BPIH from raw and precooked beans. An IC50 value represents the concentration needed to inhibit 50% of the enzyme; the lower the number, the more potent the inhibition. None of IC50 values showed a significant difference between the raw and precooked beans. This suggests that the ACE inhibitory potential of common beans was not affected by processing. Raw and processed Red beans were significantly less potent inhibiting ACE than other samples. However, these IC_{50} values (0.2 to 0.5 mg/mL) are consistent with reported IC50 values for ACE inhibition that ranged between 0.1 and 0.6 mg/mL [22,58].

5.4.8 DPP-IV inhibition activity

The DPP-IV inhibition in general presented lower IC50 values for precooked beans than the raw counterparts (Figure 15B). Red bean showed less inhibition activity with the highest IC50 for raw and processed. On the other hand, Navy bean presented the lowest IC_{50} values for precooked and raw (0.093 and 0.095mg protein/mL), respectively. Results showed better DPP-IV inhibition potential than published results for Black bean (IC_{30} = 1.4 mg/mL) [59]. Thermal aggregation and protein/polyphenols complexes could affect protein digestibility and as a consequence decreased the amount of bioactive peptides released during hydrolysis. In this case, the combination of processing conditions as bleaching, high pressure cooking, and drying affected positively the protein structure, avoiding aggregation and protein/polyphenols complexes formation. Processing conditions as boiling, germinating, and autoclaving can increase common bean protein susceptibility to hydrolysis [9,12]. Processing effect in common bean protein showed to increase hydrolysis in *in vitro* models. Further work in our laboratory considers the evaluation of the bioaccessibility and bioavailability of identified peptides and their potential delivery vehicles in the human body, as well as other mechanisms involved in the action of peptides in the management of type-2 diabetes and hypertension.

6.5 Conclusions

Processing did not affect bioactive properties of common bean protein hydrolysates. However, there was a clear tendency to increase digestibility, measured by degree of hydrolysis, in processed common beans. Protein hydrolysates presented potential to inhibit enzymes related with chronic diseases as hypertension (ACE) and type-2 diabetes (DPP-IV, and most relevant the effect on α-amylase and α-glucosidase). Peptide extracts were more efficient to inhibit DPP-IV enzyme than ACE enzyme. From the nutraceutical point of view, food industry could use this information to diversify their criteria when processing foods; focusing on the improvement of both sensory and nutraceutical properties of products through processing. The consumption of fast cooking common beans has the advantage of reducing cooking time without affecting bioactive properties.

6.6 References

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6.7 Tables and figures

Table 6. Percent of total area of peptides in a particular molecular mass range from raw and precooked bean hydrolysates obtained by MALDI-TOF.

MM (Da) Range[1]	Raw					Precooked				
	Black	Pinto	Red	Navy	Great N	Black	Pinto	Red	Navy	Great N
500-700	50.90	6.41	20.46	26.19	22.79	73.15	58.96	44.33	69.07	72.83
700-900	26.20	-	-	11.05	72.73	-	-	4.70	2.13	-
900-1100	12.23	-	-	37.82	-	18.10	19.06	1.51	17.13	15.24
1100-1300	7.98	-	-	3.83	-	-	-	-	-	-
1300-1500	0.65	11.33	9.45	1.90	-	-	-	4.68	-	-
1500-1700	1.12	31.78	59.52	4.82	2.76	-	-	-	-	-
1700-1900	0.39	9.29	-	3.84	-	-	-	-	-	-
1900-3000	0.91	50.48	10.57	14.39	1.71	8.75	21.99	44.77	11.67	11.92

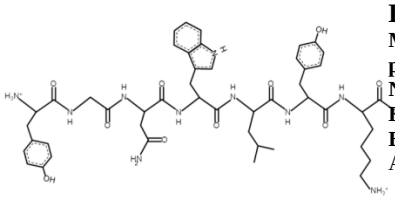
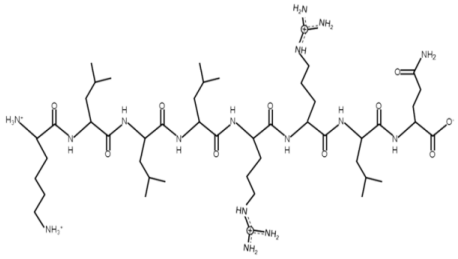
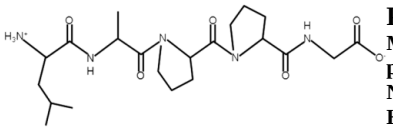
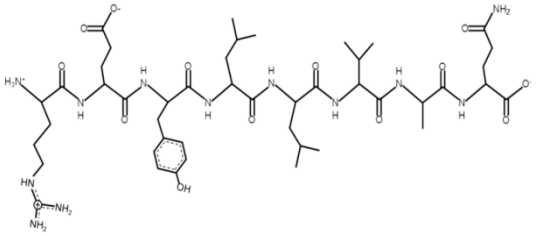
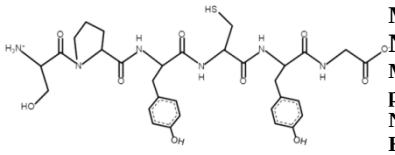
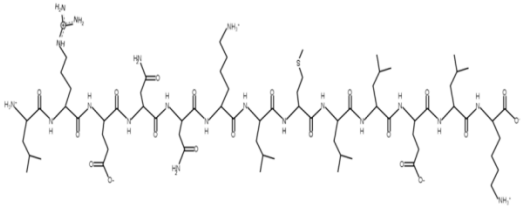
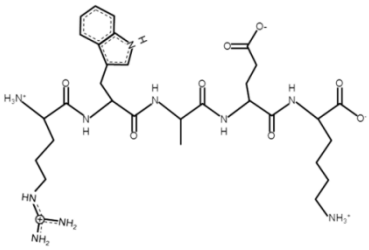
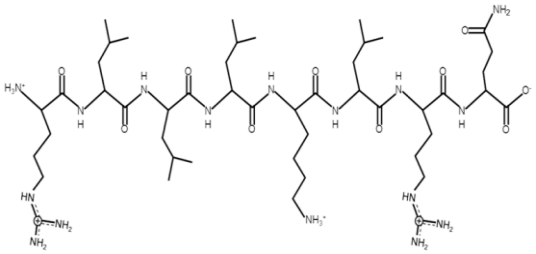
Peptides obtained from the HPLC elution profile with intensity of at least 50%; Potential bioactivities were obtained from the BIOPEP database. Sequences belonging to *Phaseolus vulgaris* protein were confirmed by BLAST tool. HPLC-MS-MS: high performance liquid chromatography-mass spectrometry-mass spectrometry; Great N: great northern; MM: molecular mass; ACE inh: angiotensin converting enzyme inhibition; DPP-IV inh: dipeptidyl peptidase IV inhibition; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine

Table 7. Peptide sequences obtained by HPLC-MS/MS in hydrolysates from raw and precooked beans and their potential biological activities

Raw					Precooked				
MM (Da)	Bean Variety	Peptide	Bioactive sequence[1]	Potential Bioactivity[1]	MM (Da)	Bean Variety	Peptide	Bioactive sequence[1]	Potential Bioactivity[1]
495	Black	YAAAT	YA, AA	ACE inh.	459	Black	EGGSF	SG,GG, EG, SF	ACE inh.
495	Black	FATGT	GT, TG, FA	ACE inh, DPP-IV inh.	517	Black	RSKK	KK	Bacterial permease ligand
521	Black	ERAF	AF, RA	ACE inh.	688	Black	FFECSG	SG	ACE inh.
728	Black	RKRAAQ	KR, RA, AA	ACE inh.	942	Black	YGNWLYK	LY, YG, YK,	ACE inh., Antioxidant
1740	Pinto	RNEQMGAGRLGRLRK	GA,AG,GR,MG,LG	ACE inh.	1038	Black	KLLLRRLQ	RL,KL, RR, LO, LLL	ACE Inh., DPP-IV inh.
1741	Red	RRQRRRRMRKDK	RR, KD	ACE inh.	453	Pinto	LAPPG	LAP,AP,LA,PG,PP	ACE inh., DPP-IV
453	Navy	SGAGY	SY, GA,AG,SG	ACE inh.	453	Pinto	YGAGS	YG,GA,AG.GS	ACE inh.
495	Navy	YAAAT	YA, AA	ACE inh.	990	Pinto	REYLLVAQ	YL, EU, LV, LL, VA	ACE inh., DPP-IV inh.
1252	Navy	QQRRLLRRK	RL, RR, LL	ACE inh., DPP-IV inh.	364	Red	SDGS	GS,DG	ACE inh.
453	Great N	YAGGS	YA,AG,GS,GG	ACE inh.	688	Red	SPYCYG	YG, YCY	ACE inh, Antioxidant
495	Great N	FTTQ	TQ	ACE inh.	1612	Navy	LRENNKLMLLELK	NKL,KL,NK,LL,EL,LK,LL	ACE inh., DPP-IV inh., Antioxidant
					453	Great N	YTGN	TG	ACE inh.
					453	Great N	YQGS	GS,QG	ACE inh.
					688	Great N	RWAEK	RW,EK,RWA,RW	ACE inh., Antioxidant
					1038	Great N	RLLLKLRQ	RL,LKL,KL,LL	ACE inh., DPP-IV
					1557	Great N	RRARGERMSRRK	RA,GE,RR,AR	ACE inh.
					1885	Great N	LSERRMLLRKEKQAQ	RR,EK,KE,LL	ACE inh., DPP-IV

[1]Values are expressed as percentage of total area for each molecular mass range; MM: molecular mass; Da: daltons; MALDI-TOF: matrix-laser desorption ionization–time-of-flight

Table 8. Amino acid sequence and structure of selected peptides with potential biological activities in hydrolysates from precooked beans

Sequence	Structure	Sequence	Structure
YGNWLYK **Black** **MW:** 942 **pI:** 9.32 **Net charge:** +1 **Hydrop:** +7.94 **Bioactivity:** ACE, AC		**KLLLRRLQ** **Black** **MW:** 1038 **pI:** 12.49 **Net charge:** +3 **Hydrop:**+10.09 **Bioactivity:** ACE, DPP-IV	
LAPPG **Pinto** **MW:** 453 **pI:** 5.58 **Net charge:** 0 **Hydrop:** +5.58 **Bioactivity:** ACE, DPP-IV		**REYLLVAQ** **Pinto** **MW:** 990 **pI:** 6.55 **Net charge:** 0 **Hydrop:** +10.9 **Bioactivity:** ACE, DPP-IV	
SPYCYG **Red** **MW:** 688 **pI:** 5.12 **Net charge:** 0 **Hydrop:** +8.21 **Bioactivity:** ACE, AC		**LRENNKL MLLELK** **Navy** **MW:** 1612 **pI:** 9.62 **Net charge:** +1 **Hydrop:** +17.3 **Bioactivity:** ACE, DPP-IV AC	
RWAEK **Great Northern** **MW:** 688 **pI:** 9.8 **Net charge:** +1 **Hydrop**: +14.5 **Bioactivity:** ACE, AC		**RLLLKLRQ** **Great Northern** **MW:**1038 **pI: 12.49** **Net charge:** +3 **Hydrop:** +10.1 **Bioactivity:** ACE, DPP-IV	

Structures obtained by PepDraw tool; pI: isoelectric point; MW: molecular weight; hydrop: hydrophobicity; P. bioactivity: potential bioactivity; ACE: angiotensin converting enzyme inhibitory activity; DPP-IV: Dipeptidyl peptidase IV inhibitory activity; AC: antioxidant capacity; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

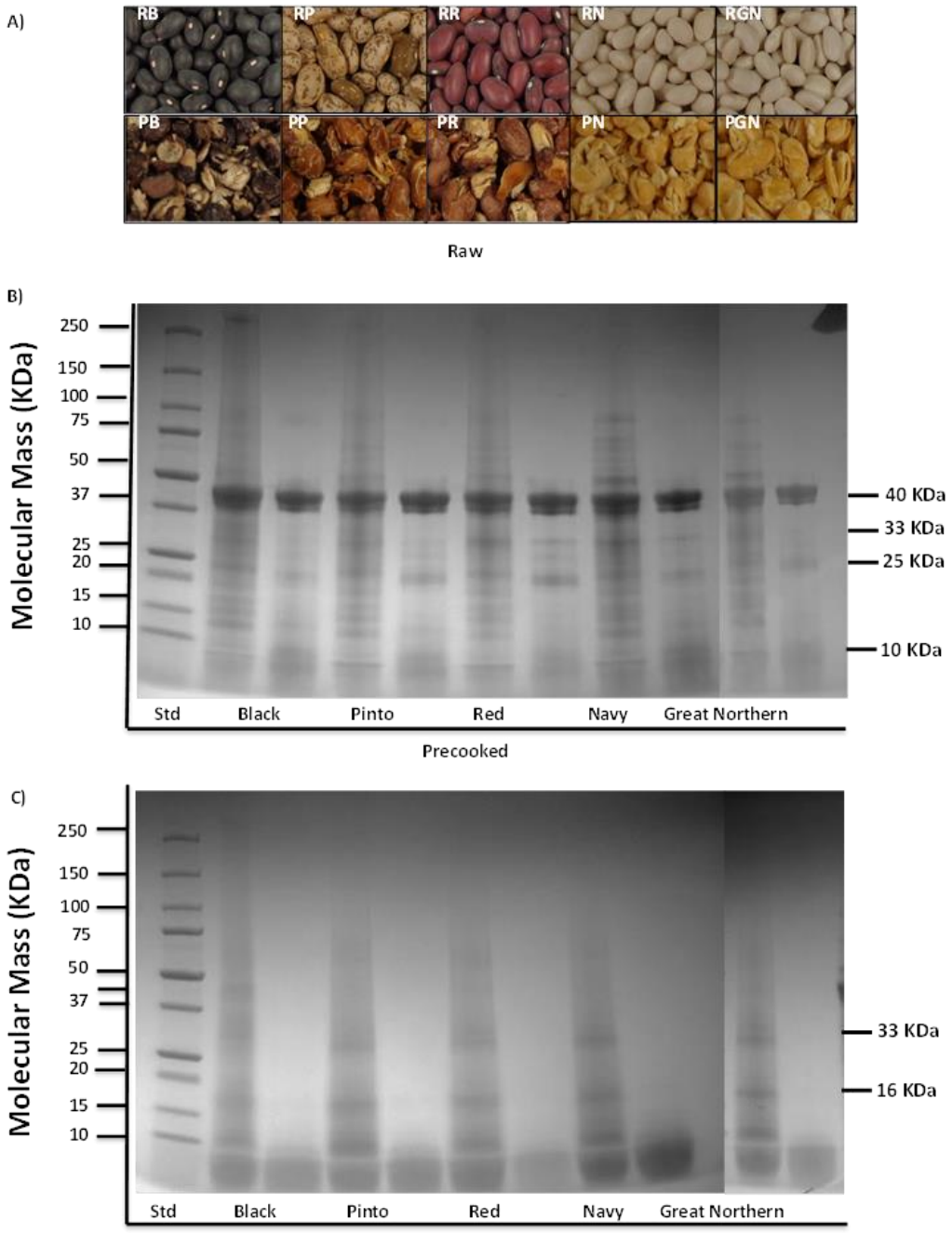
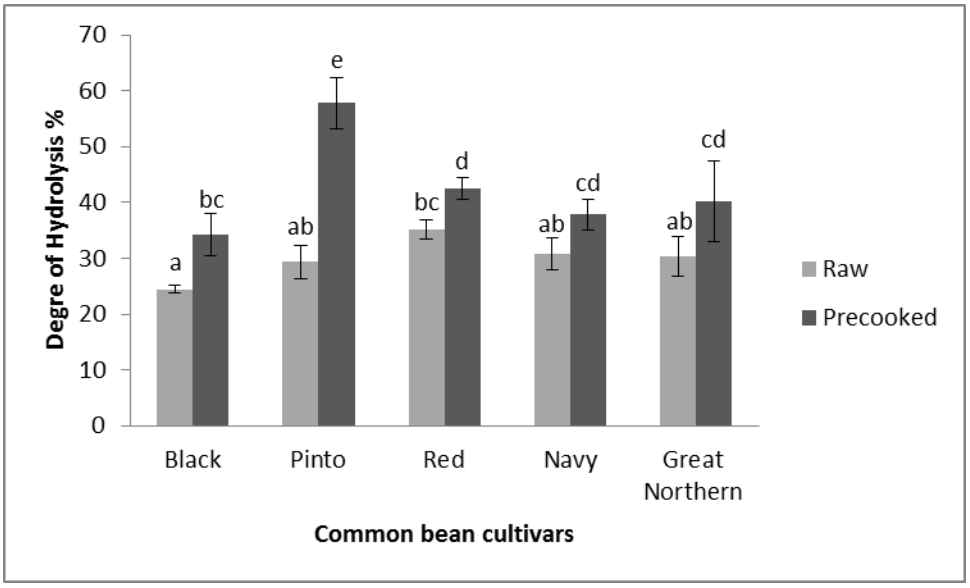


Figure 11. Electrophoretic profile of raw and precooked common beans. A. Image of raw and precooked common bean samples. RB: raw black; RP: raw pinto; RR: raw red; RN: raw navy; RGN: raw great northern; PB: precooked black; PP: precooked pinto; PR: precooked red; PN: precooked navy; PNG: precooked great northern. B. Protein profile of raw beans before and after hydrolysis. Each sample contains two bans, first belongs to protein isolate profile and second belongs to protein isolate after hydrolysis with pepsin/pancreatin. C. Protein profile of precooked beans before and after hydrolysis. Each sample contains two bans, first belongs to protein isolate profile and second belongs to protein isolate after hydrolysis with pepsin/pancreatin.

A)



B)

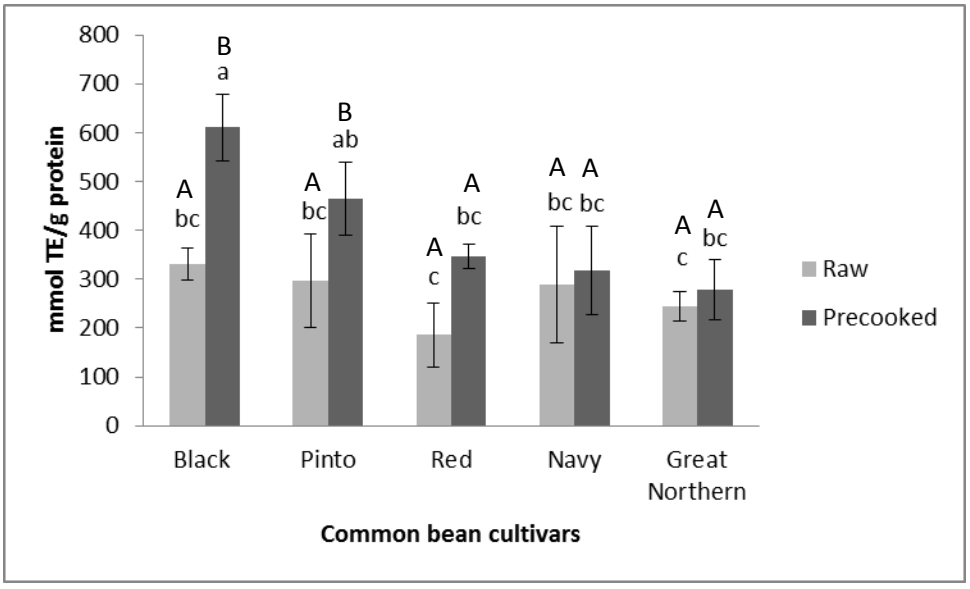


Figure 12. A. Degree of hydrolysis of BPIH from raw and precooked common beans. B. Antioxidant capacity of BPIH from raw and precooked common beans. Different capital letters indicate significant differences between raw and precooked BPIH; different lower case letters indicate significant differences ($p < 0.05$) among BPIH.

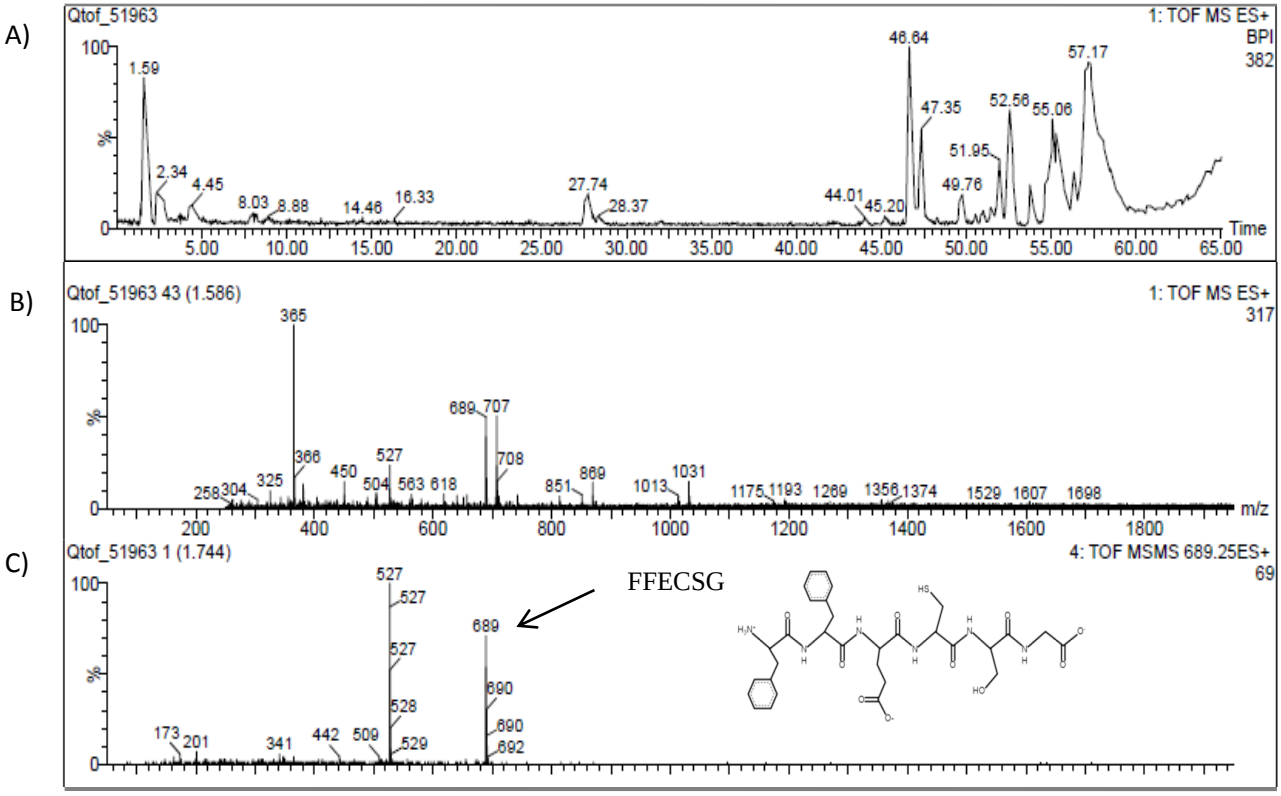


Figure 13. A. HPLC-ESI-MSMS profile for BPIH from precooked Black bean. B. Mass spectrometric fragmentation profile of first peak. C. MSMS ion at m/z 689 for sequencing FFECSG peptide.

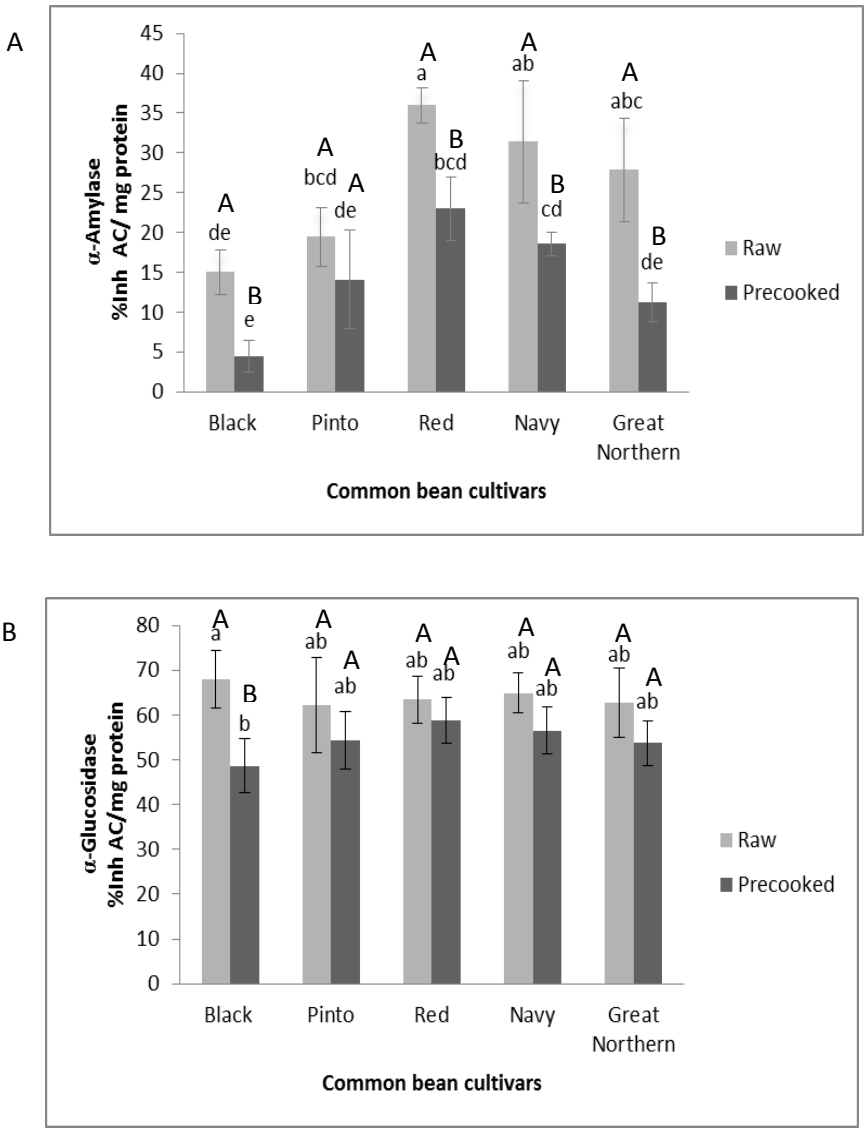


Figure 14. A. α-amylase inhibition of BPIH from raw and precooked common beans. B. α-glucosidase inhibition of BPIH from raw and precooked common beans. Different capital letters indicate significant differences between raw and precooked bean hydrolysates; different lower case letters indicate significant differences ($p<0.05$) among bean hydrolysates.

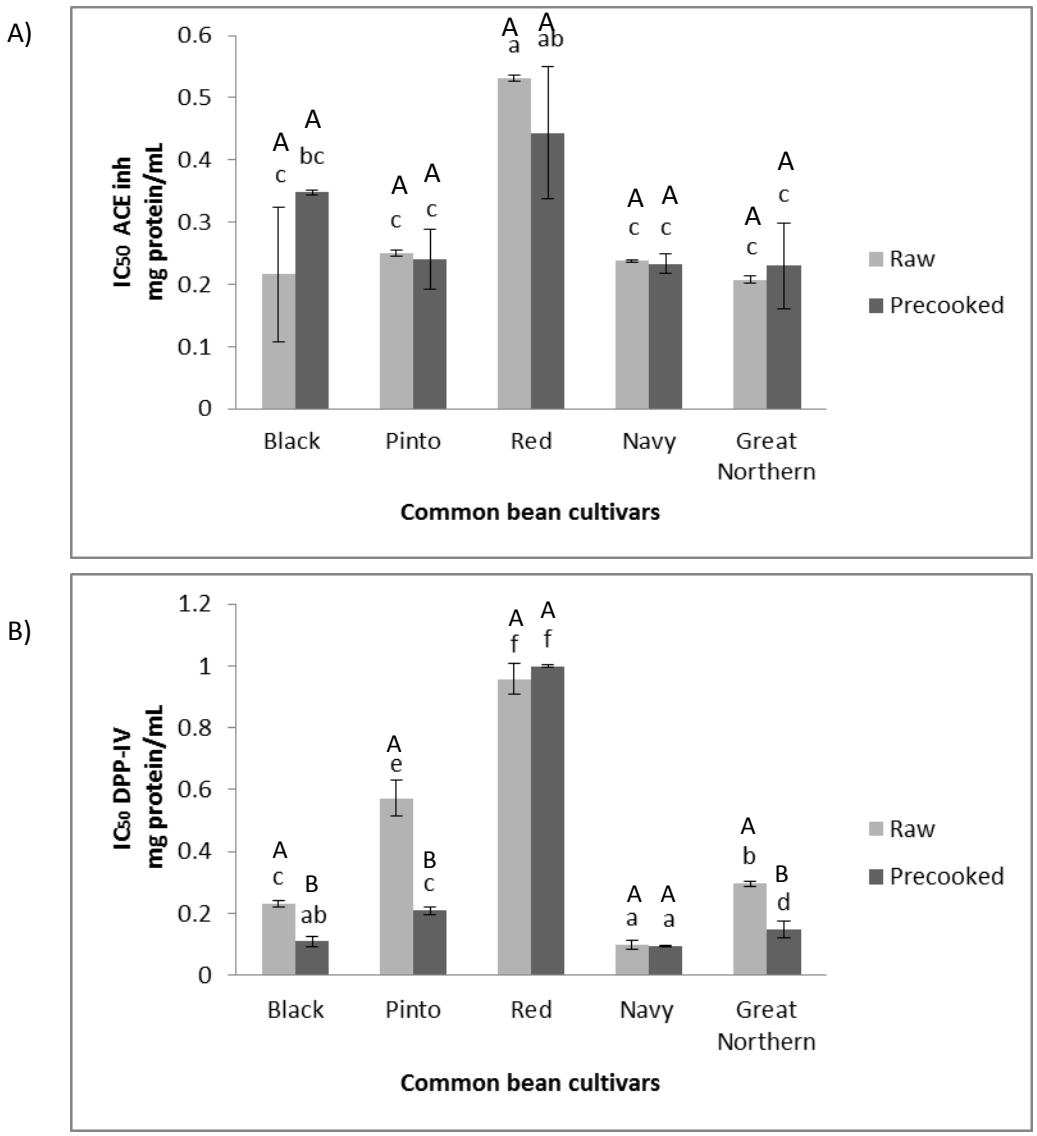


Figure 15. A. Angiotensin converting enzyme I inhibition activity of BPIH from raw and precooked common beans expressed as IC_{50}. B. Dipeptidyl peptidase IV inhibitory activity of BPIH from raw and precooked common beans expressed as IC_{50}. Different capital letters indicate significant differences between raw and precooked bean hydrolysates different lower case letters indicate significant differences ($p < 0.05$) among bean hydrolysates.

CHAPTER 7: OPTIMIZATION OF ENZYMATIC PRODUCTION OF ANTI-DIABETIC PEPTIDES FROM BLACK BEAN (*Phaseolus vulgaris* L.) PROTEINS AND THEIR CHARACTERIZATION AND BIOLOGICAL POTENTIAL

7.1 Abstract

The aim was to optimize the production of bioactive peptides from black bean (*Phaseolus vulgaris* L.) protein isolate and to determine their biological potential using biochemical and *in silico* approaches. Protein fractions were generated using eight commercially available proteases after 2, 3 and 4 h and 1:20, 1:30 and 1:50 enzyme/substrate (E/S) ratios. The best combination of conditions to generate anti-diabetic peptides were with alcalase for 2 h and E/S of 1:20; with inhibitions values for dipeptidyl peptidase IV (DPP-IV, 96.7%), α-amylase (53.4%) and α-glucosidase (66.1%). Generated peptides were characterized using LC-ESI-MS/MS. Molecular docking analysis was performed to predict individual peptide biological potential using DockingServer®. Peptides EGLELLLLLLAG, AKSPLF and FEELN inhibited DPP-IV more efficiently *in silico* through free energy interactions of -9.8, -9.6 and -9.5 kcal/mol, respectively, than control sitagliptin (-8.67 kcal/mol). Peptide TTGGKGGK (-8.97 kcal/mol) had higher inhibitory potential on α-glucosidase compared to the control acarbose (-8.79 kcal/mol). Peptides AKSPLF (-10.2 kcal/mol) and WEVM (-10.1 kcal/mol) generated a lower free energy interaction with the catalytic site of α-amylase in comparison with acarbose (-9.71 kcal/mol). Bean peptides inhibited the tested enzymes through hydrogen bonds, polar and hydrophobic interactions. The main bindings on the catalytic site were with ASP192, GLU192 and ARG 253 on DPP-IV; TYR151, HIS201 and ILE235 on α-amylase; and ASP34, THR83 and ASN32 on α-glucosidase. For the first time, a systematic evaluation and characterization of the anti-diabetic peptides from black bean protein isolate is presented with the potential to inhibit important molecular markers related to diabetes.

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7.2 Introduction

Common beans contain bioactive compounds such as polyphenols, resistant starch, oligosaccharides and bioactive peptides [1]. Their protein content range from 16 to 33% [2] with phaseolin and the lectin family of proteins representing 50-70% of total proteins [3]. Several factors can affect the hydrolysis of proteins; for instance, protease amino acid specificity, protein extraction conditions and pretreatments could have an effect on peptide profile. Processing conditions such as the relationship of enzyme/substrate, time of hydrolysis and enzyme combination play an important role in the bioactivity of generated peptides [1, 4, 5]. The mechanisms and processes involved in common bean peptide generation vary [1]. Proteases such as alcalase, flavourzyme, pepsin, pancreatin, chymotrypsin, papain, trypsin, and thermolysin have been used to produce bioactive peptides from common bean proteins [1].

Food derived bioactive peptides have gained interest due to a positive impact on body function or condition and ultimately may influence human health beyond basic nutrition [6]. Their biological potential is mainly performed by blocking specific enzymes or modulating gene expression. Moreover, peptides can act as neurotransmitters, hormones or antibiotics [7]. Due to the structural similarity with endogenous peptides, they can interact with receptors and play important roles modulating metabolic processes that can modify food intake, growth factors, immune regulators or antimicrobials [8,9]. The biological potential of common bean protein fractions has been related to antihypertensive capacity, antioxidant capacity, antifungal activity, tumor cell inhibition and recently, anti-diabetic potential [1, 10, 11].

Diabetes mellitus is a condition characterized by high blood glucose levels, with extraordinary growing rate and prevalence [12]. Some molecular targets used in the treatment of diabetes mellitus are the inhibition of enzymes dipeptidyl peptidase IV (DPP-IV), α-amylase and α-glucosidase. DPP-IV rapidly inactivates gut incretin hormones, known as insulin secretagogues [13]. Furthermore, blocking starch degradation enzymes (α-amylase and α-glucosidase) is another medical therapy in the control of glucose homeostasis in diabetic patients [14]. Due to their chemical structure, peptides can interact with amino acids in the catalytic cavity of enzymes that are related to important diseases, and inhibit their action. Peptides can also exert a protective effect based on their antioxidant potential caused by some functional groups of the amino acids [15].

The anti-diabetic potential of common bean protein fractions is a novel approach for diabetes management, with high significance for this public health problem. However, process optimization for peptide production with anti-diabetic potential and their characterization are still needed. The objective of this research was to optimize the hydrolysis conditions of black bean protein isolate using eight different commercially available proteases to generate peptides with anti-diabetic potential. Furthermore, the chemical characterization of all peptides was determined and their biological potential was evaluated using biochemical assays and *in silico* molecular interactions.

7.3 Materials and methods

Black-Otomi bean cultivar was provided by the Experimental Station of the National Research Institute for Forestry Agriculture and Livestock (INIFAP), Zacatecas, Mexico. The dry grains were stored at 4°C until use. Enzymes human dipeptidyl peptidase IV (EC 3.4.14.5), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), α-amylase (EC 3.2.1.1), and commercial proteases: 1: trypsin (EC 3.4.21.4), 2: flavourzyme (EC 3.4.11.1), 3: proteinase k (EC 3.4.21.64), 4: thermolysin (EC 3.4.24.27) 5: alcalase (EC 3.4.21.62), 6: pepsin (EC 3.4.23.1) 7: papain (EC 3.4.22.2), and 8: chymotrypsin (EC 3.4.21.1) were purchased from Sigma-Aldrich (St. Louis, MO). DPPIV-GLO® protease assay kit was purchased from Promega (Madison, WI). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

7.3.1 Protein extraction

Beans were soaked in water at room temperature for 16 h. The hull was then manually removed, and beans were ground in a commercial blender in a 1:10 bean/water ratio. The pH was adjusted to 8.0 with 0.1 M sodium hydroxide and protein extraction was carried out at 35° C with stirring for 1 h. The mixture was centrifuged at 5,000 *g* for 15 min at 25 °C. The precipitate was re-extracted under identical conditions to maximize yield and both extracts were combined. Then, the pH was adjusted to 4.3 with 0.1 M hydrochloric acid to precipitate out proteins, followed by centrifugation at 10,000 *g* for 20 min at 4° C. The supernatant was discarded and the pellet freeze-dried in a Labconco Freeze Dryer 4.5 (Kansas, MO). Dried bean protein isolates (BPI) were stored at -20° C until analysis [16].

7.3.2 Protein hydrolysis

Bean protein isolate was suspended in water (1:20 w/v) and autoclaved for 20 min at 121˚C. Enzymatic digestion was carried out using a combination of treatments;

protease/substrate ratio of 1:20, 1:30, and 1:50 (w/w), time of hydrolysis 2, 3 and 4 h, with pH and temperature optimal for each protease: proteinase K (pH: 7.5, 37˚C), pepsin (pH: 2.0, 37˚C), trypsin (pH: 7.5, 37˚C), papain (pH: 6.5, 60˚C), alcalase (pH: 7.0, 50˚C), flavourzyme (pH: 8.0, 50˚C), themolysin (pH 8, 50˚C) and chymotrypsin (pH 7.5, 37˚C). Protein hydrolysis was stopped by heating at 75 °C for 20 min, and the resulting protein fractions were centrifuged at 20,000 *g* for 15 min at 4 °C. Bean protein fractions were dialyzed to eliminate salts using a 500 Da molecular weight cut-off membrane and then freeze-dried in a Labconco FreeZone Freeze dry system (Kansas City, MO, USA). Samples were stored at -20°C until analysis.

7.3.3 Optimization of enzymatic production of anti-diabetes *peptides using response surface methodology*

Response surface methodology (RSM) was used to optimize the best conditions needed to produce anti-diabetes peptides from common bean proteins using functional relationships between the dependent variable and the independent variables as previously reported [17]. Enzyme type (x_1), enzyme-to-protein ratio (x_2) and hydrolysis temperature (x_3) were chosen for independent variables. The range and center point values with actual and coded values of variables used for the optimization of anti-diabetes peptides production were coded levels (1-8) for enzyme type (x_1); -1, 0, +1, for enzyme-to-protein ratio 1:20, 1:30, 1:50 (x_2); and time 120, 180, 240 min (x_3). Inhibition of DPP-IV, α-amylase and α-glucosidase were selected as the responses for the combination of the independent variables. The variables were coded according to the following equation:

$$x = \frac{x_i - x_o}{\Delta x}$$

where x is the coded value, x_i is the corresponding actual value, x_0 is the actual value at the center of the domain and Δx is the increment of x_i corresponding to a variation of 1 unit of x. The polynomial second degree equation is described by the following equation:

$$y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j$$

Simulated gastrointestinal digestion of alcalase protein fractions

Alcalase protein fractions were suspended with water (1:20 w/v) and sequential enzyme digestion was carried out with pepsin/alcalase hydrolysate 1:20 (w/w) (pH 2.0) followed by pancreatin/alcalase hydrolysate 1:20 (w/w) (pH 7.5) at 37˚C, for two hours each. The hydrolysis was stopped by heating at 75˚C for 20 min, and the resulting protein fractions were centrifuged at 20,000 *g* for 15 min at 4 °C. Bean protein fractions were dialyzed to eliminate salts using a 500 Da Molecular weight cut-off membrane and then freeze-dried in a Labconco FreeZone Freeze dry system. Samples were stored at -20˚C until analysis [18].

7.3.4 Dipeptidyl peptidase inhibition biochemical assay

DPP-IV inhibition was measured using the DPP-IVGLO® Protease Assay (G8351, Promega, Madison, WI). A 50 µL of DPP-IVGLO® reagent was added to a white-walled 96-well plate containing either 50 µL of blank, 40 µL enzyme control or 40 µL protein fractions. Bean protein fractions were prepared in buffer (100 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA) at a concentration of 1 mg dry weight (DM)/mL. The blank contained only buffer and DPP-IVGLO® reagent, while the enzyme control and the samples contained buffer, DPP-IVGLO® reagent and 10 µL purified DPP-IV human enzyme (10 ng/mL). Luminescence was measured after mixing and incubating for 30 min using a Synergy2 multiwell plate reader (Biotek Instruments, Winooski, VT). Percent inhibition was calculated from the blank and enzyme control for each sample.

7.3.5 α-Glucosidase inhibition

For the α-glucosidase assay, in a 96-well plate, 50 µL of protein fractions, (1 mg DM/mL buffer), or positive control (1 mM acarbose) were added to 100 µL of 1 U/mL α-glucosidase solution (0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 µL aliquot of a 5 mM p-nitrophenyl-α-D-glucopyranoside solution (0.1 M sodium phosphate buffer pH 6.9) was added to each well and incubated at 25 °C for 5 min and absorbance read at 405 nm. Results are presented as percent inhibition per mg of dry weight (% inh/mg DM) [14].

7.3.6 α-Amylase inhibition

For the α-amylase assay, 500 µL of protein hydrolysate (1 mg DM/mL buffer), or positive control (1 mM acarbose) was added to 500 µL of 13 U/mL α-amylase solution (type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 µL of 1% soluble starch solution (previously dissolved in sodium

phosphate buffer and boiled for 15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color reagent was added, and the tubes were placed in 100 °C water bath for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance read at 520 nm. Results are presented as percent inhibition per mg of dry weight (% inh/mg DM) [14].

7.3.7 Characterization of the peptides and sequence identification LC-ESI-MSMS

The peptides obtained were analyzed by LC-ESI-MSMS using a Q-tof Ultima mass spectrometer (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system. Separation of the components was performed by using a mobile phase of Solvent A (95% H_2O, 5% ACN and 0.1% formic acid) and Solvent B (95% ACN, 5% H_2O and 0.1% formic acid) using a flow rate of 200 µL/min. The elution was in a linear gradient (0 min, 90% A; 2 min, 90% A; 40 min, 65% A; 60 min, 10% A; 65 min, 10% A; 66 min, 90% A; 80 min, 90% A). The temperature was kept in 20 ºC during the whole procedure. A splitter with a split ratio of 1:10 was used, where one part went to the mass spectrometer and ten parts to the waste. The Q-tof Ultima mass spectrometer was equipped with a Z-spray ion source. Using a positive ion electrospray mode (+ESI), the analysis on the Q-tof was carried out in V-mode with an instrument resolution between 9000 and 10,000 based on full width at half maximum, with a flow rate of 20 µL/min. The source temperature was set at 80 °C and desolvation temperatures were set at 250 °C, respectively. The Q-tof was operated at a capillary voltage of 3.5 kV and a cone voltage of 35V. The final detector was a microchannel plate with high sensitivity. The MassLynx 4.1V software (Waters, Milford, USA) was used to control the instruments and to process the data to get the highest probability of the peptides sequences [19]. Confirmation of peptides sequence in common bean (*Phaseolus vulgaris* L.) proteins was performed using the BLAST® tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 06/1/2015). The potential biological activity of the peptides was predicted by using BIOPEP database (http://www.uwm. edu.pl/biochemia, accessed on 06/10/2015).

7.3.8 Computational docking

Docking calculations of peptides, discovered by HPLC-MSMS from bean proteins, and DPP-IV, α-amylase and α-glucosidase enzymes, were carried out using the DockingServer [20]. The MMFF94 force field [21] was used for energy minimization of peptides. Gasteiger partial charges were added to the peptide ligand atoms, namely, the peptides. Non-polar hydrogen atoms

were merged and rotatable bonds defined. Docking calculations were carried out on DPP-IV (3W2T), α-amylase (1B2Y) and α-glucosidase (3AJ7) protein crystal structures. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [22]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 250,000 energy evaluations. The population size was set up to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

7.3.9 Statistical analysis

The experiments were repeated at least three times with consistent results. Data are expressed as mean ± standard deviation. Statistical analyses were conducted using one-way ANOVA to compare experimental to control values SAS version 9.3 (SAS Inst. Inc., Cary, NC). Comparisons between groups were performed using Tukey test, and differences were considered significant at $P < 0.05$. Principal component analysis (PCA) and clustering analysis were performed using SPSS Statistics Premium GradPack 22. PCA was conducted on the enzyme inhibition variables (net charge, molecular weight, amino acid number, hydrogen bond interactions, polar interactions, hydrophobic interactions, free energy, *Ki*, number of total interactions with amino acids, acidic amino acids, basic amino acids, aromatic amino acids, sulfur amino acids, hydrophobic amino acids and polar amino acids) for the peptides inhibiting the different enzymes that were labeled as follows: DPP-IV (-α), α-amylase (-β), and α-glucosidase (-γ). To evaluate the similarities among inhibition variables, cluster analysis was performed on two PCs; hierarchical clustering methods were used to determine the number of clusters. Correlation analysis was performed using JMP version 7.0.

7. 4 Results and discussion

7.4.1 Optimal conditions for production of anti-diabetic peptides from common bean proteins

Table 9 shows the conditions used to optimize bean protein hydrolysis, production of bioactive peptides and means of the inhibition potential of the peptides produced from each condition. The combination of treatments using eight proteases, three hydrolysis times and three enzyme/substrate concentrations were used to model the equation using the RSM method.

Multiple regression analysis of inhibition potential of DPP-IV enzyme showed that the tested variables were related by the second-degree polynomial equation (eq 1).

$$Y= 98.058287 + 6.104394x_1 – 0.386171x_2 – 0.365865x_3 + 0.001170x_1x_2 + 0.015838x_1x_3 + 0.003245x_2x_3 – 1.097651x_1^2 + 0.000770x_2^2 - 0.008333x_3^2 \quad \text{(eq. 1)}$$

Similarly, by applying the multiple regression analysis, inhibition potential of α-amylase enzyme and the independent variables were related to the dependable variables by the second-degree polynomial equation (eq 2).

$$Y= 62.041255 – 1.052748x_1 - 0.240811x_2 - 0.502895x_3 - 0.004322x_1x_2 + 0.011166x_1x_3 + 0.000226x_2x_3 + 0.282790x_1^2 + 0.000713x_2^2 + 0.005287x_3^2 \quad \text{(eq. 2)}$$

For α-glucosidase enzyme, multiple regression analysis was applied to find the relation of the independent and dependent variables by the second degree polynomial equation (eq. 3)

$$Y= 17.484091 + 9.786276x_1 - 0.188538x_2 + 0.131795x_3 - 0.0068x_1x_2 + 0.01537x_1x_3 + 0.000584x_2x_3 - 0.636268x_1^2 + 0.000676x_2^2 - 0.004471x_3^2 \quad \text{(eq. 3)}$$

Y in equations 1, 2 and 3 represents inhibition of DPP-IV, α-amylase and α-glucosidase enzymes, respectively, x_1 protease, x_2 time and x_3 enzyme/substrate ratio. For the optimization of the production of bioactive peptides to inhibit DPP-IV, α-amylase and α-glucosidase it was found that the total model was significant only for α-glucosidase and DPP-IV parameters (P = 0.0002 and <0.0001, respectively).

Figure 16 shows the three-dimensional response surface plots. In the case of DPP-IV the inhibition slightly increased with time but decreased when increasing enzyme/substrate ratio. The optimal conditions were found for thermolysin, alcalase and pepsin for 2 h and an enzyme/substrate of 1:20 (Figure 16A, 16B, 16C). For α-amylase inhibition (Figure 16D, 16E, 16F), there was an increment in inhibition when time and enzyme substrate ratio tended to increase. The optimal conditions for inhibition of α-amylase from the optimization procedure were the enzyme chymotrypsin, papain and pepsin for 1.9 h and an enzyme/substrate of 1:35. Figure 16G, 16H, 16I shows the inhibition potential for α-glucosidase, showing a tendency to

increase inhibition with time of hydrolysis. For α-glucosidase inhibition, the optimal conditions were the enzyme pepsin, alcalase and thermolysin for 3.9 h and an enzyme/substrate of 1:35.

7.4.2 Diabetes-related enzymes inhibition and peptide fractions generated with different proteases

Table 9 presents the inhibition potential of the common bean protein fractions to inhibit DPP-IV, α-amylase and α-glucosidase expressed as percentage of inhibition per mg of dry matter. For DPP-IV, the highest inhibition was 96.7% obtained with alcalase during 2 h of hydrolysis and an enzyme substrate ratio of 1:20. For α-amylase, the highest inhibition was 64.5%, using flavorzyme during 2 h of hydrolysis and an enzyme substrate ratio of 1:20. However, only the treatment of three hours of hydrolysis and enzyme/substrate of 1:50 presented statistical differences. Similarly, in the case of α-glucosidase, the highest inhibition potential was obtained during 4 h of hydrolysis with papain and an enzyme substrate ratio of 1:30 (78.4%) with no statistical differences among treatments. Alcalase protein fractions generated during 2 h of hydrolysis and an enzyme substrate ratio of 1:20 beyond the high inhibition of DPP-IV, also presented outstanding inhibition potential for α-amylase (53.4%) and α-glucosidase (66.1%).

Thirty-four peptides were sequenced from the alcalase protein fractions; peptides identity was confirmed by Blast. Moreover, 44.1% peptide sequences belong to common bean storage proteins phaseolin, lectin, arcelin, α-amylase inhibitors (Table 10). Figure 17 shows a representative chromatogram and mass spectra of one of the most potentially bioactive peptides, AKSPLF. The physicochemical properties and biological potential of the peptides are listed in Table 10. Hydrophobicity of the peptides ranged from +4.19 kcal/mol^{-1} (LALVL) to +19.52 kcal/mol^{-1} (SRSPAGPPPTEK). The isoelectric points of peptides ranged from the acidic pI 2.82 (DLALLLLLAELG) to alkaline pI 12.96 (QQRRRLRLK). In the case of the net charge, 50% of the peptides contained a positive net charge and while 32% of the peptides had a neutral charge, 18% were negatively charged. Regarding the biological potential of the peptide sequences obtained from Biopep database, DPP-IV and angiotensin converting enzyme (ACE) inhibitory potential were the most common biological activities.

Alcalase generated peptide fractions showed stability after *in vitro* simulated gastrointestinal digestion (pepsin/ pancreatin) (Figure 18). A slight decrease in the inhibitory potential was found for DPP-IV (17%) and α-amylase (8%). On the other hand, the simulated

gastrointestinal digestion increased α-glucosidase inhibition by 3%. However, not statistical differences were found after simulated gastrointestinal digestion on any enzyme.

7.4.5 Biological potential of peptides generated by different proteases

The biological potential of peptide sequences generated by eight studied enzymes after 2 h and 1:20 E/S are shown in Figure 18. The percentage of potential bioactivity is relative to the total bioactive peptides produced by each protease. Moreover, positive correlation (r=0.54) was found between the experimental results for DPP-IV and the biological potential to DPP-IV among all the proteases used to generate the protein fractions. The highest percentages of bioactivity were found as inhibitors of ACE and DPP-IV inhibition (~40%) for the peptides produced by proteases alcalase, thermolysin and papain. In higher or smaller proportion, all proteases generated bioactive peptides that were related to potential anti-thrombotic, anti-amnestic, regulating different activities, anti-oxidative, glucose uptake promoters, among others. Table 11 shows the bioactive sequences of peptides generated using proteinase K, pepsin, trypsin, papain, flavourzyme, themolysin and chymotrypsin proteases. These peptides belong to the main common bean storage proteins (phaseolin, lectin, arcelin and α-amylase inhibitors).

7.4.6 Computational modeling inhibition constant and free energy

Peptide sequences with the highest potential to inhibit the enzymes DPP-IV, α-amylase and α-glucosidase are shown in Table 12. For DPP-IV, the low free energy values ranged from -8.6 to -9.8 kcal/mol. All the selected peptides presented higher or similar free energy value than the positive control sitagliptin (-8.6 kcal/mol). Regarding the inhibition constant (*Ki*), all peptides presented lower concentrations than sitagliptin (0.44 µM), except SGPFGPK (0.49 µM). The peptides with the highest inhibition potential were EGLELLLLLLAG, AKSPLF and FEELN with relatively low *Ki* values of 0.059, 0.083 and 0.096 µM, respectively. The peptide with highest inhibition potential for α-glucosidase was TTGGKGGK with free energy and *Ki* of -8.97 kcal/mol and 0.265 µM respectively. The inhibition constant was lower than the positive control acarbose with a *Ki* of 0.36 µM. For the inhibition of the α-amylase, two peptides presented inhibition constants lower than acarbose 0.070 µM (AKSPLF, 0.033 µM and WEVM, 0.040 µM). Moreover, peptides AKSPLF, FEELN, QTPF and LSKSVL showed good potential to inhibit the three studied enzymes. These four peptides interacted with the catalytic site of the enzymes by different type of interactions with different amino acids. Table 13 shows a summary of amino acid interactions, type of interactions and distances of the peptides AKSPLF, FEELN,

QTPF and LSKSVL interacting with specific amino acids on the polypeptide chains of the three studied enzymes. For the inhibition of DPP-IV, the four peptides presented hydrogen bond interactions and polar interactions; and AKSPLF and QTPF presented also hydrophobic interactions. The peptide KSPLF had the shortest distance of interaction (average 2.53 A˚), followed by FEELN 2.93A˚. Regarding the number of interactions, the polar interactions were more common for peptides FEELN (7), QTPF (5), LSKSVL (5) and AKSPLF (3).

For the inhibition of α-amylase, the four peptides presented hydrogen bonds, polar and hydrophobic interactions. In this case, hydrophobic interactions were the most common among the peptides, followed by polar interactions and finally the hydrogen bond interactions. AKSPLF presented the highest amount of hydrogen bond interactions with the lower average distance of 2.85A˚, which correlated with its higher inhibition potential among the peptides. Lys in AKSPLF was the amino acid that presented higher interaction with α-amylase. Phe presented most interactions with the enzyme for QTPF and FEELN. In the case of LSKSVL, the amino acid Leu showed the highest interactions with the enzyme.

The major interactions for α-glucosidase were hydrogen bonds and polar interactions, with only one hydrophobic interaction for the peptide QTPF (Pro-PRO82). The peptide AKSPLF presented interactions mainly between the amino acids Lys-ASP34, Lys-THR83 and Ala-ASP89 and Lys-ASN32 by hydrogen bonds and LysASP-34, Lys-THR83 and Ala-AS9-89 with polar interactions. Peptide QTPF presented the highest number of polar interactions with an averaged distance of 2.71A˚. Phe and Glu were the amino acids of the peptide FEELN with higher interaction of the enzyme, main interactions were with ASP34, ASN32 and THR83. In the case of LSKSVL, the principal amino acids interacting were Ser and Leu with ASP34, THR83, ASN32 and ASP89.

Bean peptides inhibited the enzymes by interacting with amino acids in their catalytic site. Figures 19A-D, 19E-H and 19I-L show the best pose of peptide AKSPLF interacting with DPP-IV, α-amylase and α-glucosidase enzymes, respectively. For DPP-IV, Figure 19A presents the complete enzyme in a dimeric natural occurring form, showing the peptide in the catalytic site of the enzyme. Figure 19B presents a zoom of the catalytic cavity of the enzyme with the peptide on the best pose. Figure 19C contains the most important linkages of the peptide with the enzyme amino acid residues which present interaction and their corresponding distances. On Figure 19D, there is a diagram of the interaction type of each amino acid in the peptide with the

amino acid residues in the catalytic site, being mainly hydrogen bonds, electrostatic or polar interactions and hydrophobic interactions. Similarly, interactions of α-amylase and α-glucosidase with the peptide AKSPLF are shown in Figures 19E-H and 19I-L, respectively. For DPP-IV, the amino acids Ala and Leu from the peptide presented the highest potential to interact in the catalytic site by hydrogen bonding, polar interactions and hydrophobic interactions. The interactions occurred primarily between amino acids GLU191, ASP192, ARG253 and LEU235 (Figure 19D). For α-amylase, all the amino acids in the peptide interacted with the enzyme, except Phe. Peptides and enzyme interactions were hydrogen bonding and hydrophobic interactions with ten different amino acid interactions in the enzyme catalytic site. For α-glucosidase, Ala and Lys were the only two amino acids interacting with ASP34, THR83, ASP89 and ASN32; showing only hydrogen bonding and polar interactions.

7.4.7 Cluster and principal component analysis of inhibition parameters for DPP-IV, α-amylase and α-glucosidase

The inhibiting mechanism and the physicochemical properties of the most active peptides were used to perform a principal component and cluster analyses. 60% of the variability was extracted by the PC1, PC2 and PC3 with score plots of 26, 19 and 15, respectively (Figure 20). Inhibition parameters that significantly affected PC1 were the basic amino acids, net charge, hydrogen bonds, sulfur containing and amino acids. PC2 was significantly ($P < 0.05$) affected by molecular weight, amino acids number, hydrophobic amino acids, and acidic amino acids. PC3 was positively affected by the number of interactions with the amino acids, and the hydrophobic interactions, contrary it was negatively affected by the free energy and inhibition constant parameters. In the combination of PCs 1 and 2 we found only one main big cluster containing 93% of the peptides (Figure 20A). EGLELLLLLLAG-γ and RKLKMRQ-β for were separated in two different clusters. This big cluster group present balanced score in both components, which means that it was affected similarly by the two PCs. In this case the peptides clustered due to presence of basic amino acids containing peptides (Arg, His and Lys) and sulfur amino acids containing peptides (Met and Cys), the net charge of the peptide and the hydrogen bonds formed. On the other hand, the PC2 was affected by the molecular weight and size of the peptide as well as the hydrophobic (Ala, Val, Ile, Leu, Met, Phe, Tyr and Trp) and acidic amino acids (Asp and Glu). EGLELLLLLLAG-γ was highly affected by PC2 which was related to size and a negative

net charge of -2. On the other hand, the peptide RKLKMRQ-β was highly affected by PC1 which was related to its high positive net charge of 4.

For cluster plots of combinations, PC1-PC3 and PC2-PC3 (Figure 20B and C), there was only one big cluster and a small one in both cases. The same peptides RKLKMRQ- β and EGLELLLLLLAG-γ were forming the small cluster in plots of PC1-PC3 and PC2-PC3. It is worth to mention that peptides characteristics and interactions with the enzyme were similar among peptides, showing similar pattern to bind and inhibit the target enzymes.

The correlation matrix of the peptides physicochemical properties and inhibition interactions of the enzymes DPP-IV, α-amylase and α-glucosidase, showed positive correlation between the molecular weight and the amino acid number (r = 0.90), net charge and hydrogen bonds (r = 0.52), number of interaction with amino acids and hydrophobic interactions (r = 0.72), net charge and basic amino acids (r = 0.85), sulfur containing amino acids and hydrogen bonds (r = 0.57).

7.4.7 Discussion

Proteases thermolysin, alcalase and pepsin generated peptides from bean protein isolate with higher potential to inhibit DPP-IV, enzyme which biological function is to inactivate the glucagon-like peptide 1 (GLP-1). GLP-1 is an important incretin hormone involved in insulin release pathway from pancreatic β-cells [23]. Alcalase has been widely used in the production of bioactive peptides [24, 25]. When comparing the overall inhibition potential with thermolysin and pepsin, at the specific processing conditions used, alcalase presented an outstanding inhibitory profile. Also these conditions generated protein fractions with a good inhibition potential for α-amylase and α-glucosidase (>50%), enzymes involved in starch breakdown [14]. Garcia-Mora et al. [26] concluded that the enzyme type and hydrolysis time are important factors influencing bioactive composition of hydrolysates; moreover they obtained peptides with antioxidant, anti-hypertensive and anti-inflammatory properties from pinto bean using alcalase and savinase proteases. On the other hand, de Castro et al. [27] evaluated the synergistic potential of using combinations of alcalase, flavorzyme and yeastmax A proteases in the production of antioxidant peptides from soy; this combination showed synergistic results in the bioactive peptides production. Oseguera-Toledo et al. [11] reported the production of anti-diabetic peptides from hard to cook common beans using alcalase and bromelain hydrolysis kinetics;

reaching the highest biological potential (50-76%) to inhibit DPP-IV, α-amylase and α-glucosidase after 2 h of hydrolysis.

Peptide fractions generated by alcalase maintain anti-diabetic biological potential after 2 h of simulated GI digestion. Resistance to GI hydrolysis is an important quality parameter in the generation of bioactive peptides since pepsin/pancreatin could decrease the biological potential by further peptide hydrolysis Figure 21.

To understand the mechanism by which peptides inhibited DPP-IV α-amylase and α-glucosidase, the alcalase generated protein fractions were investigated using computational docking. Peptide AKSPLF was interesting due to its high potential to inhibit the three enzymes at low *Ki* concentrations even lower than the positive controls, sitagliptin or acarbose; these are drugs used in the treatment of diabetes due to their potential to inhibit DPP-IV, α-amylase and α-glucosidase [28]. Free energy and inhibition constant demonstrated the strength of the interaction with the amino acids in the catalytic site.

Luna-Vital et al. [29] reported that free energy values ranged from −6.47 to −9.34 kcal/mol when peptides interacted with the catalytic site of ACE, an enzyme related to hypertension. These values are higher (therefore less potent interaction) than some of the values obtained for peptides in this study; for instance, EGLELLLLLLAG (-9.8 kcal/mol), AKSPLF (-9.6 kcal/mol) and FEELN (-9.5 kcal/mol) for DPP-IV, and AKSPLF (-10.2 kcal/mol), WEVM (-10.1 kcal/mol) and QTPF (-9.62 kcal/mol) for α-amylase. DPP-IV substrate specificity for Pro and Ala amino acids containing peptides on the N-terminal side chain make Pro containing peptides good inhibitors of the enzyme [30]. Ngoh et al. [31] reported that peptides containing Leu, Pro, Gly, and Phe are good inhibitors of α-amylase. In the case of α-glucosidase, Roskar et al. [32] suggested that sugar-mimetic structure compounds are better α-glucosidase inhibitors; however, peptides also present great inhibitory potential due to their affinity and specificity of action on molecular targets.

The mechanism of enzymatic inhibition of sequenced peptides was executed mainly by hydrogen bonds, polar and hydrophobic interactions. Other factors also played an important role in the inhibition potential of peptides, for instance in the principal component analysis performed, the size, free energy, *Ki*, number of hydrophobic amino acids, net charge, hydrogen bonds and number of basic amino acids in the peptide were significantly affected. The

combination of those properties determined the type of interaction and the strength of the binding on the enzyme catalytic site.

Molecular docking techniques have been widely used in drug discovery [30]. *In silico* approach has been used to understand the inhibitory mechanism of milk peptides on DPP-IV, which revealed that primary interaction between peptide and amino acids in the catalytic site of the enzyme are through hydrogen bonding and hydrophobic interactions [30]. The inhibitory mechanism for 93% of peptides and enzymes was similar; however, peptides with high positive or negative net charge, containing high amounts of hydrophobic and basic amino acids tend to separate in smaller clusters. Hydrogen bonds interaction and basic amino acids showed a positive correlation with net charge. This indicates that peptides that the charged peptides are more likely to for hydrogen bonds with the enzyme catalytic site.

7.5 Conclusions

It was possible to generate protein fractions with high potential to inhibit enzymatic activity of DPP-IV, α-amylase and α-glucosidase. Alcalase protein fractions, produced at 2 h and 1:20 E/S ratio showed outstanding anti-diabetic potential by inhibiting targeted enzymes through hydrogen bonds, polar and hydrophobic interactions. Peptides from black bean protein isolate have the potential to inhibit important molecular markers related to diabetes.

7.6 References

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7.7 Tables and figures

Table 9. Inhibition of DPP-IV, α-amylase and α-glucosidase of bean protein fractions generated using different proteases, time and E/S ratios.

Trypsin					**Proteinase k**		
Time (h)	*E/S*	*DPP-IV inh (%)*	*α-amylase inh (%)*	*α-glucosidase inh (%)*	*DPP-IV inh (%)*	*α-amylase inh (%)*	*α -glucosidase inh (%)*
2	1:20	90.0±1.9^{a}	13.0±1.9^{c}	19.0±5.5^{cd}	46.7±4.6^{bcd}	26.5±6.6^{a}	29.5±0.9^{abc}
2	1:30	70.0±5.2^{bc}	18.3±5.2^{bc}	12.2±1.1^{d}	55.7±8.5^{abc}	37.1±10.0^{a}	30.6±1.8^{abc}
2	1:50	42.9±3.2^{d}	19.4±3.2^{bc}	27.5±1.6^{b}	28.1±8.7^{de}	31.8±4.8^{a}	27.2±1.9^{c}
3	1:20	50.8±3.9^{cd}	14.1±3.9^{c}	23.6±0.3^{bc}	60.2±9.4^{ab}	29.4±0.8^{a}	25.0±1.0^{c}
3	1:30	76.4±6.6^{b}	33.5±6.6^{a}	11.7±6.8^{d}	70.2±6.9^{a}	34.2±9.7^{a}	30.0±1.3^{abc}
3	1:50	83.6±1.8^{b}	14.8±1.8^{bc}	3.6±0.6^{e}	16.5±2.1^{e}	28.0±6.8^{a}	27.8±0.4^{bc}
4	1:20	51.3±7.5^{cd}	28.3±7.5^{ab}	24.8±0.1^{bc}	38.9±5.1^{cd}	34.0±4.8^{a}	35.8±5.6^{a}
4	1:30	55.2±0.2^{cd}	14.6±0.2^{bc}	46.7±1.0^{a}	39.4±4.3^{cd}	35.6±9.5^{a}	34.5±1.2^{a}b
4	1:50	67.7±7.7^{bc}	26.6±7.7^{abc}	24.7±0.8^{bc}	56.2±7.8^{abc}	31.9±7.4^{a}	29.8±2.5^{abc}
Flavorzyme					**Thermolysin**		
Time	*E/S*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*
2	1:20	39.7±14.3^{cde}	64.5±2.7^{a}	25.2±0.3^{ab}	64.6±8.1^{ab}	17.5±1.9^{bc}	15.0±1.2^{cd}
2	1:30	26.3±5.4^{de}	56.8±8.4^{ab}	22.9±0.1$_{ab}$	58.1±12.7^{ab}	16.7±2.6^{c}	13.2±0.1^{cd}
2	1:50	49.5±7.6^{cd}	61.8±11.7^{ab}	22.6±2.7^{b}	60.9±14.0^{ab}	19.9±3.1^{abc}	11.2±5.9^{d}
3	1:20	56.1±4.0^{bc}	61.5±10.5^{ab}	26.3±0.5^{ab}	85.4±6.3^{a}	23.2±0.3^{ab}	19.1±0.4^{abc}
3	1:30	51.7±14.1^{cd}	39.1±1.8^{ab}	22.4±2.3^{b}	42.2±11.0^{b}	19.7±0.2^{abc}	19.2±1.1^{abc}
3	1:50	46.6±4.3^{cde}	36.1±1.8^{b}	22.8±1.6^{b}	65.0±12.6^{ab}	15.6±1.3^{c}	18.3±0.1^{bcd}
4	1:20	78.8±8.6^{ab}	60.1±8.5^{ab}	23.0±5.9^{ab}	71.5±9.8^{a}	14.3±1.3^{c}	16.9±4.5^{cd}
4	1:30	86.7±9.6^{a}	59.8±10.7^{ab}	24.3±3.3^{ab}	66.0±9.0^{ab}	18.1±2.8^{abc}	26.2±0.1^{a}
4	1:50	22.2±8.3^{e}	58.7±16.4^{ab}	30.4±0.1^{a}	87.3±2.6^{a}	22.6±1.4^{ab}	25.0±0.4^{ab}
Alcalase					**Pepsin**		
Time	*E/S*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*
2	1:20	96.7±16.0^{a}	53.4±5.4^{a}	66.1±0.8^{a}	50.1±7.1^{b}	42.4±1.8^{a}	8.90±1.1^{ab}
2	1:30	69.6±7.4^{b}	44.4±2.8^{a}	62.9±0.1^{a}	42.9±4.9^{b}	29.1±2.0^{ab}	8.2±0.6^{b}
2	1:50	86.1±5.3^{ab}	52.5±5.2^{a}	63.2±0.8^{a}	51.7±4.6^{b}	36.6±6.2^{ab}	7.6±1.0^{b}
3	1:20	83.0±6.5^{ab}	51.4±4.8^{a}	65.6±1.0^{a}	15.1±4.7^{c}	36.0±5.5^{ab}	10.9±0.8^{ab}
3	1:30	74.3±4.6^{b}	50.4±3.3^{a}	63.2±0.7^{a}	79.7±9.2^{a}	31.5±4.5^{ab}	12.1±0.9^{ab}
3	1:50	79.2±7.4^{ab}	44.6±1.9^{a}	63.5±1.3^{a}	80.3±9.4^{a}	36.0±5.5^{ab}	8.1±0.4^{b}
4	1:20	72.2±14.7^{b}	49.4±4.6^{a}	61.9±2.8^{a}	48.4±9.0^{b}	23.2±4.1^{b}	11.1±3.6^{ab}
4	1:30	79.7±15.5^{ab}	45.6±0.6^{a}	64.9±2.9^{a}	31.9±10.8^{cb}	40.6±8.8^{a}	16.3±7.1^{a}
4	1:50	84.4±6.7^{ab}	45.5±1.9^{a}	65.4±0.3^{a}	11.6±2.9^{c}	34.7±1.3^{ab}	13.2±0.4^{ab}
Papain					**Chymotrypsin**		
Time	*E/S*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*	*DPP-IV inh*	*α-amylase inh*	*α-glucosidase inh*
2	1:20	69.5±8.5^{a}	16.1±2.5^{c}	78.3±2.5^{a}	45.6±4.3^{ab}	57.3±6.7^{a}	21.5±3.6^{c}
2	1:30	52.6±6.9^{ab}	14.8±2.4^{c}	77.1±0.3^{a}	46.3±1.6^{ab}	56.9±7.1^{a}	24.1±0.7^{bc}
2	1:50	28.6±5.0^{bcd}	27.8±8.2^{ab}	76.3±1.9^{a}	24.8±4.7^{cd}	49.7±6.2^{a}	23.0±3.6^{bc}
3	1:20	20.7±12.4^{cd}	26.8±1.7^{ab}	77.4±0.2^{a}	23.6±5.2^{cd}	51.3±1.7^{a}	26.1±1.5^{bc}
3	1:30	63.8±10.2^{a}	20.9±1.5^{abc}	75.7±0.4^{a}	22.2±6.8^{cd}	48.3±1.1^{a}	21.4±0.6^{c}
3	1:50	13.9±7.1^{d}	15.4±1.0^{c}	75.3±0.7^{a}	14.5±2.7^{d}	46.6±4.3^{a}	25.0±0.1^{bc}
4	1:20	72.3±15.1^{a}	25.2±2.3^{abc}	75.9±0.9^{a}	34.4±5.8^{bc}	52.4±2.2^{a}	25.0±2.9^{bc}
4	1:30	46.6±8.3^{abc}	19.1±5.2^{bc}	78.4±0.6^{a}	58.3±9.8^{a}	51.1±4.4^{a}	29.5±3.9^{ab}
4	1:50	36.8±5.9^{bcd}	30.0±1.7^{a}	77.8±1.3^{a}	46.4±2.0^{ab}	54.5±3.5^{a}	33.6±1.1^{a}

Data is expressed as mean ± standard deviation with at least three independent replications; Values within a column followed by different letters are significant at $p < 0.05$. %: Percent inhibition of enzymes per mg of dry hydrolysate; Time in hours; E/S: enzyme/substrate; DPP-IV: dipeptidyl peptidase IV; inh: inhibition; h: hour.

Table 10. Peptide sequences obtained and their biological potential after 2 h alcalase hydrolysis and 1:20 [E/S].

MW	Sequence	Net C	pI	Hydro-phobicity	Bioactive sequences	Potential biological activity	Blast protein
469.3	LLPK	+1	9.80	+8.34	LLP, LL, LP, PK	ACE, GU, DPP-IV	Cytochrome P450 85A
491.2	QTPF	0	5.38	+7.35	TP, PF, QT	DPP-IV	Endochitinase CH5B
527.2	FFQS	0	5.39	+5.71	FQ, QS	DPP-IV	UDP-glucuronic acid decarboxylase 1
526.2	GSLGGH	0	7.69	+12.89	GH, GS, GG, LG, SL	ACE , DPP- IV	Phytohemagglutinin-L
526.3	GSRAH	+1	10.9	+14.15	RA, GS, AH	ACE, AC, DPP-IV, OT	Inositol-3-phosphate synthase
627.3	YVFLS	0	5.5	+4.23	VF, FL, YV	ACE, DPP-IV	lanC-like protein
527.3	LALVL	0	5.58	+4.19	LVL, LA, VL, LV, AL	ACE , GU, OT, DPP-IV	Lectin
563.2	WEVM	-1	3.27	+8.31	EV, WE, VM	ACE, DPP IV	Pectinesterase 3
627.3	FEELN	-2	2.97	+13.05	LN, EE, EL	ACE, DPP-IV, AC, OT	Putative resistance protein TIR 17
627.3	VYFLS	0	5.53	+4.23	VY, FL, YF	ACE, AC, DPP-IV	Phaseolin
630.3	LKEGGK	+1	9.63	+18.18	GK, GG, EG, KE, LK	ACE, AC, DPP-IV	NADP-dependent malic enzyme
645.4	LSKSVL	+1	10.1	+8.66	VL, KS, SK, SV	GU, DPP-IV	Lectin
650.3	LYELN	-1	3.20	+9.17	LY, LN, EL, YE	ACE, AC, DPP-IV	Protein kinase PVPK-1
661.3	ATNPLF	0	5.53	+6.68	LF, PL, TNP, NP, AT, TN	ACE , DPP IV	Arcelin-1
661.4	AKSPLF	+1	9.93	+8.84	LF, PL, SP, KS	ACE, DPP-IV	Wound-induced basic protein
688.3	SGPFGPK	+1	9.80	+12.03	GP, FG, SG, GP, PF, PK	AT, ACE, AA, DPP- IV	Polygalacturonase inhibiting protein
689.2	GSPVSSR	+1	10.7	+11.92	GS, SP, PV, VS	ACE, GU, DPP-IV	Arcelin-1
691.4	TTNPLF	0	5.32	+6.43	LF, PL, TNP, TTN, NP, PL	ACE, DPP-IV	Glutelin type-A 2-like protein
704.4	TTGGKGGK	+2	10.57	+18.60	KG, GK, GG, TG, TT	ACE, DPP-IV	Glycine-rich cell wall structural protein
718.3	ASATTGVL	0	5.60	+9.30	GV, TG, VL, AS, AT, TT	ACE, GU, DPP-IV	Phytohemagglutinin-L
789.4	SKGSGGGKL	+2	10.62	+17.77	KG, GS, GK, GG, KL, SK	ACE, DPP-IV	Phosphoenolpyruvate carboxylase
942.5	SAKGPPMGAK	+2	10.57	+16.87	GP, KG, MG, GPP, PP, PM	AA, ACE, AT, DPP-IV	Leucine rich protein, plant defense
942.6	SAKGPPTSAK	+2	10.57	+17.10	KG, GPP, PP, GP, PT, TS	AA, ACE, AT, DPP-IV	Arcelin-5
942.6	SARVLAAGAK	+2	11.52	+14.41	LAA, LA, AA, GA, AG, AR	ACE, GU, DPP-IV, OT	Arcelin-5
958.5	SANRLPSAGS	+1	10.85	+12.98	RL, AG, GS, LP, NR, PS	ACE, DPP-IV	Phaseolin
958.6	RKLKMRQ	+4	12.50	+15.97	KL, LK, MR, RK	ACE , AC, DPP IV	Red kidney bean purple acid phosphatase
1090.5	SLKWWDLGS	0	6.51	+9.73	KW, LG, LK, SL, WW, WD	ACE, AC, DPP-IV	Arcelin-1
1118.6	SLPAGGNRYGK	+2	10.35	+15.95	RY, AG, GK, GG, YG, PA, LP, SL, NR	ACE , OT, DPP-IV	Nitrate reductase [NADH] 2
1222.6	SRSPAGPPPTEK	+1	9.80	+19.52	GP, AG, GPP, TE, PT, PP, EL, PA, SP, EL	AA, ACE, AT, DPP-IV	Potassium-dependent plant-type L-asparaginase
1223.6	ALMLEEYLLE	-3	2.94	+12.91	YL, EY, LL, EE, AL, LM	ACE, GU, OT, DPP-V	Phaseolin
1252.8	QQRRRLRLK	+5	12.96	+16.98	RL, RR, LK, QQ, RL	ACE , AC, DPP-IV	Phaseolin
1252.8	EGLELLLLLLAG	-2	2.92	+9.21	LA, GL, AG, EG, LLL, LL	ACE, GU, OT, DPP-IV	Phaseolin
1252.8	DLALLLLLAELG	-2	2.82	+8.57	LA, LG, LLL, LL, EL, AL, AE	ACE, GU, AC, OT, DPP-IV	Lectin
1378.7	LPPSPERTAAPPF	0	6.65	+12.79	AAP, LPP, AP, AA, PP, PPSP, LP, SP, TA, PF, PS	ACE, DPP-IV	Red kidney bean purple acid phosphatase

Peptides obtained from the LC-ESI-MSMS elution profile with intensity of at least 70%; Sequences belonging *to Phaseolus vulgaris* protein was confirmed by BLAST tool; Potential bioactivities were obtained from the BIOPEP database; Physicochemical properties were obtained from Pepdraw: Net C: net charge; pI: isoelectric point; hydrophobicity: Kcal*mol^{-1}; AT: Antithrombotic; AA: Antiamnestic; AC: antioxidant; GU: Glucose uptake; OT: other activities such as activating proteolysis; neuropeptide and immunomodulation. MW: molecular weight.

Table 11. Biological potential of peptides produced by different enzymes from principal common bean proteins such as phaseolin, lectin, and α-amylase inhibitors.

Trypsin	Proteinase k	Flavorzyme	Thermolysin	Pepsin	Papain	Chymotrypsin
SHGGLGGAPSTGGV, TDNVLSSLGR, KSGGGGGGGSPVFG APLP, DPPGGAN, SPHPTPDPAPGGAG, GNSPMGGGSGSK, TGNGGAAGFPVQPPM GGLGGAGGGQ, NGEGPHSGGADSGFDQ , NSTPMGPVGSG, PALPFPDFGGSQ, DHSVGAPGSPDPAACN DSP, MGGSSK, LSGGGGWHGGAG, MPAPNPGPPNGVQ	NLHPGSAGGVL, TVPSNAGGGAGA PPAPH, DGRSVLGL, ALVLL, GSNGFPGGGGGL ,RNGPHPGGGGP VEPSL, SPMPVNGPPAGP GGAGF	YPVAL, KSSASPGATAFVLPA , NDGFLFSVSGNGAL AP, ANGAV, KSASSPGATAVELVS	LDEVED, AFFLAG	DGRSVL GL, RNKLYE AQ,FGGK, DGKDVL GL, LVLL, GRGGGL PGVS, FVLPAAY	NDPFY, VPQGLDL	LQEF, VLPAAY, RLVEF, RLPL, ANAPPAGT, VGPVQL, TGSNGA, RAELSKDDVF, APATQPVGT, NTLF, RLDLGSPLLSH, KGGSALPSGMFKPVG GGH, RRLREELRPQ, QPPVGSGVAGLGH

X: ACE; X: DPP-IV; X: glucose uptake, X: Anti-amnestic; X: anti-oxidative; X: regulating different activities; X: Antithrombotic; X other bioactivities. Peptides obtained from the LC-ESI-MSMS elution profile with intensity of at least 70%. Peptide sequences found in common bean storage proteins (phaseolin and lectin family proteins) after 2 h of hydrolysis and E/S: enzyme/substrate 1:20; Potential bioactivities were obtained from the BIOPEP database, Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

Table 12. Summary of free energy and inhibition constant (*Ki*) of ten peptides obtained by alcalase hydrolysis after 2 h at 1:20 [E/S], characterized by HPLC-MSMS, and with the highest biological potential to inhibit DPP-IV, α-amylase and α-glucosidase enzymes using computational docking.

DPP-IV				α-Glucosidase				α-Amylase			
Peptide Sequence	MW kDa	Free Energy	*Ki* μM	Peptide Sequence	MW	Free Energy	*Ki* μM	Peptide Sequence	MW	Free Energy	*Ki* μM
EGLELLLLLLAG	1252.8	-9.8	0.059	TTGGKGGK	704.3	-8.97	0.265	AKSPLF	661.5	-10.2	0.033
AKSPLF	661.5	-9.6	0.083	AKSPLF	661.5	-8.37	0.772	WEVM	563.2	-10.1	0.040
FEELN	650.2	-9.5	0.096	LKEGGK	630.3	-8.07	1.210	QTPF	491.2	-9.62	0.089
TTNPLF	691.3	-9.3	0.140	GSPVSSR	688.3	-7.27	4.712	FFQS	527.2	-9.58	0.094
TTGGKGGK	704.3	-9.2	0.164	LLPK	469.3	-7.18	5.430	YVFLS	627.3	-9.48	0.112
ATNPLF	661.3	-8.9	0.261	SGPFGPK	688.3	-7.18	5.480	LSKSVL	645.4	-9.25	0.165
QTPF	491.2	-8.9	0.262	QTPF	491.2	-6.87	9.220	FEELN	650.2	-9.08	0.222
LSKSVL	645.4	-8.8	0.344	RKLKMRQ	958.5	-6.84	9.690	LLPK	469.3	-8.89	0.302
LKEGGK	630.3	-8.7	0.384	FEELN	650.2	-6.81	10.22	LYELN	650.3	-8.28	0.851
SGPFGPK	688.3	-8.6	0.494	LSKSVL	645.4	-6.74	11.40	LALVL	527.3	-8.27	0.860
Sitagliptin	407.31	-8.6	0.440	Acarbose	645.6	-8.79	0.360	Acarbose	645.6	-9.71	0.070

Peptides obtained from the LC-ESI-MSMS elution profile with intensity of at least 70%; 34 peptides were tested by computational docking and 10 were selected due to their potency to inhibit DPP-IV, α-amylase and α-glucosidase. MW: molecular weight; kDa: kilodaltons; free energy expressed as kcal/mol; *Ki*: inhibition constant expressed in μM; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

Table 13. Amino acid interaction betwwn the peptide AKSPLF, QTPF, FEELN and LSKSVL and the enzymatic catalytic site of DPP-IV, α-amylase and α-glucosidase.

Enzyme	Peptide	Hydrogen bonds			Polar interactions			Hydrophobic interactions		
		Peptide aa	Distance (A°)	Enzyme aa	Peptide aa	Distance (A°)	Enzyme aa	Peptide aa	Distance (A°)	Enzyme aa
DPPIV	AKSPLF	LYS	[2.57]	GLU191	ALA	[2.06]	ASP192	ALA	[3.81]	LEU235
		LYS	[2.48]	ASP192	LYS	[2.09]	GLU191			
		LYS	[2.56]	ARG253	ALA	[3.47]	ARG253			
	QTPF	GLU	[2.68]	ASP192	GLU	[1.67]	ASP192	PHE	[3.39]	TRP124
		PRO	[3.47]	THR251	PHE	[3.58]	ASP192	PRO	[3.31]	VAL252
					THR	[3.63]	ARG253	PHE	[3.53]	VAL252
					GLU	[3.58]	ARG253	PHE	[3.10]	VAL254
					THR	[2.97]	ARG253			
	FEELN	PHE	[3.43]	GLU191	GLU	[3.15]	GLU191			
		PHE	[2.41]	ASP192	PHE	[3.36]	GLU191			
		ASN	[2.95]	PRO249	PHE	[1.74]	ASP192			
					GLU	[3.86]	ASP192			
					ASN	[3.41]	LYS250			
					GLU	[3.42]	ARG253			
					LEU	[2.92]	ARG253			
	LSKSVL	LEU	[2.56]	ASP192	LEU	[1.97]	ASP192			
		VAL	[3.17]	LYS250	LEU	[3.25]	ARG253			
		LEU	[3.45]	THR251	LYS	[2.77]	ARG253			
		LEU	[3.46]	ARG253	LEU	[3.53]	GLU237			
		LYS	[2.31]	GLU237	LYS	[2.12]	GLU237			
α-amylase	AKSPLF	SER	[2.95]	TYR151	LEU	[3.81]	TYR151	LYS	[3.43]	LEU162
		LYS	[2.83]	ASP197	ALA	[3.70]	ARG195	SER	[3.27]	LEU162
		ALA	[2.52]	GLU233	ALA	[1.88]	ASP197	LYS	[3.25]	ALA198
		LYS	[3.10]	GLU233	SER	[3.02]	HIS201	LYS	[3.11]	HIS201
					LYS	[2.48]	GLU233	LYS	[3.80]	ILE235
					ALA	[3.30]	HIS299	PRO	[3.57]	ILE235
	QTPF	GLN	[2.75]	ASP197	THR	[3.27]	TYR151	PRO	[3.14]	LEU162
		PHE	[3.49]	HIS201	PHE	[3.25]	HIS201	PHE	[3.33]	HIS201
					PHE	[3.05]	GLU233	PHE	[3.11]	ILE235
					GLN	[3.63]	ASP300			
	FEELN	ASN	[2.84]	TRP59	ASN	[3.20]	GLN63	ASN	[3.80]	LEU165
		GLU	[3.36]	THR163	PHE	[3.76]	TYR151	PHE	[3.32]	ILE235
					GLU	[3.40]	TYR151	PHE	[3.61]	LEU237
					LEU	[3.83]	THR163	PHE	[3.67]	ALA307
					PHE	[3.68]	LYS200			
					GLU	[3.49]	HIS201			
	LSKSVL	SER	[3.25]	TYR151	SER	[3.08]	TYR151	LEU	[3.26]	TYR62
		SER	[2.94]	THR163	LEU	[2.05]	ASP197	LYS	[3.75]	TYR151
		LEU	[2.47]	ASP197	LEU	[3.01]	GLU233	SER	[3.78]	TYR151
					LYS	[2.06]	GLU233	LYS	[3.38]	LEU162
								LEU	[3.60]	LEU165
								LYS	[3.53]	HIS201
								LYS	[3.35]	ILE235
								LEU	[3.54]	ALA307
α-glucosidase	AKSPLF	LYS	[2.56]	ASP34	LYS	[2.83]	ASN32			
		LYS	[2.80]	THR83	LYS	[2.12]	ASP34			
		ALA	[2.77]	ASP89	LYS	[3.72]	THR83			
					ALA	[1.82]	ASP89			
	QTPF	GLN	[2.75]	ASN32	GLN	[3.02]	ASN32	PRO	[3.83]	PRO82
		GLN	[2.57]	ASP34	THR	[2.91]	ASN32			
		PHE	[2.56]	THR83	GLN	[2.08]	ASP34			
					PRO	[3.43]	ASP34			
					GLN	[2.75]	ASP34			
					PHE	[3.83]	TRP36			
					PHE	[2.01]	THR83			
	FEELN	PHE	[2.76]	ASP34	GLU	[2.60]	ASN32			
		PHE	[3.10]	THR83	GLU	[3.22]	ASP34			
					PHE	[1.84]	ASP34			
					GLU	[3.72]	ASP34			
					PHE	[2.19]	ASP34			
					GLU	[3.66]	THR83			
	LSKSVL	LYS	[2.56]	ASN32	SER	[3.32]	ASN32			
		SER	[2.97]	ASP34	SER	[3.21]	ASP34			
		SER	[3.33]	THR83	LEU	[3.88]	THR83			
		LEU	[2.76]	ASP89	LEU	[1.92]	ASP89			

AKSPLF, QTPF, FEELN and LSKSVL peptides were selected based on their potential inhibitory activities of DPP-IV, α-amylase and α-glucosidase; A° : angstroms; aa: amino acids; amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; K, lysine.

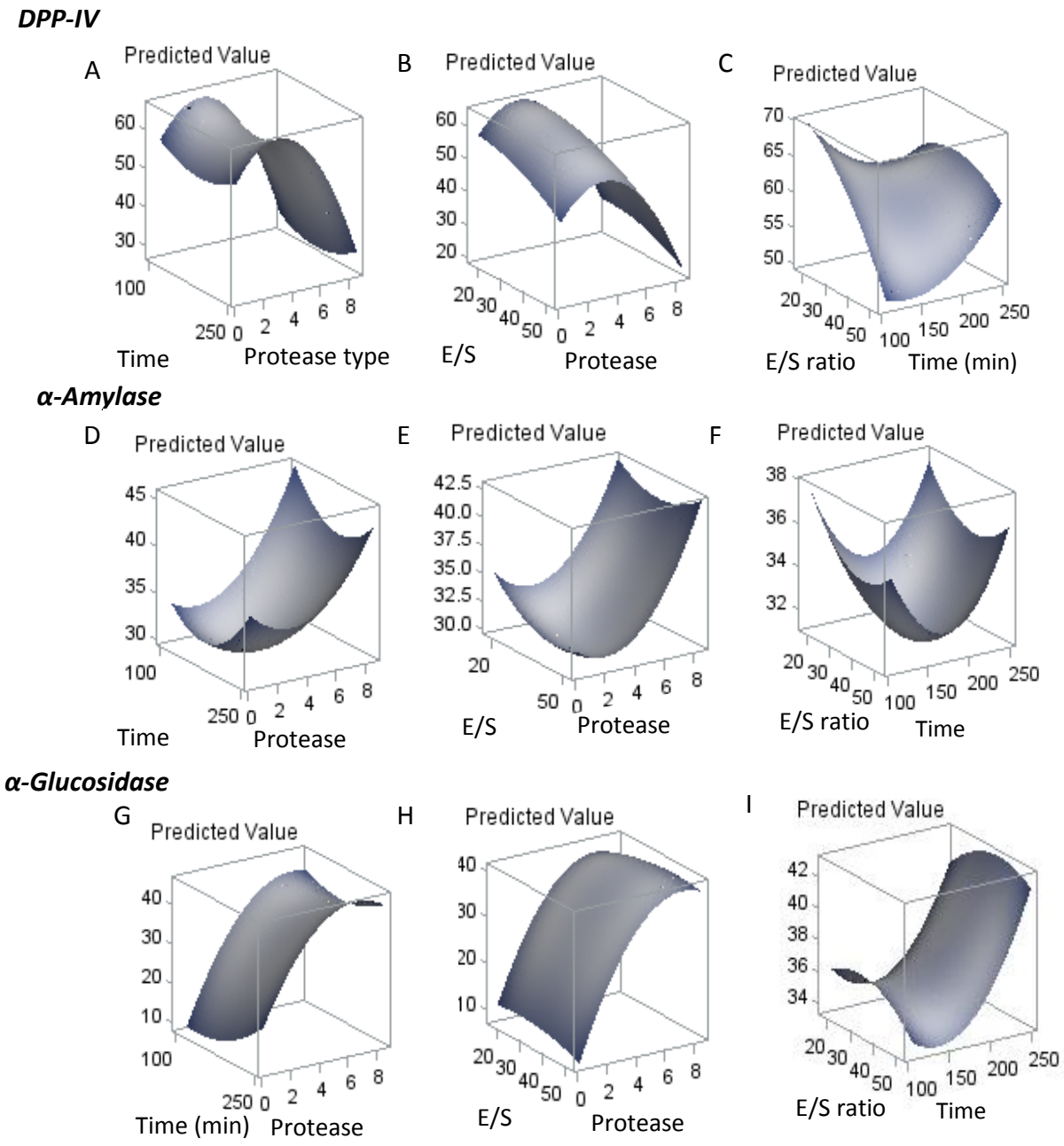


Figure 16. Surface response methodology for the optimization of bioactive peptides as affected by type of protease, time and enzyme/substrate ratio. DPP-IV (A, B and C), α-amylase (D, E and F), and α-glucosidase (G, H and I).

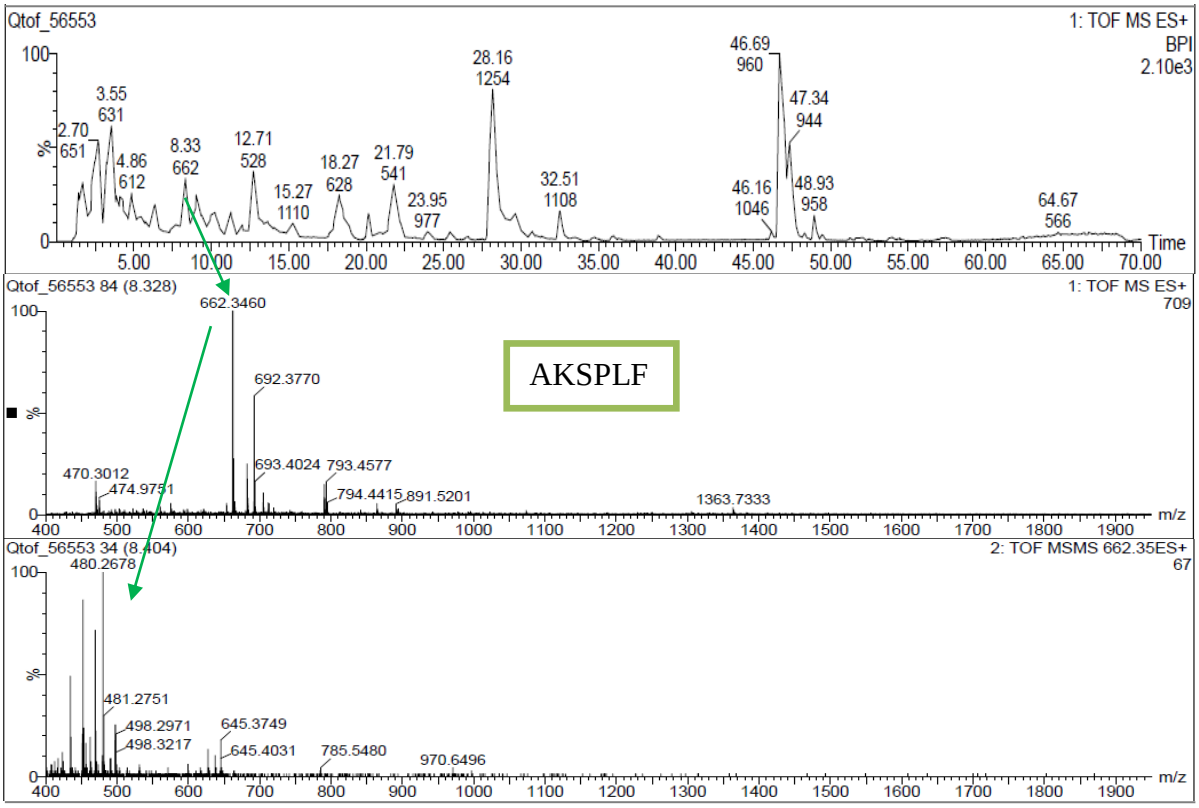


Figure 17. LC–ESI–MSMS profile for alcalase protein fractions; including mass spectrometric fragmentation profile of 1st peak chromatogram, MS first fragmentation and MSMS ion at 622 *m/z* for sequencing AKSPLF peptide.

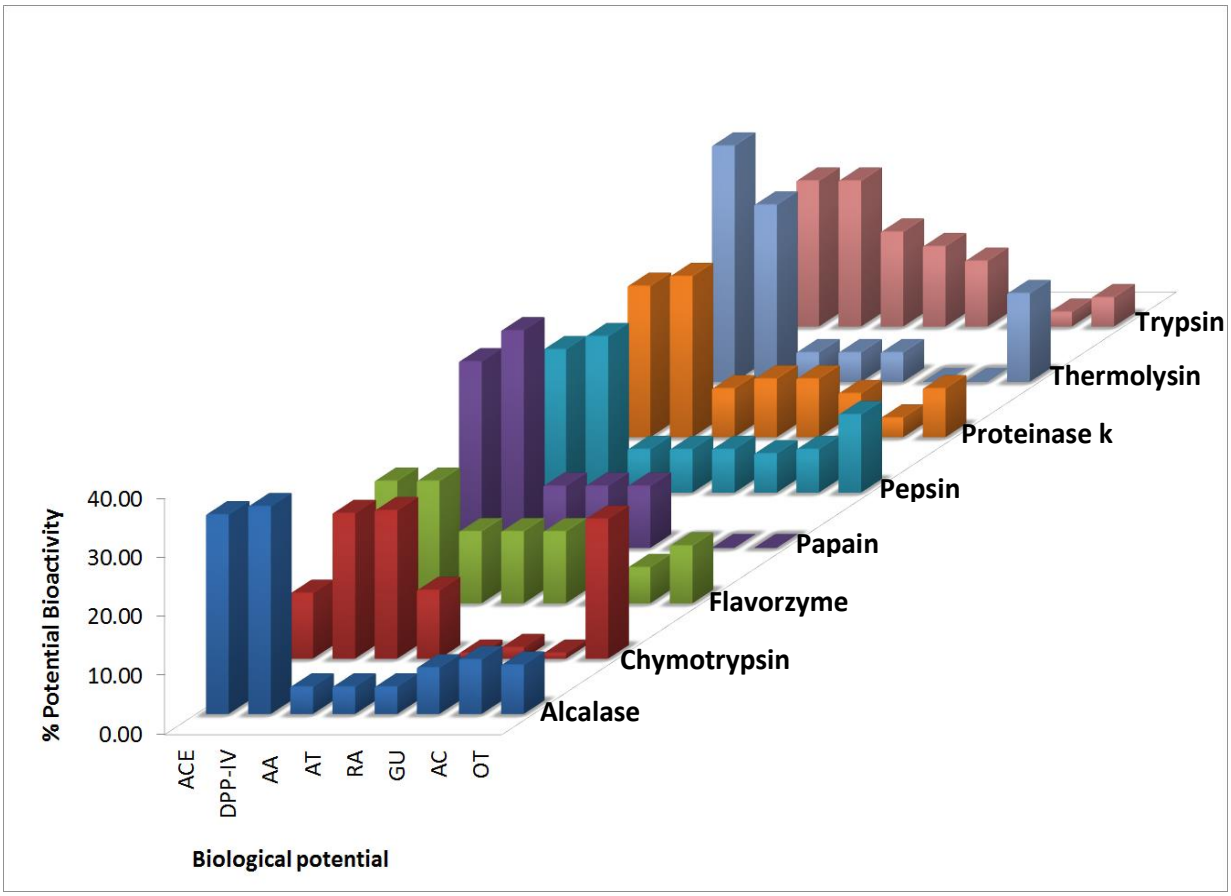


Figure 18. Biological potential of different protease generated protein fractions using *in silico* Biopep database. ACE: angiotensin converting enzyme inhibitor; DPP-IV dipeptidyl peptidase IV inhibitor; AT: Antithrombotic; AA: Anti-amnestic; RA: regulating different activities; AC: anti-oxidative; GU: Glucose uptake; OT: other bioactivities.

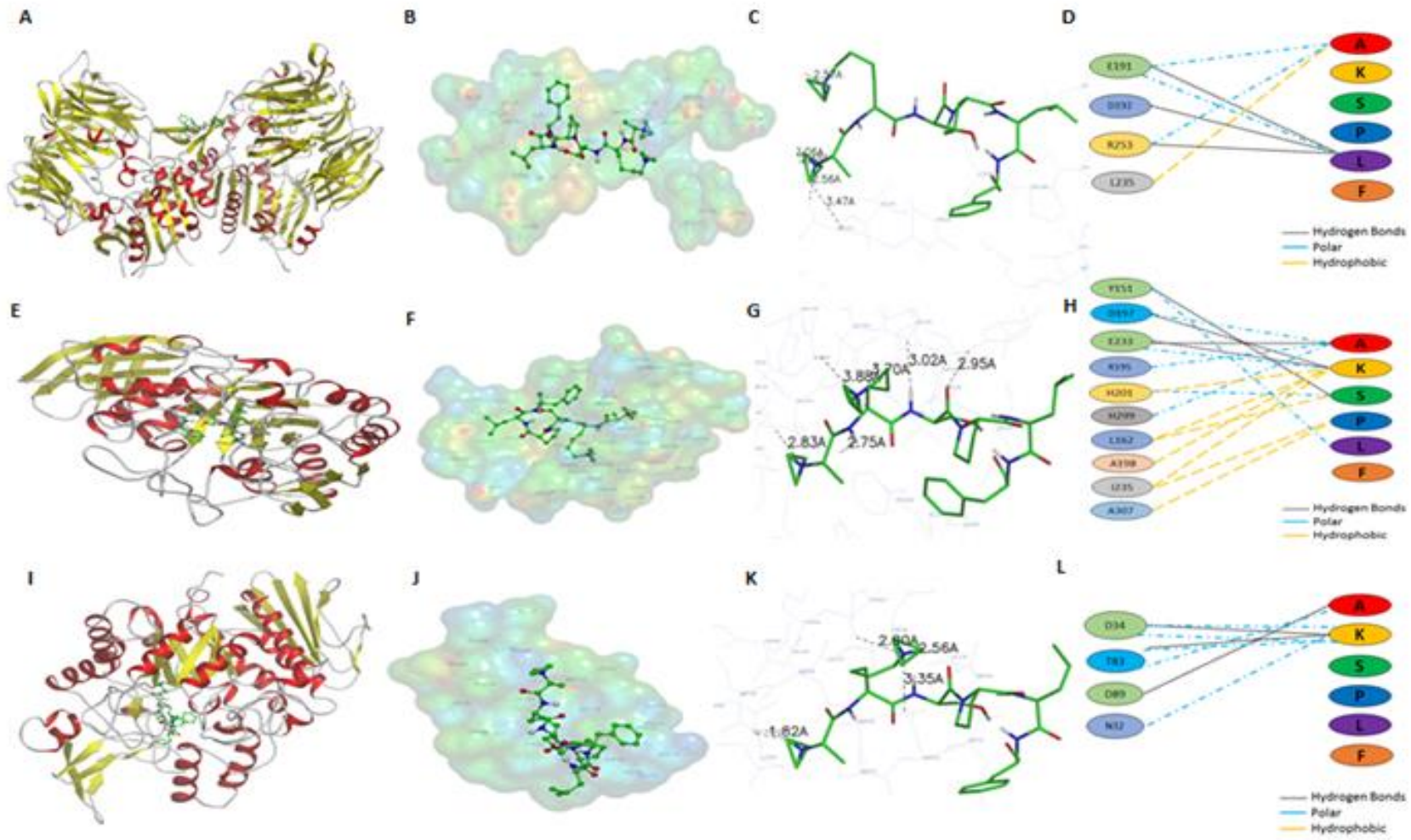
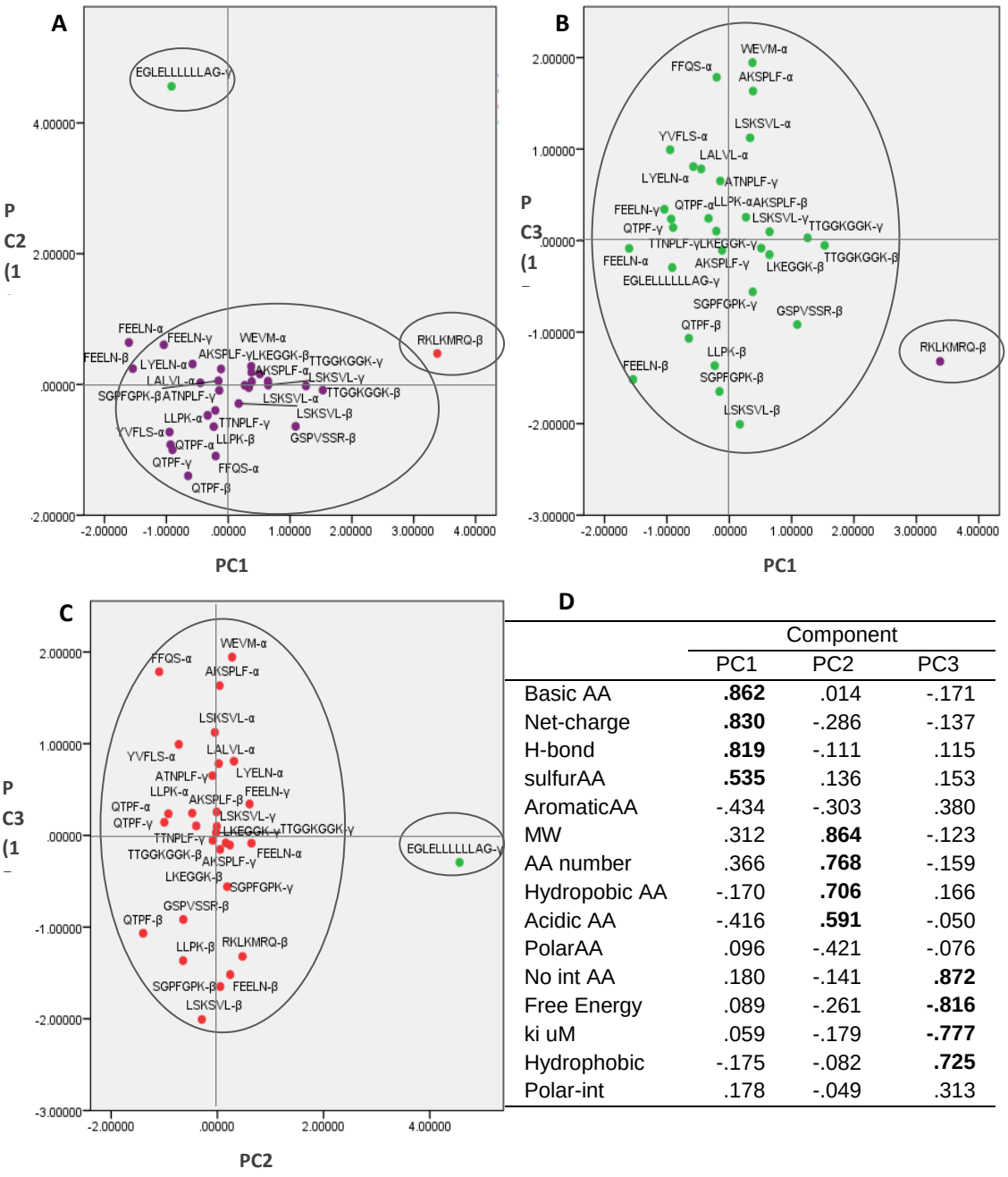


Figure 19. Molecular docking diagram exemplifying the analysis of AKSPLF peptide interacting with DPP-IV, α-amylase and α-glucosidase. A, E and I, best pose of the peptide (sticks) inside the enzyme catalytic site. B, F and J, best pose of the peptide with the interacting side chains of the catalytic site. C, G and K, distances of the peptide and the residues of the catalytic cavity wich presented ineraction. D, H and L, diagram of the interaction type of the peptides with amino acid residues of the catalytic site.



D

	Component		
	PC1	PC2	PC3
Basic AA	**.862**	.014	-.171
Net-charge	**.830**	-.286	-.137
H-bond	**.819**	-.111	.115
sulfurAA	**.535**	.136	.153
AromaticAA	-.434	-.303	.380
MW	.312	**.864**	-.123
AA number	.366	**.768**	-.159
Hydropobic AA	-.170	**.706**	.166
Acidic AA	-.416	**.591**	-.050
PolarAA	.096	-.421	-.076
No int AA	.180	-.141	**.872**
Free Energy	.089	-.261	**-.816**
ki uM	.059	-.179	**-.777**
Hydrophobic	-.175	-.082	**.725**
Polar-int	.178	-.049	.313

Figure 20. Cluster and principal component analysis score plots of peptides inhibition parameters on DPP-IV, α-amylase and α-glucosidase. Score plots for the first three principal components A: PC1 (26.0), B: PC2 (19.0) and C: PC3 (15.0). α: peptides interacting with DPP-IV; β: peptides interacting with α-amylase; γ: peptides interacting with α-glucosidase; amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

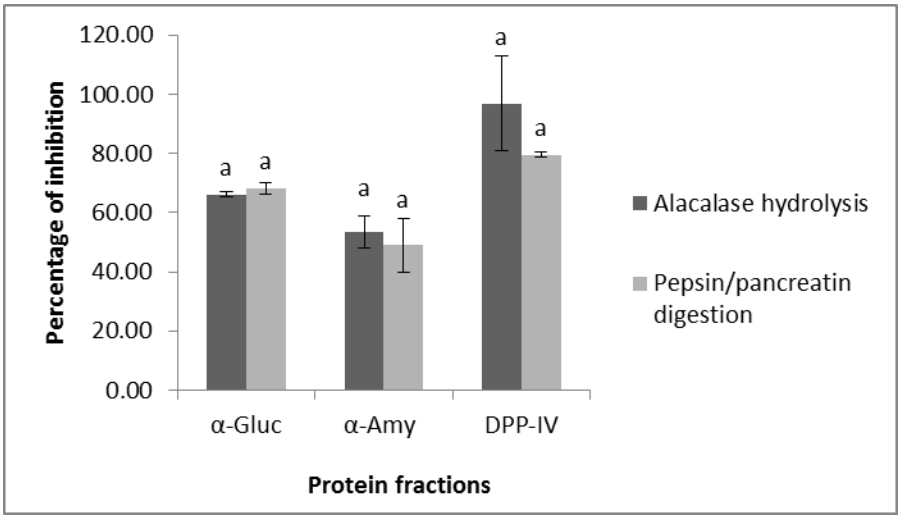


Figure 21. Effect of GI digestion over peptides biological potential to inhibit α-glucosidase, α-amylase and DPP-IV.

CHAPTER 8: BEAN CULTIVARS (*Phaseolus vulgaris* L.) HAVE SIMILAR HIGH ANTIOXIDANT CAPACITY, *in vitro* INHIBITION OF α-AMYLASE AND α-GLUCOSIDASE WHILE DIVERSE PHENOLIC COMPOSITION AND CONCENTRATION

8.1 Abstract

Common beans are a good source of essential nutrients such as protein, fiber, vitamins, and minerals; they also contain phenolic compounds and other phytochemicals. Phenolic compounds exhibit high antioxidant capacity that promotes health benefits by reducing oxidative stress. The objective was to compare the composition and quantity of anthocyanins and other non-colored phenolic compounds in fifteen improved bean cultivars from Mexico and Brazil and their relation to antioxidant capacity and enzymes related to type-2 diabetes. Samples were analyzed for total phenolic compounds (TP), flavonoids, antioxidant capacity (AC), tannins and total anthocyanins. Type and quantity were evaluated by HPLC-ESI-MS. Delphinidin glucoside (0.9–129.0 mg/100g dry coat), petunidin glucoside (0.7–115.0 mg/100 g dry coat) and malvidin glucoside (0.14–52.0 mg/100 g dry coat). Anthocyanidins were positively correlated when quantified by HPLC and colorimetric analysis (R =0.99). Cultivar Negro-Otomi presented the highest concentration of anthocyanins (250 mg/100 g dry coat). Seventeen non-colored phenolic compounds were identified among cultivars; catechin, myricetin 3-Oarabinoside, epicatechin, vanillic acid, syringic acid and o-coumaric acid, presented the highest concentrations among identified phenolic compounds. The AC of all fifteen bean cultivars did not show significant differences ($p < 0.05$) ranging from 185.2 (FM-67) to 233.9 (FM-199) mmol TE/g coat. Compounds in the coat extracts of pinto and black cultivars were the most efficient to inhibit α-amylase and α-glucosidase. Studied cultivars differed in composition and concentration of phenolics including anthocyanins; however, there was no effect on AC as measured by oxygen radical absorbance capacity. Black beans contained delphinidin and ferulic acid, compounds commonly used as ingredients in functional foods due to their associated health benefits.

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8.2 Introduction

The common bean (*Phaseolus vulgaris* L.) is an inexpensive and important source of protein, complex carbohydrates, minerals, vitamins and phenolic compounds [1, 2]. Phenolic compounds have at least one aromatic ring with one or more hydroxyl groups attached and can be classified as phenolic acids, flavones, flavanones, isoflavones, flavonols, flavanols, anthocyanins and condensed tannins [3, 4]. They are products of plant secondary metabolism and are mediators of plant stress including insect and microbial defense [5]. Phenolics in common beans are found in the seed coats with lower amounts in the cotyledons. The concentration of these compounds is influenced by growing conditions and genetic factors [5]. Health benefits of legumes are related to the dietary fiber, phenolic compounds, saponins, phytosterols, protein and peptides [6, 7]. Tannins and anthocyanins determine the color of the seed coat; the darker the coat the higher the concentration of these compounds [5].

Anthocyanins constitute one of the major groups of natural pigments and are responsible for many of the colors in fruits and vegetables [8]. Anthocyanins are polyhydroxy and polymethoxy derivatives of flavilium salts and are members of the flavonoid family, possessing a characteristic C3–C6–C3 carbon structure [9]. They are generally present in the plant tissues in the glycosylated form. Anthocyanins may provide anti-inflammatory and anti-diabetes benefits since they inhibit pro-inflammatory cytokines, decrease their production, and prevent β-cell dysfunction leading to insulin output [10]. They also work to up-regulate the insulin dependent signaling and improve glucose uptake in many cell types. It is believed that anthocyanins have multiple and simultaneous anti-diabetes effects, including reducing blood glucose and preventing free radical production [11]. The most common phenols reported in beans are phenolic acids as ferulic acid and p-coumaric acid [12,13], flavonols as kaempferol and myricetin [2,14,15], and anthocyanins including delphinidin, petunidin, malvidin and pelargonidin [2,8,16]. Phenolic compounds found in common beans have been reported to possess antioxidant, anticarcinogenic, antimutagenic and anti-inflammatory effects [17-19]. These compounds could serve as antioxidants due to their capacity to inhibit the formation of radical species, inhibit enzymes related with the production of reactive oxygen species (ROS) and superoxide anions, by chelating metal ions such as iron and copper, or as hydrogen donating radical scavengers.

Breeding modification has focused on improving the nutritional value and agronomic characteristics of cultivars. However, how these modifications affect the concentration of

secondary metabolites such as phenolics is still unclear. The objective of this research was to compare the identity and quantity of phenolic compounds in fifteen agronomically improved common bean cultivars from Mexico and Brazil and determine their relationship to antioxidant capacity and enzymes related to type-2 diabetes, α-amylase and α-glucosidase.

8.3 Materials and methods

8.3.1 Materials

Twelve common bean cultivars (*P. vulgaris* L.)were grown under the same conditions in 2012 at the Experimental Station of the National Research Institute for Forestry Agriculture and Livestock (INIFAP) in Zacatecas, Mexico (Pinto-Bayacora; Pinto-Bravo; Pinto-Centenario; Pinto-Saltillo; Flor de Junio-Leon (FJ-Leon); FJ-Marcela; Flor de Mayo-Eugenia (FM-Eugenia); FM-67; FJ-199; FM-202; Negro-Frijozac; Negro-Otomi). Three common bean cultivars grown in 2012 were provided by the Brazilian Agricultural Research Center (EMBRAPA) Brazil, (Brazilian-Horizonte, BRS-Horizonte; BRS-Pontal; Perola). Independently of the surface of the harvesting area of 1/2, 3/4, or 1 ha, a minimum of five random samples were collected from at least 5 different places in the complete surface of the field and a composite sample was obtained. Samples were collected from at least 5 m from the edges of the plantation to obtain a more representative sample. Three independent replications were performed for each analysis.

Common bean cultivars were classified according to their color pattern in four groups; group one: cream with brown dots; group two: light pink with cream lines/dots; group three: blacks; group four: brown with dark lines. Mexican cultivar names Flores de Mayo and Flores the Junio are related with the time of flowering during the months of May and June; Brazilian bean cultivars are commonly called carioca beans. The dry seeds were stored at 4 °C until separation of seed coats. Seed coats were manually removed and ground to a fine powder with an electric mill and then sieved through a number 40 mesh sieve. The seed coats were stored at −20 °C until extraction and further analysis. α-Amylase from porcine pancreas (EC3.2.1.1) and α-glucosidase from Saccharomyces cerevisiae (EC3.2.1.20), acarbose (≥95%), 3,5- dinitrosalicylic acid (≥98%), p-4-nitrophenyl-α-D-glucopyranoside (≥99%), catechin (99% purity), rutin (94% purity), gallic acid (97.5% purity), chlorogenic acid (95% purity), epicatechin (95% purity), syringic acid (95% purity), vanillin (99% purity), daidzin (95% purity), p-coumaric acid (98% purity), ferulic acid (99% purity), o-coumaric acid (97% purity), myricetin (96% purity), sodium

nitroprusside (≥99%), sulfanilic acid (≥99%), and N-(1-naphthyl)ethylenediamine dihydrochloride (≥98%) were purchased from Sigma Aldrich (St. Louis, MO).

8.3.2 Seed coat sample preparation and phenolic compounds extraction

For the determinations of total phenolic compounds, tannins, flavonoids, antioxidant capacity, α-amylase and α-glucosidase analysis, at least three independent phenolic extracts were prepared as follows. The ground seed coats (0.1 g) were placed in a flask and mixed with 5 mL 100% methanol (1:50 w/v ratio). The flask was shaken for 24 h at 20 °C while wrapped in aluminum foil to protect the extract from light. The samples were then filtered using Whatman #4 filter paper. The methanol extracts were stored at −20 °C until analysis.

For anthocyanin analysis, the three independent extractions were performed as reported by Abdel-Aal and Hucl, [20]. Briefly, 0.1 g of common bean coat was extracted with 2.4 mL of acidified ethanol (ethanol in 1 N HCl, 85:15, v/v). The solution was mixed and adjusted to pH 1 with 4 N HCl. The resulting solution was shaken for 15 min, readjusted to pH 1, and shaken for additional 15 min, centrifuged at 27,200 g for 15 min, and the supernatant made up to 5 mL volume with acidified ethanol. Sample treatment for HPLC analysis of anthocyanins and noncolored phenolic compounds was performed using a standardized method reported by Berhow et al, [21]. Between 0.05 and 0.1 g of coats were placed in a capped vial with 2–5 mL of methanol (100%). The vials were sonicated for 15 min, and allowed to stand overnight. After another brief sonication, a portion of this extract was filtered through a 0.45 μm filter into an auto sampler vial. Samples were extracted in three independent replicates.

8.3.3 Color measurements

Measurements of color space as indicated by the International Commission on Illumination (CIE), brightness (L*), redness (+a*), greenness (−a*), yellowness (+b*), and blueness (−b*) were performed using a Labscan XE Hunter Lab colorimeter. Whole beans were transferred into a cuvette to measure the color; seven measurements of each sample were performed in different sections of the sample cuvette. Chroma C* and hue angle h* were calculated [22].

8.3.4 Total phenolic compounds

Total phenolic compounds were quantified using the Folin–Ciocalteu method adapted to a microassay using gallic acid (GA) as the standard [23]. To a 96-well flat bottom plate, 50 μL of 1 N Folin–Ciocalteu's phenol reagent and 50 μL (methanol extract) of either seed coat sample

prepared as indicated in Section 2.2, standard concentrations (40–200 μg/mL) or blank were added; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na2CO3. The solution was then allowed to stand for 10 min before reading at an absorbance of 690 nm using a Synergy 2 multiwell plate reader (BioTek, Winooski, VT). The results were expressed as milligrams of gallic acid equivalents per gram of dry coat ($y = 0.0194x + 0.0449$, $R^2 = 0.99$).

8.3.5 Total anthocyanins

Total anthocyanins were determined using the AOAC Official Method (2005.02). Samples were diluted to a factor of 1:10 using two buffers (pH 1.0, 0.025 M potassium chloride buffer and pH 4.5, 0.4 M sodium acetate buffer). A 200 μL of diluted solutions, prepared as indicated in Section 2.2 of Methods, at each pH, was transferred to a 96 well clear plate and absorbance was read at 520 and 700 nm in a Synergy 2 multiwell plate reader (BioTek, Winooski, VT). The total anthocyanins were expressed as mg of cyanidin-3-glucoside (C3G) equivalents per gram of dry coat.

8.3.6 Total flavonoids

Total flavonoid concentration was quantified using an adaptation of the method used by Ooman et al. [24]. Briefly, samples (50 μL of methanolic extract prepared as indicated in Section 2.2 or rutin standard, 1–50 μg/mL) were added to a flat bottomed 96-well plate followed by the addition of 180 μL distilled water; 20 μL of a solution of 2-aminoethyldiphenol borate (10 mg/mL) was then added to each well and absorbance was read at 380nmusing a Synergy 2 multiwell plate reader (BioTek Instruments, Winooski, VT). Results were expressed as mg rutin equivalents (RE) per gram of dry coat ($y = 0.0015x + 0.0043$, $R^2 = 0.99$).

8.3.7 Total tannins

The method used for total tannins was based on the method by Aparicio-Fernandez et al. [16] with some modifications. Briefly, a 50 μL (methanol extract) of seed coat sample prepared as indicated in Section 2.2, or catechin standard (0.1–0.8 mg/mL) was added to a 96 well plate followed by the addition of 200 μL of 8% acidified methanol and 1% vanillin 1:1 solution by 50 μL until complete 250 μL. Fifty μL of methanol and 200 μL of 4% acidified methanol were used as blank. Absorbance was read at 500 nm, with filter, from 492 to 540 nm using a Synergy 2 multiwell plate reader (BioTek Instruments, Winooski, VT). The amount of condensed tannins

was calculated and expressed as mg catechin equivalents (CAE) per gram of dry coat ($y = 0.2954 + 0.0121$, $R^2 = 0.99$).

8.3.8 Identification and quantification of non-colored phenolic compounds and anthocyanins

For the identification of non-colored phenolic compounds and anthocyanin, bean seed coat extracts prepared as indicated in Section 2.2, were run on a Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer - a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high energy collision (HCD) cell - with an Ion Max electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack autoinjector, and an ACCELA 80 Hz PDA detector); all running under Thermo Scientific Xcalibur 2.1.0.1140 LC–MS software. For the quantification of non-colored phenolic compounds and anthocyanins, the samples were analyzed by HPLC using a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU 20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector (PDA), running under Shimadzu LC Solutions version 1.22 chromatography software, Columbia, MD, USA). The MS was calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the auto tune software feature as needed. The MS was run with the ESI probe in the negative mode. The source inlet temperature was typically 300 °C, the sheath gas rate was typically set at 50 arbitrary units, the auxiliary gas rate at 5 arbitrary units and the sweep gas rate at 2 arbitrary units. The maximal mass resolution was set at 30,000, the spray voltage i at 3.0 kV and the tube lens at −100 V. Other parameters were determined and set by the calibration and tuning process. For identification of compounds, the column was a 3 mm × 150 mm Inertsil reverse phase C-18, ODS 3, 3 μm column (Metachem, Torrance, CA). For non-colored phenolic compounds, the initial solvent system was 20% methanol and 80% water with 0.25% formic acid at a flow rate of 0.25 mL per min. After injection (15 μL) the column was developed with a linear gradient to 100% methanol over 50 min. The column eluate was monitored at 280 nm in the PDA detector. The software package was set to collect mass data between 150 and 1000 AMUs. Generally, the most significant sample ions generated under these conditions were $[M^{-1}]^-$ and $[M + HCOO]^-$. For anthocyanins, the MS was typically run with the ESI probe in the positive mode. The initial column conditions were 5% methanol and 0.2% acetic acid in water, at a flow rate of 0.25 mL per min. The eluate was monitored at 520nmon the PDA. After a delay of 2 min, the column was

developed to 100% methanol with a linear gradient over 60 min. For quantification the column used was an Inertsil ODS-3 reverse phase C-18 column (5 μ, 250 × 4.6 mmfromVarian). The initial column conditions for non-colored phenolic compounds were 20% methanol and 80% water with 0.05 M phosphoric acid, at a flow rate of 1 ml per min. The eluate was monitored at 325 nm on the VWD. After injection (typically 25 μL), the column was held at the initial conditions for 2 min, then developed to 100% methanol in a linear gradient over 55 min. A standard curve based on a range of 1 to 40 nanomoles injected was prepared from a standard of chlorogenic acid (Sigma, Chemical Co., St. Louis, MO). The molar extinction coefficient for chlorogenic acid (1.6×10^{-6} L mol^{-1} cm^{-1}) was used to quantify the caffeoylquinic acid compounds. The degree of substitution on the quinic acid was confirmed by purification and LC–MS. For anthocyanins, the initial column conditions were 2% acetonitrile and 0.5% acetic acid in water, at a flow rate of 1 ml per min. The eluate was monitored at 520 nm. After injection (typically 15 μL), the column was held at the initial conditions for 2 min, and then developed to 100% acetonitrile in a linear gradient over 60 min. Standard curves were based on a range 1 to 40 nanomoles and prepared from pure standards. Extinction coefficients were calculated from a linear regression formula based on four different nanomolar concentrations of anthocyanins aglycone standards (ChromaDex®, Irvine, CA) injected and determined their respectivemAbs areas. The extinction coefficient for each anthocyanin was then used to calculate respective anthocyanin glycoside concentrations in the dry bean samples by the following formula:

μg/mg or mg/g = mAbs (area) * extinction coefficient (nM=/Abs/injection volume (μL) * total volume of extract (μL) * MW of anthocyanins glucoside (μg/nM) /sample weight (mg)

The extinction coefficients for each of the anthocyanin aglycones, used to quantify anthocyanin concentrations were determined to be for delphinidin (2.1×10^{-7} L mol^{-1} cm^{-1}), malvidin (1.7×10^{-7} L mol^{-1} cm^{-1}) and petunidin (7.3×10^{-7} L mol^{-1} cm^{-1}). Addition of a glycosyl group to the anthocyanin has little effect on its absorption profile, so anthocyanin aglycones can be used to prepare standard curves for anthocyanin glycosides on a molar basis [21]. Different columns, aqueous mobile phases and gradients were used for LC–MS analysis compared to the method used for the quantification. A smaller column was needed on the LC–MS system due to lower flow rates required and used in an optimal MS system to get the best MS response. LC–

MS was used to identify anthocyanins in the extracts by molecular weight. Once the identification was completed, standards were used on the analytical system to determine both retention time and quantitation.

8.3.9 Antioxidant capacity

Bean seed coat samples were extracted as indicated in Section 2.2. Antioxidant capacity was measured by the oxygen radical absorbance capacity (ORAC) assay as described by Prior and Cao et al. [25] to obtain a comparative evaluation, using 20 μL Trolox (6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid, 4-240 μM), samples (20 μL of methanol extract), or blank (75 mM phosphate buffer, pH 7.4), 120 μL of 116.9 nM fluorescein (final concentration 70 nM/well), and 60 μL of 40 mM α,α′-azodiisobutyramidine dihydrochloride (AAPH) per well. A black walled 96-well plate was read at excitation wavelength of 485 and an emission wavelength of 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multiwell plate reader (BioTek). Results were expressed as mmol Trolox equivalent (TE) per gram of dry coat ($y= 0.124x - 2$, $R^2=0.97$). Also, nitric oxide radical scavenger was determined to evaluate the antioxidant activity against reactive species with biological significance. To establish the ability of the extracts to capture nitric oxide (NO), the methodology by Giraldo et al, [26] was followed with some modifications. A 125 μL of samples and control (water) were added to a 96 well plate; to one set, sodium nitroprusside (25 μL, 113 mM) was added, and to the second set only water (25 μL). The plate was allowed to incubate at room temperature (120 min), and the Griess reagentwas added. First, 50 μL of reagent A (1% sulfanilic acid in 5% phosphoric acid) and 10 min later 50 μL of reagent B [N-(1- naphthyl) ethylenediamine dihydrochloride 0.1% in water]; the plate was allowed to stand 15 min, and the absorbance was measured at 546 nm. The second set was used as a blank and the antinitrosative activity was expressed as percentage of inhibition.

8.3.10 α-Amylase inhibition and α-glucosidase inhibition by bean phenolic extracts

For α-amylase and a-glucosidase assays, the methods described by Johnson et al. [23] were followed. For the α-amylase assay, 500 μL of the phenolic extracts (methanol extract), prepared as indicated in Section 2.2, or positive control (1 mM acarbose) was added to 500 μL of 13 U/mL α-amylase solution (type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 μL of 1% soluble starch solution (previously dissolved in sodium phosphate buffer and boiled for 15min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color

reagent was added and the tubes were placed in 100 °C water bath for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100% inhibition. For the α-glucosidase assay, in a 96-well plate, 50 μL (methanol extract) of phenolic extracts, prepared as indicated in Section 2.2, or positive control (1 mM acarbose) was added to 100 μL of a 1 U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 μL aliquot of a 5 mM p-nitrophenyl-α-Dglucopyranoside solution (in 0.1 M sodium phosphate buffer pH 6.9) was added briefly to each well and incubated at 25 °C for 5 min before the absorbance was read at 405 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100% inhibition.

8.3.11 Statistical analysis

The experiments were repeated at least three times with consistent results. Data are expressed as the mean± standard deviation. The data obtained were analyzed using one-way ANOVA to compare experimental to control values (StatGraphics plus 5.0). Comparisons between groups were performed using LSD test, and differences were considered significant at p b 0.05. Principal component analysis (PCA), clustering and MANOVA analysis and correlations were performed using SPSS Statistics Premium GradPack 22. PCA was conducted on eight dependent variables (total phenolic compounds, flavonoids, tannins, anthocyanins, antioxidant capacity, L, chroma and hue). To evaluate the similarities among the fifteen cultivars on dependent variables, cluster analysis was performed on two PCs; hierarchical clustering methods were used to determine the number of clusters. Agglomeration schedule and Euclidean distances methods were used for the proximity matrix. Multivariate analyses of variance (MANOVA) were performed to investigate how the independent variable (bean variety) impacted the dependent variables.

8.4 Results and discussion

8.4.1 Common bean cultivar characteristics and color

Cultivars studied have been modified by breeding in order to improve agronomical and nutritional characteristics. Main modifications relied on the plant defense capacity, against bacteria and viruses that commonly affect this crop. For example most of the cultivars present resistance to anthracnose, rust, halo bright, mosaic virus or bacteriosis in different levels. On the other hand, other cultivars as Pinto-Bayacora present the characteristic of fast growth. FJ-Leon,

Negro-Otomi and BRS-Pontal present high seed yields. BRS-Horizonte contains high protein and BRS-Pontal and FM-Eugenia are high in iron content. New improved cultivars are now available for consumers; however there are no previous studies investigating the phenolic profile and potential health benefits associated with those cultivars.

Common beans were grouped according to color and characteristics in four groups: Pintos, Flor de Mayos and Flor de Junios, Blacks and Cariocas (Table 14). Pinto beans did not present significant differences in hue values, within this cultivar group, which were related to color pattern. However, there were differences in the chroma values (among cultivars and within a cultivar group), which were related with the saturation or intensity. Similarly, the Flor de Mayo and Flor de Junio as well as the Carioca cultivars did not have significant differences in hue, but there were also differences in the saturation or chroma. Black beans presented no differences in either chroma or hue.

In general, few differences were noticed in the color and dotted pattern among groups of cultivars. Bean seed color was determined by the presence of flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins) [16]. Tannins and anthocyanins content are important during breeding since those compounds determine seed color and also the proportion of the antinutritive tannins with the antioxidant anthocyanins. Diaz et al. [1] found that the inheritance of bean color with tannins and anthocyanins are mainly related to seed color genes. However, anthocyanin concentration can be modified by a few genes related with color pattern and intensity. Rocha-Guzmán et al. [5] evaluated how antimutagenic and antioxidant capacity of common beans was affected by color, and found a positive correlation between phenolic content and between phenolic concentration and seed color. Spectrophotometric analyses provided information about total concentrations of main groups of interest; in this case, phenolic compounds. However, for accuracy data, techniques as LC–MS and HPLC were needed to identify and quantify specific compounds.

8.4.2 Anthocyanins in common beans

Three anthocyanins were identified and quantified in ten out of fifteen common bean cultivars. Black bean cultivars showed the highest concentrations of anthocyanins (Table 15). Anthocyanin concentration ranged from 0.01 mg/g coat (1.0 mg/100 g) in Perola cultivar to 2.5 mg/g coat (250 mg/100 g) in Negro-Otomi cultivar and correlated with anthocyanin concentrations quantified by the AOAC official colorimetric method ranging from 0.012 mg

cyaniding 3 glucoside equivalents (C3GE)/g coat in Pinto-Bravo to 2.3 mg C3GE/g coat in Negro- Otomi (Figure 22A). Diaz et al. [1] reported anthocyanin concentrations ranging from 0.13% to 0.21% on bean coats. Health benefits of anthocyanins are related with anti-inflammatory, vasotonic and antioxidant capacity; however, their industrial application is limited due to their low stability in the food matrix [8]. Anthocyanins were identified among cultivars using LC-ESI-MS (Figure 23). Delphinidin glucoside was identified in cultivars Negro-Frijozac, Negro-Otomi and BRS-Horizonte; the Negro-Otomi cultivar had the highest concentration (1.29 mg/g coat). Petunidin glucoside was identified in all ten cultivars; its concentration ranged from 0.007 mg/g coat (Perola) to 1.15 mg/g coat (Negro-Frijozac). Malvidin glucoside was identified in ten cultivars, ranging from 0.014 mg/g coat (Perola) to 0.52 mg/g coat (Negro-Frijozac). Delphinidin, petunidin and malvidin aglycones have been reported by Aparicio-Fernandez et al. [2] and Lin et al. [16] in black jamapa bean and in US commercial common beans, respectively.

8.4.3 Non-colored phenolic compounds in common beans

Seventeen non-colored phenolic compounds were identified in bean coats from all the studied cultivars (Table 16). The percentage of total area represents the relative abundance of one specific compound related with the sum of the areas of all peaks in the chromatogram for each cultivar. Percentage of total area was calculated for the already identified phenolics (Figure 24). These values provided information about relative amount of the compound in the cultivar. Identification was based in the use of pure standards and comparing spectra m/z (−) ionization with available online database (http://www.phenol-explorer.eu) (Table 16). Catechin (1.75–5.42%) and epicatechin (3.80–12.48%) were found in all samples. Flavonols as catechin, quercetin, myricetin and kaempferol were present as aglycones. Those compounds have been identified in common bean cultivars [2,12,15]. Proanthocyanidin dimers (0.24–0.8%) were found in ten cultivars. Aparicio-Fernandez et al. [16] identified proanthocyanidin monomers, dimers, trimers, tetramers, pentamers, and hexamers in black bean seed coat. Phenolic acids as syringic acid, ferulic acid, p-coumaric acid, and o-coumaric acid, vanillic acid were found in the studied cultivars; those compounds have been reported in common beans [12,13]. Myricetin and kaempferol 3-O galactoside were identified only in black beans (1.5%). Vanillin (0.80–7.9%) and daidzin (2.29–1.18%) were found in eight and nine cultivars, respectively. Standard analytical techniques were used in this study based on precision instruments; sensitivity, accuracy, and precision are given in the instruments specifications. Phenolics are generally

detected at 260–285 nm, and non-conjugated phenols absorb at 340 nm [2,16,27]. Detection limits varied from compound to compound and from wavelength to wavelength detecting down to 0.1 nM for both anthocyanins and phenolics.

8.4.3 Flavonoids, total phenolic compounds and tannins in common beans

Brazilian and Mexican beans presented higher concentration of flavonoids ranging from 0.083 mg RUE/g coat in Pinto-Saltillo to 0.694 mg RUE/g coat in Perola (Figure 22B); Carioca and black beans presented the highest concentrations of flavonoids among other cultivars. Xu et al. [15] reported similar flavonoid content 0.98 mg CAE/g in black beans. The consumption of these compounds protects against oxidative stress [15]. Figure 22C shows the total polyphenol concentration of fifteen common bean cultivars. Pinto-Saltillo was the cultivar with the lowest concentration (5.46 mg GAE/g coat) in comparison to Negro-Frijozac (15.5 mg GAE/g coat). Other studies reported higher and lower concentrations of total phenolic compounds; Guajardo-Flores et al. [28] reported 30 mg GAE/g coat in black beans, while [5] reported 11.23–16.94 mg GAE/g bean, and Xu et al. [15] reported 0.57–6.99 mg GAE/g bean. The difference in total phenolic compounds concentration could be due to genotype, and not growing conditions of the beans, since all cultivars from Mexico were grown and handled under the same conditions. Condensed tannin concentrations in the studied bean cultivars ranged from 60.6 mg GAE/g coat to 369.3 mg GAE/g coat in Pinto-Saltillo and BRS Horizonte, respectively (Figure 22D). Higher concentrations of tannins were observed in cultivars from Brazil and FJ-Leon and FM-Eugenia from Mexico. Tannins are related to color and also believed to be associated with antinutritional properties of foods. Tannins can precipitate proteins and bind to minerals thus limiting their absorption. Reported tannins in whole beans are lower since those compounds are concentrated in the coat; ranging from 0.47 to 5.73 CAE/g bean [15]. Higher concentrations of these compounds could be attributed to improvements to resistance to biotic and abiotic stress, in the studied cultivars.

8.4.4 Antioxidant capacity of common beans

AC ranged from185.2mmol TE/g coat to 233.9mmol TE/g coat with not significant differences among studied cultivars (Table 14). Other authors have reported lower AC values in different beans ranging from 13.3 to 92.7 μmol TE/g bean [15], and 132 μmol TE/g coat [28]. Nitric oxide (NO) scavenging capacity of phenolic extracts is presented in Table 14. Sodium nitroprusside produced nitric oxide in an aqueous solution at physiological pH when it reacted

with oxygen [29]. Phenolic compounds in beans presented a range of NO inhibition from 5.73 (cultivar Perla) to 85.42% (cultivar Pinto-Saltillo). Pinto-Saltillo (85.42%), Negro-Frijozac (68.15%), FJ-Leon (65.60%), and Negro-Otomi (60.79%) presented some of the highest values. High concentrations of nitric oxide can activate NF-κB in peripheral blood mononuclear cells, an important transcription factor in iNOS gene expression in response to inflammation and also related to rapid tumor growth [30,31]. Figueroa et al. [29] reported that phenolic compounds from peppermint infusions were able to counteract the effect of NO formation with a maximum inhibition of nitric oxide of 82%. Table 17 shows the correlations among different evaluated parameters. AC did not correlate with tannins, anthocyanins and flavonoids; indicating that AC is attributed to all the phenols present in the bean coats. The luminosity values positively correlated with chroma which is related with the saturation or intensity of the color. In order to understand how bean cultivar (Pinto, Flor de Junio and Flor de Mayo, Negro and Carioca) affected color and phenolics, multivariate analysis of variance was performed. Multivariate results showed that independent variables have significant effect of the dependent variables as a group. The test between-subject effects indicated how the independent variables affected each of the six dependent variables (Table 17). From the output, it can be observed that bean cultivar had significant effect on flavonoids, tannins, anthocyanins, L, chroma and hue. However, total phenolic compounds in the common bean coat, as well as their AC were not significantly affected. Despite the different concentrations of phenolics in the different cultivars present, when measured in as totals, all contribute to the AC of the beans. Several authors have reported positive correlation of total phenolic compounds and antioxidant capacity [5,24,32].

8.4.5 Inhibition α-amylase and α-glucosidase

α-Amylase and α-glucosidase are key enzymes linked with type-2 diabetes. Inhibition of these enzymes has an impact on the sugars available to be absorbed during digestion and can be used as strategy in early treatment to type-2 diabetes. Phenolic compounds have the capacity to bind to digestive enzymes modifying their activity. α-Amylase inhibition ranged from 25.8% in BRS-Pontal to 74.2% in Pinto-Saltillo, at the same phenolic concentration of extracts obtained under exactly the same extraction conditions (Table 14). In the case of α-glucosidase, the lowest inhibition was for BRS-Pontal (20.8%) and the highest for Negro-Otomi (82.5%). The cultivars with higher potential to inhibit both enzymes were Negro-Otomi, Pinto-Saltillo, Pinto-Centenario, and Pinto-Bayacora; all of them with inhibitions between 66 and 82%. The

inhibition of α-amylase and α-glucosidase using phenolic extracts could be affected by the type of extracts, concentrations, sample pretreatment and sample composition, making comparison among different studies complicated. However, Ranilla et al. [33] reported 90% inhibition for α-amylase from Brazilian and Peruvian common bean phenolic extracts. In the same study they reported lower inhibitions of α-glucosidase ranging from 16 to 31% using whole bean phenolic extracts after heat treatment. A significant reduction in the inhibitory properties of phenolic extracts after thermal treatment was reported. Phenolic compounds from common beans have high potential to inhibit enzymes related to carbohydrate metabolism and reduce availability ofα-amylase and α-glucosidase. Nowadays type-2 diabetes therapies involve the use of drugs as gastric enzymes inhibitors in order to decrease glucose adsorption in the gut [23]. However, certain foods as common beans contain bioactive compounds as phenolics that can inhibit those enzymes, representing a natural source of inhibitors.

Flavonoids and other phenolic compounds present on extracts from different sources (brown seaweed, tea fruit peel, blueberries and their fermented extracts, red pepper extracts and soybean) have been reported to inhibit gastric enzymes [34-39]. Phenolic compounds in common bean could contribute to ameliorate complications of type-2 diabetes.

Based on principal component analysis conducted to reduce and interpret data variability between dependent variables, the first two PCs had eigenvalues that explained 74% of the total variance (PC1 =47.5; PC2 = 26.5) (Figure 25). Cluster analysis on the two PCs, evaluated the similarities among the fifteen cultivars according to the dependent variables, and formed clusters (Figure 25). Group one contained the Brazilian beans, FM-Eugenia and FJ-Marcela; this cluster contained large scores in PC2; they are positively correlated with AC, tannins, total phenolic compounds and flavonoids. Similarly, the cluster containing Pinto-Bayacora, FJ-67, FJ-202, FJ-199 and FJ-Leon presented positive but lower values than group one based on PC2 score. Cluster three presented low score in PC2 and a positive score in PC1. PC1 was positively related with L*, C*and tannins; cultivars in this group are Pinto-Saltillo, Pinto- Centenario and Pinto-Bravo. Cluster four contains the black beans; this group presented low PC1 scores, associate with color, and positive scores on PC2, related with phenolics. In general, there was a tendency of cultivars to group according to variety based on their phenolic concentration and seed color.

Common bean consumption has been encouraged because of its high fiber and relatively high concentration of phytochemicals associated with a lower risk of cardiovascular disease [5-7].

Those phytochemicals in beans are mainly phenolic compounds including flavonoids, anthocyanins, phenolic acids and tannins present principally in the bean coat that represents only the 10% of the whole bean [5]. The mechanisms of action of those compounds to promote health appear to be related to their antioxidant capacity and their capacity to inhibit certain enzymes related to chronic diseases. Differences in the profile of phenolic compounds and their concentrations could be influenced by breeding modification to improve agronomical characteristics of evaluated cultivars. More studies are needed to evaluate in humans if bean intake may be considered a good source of bioactive compounds to inhibit the activity of enzymes related to type-2 diabetes.

8.5 Conclusions

Beyond the nutritional value of common beans, their bioactive components such as phenolic compounds have potential to contribute to the management of type-2 diabetes due to their capacity to inhibit enzymes related to glucose absorption; bean phytochemicals can be used as complementary strategy to this devastating disease. Common beans are a good source of phenolic compounds that are responsible for their antioxidant capacity. Phenolic compounds present in the extract of Pinto-Saltillo bean coat demonstrated high antioxidant activity against reactive species with biological significance such as nitric oxide. Also, this extract presented the highestα-amylase inhibition. These results may have important implications related to reduction of inflammatory diseases, such as type-2 diabetes. Black beans contain high concentration of anthocyanins, especially delphinidin, and also important phenolics such as ferulic acid. This study has demonstrated that even though, phenolic concentration varied among cultivars, this factor did not affect the potential health benefits associated with their oxygen radical absorbance capacity and inhibitory activity of enzymes related to glucose metabolism. Bean cultivars and their phenolic compounds are good natural sources of antioxidants and starch-degrading enzyme inhibitors important for type-2 diabetes management.

8.6 References

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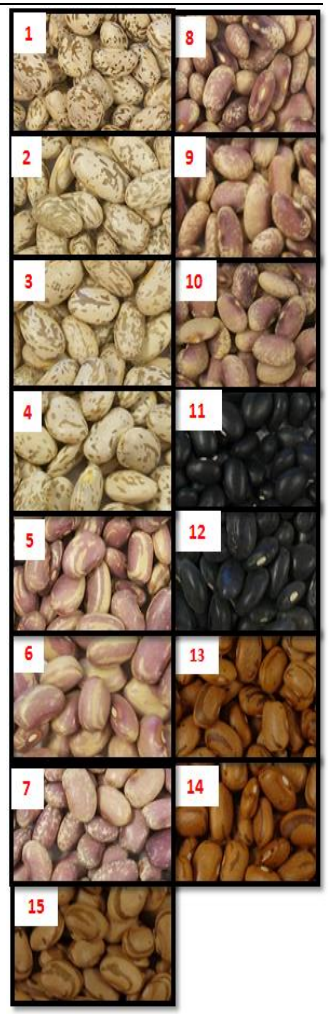
8.7 Tables and figures

Table 14. CieLab measurement and antioxidant capacity and α-amylase and α-glucosidase inhibition of common bean cultivar.

Cultivar	L*	a*	b*	C*	H*	Coat color	AC mmoles TE/g	% α-Amylase inhibition	% α-Glucosidase inhibition
1. Pinto-Bayacora	50.7±1.4g	7.7±0.5d	15.1±0.5g	17.0±0.5gh	62.8±5.1cde	1	225.5±28.5a	66.0±1.9de	67.4±1.2efgh
2. Pinto-Bravo	54.7±2.8h	5.9±0.5c	14.0±1.1g	15.2±1.1fg	67.0±1.4cde	1	197.5±44.4a	40.3±7.9abc	77.6±3.9gh
3. Pinto-Centenario	55.2±2.5h	6.1±0.7c	16.2±0.7h	17.3±0.9h	62.1±1.5de	1	187.2±55.0a	67.1±1.7de	75.3±6.7fgh
4. Pinto-Saltillo	58.2±1.3i	4.7±0.2b	13.2±0.9f	14.0±0.9c	70.1±0.9de	1	185.2±17.8a	74.2±1.9e	68.5±1.9efgh
5. FJ-Leon	45.0±1.7e	9.5±0.7e	10.4±1.2cd	14.1±0.7c	47.5±4.8bcd	2	221.4±30.2a	31.0±7.7a	58.1±8.5de
6. FJ-Marcela	46.6±0.9e	10.2±0.6fg	13.1±1.9f	16.6±1.4gh	51.7±5.1bcd	2	228.2±29.5a	58.5±12.2cde	66.1±3.1efg
7. FM-Eugenia	49.8±1.5fg	9.9±0.84g	6.6±0.5b	11.9±0.8b	33.7±2.5ab	2	223.7±31.7a	59.1±1.2de	61.1±4.9def
8. FM- 67	46.7±1.3e	10.6±0.5g	9.9±1.1c	14.6±0.8cd	43.0±3.7abc	2	218.5±32.0a	54.1±11.9a	31.7±1.5ab
9. FM- 199	46.9±1.9ef	10.6±0.2g	12.3±0.8ef	16.2±0.7fg	49.3±1.7bcd	2	233.9±38.6a	30.1±5.0a	46.4±1.8cd
10. FM- 202	46.9±1.4e	10.1±0.6fg	11.2±0.9de	15.1±1.1ef	47.7±1.6bcd	2	225.7±30.1a	32.8±4.6a	70.9±9.2efgh
11. Negro-Frijozac	19.6±1.1a	-0.1±0.06a	-0.8 ±0.1a	0.8±0.1a	81.1±3.8e	3	223.4±33.7a	67.8±4.9de	38.3±3.9b
12. Negro-Otomi	23.9±0.8b	-0.1±0.05a	-1.0±0.2a	1.0±0.2a	76.3±9.5e	3	215.0±24.2a	73.8±3.2e	82.5±6.9h
13. BRS-Horizote	45.6±1.2e	9.5±0.5ef	16.6±0.7i	19.1±0.7i	60.1±1.6cde	4	218.5±24.9a	55.2±0.7bcd	58.3±3.3de
14. BRS-Pontal	39.2±2.12d	12.5±0.6h	18.9±0.6h	22.7±0.8j	56.4±0.8bcde	4	209.9±28.2a	25.8±5.8a	20.7±7.6a
15. Perola	35.5±1.3c	14.3±0.4i	20.3±0.7j	24.9±0.8k	54.7±0.3bcd	4	200.4±32.6a	39.0±3.7ab	46.4±0.9bc



Data represent the mean ± SD from at least seven independent studies. Values within a column followed by different letters are significant at $p < 0.05$. Percentages of inhibition for α-amylase and α-glucosidase are expressed as percentage relative to positive control acarbose per g of coat. L*: brightness; a*: (+) redness to (-) greenness; b* yellowness to blueness; C*: chroma; H*: hue angle. AC: antioxidant capacity mmoles TE/g coat; TE: trolox equivalents; RAC: relative to acarbose.

Table 15. Concentration of identified anthocyanins in common bean cultivars by LC-ESI-MS and HPLC

Cultivar	Delphinidin gluc mg/g coat	Petunidin gluc mg/g coat	Malvidin gluc mg/g coat	Total Anthocyanins mg/g coat
FJ-León	-	0.05±0.00ab	0.0043±0.00a	0.06±0.00a
FJ-Maricela	-	0.04±0.00ab	0.004±0.00a	0.05±0.00a
FM-Eugenia	-	0.03±0.02ab	0.002±0.00a	0.03±0.01a
FM-67	-	0.077±0.00ab	0.006±0.00a	0.08±0.00a
FM-199	-	0.06±0.00ab	0.002±0.00a	0.06±0.00a
FM-202	-	0.08±0.00ab	0.004±0.00a	0.08±0.00a
Negro-Frijozac	0.38±0.04b	1.15±0.04c	0.52±0.01d	2.06±0.01b
Negro-Otomí	1.29±0.15c	1.11±0.14c	0.09±0.01c	2.5±0.09c
BRS Horizonte	0.009±0.00a	0.09±0.01b	0.035±0.01b	0.13±0.00a
Perola	-	0.007±0.00a	0.0014±0.00a	0.01±0.00a

The data represents the mean ± SD from at least three independent replicates done in triplicate. Values within a column followed by different letters are significant at $p < 0.05$. gluc: glucoside. Concentration of anthocyanins were calculated using standard courve of pure compounds. Total anthocyanins are the sum of all quantified anthocyanins within a bean cultivar.

Table 16. Phenolic compounds identified by LC-ESI-MS and percentage of total area under the curve in common bean cultivars

Peak	Compound	t_R min	m/z	Pinto-Bayacora	Pinto-Bravo	Pinto-Centenario	Pinto-Saltillo	FJ-Leon	FJ-Marceka	FM-Eugenia	FM-67	FM-199	FM-202	Negro-Otomi	Negro-Frijozac	BRS-Horizonte	BRS-Pontal	Peroka
1	Catechin 3-O-glucoside[1b]	6.33	527.14	0.84	0.90	-	1.52	-	0.62	0.9	0.74	0.60	0.60	-	-	0.42	0.27	0.29
2	Proanthocyanidin dimer[2b]	7.72	452.13	-	-	-	-	-	0.35	0.38	0.34	0.39	0.30	0.58	0.53	0.24	0.38	0.33
3	Quercetin 3-O-glucoside[3,4a]	8.10	577.13	-	2.25	-	-	-	-	4.0	-	-	-	-	-	-	1.58	1.48
4	Catechin[2b]	10.60	463.08	5.42	3.51	4.91	2.88	3.02	4.34	3.72	3.99	3.80	3.30	4.34	4.47	4.15	3.11	1.75
5	Chlorogenic acid[5a]	13.40	289.07	14.8	1.69	4.48	5.14	12.0	-	11.0	10.10	11.0	10.0	4.29	-	8.07	6.06	4.45
6	Myricetin 3-O-arabinoside[1b]	14.60	354.31	7.15	2.70	11.80	-	7.0	15.32	14.0	12.9	18.0	14.0	26.70	21.20	2.04	-	6.89
7	Epicatechin[2b]	14.70	449.10	6.86	6.71	3.80	4.03	4.49	4.54	8.32	11.11	7.05	7.37	4.54	7.64	9.90	12.48	9.73
8	Vanillic acid[3,6,7a]	15.50	290.26	12.5	4.59	3.80	-	6.42	3.29	0.66	4.75	5.05	4.22	-	-	3.68	3.18	3.58
9	Syringic acid[6,7a]	16.30	198.17	0.77	11.10	14.80	1.70	1.70	16.50	16.0	-	14.0	15.0	11.40	10.60	-	11.90	-
10	Vanillin[6,7a]	17.70	152.15	-	4.18	4.19	-	-	-	5.0	3.62	-	0.80	1.150	1.60	7.98	-	-
11	Daidzin[4,7a]	17.90	254.23	2.29	8.31	14.18	-	3.60	2.92	-	-	2.90	3.20	-	5.70	-	9.72	-
12	P-coumaric acid[3,6,7,8a]	20.10	164.16	-	3.80	-	-	-	-	-	-	-	-	-	-	4.31	-	-
13	Myricetin 3-O-glucoside[1,8a]	21.00	479.08	-	0.93	1.62	-	-	0.35	-	-	0.30	0.30	-	-	-	1.74	2.19
14	Ferulic acid[3,6,7,8a]	21.10	197.17	-	1.12	-	-	-	-	0.90	-	-	-	2.02	1.53	0.39	-	-
15	0-coumaric acid[8a]	25.00	164.23	-	-	-	-	0.20	0.93	-	0.41	0.40	0.50	1.10	0.83	0.29	0.23	0.28
16	Myricetin[1,8a]	26.50	318.24	-	-	-	-	-	-	-	-	-	-	1.46	0.88	-	-	-
17	Kaempferol 3-O-galactoside[9a]	26.50	447.09	-	-	-	-	-	-	-	-	-	-	0.05	0.34	-	-	-
18	Others	-	-	49.47	51.30	35.80	84.73	61.57	50.84	35.12	52.04	36.51	40.14	43.37	44.68	58.53	49.35	69.03

Results are expresed as % of total area. *m/z* values are related to negative ionization; ID: Identification method based on pure standardads (a) and and m/z identification using online available library database (b) (Phenol-Explorer database: http://www.phenol-explorer.eu/ reviewed on 06/23/2014). t_R: retention time. 1: Lin et al. [2]; 2: Aparicio-Fernandez et al. [16]; 3: Diaz-Batalla et al. [12]; 4: Doria et al., [14]; 5: USDA, [40]; 6: Xu et al. [32]; 7: Espinosa-Alonso et al. [41]; 8: Luthria et al., [13]; 9: Xu et al. [15].

Table 17. Correlations among phenolics, color and antioxidant capacity, and effect of bean variety

	Polyphenols	Flavonoids	Tannins	Anthocyanins	Antioxidant Capacity	L*	C*	H*
Polyphenols	1	0.46	0.51	0.46	0.60	-0.65	-0.28	0.03
Flavonoids		1	0.26	0.43	-0.06	-0.75	0.02	0.27
Tannins			1	-0.19	0.45	-0.13	0.26	-0.46
Anthocyanins				1	0.15	-0.84	-0.86	0.65
Antioxidant Capacity					1	-0.31	-0.21	-0.37
L*						1	0.53	-0.40
C*							1	-0.46
H*								1

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	p
Bean Variety	Polyphenols	59.6	4	14.9	2.4	0.116
	Flavonoids	0.37	4	0.09	10.0	0.002
	Tannins	60410.9	4	15102.7	4.3	0.027
	Anthocyanins	9.0	4	2.2	10759.0	0.000
	Antioxidant capacity	1788.2	4	447.0	3.2	0.060
	L*	1543.2	4	385.8	40.3	0.000
	C*	556.4	4	139.1	37.3	0.000
	H*	2071.9	4	517.9	23.1	0.000

Bean Variety (Pintos, Flores de Junio and Flores de Mayo, Negros, Cariocas); df: degrees of freedom. L*: brightness; C*: chroma; H*: hue angle. Bold numbers are statistically different ($p<0.05$) among bean varieties.

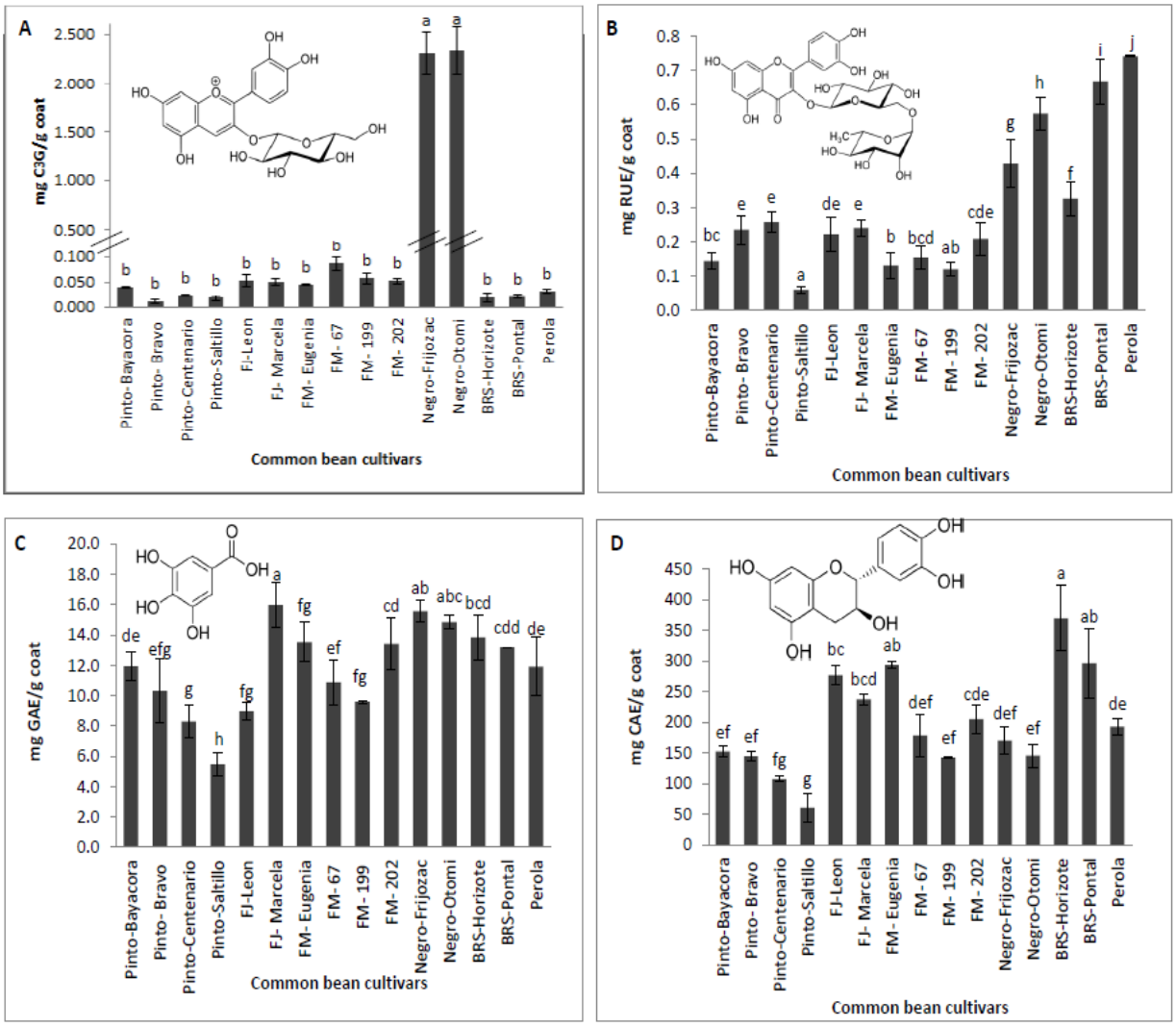


Figure 22. Concentration of phenolics in common beans cultivars. A. Total anthocyanins expressed as mg of cyanidin-3-glucoside equivalents per gram of dry coat. B. Flavonoids expressed as mg of rutin equivalents per gram of dry coat. C. Total polyphenols expressed as mg of gallic acid equivalents per gram of dry coat. D. Tannins expressed as mg of catechin equivalents per gram of dry coat. Different letters indicate significant differences among common bean cultivars ($p<0.05$). Data represent the mean ± SD from at least three independent studies.

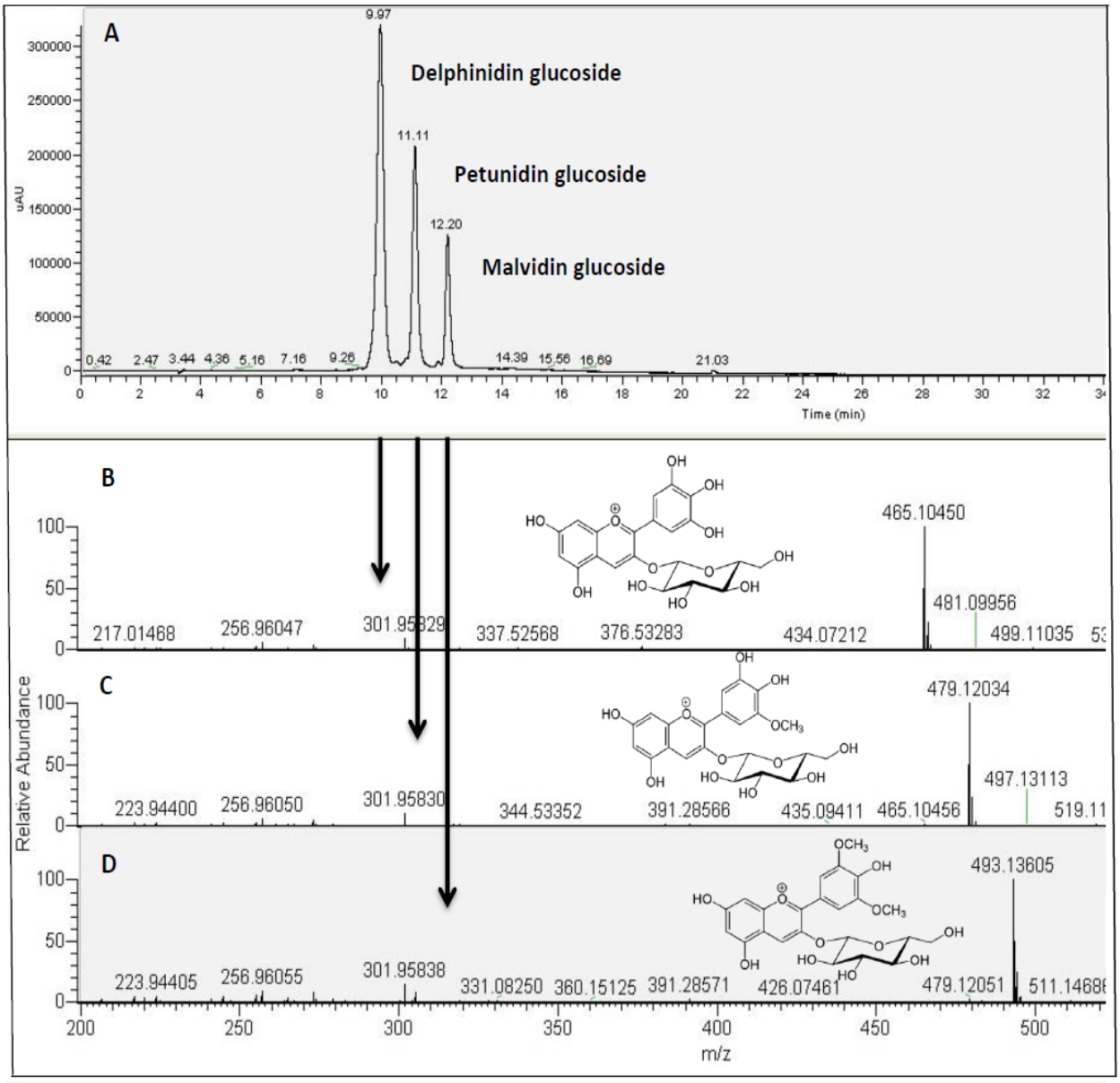


Figure 23. Representative chromatograms of anthocyanins in Negro-Otomi cultivar by LC-ESI-MS in positive mode. A. Absorbance at 520 nm. B. Mass spectrum delphinidin glucoside. C. Mass spectrum petunidin glucoside. D. Mass spectrum malvidin glucoside.

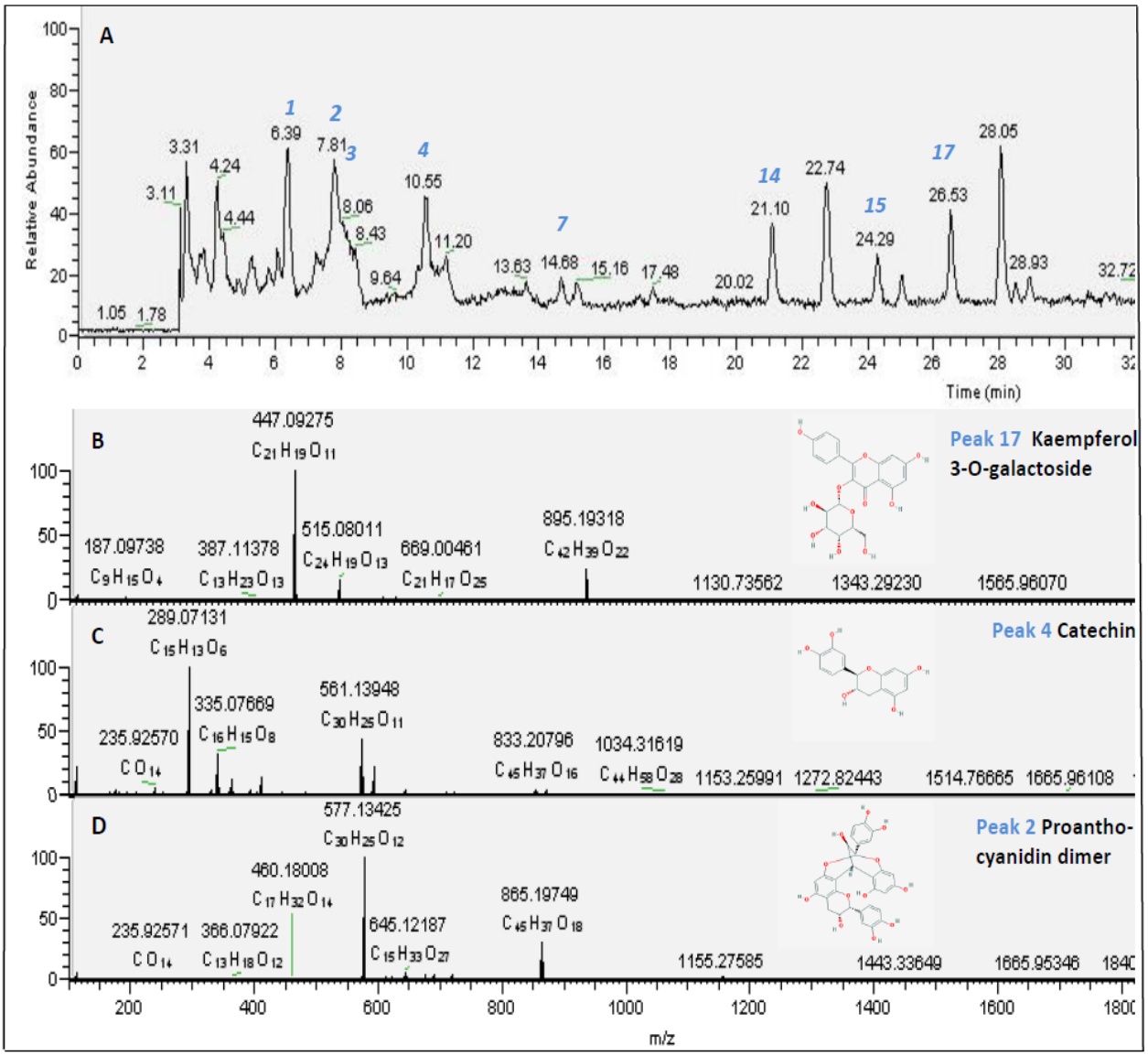


Figure 24. Representative chromatogram for phenols in Negro-Otomi cultivar by LC-ESI-MS in negative mode. A. Absorbance at 280 nm. B. Mass spectrum for kaempferol-O-galactoside. C. Mass spectrum for catechin. D. Mass spectrum for proanthocyanidin dimer. Peak 1: chatechin 3-O-glucoside; 2: proanthocyanidin dimer; 4: catechin; 7: epicatechin; 14: feluric acid; 15: O-coumaric acid; 17: kaempferol 3-O-galactoside.

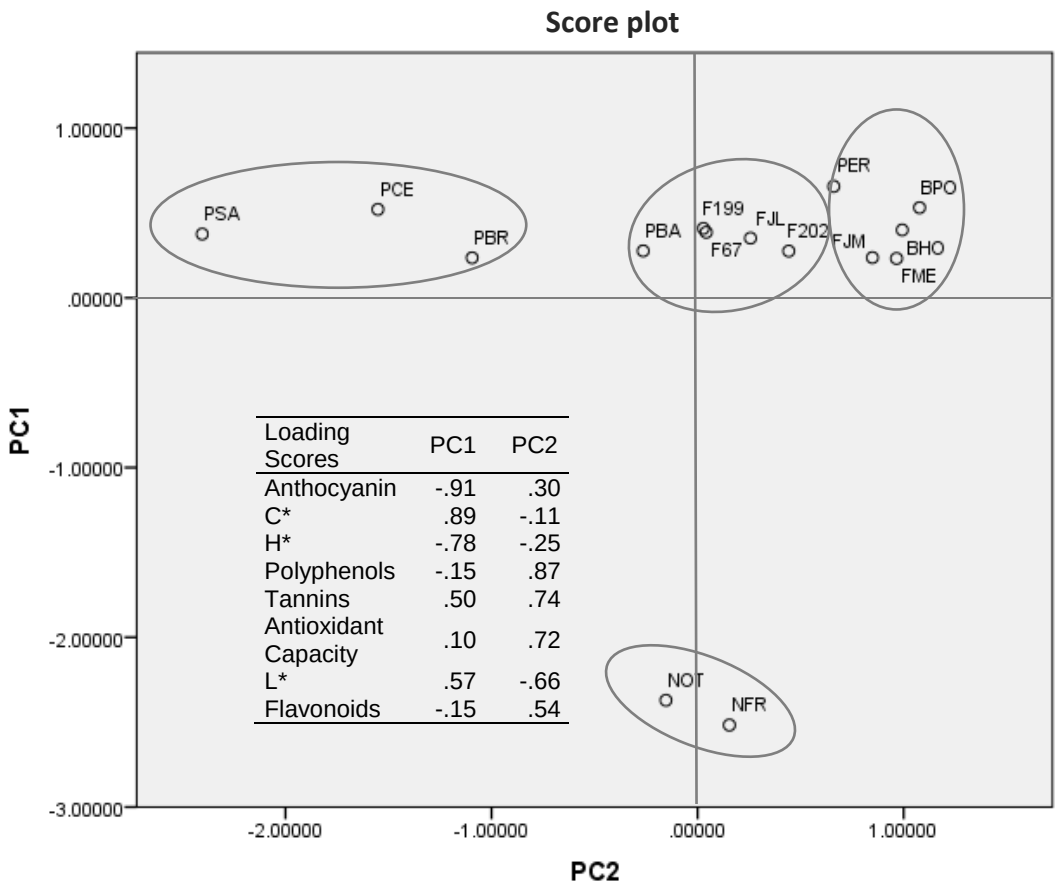


Figure 25. Cluster and principal component analysis score plot of fifteen common bean cultivars. Score plots for the first two principal components PC1 (47.5) and PC2 (26.53). PBA: Pinto-Bayacora; PBR: Pinto-Bravo; PCE: Pinto-Centenario; PSA: Pinto-Saltillo; FJL: Flor de Junio Leon; FJM: Flor de Junio Marcela; FME: Flor de Mayo Eugenia; F67: Flor de Mayo 67; F199: Flor de Mayo 199; F202: Flor de Mayo 202; NFR: Negro Frijozac, NOT: Negro Otomi, BPO: Brazilian Pontal; BHO: Brazilian Horizonte and PER: Perola.

CHAPTER 9: OPTIMIZATION OF EXTRACTION, THERMAL STABILITY, COLOR REACTION KINETICS AND SHELF-LIFE OF ANTHOCYANINS FROM BLACK BEAN (*Phaseolus vulgaris* L) SEED COATS UNDER DIFFERENT pH AND TEMPERATURE CONDITIONS

9.1 Abstract

Natural pigments have gained importance in the food industry due to consumer preferences. Black beans contain anthocyanins that could be used as colorants in foods. The objective was to optimize anthocyanins extraction from black bean coats and evaluate their thermal and shelf-life stability at different conditions of pH and temperature, and relate this information to color parameters. Extraction yield was 26.4 mg/g bean coat (w/w) after three consecutive extractions with ethanol. Optimal extraction conditions were 24% ethanol, 1:40 solid-to-liquid ratio and 29 ˚C ($P < 0.0001$). Three anthocyanins were identified by MS ions, delphinidin glucoside (465.1 *m/z*), petunidin glucoside (479.1 *m/z*) and malvidin glucoside (493.1 *m/z*). A total of 32 mg of anthocyanins were quantified per gram of dry extract. Bean anthocyanins were more stable at pH 2.5 and low temperature 4 ˚C (89.6%), in comparison to 19.3% at 22 ˚C, with a half-life of 277 and 56 days, respectively. Anthocyanins presented the lowest degradation rate value at 70 ˚C (k = 0.061/h) and pH 3.0 and the highest half-life values (11.29 h). Black bean coats are a good source of anthocyanins with potential to be used as natural-source of food colorants.

This chapter is part of the publication: Mojica L, Tan A, Berhow MA, de Mejía EG (2016). Optimization of extraction, shelf-life and anti-diabetes potential of anthocyanin rich extract from black bean (*Phaseolus vulgaris* L.) seed coats (*to be submitted*).

9.2 Introduction

Anthocyanins represent the largest group of phenolic pigments and the most important group of water-soluble pigments in plants, responsible for colors in fruits, vegetables, cereal grains, and flowers [1]. They are formed by two or three chemical units: an aglycon base or flavylium ring (anthocyanidin), sugars, and possibly acylation groups. Cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin are the most frequently occurring anthocyanidins, which may be glycosylated or acylated by different sugars and aromatic or aliphatic acids on their aglycon unit to yield anthocyanins in the plan [2].

Anthocyanins are very unstable and susceptible to degradation. Its color stability is affected by pH, their own chemical structure, concentration, storage temperature, light, oxygen, and the presence of enzymes, flavonoids, proteins and metal ion [3,4]. Anthocyanins are usually stable at pH 1 to 4 and degrade above pH 7. At pH 1, the predominant structure corresponds to the flavylium cation, conferring red and purple colors, whereas at values between pH 2 and 4, blue quinoid bases predominate [4]. Some of the ways to optimize anthocyanin stability during storage is to increase anthocyanin concentration, remove oxygen and inactivate enzymes [4].

There are nine chemically synthetized colorants approved in the United States to be used in foods; they are applied to dye beverages, foods and sweets worldwide [5]. These chemical compounds present high stability in the food matrix. However, the food industry is trying to substitute synthetic colorants by using pigments from natural sources. Anthocyanins in legumes have been previously reported; for example, Korean black soybean contains 15.54 mg anthocyanin/g [6]. Furthermore, anthocyanins were found in the skin of peanut varieties with purple skin at a concentration of 127.2 mg cyaniding 3-*O*-glucoside equivalents per L (C3GE/L) [7]. On the other hand, in common beans, anthocyanins are usually located in the seed coat. In previous studies, we found that Negro-Otomi cultivar (black bean), had the highest anthocyanin concentration (2.5 mg/g coat) [8] among other 14 common bean cultivars. Anthocyanins may provide anti-inflammatory and anti-diabetes benefits since they inhibit pro-inflammatory cytokines, decrease their production, and prevent β-cell dysfunction leading to insulin output [9]. Moreover, anthocyanins have a wide range of health benefits for the human body such as antioxidant, anticancer, anti-cardiovascular disease, and hepatoprotective activity [10].

Consumers may have a preference towards natural pigments versus synthetic colorants due to their perception of being a healthier and safer option. Besides, anthocyanins exert a wide

range of colors and hydro-solubility, making them an important alternative as a food pigment. Therefore, the objective of this study was to optimize the extraction conditions of anthocyanins from common bean coats, evaluate their shelf-life and thermal stability at different pH and temperatures and relate this information to color parameters such as Chroma (C*), Hue (h˚) and ΔE* calculated based on L*, a* and b* values. Furthermore, to obtain relevant information regarding bean anthocyanins reaction kinetics and provide an Arrhenius model.

9.3 Materials and methods

9.3.1 Materials

Black bean "Negro Otomi" cultivar was obtained from INIFAP research center in Zacatecas, Mexico. Chemicals used for extraction were all ACS grade and purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). All solvents for chromatographic techniques were of HPLC-grade. For sample preparation, common bean coats were removed from cotyledons after 16 h of soaking at room temperature, dried at 50 ºC in a conventional oven, ground, sieved in mesh 40 and store at 4 ºC until analysis.

9.3.2 Optimization of Extraction of Anthocyanins from Bean Coat by Response Surface Methodology (RSM)

Anthocyanins and total polyphenols were extracted from bean coats using either only water or two different concentrations of ethanol (0, 12.5 and 25%) in acidified water with 2% formic acid (pH = 2.0). Extractions were performed by stirring coat beans and the respective solution at 600 rpm for 2 h at different temperatures (4, 22 and 40 °C), and different solid-to-liquid ratios (1:30, 1:40 and 1:50). After extraction, the mixtures were filtered using Whatman No. 1 filter paper. All extracts were immediately analyzed for total monomeric anthocyanins, color, and total polyphenols.

Response surface methodology was used to optimize the extraction of anthocyanin and polyphenols by using functional relationships between the dependent variable and the independent variables as previously reported [11]. Ethanol concentration (x1), solid-to-liquid-ratio (x2) and extraction temperature (x3) were chosen for independent variables. The range and center point values with actual and coded values of variables used for the optimization of anthocyanins and total polyphenols extraction from black bean coat were coded levels -1, 0, +1, for ethanol concentration (x1, %), 0, 12.5 and 25; for solid-to-liquid ratio (x2, mL/g), 1:30, 1:40

and 1:50; and for extraction temperature (x3, °C), 4, 22 and 40. Anthocyanin concentration and total polyphenols were selected as the responses for the combination of the independent variables as presented in Table 18. The variables were coded according to the following equation:

$$x = \frac{x_i - x_o}{\Delta x}$$

where x is the coded value; x_i, the corresponding actual value; x_0, the actual value at the center of the domain; and Δx, the increment of x_i corresponding to a variation of 1 unit of x. The polynomial second degree equation is described below:

$$y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j$$

9.3.3 Anthocyanins Extraction and Measurement of Total Anthocyanin Concentration

Anthocyanins were extracted from black bean coats using the parameters obtained from the optimization analysis previously mentioned. Three consecutive extractions were performed to evaluate the percent recovery of anthocyanins and polyphenols after extraction with 24% ethanol in acidified water (2% formic acid), 1:40 solid-to-liquid ratio and 29 ˚C during 2 h. Ethanol was removed using a rotary vacuum evaporator at 40 ˚C. Anthocyanin extracts were freeze-dried in a Labconco Freeze Dryer 4.5 (Kansas, MO). The obtained powder was recovered and stored at -20 ˚C until analysis. Total monomeric anthocyanin was determined by pH differential method as previously reported [12]. Samples were diluted to a factor of 1:5 using two buffers (pH 1.0, 0.25 M KCl buffer and pH 4.5, 0.40 M sodium acetate buffer). Two hundred microliters of diluted solutions at each pH were transferred to a 96-well plate, and the absorbance was read at 520 and 700 nm using a Synergy 2 multiwell plate reader (Biotek, Winooski, VT). The total monomeric anthocyanin concentration was calculated as cyanidin-3-*O*-glucoside (C3G) equivalents per L as below:

$$\text{Total monomeric anthocyanins (mg/L)} = \frac{A \times MW \times D \times 1000}{\varepsilon \times PL \times 0.45}$$

Where: A = (A520 – A700) at pH1.0 – (A520 – A700) at pH4.5; MW = 449.2 143 g/moL for C3G; D = dilution factor; PL = constant path length 1 cm; ε = 26900 L/moL-cm the molar extinction coefficient for C3G, 1000= conversion factor from grams to milligrams and 0.45= conversion factor from the established method to the plate reader method. Final concentrations were expressed as mg C3G equivalents per g coat.

9.3.4 Measurement of Total Polyphenol Concentration

Total polyphenols were measured using the Folin-Ciocalteu method adapted to a microassay [13]. Samples were diluted to a factor of 1:10 with deionized water. Fifty microliters of these diluted samples, standard or blank (deionized water) were placed in a 96-well plate and then added with 50 µL of 1N Folin-Ciocalteu's phenol reagent. After 5 min, 100 µL of 20% Na_2CO_3 were added, and the mixture was incubated for 10 min. The absorbance was read at 690 nm using a Synergy multiwall plate reader (Biotek, Winooski, VT) and the results were expressed as mg gallic acid equivalents per g of coat.

9.3.5 Analysis of Anthocyanins by LC-ESI-MS and HPLC

Anthocyanin solutions were run on a Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer -- a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high energy collision (HCD) cell -- with an Ion Max electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack autoinjector, and a ACCELA 80 Hz PDA detector); all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software. The MS was calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the autotune software feature as needed. The MS was run with the ESI probe in positive mode. The source inlet temperature was 300 ˚C, the sheath gas rate was set at 50 arbitrary units, the auxiliary gas rate was set at 5 arbitrary units, and the sweep gas rate was set at 2 arbitrary units. The maximal mass resolution was set at 30,000, the spray voltage at 3.0 kV, and the tube lens at -100 V. Other parameters were determined and set by the calibration and tuning process. The column was a 3 mm x 150 mm Inertsil reverse phase C-18, ODS 3, 3 µ column (Metachem, Torrance, CA). For anthocyanin LCMS analysis, the initial conditions were 5% methanol and 0.2% acetic acid in water, at a flow rate of 0.25 ml/min. The effluent was monitored at 520 nm on the PDA. After a delay of two minutes, the column was developed to 100% methanol with a linear gradient over 60 min. HPLC analysis was performed to quantify the concentration of anthocyanins and conducted on a Shimadzu LC-20 HPLC system, LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LC Solutions version 1.22 chromatography software (Columbia, MD, USA). The column used was an Inertsil ODS-3 reverse phase C-18 column (5 µ, 250 x 4.6 mm from Varian). For anthocyanin analysis,

the initial conditions were 2% acetonitrile and 0.5% acetic acid in water, at a flow rate of 1 ml per minute. The effluent was monitored at 520 nm on the PDA. After injection (typically 15 µL), the column was held at the initial conditions for 2 min and then developed to 100% acetonitrile in a linear gradient over 60 min. Standard curves were based on a range 1 to 40 nanomoles were prepared from pure standards. The molar extinction coefficient of delphinidin (2.1×10^{-7} L mol^{-1} cm^{-1}), malvidin (1.7×10^{-7} L mol^{-1} cm^{-1}) and petunidin (7.3×10^{-7} L mol^{-1} cm^{-1}) were used to quantify anthocyanin concentrations [8].

9.3.6 Color Measurements

Color was measured using a Color flex Hunter Lab colorimeter (Reston, VA). The instrument was calibrated as manufacturer recommended and the following parameters were used: L*, a*, and b*; observer/illuminant: 10° and D_{65} and pathlength: 1 cm. Briefly, 3 mL of extracts were placed in a disposable Petri dish and the color parameters L*, a*, and b* measured and recorded. Color squares were generated by converting L*, a*, and b* values to R, G and B values using the color converter website (http://colormine.org/ convert/rgb-to-lab) and Microsoft Power Point Software. Color parameters Chroma (C*), Hue (h˚) and ΔE* were calculated using the L*, a*, and b* values.

9.3.7 Shelf-Life Studies

Extracted anthocyanins were stored in the dark for 5 weeks to assess degradation kinetics. The experiment was performed using different pH found in different commercially available beverages such as pH 2.5 (soda), 3.0 (sparkling flavored water), 3.5 (energy drink) and 4.3 (iced tea), and two storage temperatures, refrigeration (4˚C) and room temperature (22˚C). The evaluated parameters were anthocyanin concentration and variation of a* color parameter.

3.3.8 Anthocyanins and Color Shelf-Life Stability and Degradation Kinetics Study

Anthocyanin solutions were prepared using 1 mg/mL of the anthocyanin powder extract at different pH as described above according to pH in commercial beverages. The pH was adjusted using formic acid 2%. Anthocyanin and color were measured at time zero, and independent aliquots were stored for 5 weeks at 4 and 22 °C to be evaluated every week.

9.3.9 Anthocyanin and Color Thermal Stability Study.

Anthocyanin solutions at different pH (2.5, 3.0, 3.5, and 4.3) were exposed to 70, 80 and 90 °C in a water bath for 5 h and sampled each hour. After exposure at specific temperature and time conditions, samples were removed from the water bath and placed in an ice bath to

minimize further degradation. These conditions were selected based on shelf-life accelerated storage studies [14].

9.3.10 Reaction kinetics and Arrhenius model

Shelf-life and thermal stability data on anthocyanin concentration and individual anthocyanins were plotted using the first order reaction rate kinetics using the following equation:

$\ln A_t = \ln A_0 - kt$

where A_t is the total monomeric anthocyanin or anthocyanin abundance at time t, A_0 is the total monomeric anthocyanin or anthocyanin abundance at time zero, k is the reaction rate constant in h^{-1} and t is the time of heating in hours.

Activation energy for total monomeric anthocyanin and individual anthocyanins was calculated using the Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{R}\left(\frac{1}{T}\right)$$

where k is the reaction rate constant, A is the Arrhenius pre-exponential factor, Ea is the activation energy (kJ/moL), R is the gas constant (8.314 J/moL-K), and T is temperature in K.

9.3.11 Statistical Analysis

Each assay was run in triplicate, and all analyses were performed in three independent replicates. The data obtained were analyzed using one-way ANOVA to compare experimental to control values using SAS version 9.4 Software (Cary, NC); statistical differences among independent variables were determined using the Proc GLM procedure and Tukey Posthoc Test ($P < 0.05$). RSM analysis was performed using the Proc Rsreg procedure. Correlation among parameters measured was performed using the GraphPad Prism software (Version 5.02; GraphPad Software, Inc.; San Diego, CA).

9.4 Results and discussion

9.4.1 Optimization of Extraction of Anthocyanins and Total Polyphenols from Black Bean Coats.

Table 18 shows the conditions used to extract phenolic compounds and optimize the extraction yield. A combination of twenty-seven treatments was used to model the equation using the RSM method. Multiple regression analysis of anthocyanin concentration from black bean coat showed that the test variables were related by the second-degree polynomial equation (eq 1).

$Y= 0.0586 + 0.0268x_1 + 0.0074x_2 + 0.027x_3 - 0.0001x_1x_2 + 0.00023\ x_1x_3 + 0.0000065x_2x_3 + 0.000118x_1^2 + 0.000522x_2^2 - 0.002089x_3^2$ (*eq. 1*)

Similarly by applying the multiple regression analysis, total polyphenols concentration and the other independent variables were related to the dependable variables by the second-degree polynomial equation (eq 2).

$Y= 2.73 + 0.174829x_1 - 0.00977x_2 + 0.1590x_3 -0.000395x_1x_2+ 0.00372x_1x_3 + 0.000469x_2x_3 + 0.00319x_1^2 + 0.000522x_2^2 - 0.002089x_3^2$ (*eq. 2*)

Y in equations 1 and 2 represents anthocyanin or total polyphenols concentration, x_1 ethanol concentration, x_2 solid-to-liquid ratio and x_3 extraction temperature. For the optimization of the extraction of anthocyanins and total polyphenols from black bean coats it was found that the total model was significant for both parameters ($P < 0.0001$). For anthocyanins, ethanol concentration and temperature were highly significant ($P < 0.0001$) and solid-to-liquid ratio presented a *P*-value of 0.047. On the other hand, for total polyphenols also ethanol concentration and temperature were highly significant ($P < 0.0001$), and solid-to-liquid ratio presented a *P*-value of 0.049.

Figure 26 shows the three-dimensional response surface plots. Figure 26A, 26B, 26C shows a tendency of increasing the anthocyanins extraction as the concentration of ethanol and temperature increased. Regarding the solid-to-liquid ratio, there was a tendency of increasing the extraction as the ratio was smaller. For anthocyanin extraction, the optimal conditions found were ethanol 24%, solid-to-liquid ratio of 1:40 and 29 ˚C. Similarly, for total polyphenol extraction (Figure 26D, 26E, 26F), there was an increment in the extraction when ethanol concentration and temperature increased. On the other hand, the solid-to-liquid ratio showed a small increase in total polyphenol concentration when the solution tended to be more diluted. The optimal conditions for total polyphenol extraction from the optimization procedure were 23% ethanol, solid-to-liquid ratio of 1:40 g coat/mL and temperature of 30 ˚C.

The highest anthocyanins and polyphenols concentrations were obtained at 40 ˚C with a solid-to-liquid ratio of 1:50. On the other hand, the lowest anthocyanins concentration was extracted at 4 ˚C with 1:30, solid-to-liquid ratio. This condition also generated the lowest concentration of total polyphenols. The difference between highest and lowest anthocyanin concentration was about 4.5 fold yield while the difference between highest and lowest polyphenol concentration was about 4.8 fold. In general, a tendency was observed of increasing

the yield of anthocyanins and total polyphenols when ethanol concentration and temperature increased. Regarding the solid-to-liquid ratio, more anthocyanins were extracted when the solvent proportion increased. Samples with higher anthocyanin yield tended to have a higher Chroma value, color intensity and lower Hue value, or color tone. This was related to the angle on the chromaticity diagram and to low values of Hue positioned in an intense red zone. Correlations of these parameters were performed (Figure 27A-F), and positive correlations were found for the concentration of anthocyanins and total polyphenols ($P < 0.0001$, r = 0.99). In addition, the anthocyanin concentration positively correlated with delphinidin concentration, a known biologically active phenolic compound ($P < 0.0001$, r = 0.73). Anthocyanins revealed a significant positive correlation with Chroma and significantly negative correlation with Hue ($P < 0.0001$ and r = 0.894 and r = -0.797, respectively). Similarly, the total polyphenols concentration correlated positively with Chroma and negatively with Hue ($P < 0.0001$, r = 0.087 and r = -0.900, respectively).

9.4.2 Extraction Yield and Characterization

The maximum extraction yield of total phenolic compounds and anthocyanins in solution was 17.3 mg GAE/g dry coat and 1.7 mg C3GE/g dry coat respectively. Figure 28A presents the characterization and quantification of dried anthocyanin extract using LC-ESI-MS and HPLC. Three main anthocyanins were found by their respective ions [M+] 465.1 for delphinidin 3-*O*-glucoside, [M+] 479.12 for petunidin 3-*O*-glucoside and [M+] 493.13 for malvidin 3-*O*-glucoside. The three main anthocyanins were quantified by HPLC using pure standards. A total of 32 mg of anthocyanins were quantified per gram of dry extract. Petunidin 3-*O*-glucoside represented the highest proportion with 56% of the total, followed by delphinidin 3-*O*-glucoside with 34% and malvidin 3-*O*-glucoside with 10% of the total. A representative HPLC chromatogram and chemical structures are shown in Figure 28A and 28B.

9.4.3 Shelf Stability of Black Bean Anthocyanins

Table 19 shows the degradation kinetics values for anthocyanins and a* color parameter, which is related with the redness of the sample. The anthocyanin stability was higher at 4 ˚C and pH 2.5 with a half-life of 277 days. In contrast, at 22 ˚C, the half-life was only 43 days. Half-life of the anthocyanins decreased as the pH increased from 2.5 to 4.3 at 22 ˚C from 43 days to 16 days, respectively. In contrast, 4 ˚C and pH 3.5 offered a protective effect to anthocyanins showing a half-life of 172 days, compared to pH 3.0 with a half-life of 139 days and 56 days at

pH 4.3. Similarly, for a* color parameter, refrigeration offered a protective effect compared to room temperature. For redness stability, pH 3.5 improved the stability and half-life at 4 ˚C (151 days) compared with 124, 50 and 45 days at pH 2.5, 3.0 and 4.3, respectively. Table 19 shows the color of anthocyanins in solutions at different pH using 1 mg/mL of dry extract at time zero and five weeks of storage in comparison to cherry flavor soda.

9.3.4.4 First Order Reaction Kinetics and Arrhenius Model for Black Bean Anthocyanins and a Color Parameter*

Reaction rate constants and half-lives for anthocyanins and a* color parameter are summarized in Table 20 for anthocyanins exposed to high temperatures (70, 80 and 90 ºC) and different pH during 5 h. Anthocyanins and a* color followed a first order kinetics for thermal degradation. Plots of anthocyanin concentration indicated that degradation followed a first order reaction kinetic (Figure 29A-D). The highest reaction rate constants were observed at 90 ˚C and pH 4.3 (0.387/h). The increase in the temperature had an increase in the rate constants; however, pH 3.0 and 3.5 showed lower thermal degradation by having lower k values compared to pH 2.5. The longer half-lives (11.29 h, 3.86 h and 2.5 h) were for pH 3.0 at 70, 80 and 90 ˚C, respectively. On the contrary, pH 4.3 showed the shortest half-lives for all temperatures (1.84 h, 2.11 h and 1.79 h, respectively). The highest Q_{10} change in the reaction rate constant for 10 ˚C of temperature was observed at pH 2.5 with a value of 2.99 indicating that thermal degradation was three-fold higher at this temperature range and pH while the lowest Q_{10} was found at pH 4.3 (1.18) for 80 to 90 ˚C change. Arrhenius modeling of anthocyanin degradation showed a temperature and pH dependent change with activation energies of 84.76, 78.32, 75.28 and 55.92 kJ/mol and regression coefficients R^2 of 0.97, 0.95, 0.97 and 0.88 at pH 2.5, 3.0, 3.5 and 4.3, respectively.

Regarding the color parameter a*, there was a tendency to increase the reaction rate k as the temperature and the pH increased; however, similarly to degradation of anthocyanin, pH 3.0 seemed to have a protective effect. The highest k value was found at 90 ˚C and pH 4.3 (1.618/h), and the lowest k value was found at 70 ˚C and pH 2.5 (0.097/h). As expected the half-lives of anthocyanin solutions decreased as the temperature and pH increased; from 7.1 h at 70 ˚C and pH 2.5 to 0.43 h at 90 ˚C and pH 4.3. The Q_{10} change in reaction rate constant k values remained around 2 fold for pH 2.5, 3.0 and 3.5 whereas pH 4.3 presented Q_{10} values around 1.5 for both increases of temperature 70-80 ˚C and 80-90 ˚C. The Arrhenius modeling also showed

temperature dependent color a* degradation; however, pH 3.5 presented the highest activation energy (79.6 kJ/mol) compared to 76.34, 68.19 and 41.22 kJ/mol for pH 2.5, 3.0 and 4.3, respectively, correspondingly with R^2 values of 0.99 for all the treatments.

9.4.5Chroma, Hue, and ΔE Color Changes.*

The color of the anthocyanin solutions showed different trends depending on pH during the evaluated times and temperatures. In Figure 30A-D**,** the color parameter Chroma showed no statistical differences ($p < 0.05$) among temperatures; however, for pH 3.5 and 4.3 there was an increasing tendency for the Chroma value with time. On the other hand, hue color increased when increasing the temperature from 70 to 90 ˚C at pH 2.5; at pH 4.3 there was no change in the hue value between time and temperature (Figure 30E-H). Figure 31A-C shows the total change of color (ΔE*) at different pH in comparison with time zero at 70, 80 and 90˚C. The pH 2.5 showed the highest color changes at 80 and 90˚C; pH 3.0, 3.5 and 4.3 showed a slight increase on ΔE* after 2 h of heating. In general, the highest color change was observed at 90 ˚C. While the color was more stable at 70 ˚C, after 3 h of heating, not significant ($p > 0.05$) changes were observed on pH 2.5 and 3.5.

9.4.6 Discussion

Common beans, especially black beans contain significant amount of anthocyanins, mainly located in the coats. Common bean is the second most important cultivated legume, only after soybean, and it has been typically used for human consumption. Nowadays, some companies have developed edible beans product lines such as powders, grits, pieces and precooked beans for use as ingredients in the food industry [15]. No studies have reported on the optimization of extraction and stability of anthocyanins from black bean seed coats. In this study, we evaluated the potential of common beans coats as a source of natural food colorants and their shelf and thermal stability. At optimal extraction conditions, the anthocyanins concentration was 1.7 mg C3GE/g of coat. This is a lower value than the one reported by Mojica et al. [8] for Negro Otomi cultivar; probably due to different extraction methods since the investigators used acidified ethanol 85:15 ethanol: HCl. Optimization conditions were used to extract the coat pigments that were freeze dried for easy handling; anthocyanins enriched dry powder (1 mg/mL) was used to prepare the solutions at different pH that were found in commercially available beverages. The enriched anthocyanins powder represented 26.4% of yield from the bean coat after 3 consecutive extractions; 32.8 mg/g dry extract belonged to the three identified

anthocyanins (delphinidin glucoside, petunidin glucoside and malvidin gucoside). These results are in agreement to previous findings on anthocyanin composition in Mexican black bean cultivars [8].

Several factors affect anthocyanin stability, besides pH and temperature which are the most significant factors; processing and storage conditions, pressure, light, O_2, enzymes, ascorbic acid, sulfur dioxide, sulfite salts, metal ions, sugars and some co-pigments contribute to their degradation [3, 16,17]. All of these factors can cause oxidation and cleavage of covalent bonds that generate colorless smaller molecules [17]. In addition, the stability of anthocyanins can be influenced by the ring B substituents and the presence of additional hydroxyl or methoxyl groups [4]. Moreover, the solvent used for extraction and concentration exert an effect on color, solutions of synthetic flavylium salts showed changes on color such as changing from red in protic solvents to yellow in aprotic solvents [18].

Stability plays a fundamental role on evaluating natural compounds with potential as colorants. Some studies report that during refrigeration anthocyanin increased their stability. This important parameter is evaluated by the half-life ($t_{1/2}$) that represents the time needed for 50% of their degradation [16]. Shelf-life stability was monitored during 5 weeks at 4 ºC and 22 ºC, and anthocyanins were more stable under refrigeration conditions and low pH. This is in agreement with previously reported data [14, 16]. For example, Kirca et al. [14] reported lower half-life of 116 days at 5 °C for anthocyanins in orange juice and orange juice concentrate. Furthermore, Liu et al. [20] evaluated stability of anthocyanins from Chinese red radish and quantified their half-life using different fruit juice models at 4 °C. Results obtained were lower in all models (130.9 - 259.1 days) compared with 277 days of black bean anthocyanins in our study. Regarding color, Hernandez-Herrero et al. [3] reported that the color of grape and plum peel remained stable during 8 weeks of storage at 6 ˚C and 23 ˚C. We observed the highest stability of a* color parameter at pH 3.5 (151.1 days).

During the thermal stability assays all anthocyanin solutions followed a first order reaction kinetics. The energy of activation (Ea) is a thermodynamic parameter that indicated thermal stability in which the higher Ea represented the most stable. Kirca et al. [14] observed an increase in Ea when increasing the concentration of sugar in juices from 11.2 to 69 ˚Brix (73.6 to 89.5 kJ/mol). Liu et al. [19] reported higher Ea 47.94 kJ/mol on the anthocyanins from an apple juice model. Ahmed et al. [20] reported an Ea 37.48kJ/mol of anthocyanin on plum puree, in

addition to a linear correlation between color and anthocyanin concentration. Cisse et al. [21] evaluated the activation energy for different roselle cultivar extracts, finding Ea from 47.48 to 61.60 kJ/mol. On the other hand, Zoric et al. [17] reported values of Ea ranging from 42 to 55 kJ/mol on single anthocyanins from Marasca paste, with cyanidin-3-*O*-glucoside the most stable. When comparing the results of the current study with the literature, only pH 4.3 gave a lower Ea value. Q_{10} indicates the degradation rate; the higher the value, the more temperature dependent is the reaction. The Q_{10} values in the current study were higher than the values reported by Liu et al. [19], 1.70 from 70-80 ˚C and 1.48 from 80-90 ˚C in an apple juice model.

Color is an important sensory property when product quality is evaluated. Color parameters Chroma and Hue are commonly used to set the color of samples. The Chroma describes the saturation of a surface color, in other words, C* represents color intensity which is the distance of a color from the origin. On the other hand, Hue forms a continuous circular scale and is indicated in angles from 0° to 360°. The H˚ is expressed in degrees and ranges from 0˚ to 360˚, where 0˚ (red) locates on the +a* axis and then rotates anticlockwise to 90˚ (yellow) for the +b* axis, 180˚ (green) for -a* and 270˚ (blue) for -b* [22]. Plotting both parameters in the polychromatic plate the final color is matched. Changes on those parameters indicate change in the color of the sample solutions. C* values were more stable at pH 2.5 and 3.0 due to higher stability of anthocyanins at low pH; therefore, higher redness of the solutions at low pH. Regarding the H˚ value, pH 4.3 showed more stability at different times and temperatures. This stability could be associated to pale colors of the solutions that are more stable when compared with bright red colors at lower pH values.

The color of the solutions was evaluated by the color parameter a* which is related to the redness on the chromaticity dimensions of the samples. Black bean anthocyanins a* color parameters were more stable than plum puree anthocyanins when their activation energy (27.78 kJ/mol) [23] was compared; this value was lower than the Ea from all pH of bean extracts. The changes in color ΔE* are important because is a measurement of chemical stability and molecular integrity by measuring stability of the color it can directly be related with sensory quality. Ma et al. [23] evaluated the color changes at pH from 1 to 12, and they found that the red color was more stable under acidic conditions. They also found an increase in ΔE* with time and temperature; those results are similar to the tendency we found in the change of color of the samples; however the lowest pH presented the highest change in color at 90 °C.

Beyond the technological application of anthocyanins in the food industry, its use as beverage natural colorant present the advantage of their multiple health benefits associated with their consumption; for instance, these compounds can decrease oxidative stress, protect against coronary heart disease, exert anti-inflammatory and anti-carcinogenic activities and can help to control obesity and diabetes [24]. Moreover, dietary polyphenols have been associated to a prebiotic effect on microbiota modulation with beneficial health effects [25].

Common bean coats represent approximately 10% of the total seed; coats contain the highest concentration of phenolic compounds in beans. In general, common beans are consumed as a whole food. However, there is an increasing market of processed common beans as ingredients for the food industry [15]. Phenolic concentration and composition can be affected by cultivar, soil type, growing conditions, among others [8]. In addition, extraction conditions can affect their yield and composition. For optimal extraction of anthocyanins, solvents as methanol, ethanol, acetone and acid solutions are commonly used; however, several solvents are prohibited to be used during food processing due to their high toxicity to humans. In this sense, the extraction of pigments from natural sources must be performed using food grade materials, such as acidified mixtures of ethanol and water are generally recognized as safe (GRAS). Moreover, this work present for the first time the potential of black beans anthocyanins to be used as natural food colorant and their shelf-life and thermal stability under different conditions.

9.5 Conclusions

In conclusion, extraction using food grade ethanol was technically feasible to obtain stable anthocyanins from black bean coats. At pH 2.5 and refrigeration temperatures of storage (4 ˚C) anthocyanins stability was promoted up to ($t_{1/2}$) 277 days; moreover under same conditions following the Q rule, the projected shelf life is around 2.8 years [26]. Anthocyanins have outstanding potential to be used as food pigments; however, stability and feasibility are one of the main challenges to overcome. Black beans are a good source of natural pigments that could replace synthetic colorants commonly used in the food industry increasing the potential health benefits associated with the consumption of anthocyanins.

9.6 References

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9.7 Tables and figures

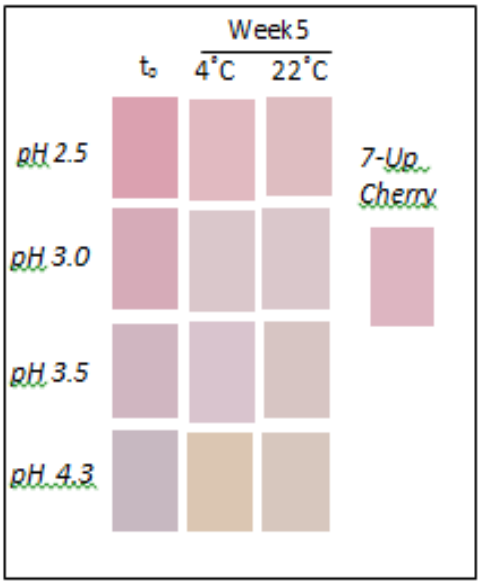
Table 18. Anthocyanin and total polyphenols concentrations, and color parameters of black bean extracts obtained under different extracting conditions[a]

Treat.	EtOH	S/L	Temp ° C	Anthocyanins mg C3GE/g coat	Total Polyphenols mg GAE/g coat	Chroma C*	Hue h°	Color
1	0	1:30	4	0.38±0.04m	3.63±0.62k	7.08±1.03l	25.01±3.98a	
2	12.5	1:30	4	0.67±0.08jklm	6.25±1.29hijk	10.49±1.16lijkl	14.38±0.74cdefgh	
3	25	1:30	4	1.03±0.17defgh	9.95±2.13efg	16.24±1.99bcdef	5.81±0.17kl	
4	0	1:40	4	0.44±0.05lm	4.205±0.76jk	8.33±1.63kl	21.14±4.48ab	
5	12.5	1:40	4	0.70±0.04jkl	6.40±1.02hijk	11.87±1.75hijk	12.74±0.90defghi	
6	25	1:40	4	1.06±0.11defg	10.15±1.57ef	15.69±2.04bcdefgh	4.67±0.19l	
7	0	1:50	4	0.46±0.02klm	4.49±0.39jk	7.13±1.32 l	24.74±3.33a	
8	12.5	1:50	4	0.73±0.02ijkl	6.65±0.39hijk	10.47±0.91lijkl	13.23±0.66cdefghi	
9	25	1:50	4	1.07±0.12def	10.31±1.61ef	14.37±1.40defgh	5.15±0.68l	
10	0	1:30	22	0.64±0.08jkl	5.34±0.18ijk	12.11±0.21ghijk	15.57±0.32cdef	
11	12.5	1:30	22	1.02±0.05defgh	9.02±0.67efgh	17.07±0.47abcde	11.72±0.17efgihj	
12	25	1:30	22	1.49±0.10abc	14.12±1.82bcd	20.36±0.70a	8.53±0.91lijkl	
13	0	1:40	22	0.69±0.12jkl	5.86±0.16ijk	10.57±0.43lijkl	17.63±0.42bcd	
14	12.5	1:40	22	1.12±0.04de	9.83±0.89efg	15.97±0.56bcdefg	11.38±0.01efghij	
15	25	1:40	22	1.54±0.05ab	14.45±1.46abc	19.37±0.69ab	6.96±0.25jkl	
16	0	1:50	22	0.75±0.14hijk	6.22±0.22hijk	10.15±0.02jkl	18.19±0.57bc	
17	12.5	1:50	22	1.15±0.14de	9.92±0.15efg	14.02±0.38efghi	11.46±0.33efghij	
18	25	1:50	22	1.54±0.01ab	14.78±0.66ab	17.64±0.43abcde	4.73±0.08l	
19	0	1:30	40	0.76±0.01ghijk	6.38±0.29hijk	12.85±0.82fghij	15.01±0.27cdef	
20	12.5	1:30	40	1.25±0.13bcd	11.72±0.59cde	17.79±0.55abcde	11.62±0.22efghij	
21	25	1:30	40	1.63±0.11ab	15.87±1.50ab	18.36±1.73abcd	9.33±1.34hijkl	
22	0	1:40	40	0.82±0.09fghik	7.05±0.22ghij	12.38±0.23fghij	14.87±0.16cdefg	
23	12.5	1:40	40	1.22±0.08dc	11.13±0.25de	18.74±1.41abc	9.69±1.85ghijkl	
24	25	1:40	40	1.64±0.04a	16.1±0.70ab	15.81±3.67bcdefgh	10.52±3.91fghijk	
25	0	1:50	40	0.88±0.17efghij	7.73±0.55fghi	12.06±0.20ghijk	16.41±0.67bcde	
26	12.5	1:50	40	1.24±0.08bcd	11.57±0.03cde	15.10±0.03cdefgh	11.55±0.54efgihj	
27	25	1:50	40	1.70±0.01a	17.33±0.15a	17.23±0.48a	5.27±0.46 l	

[a]Data represent the mean ± SD from at least three independent replicates. Values within a column followed by different letters are significant at $p < 0.05$; Treatment; EtOH: ethanol concentration; S/L: solid-to liquid ratio; C3GE: cyaniding 3 glucoside equivalents; GAE: gallic acid equivalents; C*: chroma = sqrt (a*2 + b*2); h°: hue angle = sqrt (a*2 + b*2) .

Table 19. Shelf–life stability of black bean anthocyanins and color a* parameter at refrigeration and room temperature and Red, Green, Blue (RGB) color parameter of commercial cherry soda and anthocyanin extract solutions (1 mg/mL) at different pH after 5 weeks of storage[a].

Anthocyanins					
Parameter	Temperature	pH 2.5	pH 3.0	pH 3.5	pH 4.3
Rate (k, d^{-1})	4°C	0.017	0.034	0.028	0.086
	22°C	0.113	0.127	0.130	0.304
Half-life($t_{1/2}$, days)	4°C	277.2	139.8	172.6	56.0
	22°C	43.4	37.94	37.17	15.89
Color a*					
Parameter	Temperature	pH 2.5	pH 3.0	pH 3.5	pH 4.3
Rate (k, d^{-1})	4°C	0.039	0.095	0.032	0.106
	22°C	0.144	0.307	0.172	0.630
Half-life ($t_{1/2}$, days)	4°C	124.6	50.7	151.1	45.7
	22°C	33.6	15.75	60.7	7.6



[a]k: rate constant; d: days; $t_{1/2}$: half-life.

Table 20. First order reaction kinetics and Arrhenius parameters for anthocyanins and color a* parameters degradation.[a]

		Anthocyanins			
Parameter	Temperature	pH 2.5	pH 3.0	pH 3.5	pH 4.3
Rate (k, h^{-1})	70ºC	0.072	0.061	0.071	0.143
	80ºC	0.216	0.180	0.184	0.329
	90ºC	0.370	0.277	0.303	0.387
Half-life($t_{1/2}$, h)	70ºC	9.60	11.29	9.75	4.84
	80ºC	3.21	3.86	3.77	2.11
	90ºC	1.88	2.50	2.29	1.79
Q_{10}	(70-80ºC)	2.99	2.93	2.59	2.30
	(80-90ºC)	1.71	1.54	1.65	1.18
Energy of activation, Ea (kJ/mol)		84.76	78.32	75.28	55.92
Regression coefficient, R^2		0.97	0.95	0.97	0.88

		Color a*			
Parameter	Temperature	pH 2.5	pH 3.0	pH 3.5	pH 4.3
Rate (k, h^{-1})	70ºC	0.097	0.162	0.155	0.717
	80ºC	0.214	0.328	0.341	1.093
	90ºC	0.440	0.624	0.746	1.618
Half-life($t_{1/2}$, h)	70ºC	7.10	4.26	4.47	0.97
	80ºC	3.24	2.11	2.04	0.63
	90ºC	1.58	1.11	0.93	0.43
Q_{10}	(70-80ºC)	2.19	2.02	2.20	1.52
	(80-90ºC)	2.06	1.90	2.19	1.48
Energy of activation, Ea (kJ/mol)		76.34	68.19	79.6	41.22
Regression coefficient, R^2		0.99	0.99	0.99	0.99

[a]k: rate constant; d: days; $t_{1/2}$: half-life; Ea: activation energy; Q_{10}: change in the reaction rate constant for 10˚C.

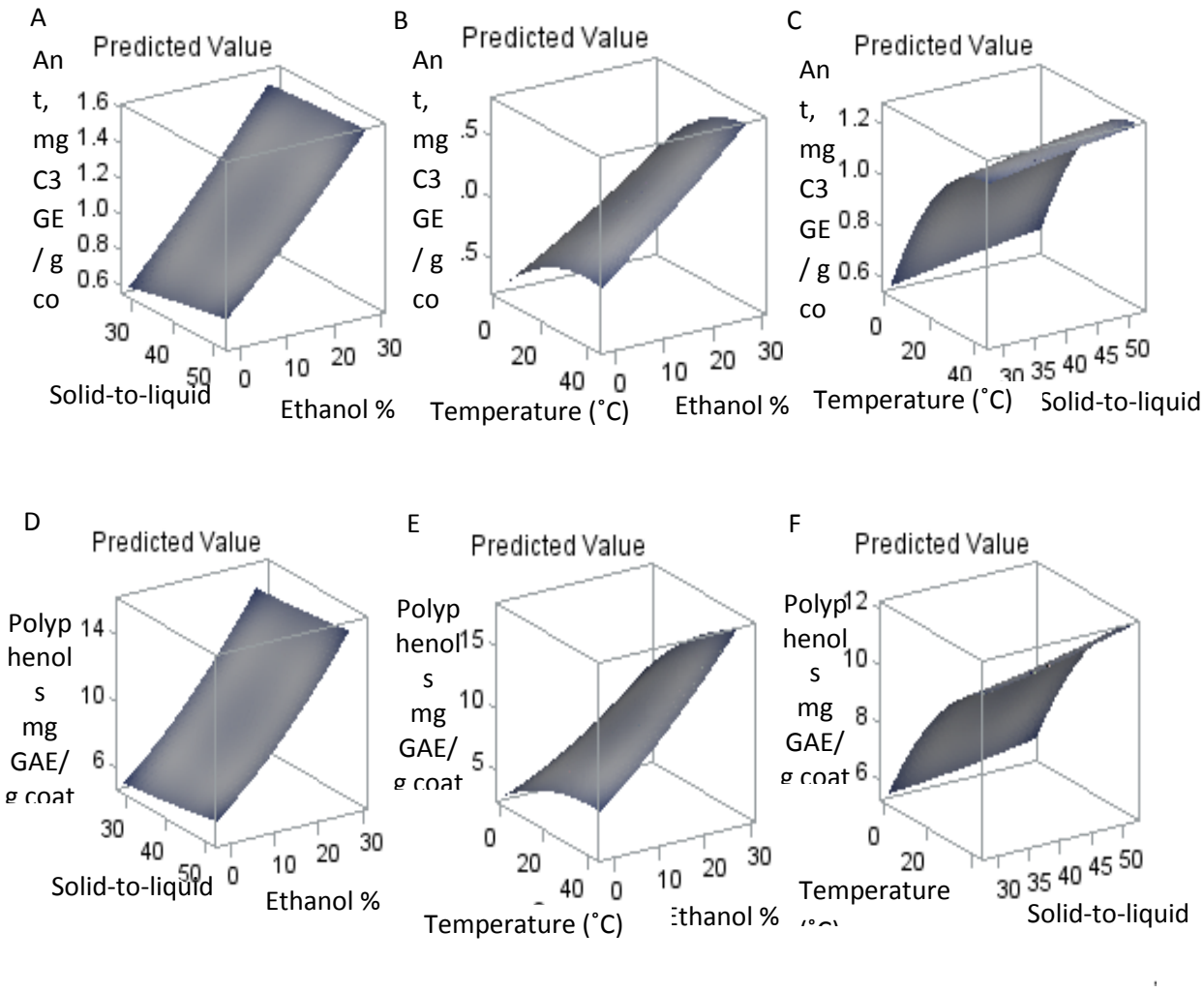
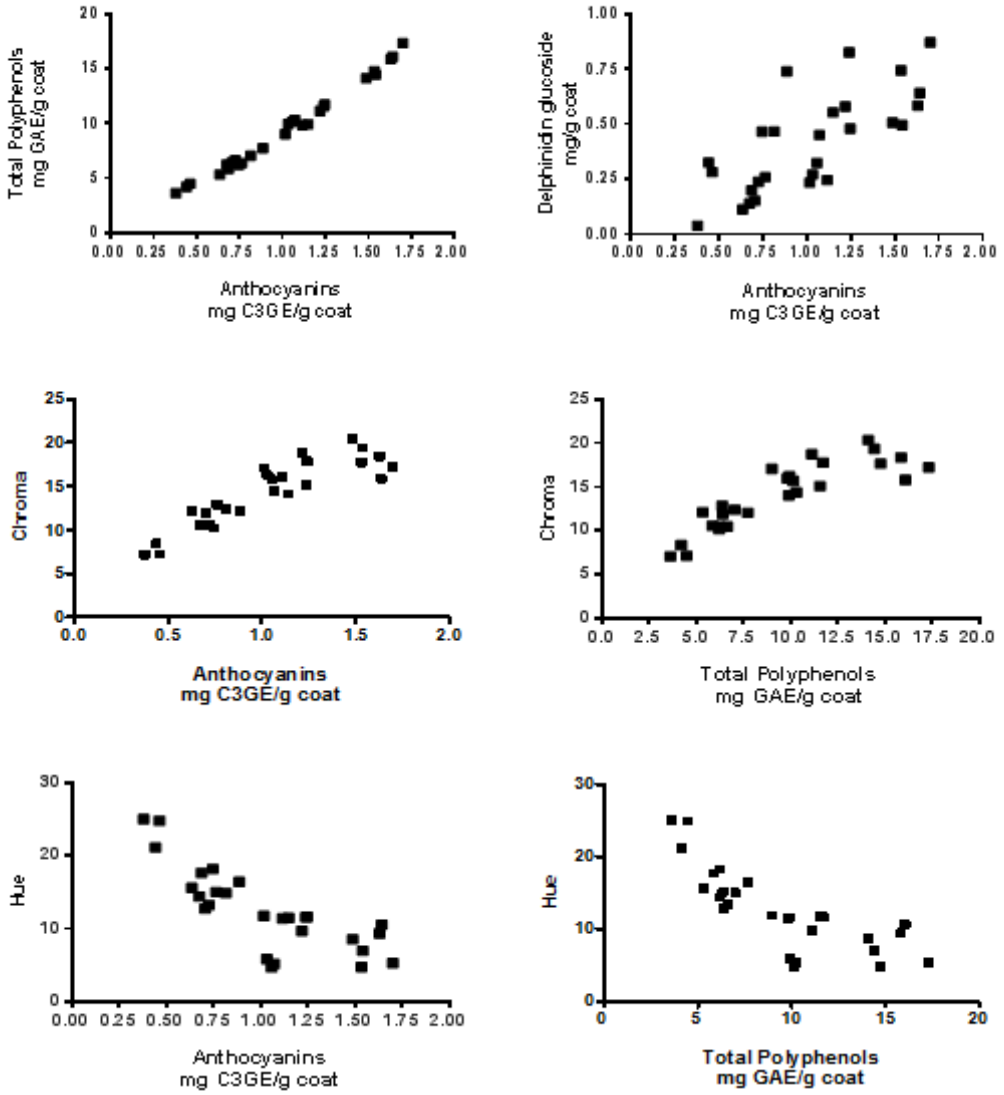


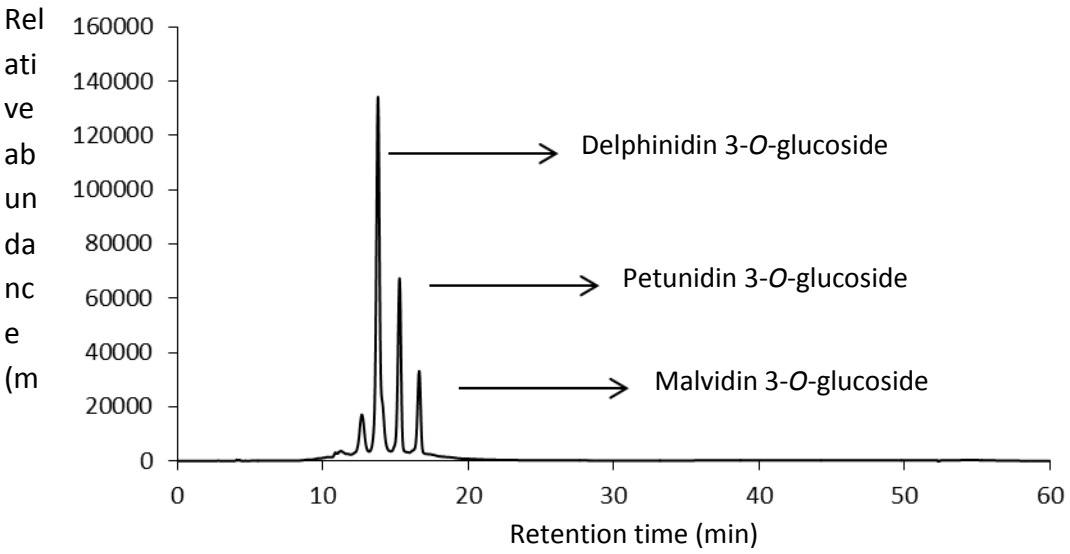
Figure 26. Surface response methodology for the extraction of anthocyanins and total polyphenols from black bean coats as affected by ethanol concentration, solid-to liquid ratio and temperature. A. Solid-to-liquid ratio vs % ethanol for anthocyanins; B. Temperature vs % ethanol for anthocyanins; C. Temperature vs solid-to-liquid ratio for anthocyanins; D. Solid-to-liquid ratio vs % ethanol for polyphenols; E. Temperature vs % ethanol for polyphenols; F. Temperature vs solid-to-liquid ratio for polyphenols.



Correlation of common bean coat extract parameters			
Comparison	P-value	Pearson r	95% Confidence interval
Anthocyanins vs Polyphenols	P<0.0001	0.993	0.986 to 0.997
Anthocyanins vs Delphinidin	P<0.0001	0.739	0.499 to 0.873
Anthocyanins vs Chroma	P<0.0001	0.894	0.779 to 0.951
Polyphenols vs Chroma	P<0.0001	0.868	0.728 to 0.938
Anthocyanins vs Hue	P<0.0001	-0.797	-0.903 to -0.599
Polyphenols vs Hue	P<0.0001	-0.791	-0.900 to -0.587

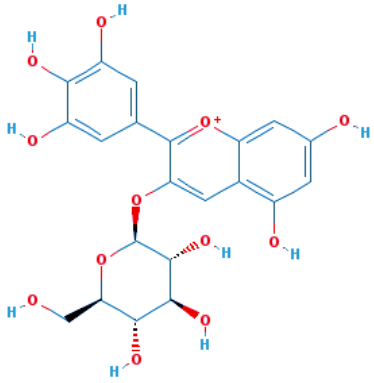
Figure 27. Correlation parameters of anthocyanins and total polyphenols with color parameters Hue and Chroma. A. Anthocyanins vs total polyphenols; B. Anthocyanins vs delphinidin glucoside; C. Anthocyanins vs chroma; D. Polyphenols vs chroma; E. Anthocyanins vs hue; F. Polyphenols vs hue.

A



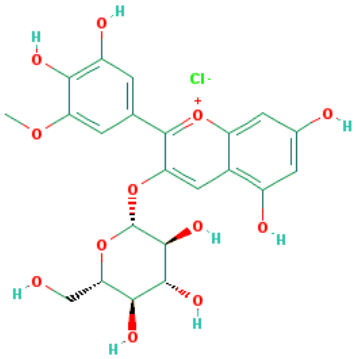
Anthocyanins	Concentration mg/g dry extract	1st Ion (*m/z*)	2nd Ion (*m/z*)	t_R (min)	Chemical formula
Delphinidin 3-*O*-glucoside	11.15 ± 0.44	465.1 $[M]^+$	391.28$[M]^+$	9.94	$C_{21}H_{21}O_{12}$
Petunidin 3-*O*-glucoside	18.32 ± 0.88	479.1[M]+	391.28$[M]^+$	11.12	$C_{22}H_{23}O_{12}$
Malvidin 3-*O*-glucoside	3.35 ± 0.32	493.1[M]+	391.28$[M]^+$	12.23	$C_{23}H_{25}O_{12}$
Total	32.82 ± 1.64				

Delphinidin 3-*O*-glucoside



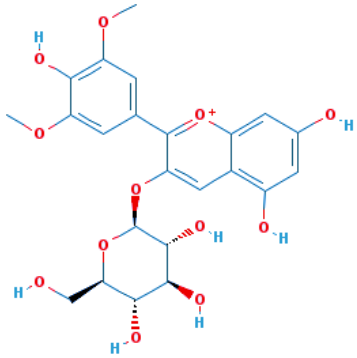
PubChem CID: 443650

Petunidin 3-*O*-glucoside



PubChem CID: 176449

Malvidin 3-*O*-glucoside



PubChem CID: 443652

Figure 28. A. Representative HPLC chromatogram of the anthocyanin extract, showing the anthocyanin characterization and concentration of individual anthocyanins; B. Chemical structure of identified anthocyanins.

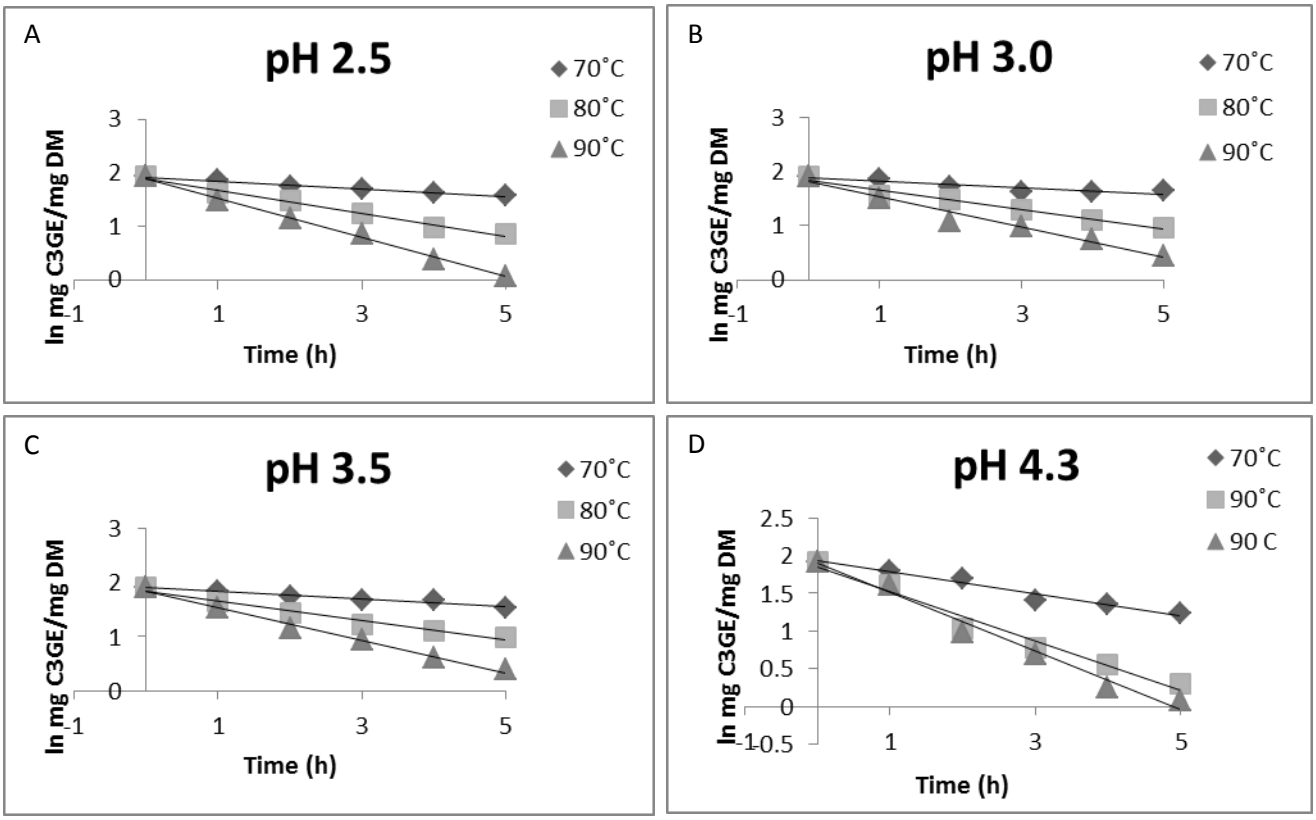


Figure 29. First order reaction kinetics of anthocyanins at different pH and temperatures for black beans anthocyanins. A. pH 2.5; B. pH 3.0; C. pH 3.5; D. pH 4.3.

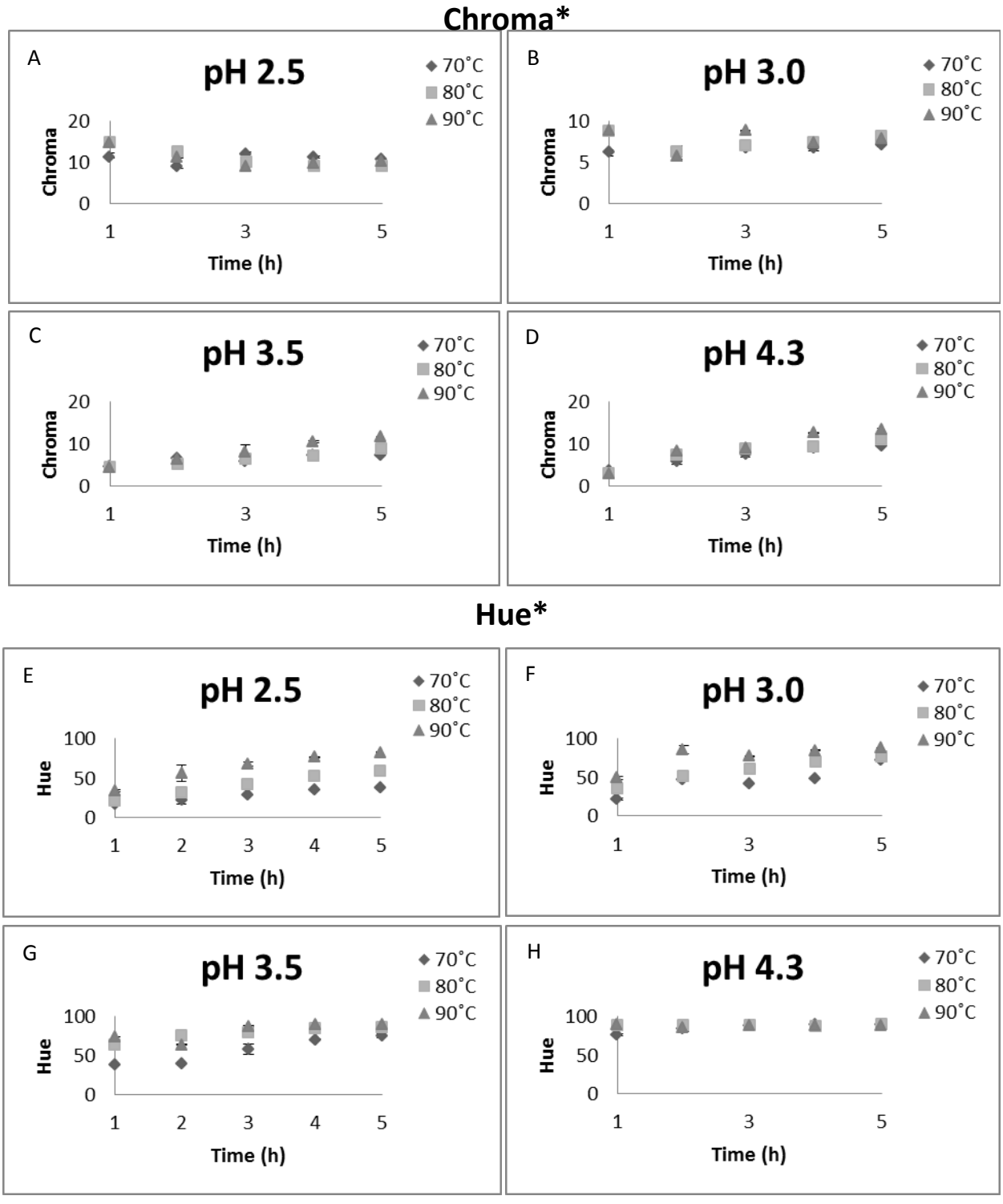


Figure 30. Chroma and hue variations at different pH and temperatures as time progresses. A. Chroma and pH 2.5; B. Chroma and pH 3.0; C. Chroma and pH 3.5; D. Chroma and pH 4.3; E. Hue and pH 2.5; F. Hue and pH 3.0; G. Hue and pH 3.5; H. Hue and pH 4.3.

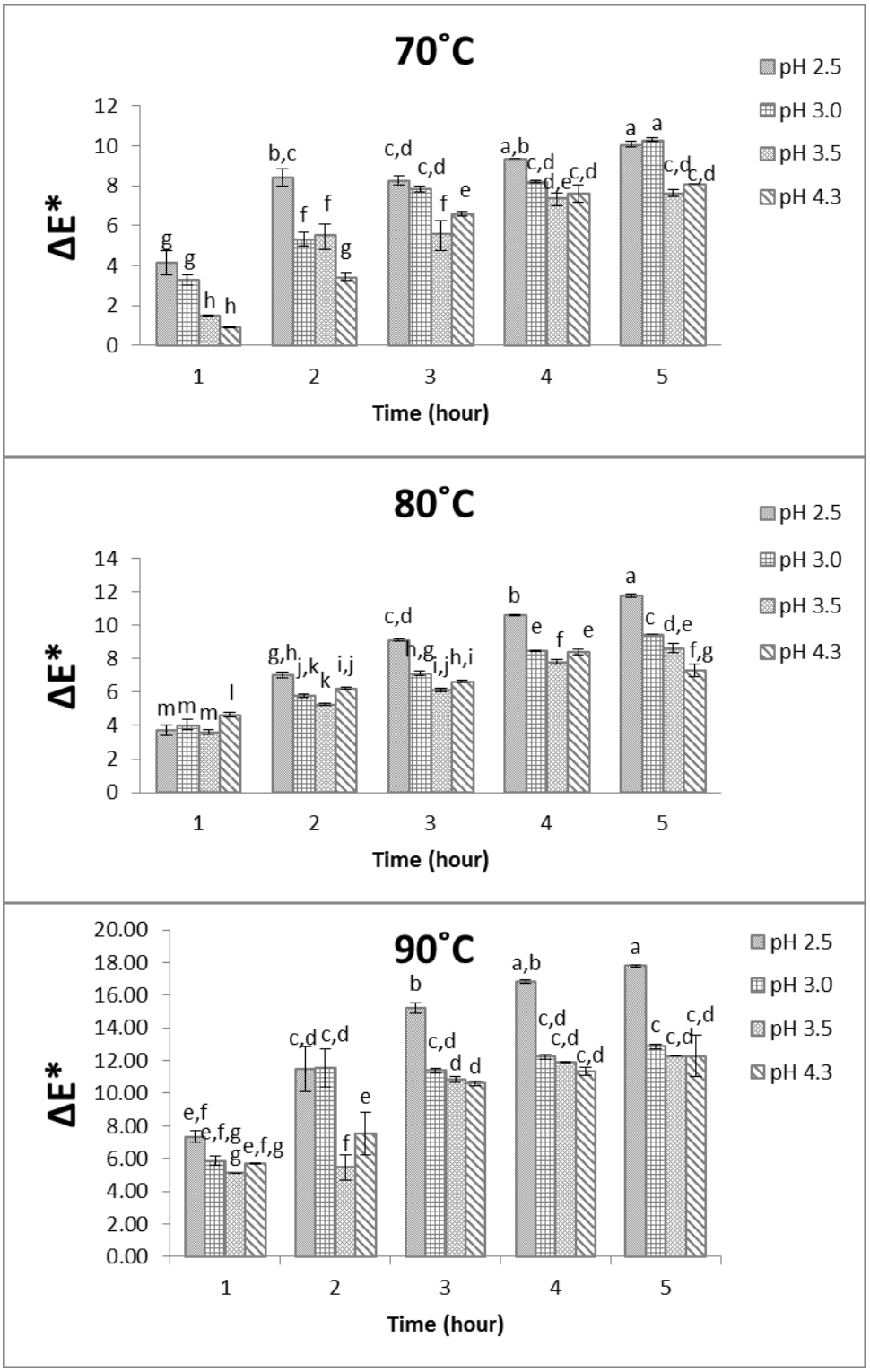


Figure 31. Color change of the anthocyanin solutions; effect of pH, time and temperature. A. 70 ˚C; B. 80 ˚C; C. 90 ˚C.

CHAPTER 10: ANTI-DIABETES BLACK BEAN PEPTIDES REDUCE GLUCOSE ABSORPTION THROUGH INTERACTION WITH GLUCOSE TRANSPORTERS: IN *in silico*, *in vitro* AND *in vivo* MODELS

10.1 Abstract

The objective was to evaluate the *in vitro* and *in vivo* effects of fully characterized black bean protein fractions (PF) pure peptides and on glucose absorption and markers of diabetes on epithelial Caco-2 cells and in healthy and hyperglycemic Wistar rats. *In silico* studies of GLUT2 and SGLT1 and peptides interaction, *in vitro* protein and gene expression and glucose absorption/uptake, DPP-IV inhibition and ROS inhibition in Caco-2 cells, oral glucose tolerance test (OGTT) and hyperglycemic streptozotocin-induced rats *in vivo* were evaluated. PF contained thirty-three peptides identified by LC-ESI-MSMS. AKSPLF, ATNPLF, FEELN and LSVSVL peptides blocked glucose transporters by interacting in their protein motif. PF (10 mg/mL) reduced protein expression of SGLT1, GLUT2, β-tubulin and AMPKα at 30 min and 24 h. Downregulation of genes SLC2A2, SLC5A1, GCG, and DPP4 was observed after PF treatments. Inhibited DPP-IV by 51.2 and 29.8 % at 30 min and 24 h, respectively ($p<0.05$) and reduced intracellular oxygen reactive species formation by 70.9 %. Glucose absorption was decreased by 6.5 and 21.5 % after 30 min and 24 h of treatment, respectively, in the Caco-2 cells model. In the OGTT experiment, the postprandial glucose significantly decreased (24.5% $p<0.05$) at a dose of 50 mg PF/kg BW compared to the control. In the hyperglycemic model, PF showed a reduction of postprandial glucose in a dose-dependent manner during treatment compared to non-treated control. Moreover, lowest fasting glucose was found in group PF 150 mg/kg (200±50 mg/dL), compared to control groups ($p<0.05$). Black common bean PF and its pure peptides showed significant hypoglycemic effects mainly by blocking the glucose transporters and decreasing the expression and translocation to the apical membrane of GLUT2 and SGLT1.

This chapter is part of the publication: Mojica L, Menjivar M, Granados-Silvestre A, de Mejía EG (2016) Anti-diabetes black bean peptides reduce glucose absorption through interaction with glucose transporters: in *in silico, in vitro* and *in vivo* models (*to be submitted*)

10.2 Introduction

Bioactive Peptides are short sequences of amino acids that show physiological benefits when consumed [1]. Peptides are generated from dietary proteins during gastro-intestinal digestion or may be consumed as an ingredient from processed protein fraction extracts. Their bioavailability after oral administration depends on their capacity to resist further digestive hydrolysis, absorption, and distribution to reach the target organ [2]. The specific amino acid functionality and position within the sequence of the peptides determines their bioactivity. Amino acids at the C-terminal region exert higher antioxidant activity than those in the N-terminal regions which correlates with the electronic, hydrophobic, steric, and hydrogen bonding properties of amino acids in those regions [3].

Bioactive peptides from milk and other food sources have been reported to modulate molecular targets of diabetes, for instance, inhibition of α-glucosidase and dipeptidyl peptidase IV (DPP-IV), stimulation of insulin secretion, a decrease of glucose absorption in the gut and improvement of glucose uptake in peripheral tissues has been observed [2,4]. Besides, Vernaleken et al. [5] reported two tripeptides capable of inducing posttranscriptional down-regulation of glucose transporter SGLT1, leading to a decrease of 30% of glucose absorption. Furthermore, common bean peptides have been reported to exert important antioxidant activity [6-8].

Diabetes is a chronic noncommunicable disease and a multifactorial disorder characterized by the inability of the body to produce insulin (type 1 diabetes) or by defects in insulin secretion and action (type 2 diabetes) [9]. Diabetes treatment consists of dietary changes, weight loss, exercise and the use of pharmaceuticals to improve glucose homeostasis. Approved drugs for the management of diabetes include sulfonylureas, meglitinides, biguanides, thiazolidinediones, α-glucosidase inhibitors, glucagon-like peptide (GLP)-1 receptor agonists, dipeptidyl peptidase-IV inhibitors, amylin analogs, dopamine D2-receptor agonists, bile acid sequestrants, and insulin and its analogs [10].

Most glucose absorption takes place in the small intestine, which is linked to endocrine regulation, immune surveillance, interaction with enteric microbiome, and ADME of nutrients [11]. Products of digestion reach the apical membrane of the jejunum 30 min after food consumption. After an increase in free glucose concentration, the absorption in the apical membrane occurs through sodium glucose linked transporter-1 (SGLT1), causing activation of

protein kinase C II (PKC) leading to activation of the apical glucose transporter 2 (GLUT2) already in the cytoplasm and its translocated to the apical membrane from intracellular vesicles [12,13].

Common bean bioactive peptides have been reported to exert antihypertensive, anti-inflammatory, antioxidant and anticancer potential [14]. However, few studies have evaluated the anti-diabetes potential of common bean peptides [8,15]. The objective of this research was to evaluate the mechanism of the hypoglycemic action of PF and pure peptides, originally found in black bean hydrolysates, using *in silico, in vitro* and *in vivo* approaches.

10.3 Materials and methods

10.3.1 Materials

Black-Otomi bean cultivar was provided by the Experimental Station of the National Research Institute for Forestry Agriculture and Livestock (INIFAP), Zacatecas, Mexico. The dry seeds were stored at 4°C until use. Alcalase (EC 3.4.21.62) and phloretin (P7912-100 mg) were purchased from Sigma-Aldrich (St. Louis, MO). DPPIV-GLO® protease assay kit was purchased from Promega (Madison, WI). Human colon epithelial cells Caco-2 [Caco2] (ATCC®HTB-37], Eagle's Minimum Essential Medium (EMEM), and 0.25% (w/v) trypsin-0.53 mM EDTA were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Penicillin-streptomycin was purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Thermo Scientific Hyclone, Logan, UT, USA). Primary rabbit polyclonal antibodies GLUT2 (sc-9117), SGLT1 (sc-98974), GADPH (sc-47724), primary goat polyclonal antibody GLUT2 (sc-7580), protein kinase C II (PKCβII) (sc-13149), β-Tubulin (sc-9104), 5' adenosine monophosphate-activated protein kinase (AMPKα1) (sc-398861), phosphoinositide-specific phospholipase (PLCβ2) (sc-206) and radioimmune-precipitation assay (RIPA) buffer were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies and anti-rabbit IgG horseradish peroxidase conjugated were purchased from GE Healthcare (Buckinghamshire, UK). Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 donkey anti-goat IgG were purchased from Life Technologies (Gaithersburg, MD). Human simpleStep ELISA^{TM} kit for GLP-1 (7-36) (AB184857) and cellular reactive oxygen species detection assay kit (Abcam®, ab113851) were purchased from Abcam (Cambridge, UK), and glucose colorimetric assay kit (100009582) from Cayman Chemical (Ann Arbor, MI). Pure peptides AKSPLF, ATNPLF, FEELN, LSVSVL (originally found in bean

protein fractions by LC-ESI-MSMS and selected based on their best performance of computational docking models) with purity higher than 98% were synthesized by GenScript (Piscataway, NJ). Falcon™ HTS Multiwell Insert System was purchased from Fisher (Waltham, MA). Male Wistar rats and standard diet (Teklad LM-485) were purchased from Harlan Inc (Indianapolis, IN) and streptozotocin from Sigma-Aldrich (St. Louis, MO). Insulin kit from ALPCO (Salem NH), insulin N human DNA recombinant (Wockhardt L., India) 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) was purchased from ThermoFisher (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

10.3.2 Protein extraction, hydrolysis, and LC-ESI-MSMS peptide characterization

Five kg of beans were soaked in water at room temperature for 16 h. The hull was then manually removed, and beans were ground in a commercial blender at a 1:10 bean/water ratio. The pH was adjusted to 8.0 with 1 M NaOH and protein extraction was carried out by stirring at 35° C with stirring for one h. The mixture was centrifuged at 5,000 g for 15 min at 25 °C. The precipitate was re-extracted under identical conditions to maximize yield, and both extracts were combined. Then, the pH was adjusted to 4.3 with 1 M hydrochloric acid to precipitate protein, followed by centrifugation at 10,000 g for 20 min at 4° C. The supernatant was discarded and the pellet freeze-dried in a Labconco Freeze Dryer 4.5 (Kansas, MO). Dried bean protein isolates (BPI, 0.5 kg) were stored at -20° C until use. A 0.5 kg bean protein isolate was suspended in water (1:20 w/v) and autoclaved for 20 min at 121˚C. Enzymatic digestion was carried out using protease/substrate ratio of 1:20 (w/w), time of hydrolysis 2 h, with pH and temperature optimal for alcalase (pH: 7.0, T: 50˚C). Protein hydrolysis was stopped by heating at 75 °C for 20 min, and the resulting protein fractions were centrifuged at 20,000 *g* for 15 min at 4°C. Bean protein fractions were filtered through a stirred ultrafiltration cell (Millipore, MA) to eliminate salts using a 300 Da molecular weight cut-off membrane and then freeze-dried in a Labconco FreeZone Freeze dry system (Kansas City, MO, USA). Protein fractions (68 g) were stored at -20°C until use and analyzed by LC-ESI-MSMS using a Q-tof Ultima mass spectrometer (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system as reported by Mojica et al. [16].

10.3.3 In vitro studies

10.3.3.1 Caco-2 cell proliferation

Caco-2 cells (HTB-37 from ATCC, Manassas, VA) were subcultured using Eagle's Minimum Essential Medium (EMEM) ATCC ® 30-2003 media supplemented with 20% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate. Cells were maintained at 37°C in 5% CO_2/95% air using a CO_2 Jacketed Incubator (NuAIRE DH Autoflow, Plymouth, MN). Cell proliferation was measured using a CellTiter 96® AQueous One Solution Proliferation assay kit; cell viability > 80% within all treatments (Promega, Madison, WI).

10.3.3.2 Dual-layered simulated glucose absorption

Simulates gastrointestinal glucose absorption, 20 mM glucose was applied to the apical side of human epithelial Caco-2 cells grown in a monolayer on hanging cell culture inserts containing a polystyrene membrane with 0.4 μM pores. Caco-2 cells were seeded on the apical side (above insert) of 24-well cell culture inserts at a density of 5 $x10^4$ cells /cm^2, with a volume of culture phenol red free media at 0.4 mL. Only those cells that successfully formed a monolayer, as determined by the Transepithelial Electrical Resistance (TEER) measurement to ensure the integrity of the monolayer were used for the experiment. TEER measurement with Millicel-ERS volt-ohm-meter (Millipore, Billerica, MA) was taken daily and before and after the experiment to ensure the integrity of the monolayer in each well. After 20 days, Caco-2 cells reached confluence (average TEER for all wells was 524.2 ± 66.7 Ω*cm^2). A 0.4 mL of glucose free media was placed in the receiver plate in order the collect the glucose that was absorbed. Cells were pretreated with glucose free media for two h and treatments were applied with either phloretin (PHL) (100 μM), pure peptides AKSPLF, ATNPLF, FEELN, LSVSVL (100 μM) or protein fractions (10 mg/mL) to the apical side for 30 min and 24 h. After treatments, the media from both the apical and the basolateral side (below insert) were collected, centrifuged at 4°C for 10 min at 1000 g to remove cell debris, and stored for glucose analysis. Glucose was quantified using a glucose colorimetric assay kit (100009582) from Cayman Chemical (Ann Arbor, MI).

10.3.3.3 Glucose uptake in vitro

Caco-2 cells were seeded in 24-well plates at the density of 2 × 10^5 cells/well. The medium was changed every 2 days, and the culture was carried out for 13 days. For uptake studies, Caco-2 cells were placed in glucose free media for two h. Then cells were exposed to 400 μL glucose-free media containing either pure peptides (100 μM), phloretin (100 μM), or protein fractions (10 mg/mL), and a fluorescent D-glucose derivative, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), (100 μM) for 10, 30, 60 and 180 min at

37 °C. Glucose uptake was stopped by washing three times with two fold volume of ice-cold PBS. Fluorescent intensity was measured by a Synergy2 multi-well plate reader (Biotek, Winooski, VT) at 485 nm excitation and 535 nm emission filter. The cells were lysed in 100 µL RIPA lysis buffer and. Lysates protein concentration was measured using DC protein assay (Bio-Rad Laboratories, Hercules, CA). Results were express as % of glucose uptake relative to the untreated control and normalized to protein concentration.

10.3.3.4 Western blot analysis of makers related to type-2 diabetes

To obtain the protein expression of GLUT2, SGLT1, PKCβII, β-Tubulin, AMPKα1 and PLCβ2 and GAPDH 20 µg of cell lysate protein were loaded in each well of 4-20% gradient SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and blocked with 3% non-fat dry milk in 0.1% TBST for one h at room temperature. After blocking, the membranes were washed with 0.1% TBST (3 times, 5 min each) and incubated with primary antibodies (1:500) overnight at 4°C. The membranes were washed again and incubated with anti-IgG horseradish peroxidase conjugated secondary antibodies (1:2500) for 2 h at room temperature. After incubation and repeated washing, the membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagents A (luminol solution) and B (peroxide solution) (GE Healthcare Biosciences, Pittsburgh, PA). The membrane pictures were taken on a GelLogic 4000 Pro Imaging System (Carestream Health, Inc., Rochester, NY). Relative expression of all proteins was normalized to GAPDH.

10.3.3.5 Fixed-cell immunostaining and confocal microscopy

Caco-2 cells were seeded at a density of 50,000 cells/well and 400 µL of phenol red-free OptiMEM medium containing 20% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate in ibiTreat microscopy chambers (Ibidi, Munich, Germany) at 37°C in 5% CO_2/95% air. Cells were pre-treated for 2 h with glucose free media and pure peptides either AKSPLF, ATNPLF, FEELN, LSVSVL (100 µM), PF (10 mg/mL) or PHL (100 µM). Media was removed and new media containing 20 mM glucose and treatments with either pure peptides AKSPLF, ATNPLF, FEELN, LSVSVL (100 µM), PF (10 mg/mL) or PHL (100 µM) for 30 min and 24 h were added. Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde aqueous solution (Electron Microscopy Sciences) for 30 min at 25°C; washed 3 times (5 min each) with PBS, and permeabilized with 0.1% Triton-X 100 in PBS for 15 min at 25°C. Cells were washed once with

PBS and incubated with ultracold HPLC-grade methanol for 15 min at −20°C. Methanol was removed, replaced with PBS, and incubated at 25°C for 30 min. Cells were blocked with Image-iT FX Signal Enhancer (Life Technologies) for 30 min at 25°C, washed once with PBS, and incubated with either GLUT2 or SGLT1 antibodies (1:500) overnight at 37°C. Cells were washed 3 times (5 min each) with PBS and incubated with Alexa Fluor 568 goat-anti-rabbit IgG for SGLT1 (578 nm excitation and 603 nm emission) and Alexa Fluor 488 donkey anti-goat for GLUT2 (495 nm excitation and 519 nm emission IgG (Life Technologies) secondary antibodies (1:200) for 3 h at 25°C in the dark. Cells were washed 3 times (5 min each) with PBS and cured with ProLong Gold antifade reagent with DAPI (Life Technologies) for 24 h at 25°C in the dark. The microscopy chamber plate was stored at 4°C in the dark until analysis. Samples were imaged using a 63×/1.4 Oil DIC M27 objective with a Zeiss LSM 880 laser scanning confocal microscope (Carl Zeiss AG, Germany). The images were obtained using a 405 nm (10 mW) (415-470 nm emission), a 488 nm (10 mW) (500–550 nm emission) and a 568 nm (5 mW) laser line (600–650 nm emission). The individual channels were obtained using a sequential scanning mode to prevent bleed-through the excitation signal. Laser power, gain and offset were kept constant across the samples, and the samples were scanned in a high-resolution format of 1024×1024 pixels averaging 4 frames. Single optical planes of the individual channels were captured and all of the optical planes were displayed as a gallery. The expression and area sums (μm^2) of the raw images were quantified with Zen 2 lite Blue edition (Carl Zeiss AG, Germany) software. All of the image panels were resized and consolidated using the GNU Image Manipulation Program, and the brightness of the final collage of images displayed was increased by 20%, as a whole. The xy axes depict localization of glucose transporters in the cell cytoplasm and membrane, and z axes show a transversal cut of the cell, where localization of glucose transporters can be observed in cells being the lower part consider apical and the upper part considered basolateral. Data was expressed as the mean ± SD of four independent fields of view from two independent cellular replicates. Means with different letters were significantly different ($p < 0.05$) according to Tukey's test.

10.3.3.6 Glucagon-like peptide 1 and dipeptidyl peptidase-IV activities

Active GLP-1 was quantified in the cell media from Caco-2 cells, plated at 2 x 10^5 cells/well in 6-well plates and incubated for 24 h, following direct 2 h pre-treatment with glucose and phenol red free media and pure peptides either AKSPLF, ATNPLF, FEELN, LSVSVL (100

μM), PF (10 mg/mL) or PHL (100 μM), then 20 mM glucose and phenol red free media and same treatments for 30 min and 24 h were added. Media was collected after treatments and GLP-1 was measured using a Human simpleStep ELISA^{TM} kit (AB184857), from Abcam (Cambridge, MA) following manufacturer's instructions. A 50 μL of media and 50 μL of antibody cocktail were added to the wells. After one h of incubation at room temperature, the wells were washed to remove unbound materials. Next, the unbound conjugate was washed, and bound detection conjugate was quantified by addition of 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate, the reaction was stopped by adding 100 μL of stop solution after 15 min. Optical density was measured using a Synergy2 multi-well plate reader (Biotek, Winooski, VT) at 450 nm and quantified using GLP-1 standard curve ranging from 29.8-500 pg/mL with a standard equation of $y = 0.0009x - 0.0547$, $R^2 = 0.97$.

The activity of DPP-IV was measured using DPP-IV Glo ™ protease assay (Promega, Madison, WI) following manufacturer's instructions. Cells media was collected from Caco-2 cells, plated at 2 $x10^5$ cells/well in 6-well plates and incubated for 24 h, following direct treatment for 30 min and 24 h. After treatments, as indicated for GLP-1 above, normalized for protein concentration, and diluted to a final concentration of 10 ng of protein per well, the Glo-substrate was added. The 96 wells plate was gently mixed using a Synergy2 multi-well plate reader (Biotek, Winooski, VT) at medium intensity for 4 s and read after 30 min of incubation. DPP-IV cleavage of the provided Gly-Pro-amino methyl coumarin (AMC) substrate generated a luminescent signal by luciferase reaction with the amount of DPP-IV enzyme available to bind Gly-Pro-AMC proportional to relative light units (RLU) produced and treatments were compared to wells containing cell media only as control of no DPP-IV enzyme inhibition.

10.3.3.7 Reactive oxygen species

Independent cell treatments were performed in 96 well plate for the ROS inhibition assay, using the cellular reactive oxygen species detection assay kit (Abcam®, ab113851, Cambridge, MA). Caco-2 cells (1 x 10^4 cells/well) were incubated for 24 h before treatments. Cells were treated with 100 μM H_2O_2, 2',7'- dichlorofluorescein diacetate (DCFDA) (25 μM) and either pure peptides (AKSPLF, ATNPLF, FEELN, or LSVSVL, 100 μM), or PF (10 mg/mL) or PHL (100 μM). Followed by four h of incubation, after this period of time the plate was read in the Synergy2 multi-well plate reader (Biotek, Winooski, VT) with excitation wavelength at 485 nm

and emission wavelength at 535 nm. The results were expressed as a percentage of fluorescence inhibition relative to the untreated control.

10.3.3.8 Gene expression

Gene expression analyzes were carried out on Caco-2 cells in order to determine the direct action of the compounds tested on gene expression. Caco-2 cells growing at 2 x 10^5 cells/well in a 6-well plate were treated with either pure peptides (AKSPLF, ATNPLF, FEELN, or LSVSVL, 100 μM), or PF (10 mg/mL) or PHL (100 μM). After pre-treatment for two h in glucose free media, cells were stimulated with 20 mM glucose during 30 and 24 h; then, RNA was collected using a RNAeasy kit (Qiagen, Germantown, MD), quantified, and checked for quality. cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA). Quantitative RT-PCR was performed using TaqMan® gene expression assay in a 7900HT Fast Real-Time PCR System Cycler (Applied Biosystems, Foster City, CA) and targeted human SGLT1 (Hs0173790_m1), GLUT2 (H01096908_m1), GAPDH (Hs02758991_g1), DPP-IV (Hs00897391_m1) and GLP-1 (Hs01031536_m1) genes (ThermoFisher Scientific, Waltham, MA) were used. Thermocycling conditions were: 1 cycle at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min before a dissociation stage. The relative mRNA expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method in reference to Gapdh2.

10.3.3.9 Molecular Docking

The protein sequence of GLUT2 (P12336, UniProtKB database) was used for homology modeling of GLUT2 and was carried out by using template threading alignments by I-TASSER server [17]. The template structure used by I-TASSER for the modeling of GLUT2 was the crystal structure of maltose-bound human GLUT3 (PDB ID: 4ZWB), C- score= -0.01, estimated TM-score = 0.71±0.11, estimated RMSD = 7.4±4.3 Å. This model was used for loop refinement and energy minimization in the Discovery Studio 3.0 (Accelrys Software). The energy minimization was carried out in the Smart Minimizer algorithm by applying CHARMm force field. Finally, the model was validated by analyzing stereochemical quality using a Ramachandran plot [18]. The modeled protein structure of GLUT2 was prepared using protein preparation wizard of the Maestro 9.1 software (Schrödinger Software Suite 2010). Docking calculations of peptides, originally sequenced by HPLC-MSMS from bean protein fractions, and

GLUT2 and SGLT1, were carried out using DockingServer [20]. The MMFF94 force field [21] was used for energy minimization of peptides. Gasteiger partial charges were added to the peptide ligand atoms. Non-polar hydrogen atoms were merged and rotatable bonds defined. Docking calculations were carried out on modeled GLUT2 transporter and SGLT1 (3DH4) protein crystal structure. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools [22]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 250,000 energy evaluations. The population size was set up to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of five were applied.

10.3.4 Animal care and diet

Four weeks old male Wistar rats weighing 250 g were purchased from Harlan Laboratories in Mexico, City; animals were housed in plastic cages at 22°C with a 12 h light/12 h dark cycle. The animals had *ad libitum* food and water access, standard diet (protein 19.1%, fat 5.8%, carbohydrate 44.3%, fiber 4.6%, ash 6.1%) provided by Teklad LM-485 (ENVIGO, Madison, WI). All experiments were approved by the Experimental Autonomous University of Mexico Animal Ethics Committee (OFICIO/FQ/CICUAL/098/15).

10.3.4.1 Oral glucose tolerance test

After 12 h fasting, animals were randomly divided into 3 groups: CO, control group, (n=10) rats treated with the vehicle (water); PF30 group, (n=4) rats treated with 30 mg of protein fractions (PF) per kg of body weight (BW), and PF50 group, (n=10) rats treated with 50 mg PF per kg of BW. Treatments were administered by oral gavage and tail blood samples were collected before treatment administration, and after oral gavage with a glucose load (3.5 g of glucose per kg of BW). Tail blood samples were collected to measure postprandial glucose after 30, 60, 90, 120 and 150 min of treatments. Briefly, the tail was lanced with a sterile scalpel blade and blood was measured directly onto glucose testing strips with a handheld glucometer (FreeStyle Freedom, Abbott Laboratories, Abbott Park, IL).

10.3.4.2 Hyperglycemic rat model, treatments and plasma analyses

Animals were randomized into five groups (Figure 32). Hyperglycemia was induced in seven of the groups by two intraperitoneal injections of streptozotocin (STZ) after 12 h fasting: a 30 mg/kg BW (day 1) and 15 mg/kg BW (day 4) of diluted STZ in 1 mL of 0.1 mol/L citrate buffer, pH 4.5; and four healthy control groups were tested as follows: **1**) healthy control group (HC) non-hyperglycemic and no-treat (n = 3); **2,3,4**) healthy control group, non-hyperglycemic, receiving 50, 75 or 100 mg of PF per kg BW twice daily (HP100, HP150 and HP200, respectively) (n = 3,2,3) (100, 150 and 200 mg PF/day total); **5**) Streptozotocin group with no treatment (STZ) (n = 5); **6**) Streptozotocin and 2 U insulin/ kg BW twice daily (long-acting) (SIN) (n=5) (4 U insulin/ kg BW day total); **7**) Streptozotocin and 500 mg metformin/ kg BW (SME) (n=7); **8**) Streptozotocin and 2.5 mg glibenclamide/ kg BW (SGI) (n=6); **9,10,11**) Streptozotocin and 50, 75 or 100 mg PF/kg BW twice daily (SP100, SP150, SP200, respectively) (n=5,6,6) (100, 150 or 200 mg PF day total). Treatments were administered at 8:00 am and 5:00 pm daily. Tail blood glucose was measured every morning during the experiment. After 10 days of treatments and after 12 h of fasting the animals were euthanized by decapitation. Blood was collected in tubes with serum separation gel (Becton, Dickinson and Company Franklin Lakes, NJ) and stored frozen at -80°C. Immediately following euthanasia, tissues were collected and weighed including pancreas, liver, kidneys and heart and stored frozen at -80°C for further analysis.

Plasma analysis of liver function was screened for enzymes alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein and serum albumin. A blood chemistry panel was performed at the Molecular Endocrinology Laboratory of Hospital Juárez México City by commercially available and standardized methods according to manufacturer instructions. Determinations of glucose, triglycerides, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were performed by commercially available standardized methods according to manufacturer instructions. Analytical quality determinations were monitored by utilizing of an internal quality control system and the participation of an external quality assurance program [19]. Insulin levels were determined by electrochemiluminescence using an Elecsys 2010 equipment (Roche Hitachi ELECSYS 2010) Immunoassay analyzer, Hoffmann La-Roche, Ltd.). Analysis of kidney function; creatinine, blood urea nitrogen (BUN), and uric acid were determined in the Molecular

Endocrinology Laboratory of Hospital Juárez México, City by commercially available standardized methods according to manufacturer instructions.

10.3.5 Statistical analysis

The experiments were performed with at least three independent replicates. Data are expressed as the mean ± standard deviation. Normality of data was tested using Shapiro-Wilk W. test and non-normal data was transformed to a new scale. Kruskal-Wallis test, and Dunn's comparisons were performed for non-parametric data. Statistical analyzes on normal data were conducted using one-way ANOVA and comparisons between groups were performed by Tukey test using JMP 8.0. (SAS Inst. Inc., Cary, NC). Differences were considered significant at $p < 0.05$. Correlations among parameters measured were performed using the GraphPad Prism software (Version 5.02; GraphPad Software, Inc.; San Diego, CA).

10.4 Results and discussion

10.4.1 Black bean protein fractions with anti-diabetes *potential*

Protein fractions (PF) were characterized and thirty-three peptides were sequenced (Table 21). Peptides were classified by molecular mass ranges starting from 400 Da to 1400 Da. The 501 to 600 Da range presented the highest amount of peptides (12). Table 21 also shows the chemical structures and characteristics of the four peptides with the highest *in silico* anti-diabetes potential.

10.4.2 In vitro experiments

10.4.2.1 Cell monolayers glucose absorption and glucose uptake

Figure 33A and B presents the effect of pure peptides and bean PF on glucose absorption using the Caco-2 monolayer insert system. After 30 min of glucose (20 mM) stimulus, pure peptide FEELN (100 μM), PF (10 mg/mL) and PHL (100 μM), showed a significant decrease in glucose absorption compared to the stimulated untreated control, 5.1, 6.5, and 10.2% respectively. On the other hand, after 24 h of glucose stimulus, all treatments significantly decreased glucose absorption ranging from 8.5% for peptide AKSPLF (100 μM) to 21.5% for PF (10 mg/mL). Glucose uptake was also measured in Caco-2 cells at different times using fluorescently labeled glucose and same treatments indicated above (Figure 33C). At 10 min of exposure, the inhibition of glucose uptake ranged from 3.8% for the PF (10 mg/mL) to 27.5% for AKSPLF (100 μM). After 30 min of exposure, the inhibition of glucose uptake ranged from 16.1% for AKSPLF (100 μM) to 64.1% for LSVSVL (100 μM), however, peptide ATNPLF

(100 µM) was not able to inhibit the uptake of glucose. After 60 and 180 min of exposure to the fluorescent glucose, peptide FEELN (100 µM) seems to stimulate glucose uptake. After 60 min peptide LSVSVL (100 µM), was the most potent inhibitor of glucose uptake up to 61.8%. PF (10 mg/mL) showed important potential to inhibit glucose uptake up to 48.6% after 180 min; this value did not have a statistical difference ($p > 0.05$) with the control PHL (100 µM).

10.4.2.2 Protein expression by Western blot and cytoplasm localization of GLUT2 and SGLT1 by confocal laser scanning microscopy in Caco-2 cells

PF and pure peptides and originally found in black bean (AKSPLF, ATNPLF, FEELN and LSVSVL), were able to decrease the expression of GLUT2 and SGLT1 glucose transporters (Figure 34A and B). In the case of GLUT2 after 30 min of treatment, the PF (10 and 5 mg/mL, and the peptide ATNPLF (100 µM) significantly decreased protein expression (28.2, 29.8 and 37.3%, respectively) compared to the negative control (untreated). Moreover, the positive control PHL decreased 46.2% the protein expression of GLUT2. After 24 h of treatment, the expression of GLUT2 decreased significantly (ranging from 21.7 to 42.8%) for all treatments except for the PF at 5 mg/mL. On the other hand, SGLT1 expression decreased significantly compared to the negative control after 30 min of treatment with AKSPLF, FEELN, PF (10 mg/mL) and ATNPLF (range from 22.0 to 42.0%) except LSKSVL, and protein fraction at 5 mg/mL. After 24 h, all treatments were capable to significantly ($p < 0.05$) decrease SGLT1 protein expression from 32 to 64% compared to the untreated control.

Protein expression of markers related to the mechanism of glucose transporters membrane translocation after 30 min treatment showed that AMPKα1 decreased from 15.9% (PHL) to 59.8% (PF 10 mg/mL), except for peptides AKSPLF and ATNPLF. β-Tubulin protein expression decreased in all treatments after 30 min from 5.1% (ATNPLF) to 51.3% (LSVSVL). PLCβ2 decreased in PHL (17.7%), LSVSVL (12.8%), PF 5 mg/mL (13.1%) and PF 10 mg/mL (42.0%). PKCβII protein expression did not show decrease after 30 min treatments; however, after 24 h treatment protein expression showed a decrease in all treatments ranging from 1.8 to 25.5% for PF 5 mg/mL and PF 10 mg/mL, respectively; except for AKSPLF. β-Tubulin protein expression decreased in all treatments after 24 h treatment and ranged from 30.8 to 50.3% in FEELN and PF (5 mg/mL), respectively. PLCβ2 protein expression decreased after 24 h only in the treatments PHL (52.5%), AKSPLF (47.2%) and LSVSVL (7.3%) (Figure 34). According to these results and the previous reports in literature, the mechanism of action of the PF and pure

peptides may be potentially related to the energy sensing pathway related to AMPKα1 and depolarization of cytoskeleton microtubule.

Two-dimensional optical planes demonstrated the immunocytochemical localization of glucose transporters GLUT2 and SGLT1 in Caco-2 cell cytoplasm using confocal laser scanning microscopy (Figure 35A and B). For GLUT2 and SGLT1, after 30 min or 24 h, the proteins were clearly translocated to the cell membrane in the untreated sample (Figure 35A); in comparison to the PHL, pure peptides and PF treated cells where the expressions were lower in the cell membrane and were localized in specific areas within the cytoplasm. Quantification and comparison of GLUT2 and SGLT1 fluorescence intensity in the cytoplasm and membrane after 30 min and 24 h of treatments are shown in Figure 35C. For GLUT2, after 30 min the positive control, PHL, significantly decreased (77.8%) the translocation to the membrane, similar to the PF (77.7%). Pure peptides also significantly decreased the translocation to the membrane (70.0 to 86.6%). On the other hand, PHL, pure peptides (AKSPLF, ATNPLF, FEELN, and LSVSVL) and PF significantly decreased the expression in the cytoplasm (55.5, 27.8, 29.6, 46.5, 63.8, and 52.4%, respectively). After 24 h treatment, the translocation of GLUT2 to the membrane was significantly decreased by all treatments (71.4 to 85.2%). Moreover, the expression in the cytoplasm was significantly decreased by all treatments (34.5 to 43.3%) except by AKSPLF (100 μM). In Figure 35B, the localization and expression of GLUT2 and SGLT1in the cells after 24 h treatment are shown. Similar to the 30 min treatment, the glucose transporters were more expressed and translocated to the membrane in the untreated cells, compared to PHL, pure peptides and protein fraction. SGLT1 was highly expressed in the cell membrane of the untreated control and the treatments significantly decreased the membrane expression (81.4 to 91.5%) after 30 min treatment. In the same way but to a lesser extent, the treatments also significantly reduced the cytoplasm expression of SGLT1 after 30 min (41.8 to 70.4%). Additionally, the treatments after 24 h significantly decreased SGLT1 protein expression in the membrane in all treatments (range 60.8 to 81.4%). Conversely, the reduction in the expression in the cytoplasm for SGLT1 was only significant for PHL (46.7%), ATNPLF (45.2%), and FEELN (42.3%). Three-dimensional optical planes demonstrated the immunocytochemical localization of glucose transporters GLUT2 and SGLT1 in Caco-2 cells in a transversal view (Figure 35D). Untreated cells showed high apical localization of GLUT2 and SGLT1, compared to positive control PHL

and PF where protein expression of glucose transporters decreased and the localization of these proteins was distributed in the cytoplasm.

10.4.2.3 GLP-1 concentration and DPP-IV activity in Caco-2 cell media

Results of GLP-1 measured in cell media after 30 min and 24 h treatments are shown in Figure 36A and B. At 30 min, peptides ATNPLF, AKSPLF, LSVSVL and PF treatments showed a significant reduction in the concentration of GLP-1 (89.9, 89.6, 83.3 pg/mL, 71.1 pg/mL, respectively) compared to untreated control (113.3 pg/mL). Similarly, after 24 h, treatments with either peptides ATNPLF, LSVSVL or PF showed a significant reduction in the concentration of GLP-1 (75.5 pg/mL, 67.1 pg/mL, 63.8 pg/mL, respectively) compared to untreated control (104.1 pg/mL). Contrarily, peptide FEELN showed an increase in concentration after 30 min treatment (117.4 pg/mL) and not a significant reduction after 24 h treatment (89.4 pg/mL). After 30 min treatment, DPP-IV was inhibited significantly ($p < 0.05$) by PHL (100 μM), LSVSVL (100 μM) and PF 10 mg/mL (Figure 36C); however, PHL (100 μM) was the treatments with the highest inhibition, 83.1%, followed by PF 10 mg/mL (51.24%). Peptide LSVSVL was the only one of pure peptide able to significantly inhibit DPP-IV (31.7%). Similarly, after 24 h treatment, PHL (100 μM) and PF (10 mg/mL) showed DPP-IV inhibition of 71.4 and 29.8%, respectively.

10.4.2.4 Reactive oxygen species scavenger activity

PF decreased the intracellular oxygen reactive species in a dose dependent-manner (Figure 37). At 10 mg/mL, PF inhibited 70.9% ROS activity, which was not statistically different ($p > 0.05$) to the positive control PHL (71.7% ROS inhibition). On the other hand, pure peptides showed lower potential to inhibit ROS at 100 μM (7.3 to 13.3% ROS inhibition).

10.4.2.5 Gene expression

Relative gene expression of GLUT2, SGLT1, GLP-1 and DPP-IV decreased less than 0.5 fold change in treatments with peptides AKSPLF, LSVSVL and PF 10 mg/mL after 30 min treatment. However, after 24 h the relative expression was clearly downregulated up to 2 fold change relative to the untreated control for all genes. The peptide ATNPLF only affected the expression of GLP-1 after 24 treatments (Table 22).

10.4.2.6 Molecular docking

Binding free energy, predicted inhibition constant and type of interaction of the peptides with GLUT2 and SGLT1 are shown in Table 23. GLUT2 interaction with the peptides FEELN and LSKSVL showed lower free energy (-7.91 and -5.24 kcal/mol, respectively) than the

positive control, PHL (-4.35 kcal/mol). The interaction of the peptides with GLUT2 was primarily by hydrophobic intercalations, followed by polar interactions in the protein motif. The peptide FEELN showed the lowest inhibition constant (1.52 μM) for GLUT2. On the other hand, peptides presented better binding to SGLT1; the free energy for AKSPLF (-8.66 kcal/mol), ATNPLF (-7.20 kcal/mol), and FEELN (-6.44 kcal/mol), these values were lower than the PHL (-4.44 kcal/mol). In this case the interactions were primarily polar followed by hydrophobic interactions. The peptide AKSPLF showed the lowest inhibition constant (Ki) for SGLT1 (0.452 μM). Representative illustration of peptide AKSPLF interacting with the catalytic cavity of glucose transporters GLUT2 and SGLT1are shown in Figure 38A and B. Peptides were able to bind to glucose transporters due to hydrophobic, polar, cation pi, and pipi interaction between protein motif and amino acids in the peptide.

10.4.3 In vivo experiments

10.4.3.1 Postprandial glucose decreased after a load of protein fraction in an oral glucose tolerance test

Figure 39A presents the hypoglycemic potential of black bean PF using an oral glucose tolerance test (OGTT). The PF30 and PF50 groups showed lower postprandial glucose after 90 min of treatment; however only the PF50 showed significant decrease in postprandial glucose compared to control. Both treatments reduced the area under the curve (AUC) of postprandial glucose by 16.9 and 24.5% at 30 and 50 mg PF/kg BW, respectively.

10.4.3.2 Hypoglycemic potential of common bean protein fraction in a hyperglycemic animal model

The hypoglycemic potential of PF was performed over 10 days (Figure 39B). The SGI, STZ, SME groups showed the higher glucose levels compared to other groups. A total dosage from 100 to 200 mg of PF/ kg BW/day on hyperglycemic animals (SP100, SP150 and SP200) reduced postprandial glucose levels from 22.7 to 47.7% compared to the non-treatment group (STZ). SIN group reduced postprandial glucose levels by 49.5%. Contrary, hypoglycemic drugs metformin and glibenclamide did not showed good performance in decreasing hyperglycemia. On the other hand, the healthy control group HC and healthy peptide control groups HP100, HP 150 and HP200 having standard diet did not show statistical difference among them on postprandial glucose levels ($p > 0.05$). Table 24 shows animal and organ weights, hyperglycemic groups SGI, SME, STZ and SP100 presented body weight loss at the end of the experiment.

However, hyperglycemic groups with PF dose higher 150 mg/kg BW per day presented the highest weight gain. On the other hand, in hyperglycemic group receiving glibenclamide liver weight was significantly higher (40 mg/kg BW) compared to the HC (29 mg/kg BW); the rest of the groups showed no significant difference in liver weight compared to HC. Regarding the pancreas, only the SGI group showed a significant increase in organ weight, 8.4 mg/kg BW respectively, compared to the HC 5.7 mg/kg BW. Moreover, SME group showed a significant increase in kidney weight. Table 25 presents the results of the serum biochemical markers after 10 days of treatment; the lowest fasting glucose was shown by SP75 group (200 mg/dL), with no significant differences between the INS group (251 mg/dL) ($p > 0.05$). Furthermore, the SP100, SP150 and SP200 fasting glucose was not significant different with HC and INS groups. The healthy groups did not show statistical differences in fasting glucose levels 123 mg/dL (HP100), 125 mg/dL (HP150), 117 mg/dL (HP200) and 126 mg/dL (HC). Blood urea nitrogen (BUN), creatinine and uric acid, cholesterol, triglycerides, HDL, LDL, sera proteins, albumin and globulins, AST and ALT concentrations did not show significant differences on the treatments when comparing to HC group ($p > 0.05$). Liver enzymes ALP was significantly higher in SGI group (446 U/L) than HC (159 U/L), the groups receiving PF were not different. For LDH the group with the lowest concentration was the STZ (231 U/L) compared to HC (992 U/L), besides there was not difference with other groups. All hyperglycemic groups showed significant lower insulin concentrations (0.5 to 0.9 ng/mL) compared to the HC (2.96 ng/mL/) except the INS group 2.1 ng/mL. GLP-1 ranged from 0.5 pcm/L in HP100 to 2.6 pcm/L in SP150. All the hyperglycemic groups presented higher concentration of GLP-1 than the HC.

10.4.3.3 Discussion

Common beans contain several bioactive components that have been evaluated using *in vivo* and clinical trials. These compounds seem to play a role in managing diseases such as diabetes [14]. Common bean PF contains peptides ranging from 4 to 7 amino acids with molecular masses between 400 and 600 Da. The role of common bean protein fractions on diabetes markers had been reported using only in *in silico*, biochemical and *in vitro* assays [20,21].

From *in silico* assays the peptides AKSPLF, ATNPLF, FEELN and LSKSVL showed high potential to inhibit enzymes related to diabetes treatment (DPP-IV, α-amylase and α-glucosidase) by interacting with their catalytic site [15]. Besides, bigger peptides can also be

absorbed by enterocytes thought paracellular transport and endocytosis, once they reach circulation and ant the target organ can exert different biological functions [22].

In the present study, PF was capable of significantly reduce glucose absorption and uptake. Results suggested that the principal mechanisms were the blockage of glucose transporters GLUT2 and SGLT1 and the inhibition of the translocation of GLUT2 and SGLT1 from the cytoplasm to the apical membrane. Kwon et al. [23] found a reduction of glucose absorption in Caco-2 cells by blockage of GLUT2 by quercetin and phloretin. Similarly, Johnston et al. [24] reported reduction in glucose uptake by the use of flavonoid glycosides and non-glycosylated polyphenols in sodium-dependent conditions and also a reduction in glucose uptake by aglycones and non-glycosylated polyphenols under sodium free conditions. In another study, Alzaid et al. [25] reported a decrease in sodium-dependent and independent glucose uptake in Caco-2 cells. They also found a reduction in the expression of GLUT2 mRNA and protein expression. Besides, Vernaleken et al. [5] reported a decrease in 30% of glucose absorption using a tripeptide Gln-Ser-Pro, by downregulation of SGLT1 gene. Expression and translocation of GLUT2 and SGLT1 to the apical membrane is mediated by intact cytoskeleton and activated PKC; Also, luminal glucose can also stimulate apical sweet taste receptors which activate PLCβ2 which therefore activate PKC protein downstream [26,27]. It has been found that compounds such as cytochalasin B, alphostin C and chelerythine can block the expression of the signaling pathway and inhibit the translocation of glucose transporters to the apical membrane from the cytoplasm [28]. Apical GLUT2 is a target of an energy sensing mechanism in which low energy detecting activates AMP protein kinase (AMPK) and as a consequence the insertion of the glucose transporter to the apical membrane [28].

From the western blot protein expression results, proteins such as β-tubulin and AMPKα were significantly down-regulated after 30 min and 24 h. Protein expression of PCKβ only showed a significant reduction after 24 h treatment with PF 10 mg/mL. PLCβ2 only showed a significant decrease in protein expression by PF 10 mg/mL after 30 min and AKSPLF after 24 h treatment. These results suggest that the potential mechanism of action of PF and pure peptides could be related to the depolarization of the cytoskeleton structure and the energy sensing pathway; both mechanisms are related to the translocation of glucose transporters from the cytoplasm to the apical membrane in enterocytes. Common bean PF and pure peptides had similar mechanisms to inhibit the expression and translocation of GLUT2 and SGLT1 to the

membrane. Using computational modeling, we evaluated the potential of the peptides AKSPLF, ATNPLF, FEELN and LSKSVL to block glucose transporters GLUT2 and SGLT1 by interacting with their protein motifs. Principal interactions were hydrophobic, followed by polar and hydrogen bonding. Pure peptides showed lower or similar free energy to interact with amino acids on GLUT2 and SGLT1 to the phenolic compound phloretin. Other *in silico* studies have shown the potential of bioactive compounds, such as bisphenol and quercetin to interact and block the protein motif of GLUT2 and SGLT1 [29-31].

GLP-1 concentration only showed differences among treatments for LSVSVL and PF after 30 min, and ATNPLF, LSVSVL and PF after 24 h treatments. No correlation was found between active GLP-1 concentration and DPP-IV activity after 30 min and 24 h. This could be due to incretins release is a process dependent on the glucose that was internalized in the cells. Incretin GLP-1 is secreted in the gut as a response to glucose absorption; glucose stimulates incretin secretion by depolarization of membrane caused by uptake of Na ions (SGLT1) and by membrane depolarization caused by the intracellular metabolism to ATP and closure of ATP sensitive K channels. Both pathways stimulates secretion by activation voltage-gated Ca channels stimulating uptake of extracellular Ca and mobilization from intracellular stores; causing collectively the activation of the exocytotic machinery and GLP-1 secretion [32]. Thus, lower glucose absorption may be linked lower GLP-1 release. Results showed that common bean PF anti-diabetes action may be associated with its effect at the gastrointestinal-glucose transporter level. Common bean PF showed an outstanding antioxidant potential, similar to the results observed by Torres-Fuentes et al. [33] in other legumes, who found that protein fractions from chickpea exerted antioxidant potential by different modes of action such as donating electrons and hydrogen and scavenging peroxyl-like radicals. PF was able to decrease postprandial glucose in the OGTT after 150 min showing a dose response. This reduction could be due to the blockage, inhibition of protein expression and translocation of glucose transporters GLUT2 and SGLT1, as no significant changes in insulin secretion were observed on healthy rats receiving 50 mg PF/Kg BW compared to control. Other studies have reported the hypoglycemic effect of food-derived components, such as phenolic compounds and alkyl amides [34-36]. However, no studies of common bean protein hydrolysates have been reported to decrease glucose absorption. Similarly, common bean PF lowered postprandial glucose levels in a dose-response form in the hyperglycemic animal model when compared to a non-treated

hyperglycemic control group. Furthermore, fasting glucose levels of rats after they were euthanized were lower in hyperglycemic groups receiving the PF compared to hyperglycemic groups having metformin (SME), glibenclamide (SGI), and without treatment (STZ). Several anti-diabetes mechanisms had been suggested for plant bioactive components on hyperglycemic STZ-induced rat model. For instance, phenolic acids such as ursolic acid improved pancreatic β-cell function [37]; water-soluble polysaccharides from *Opuntia dillenii* H. decreased oxidative stress and improved pancreatic islet tissue integrity [38]; phenolic compounds such as tyrosol ameliorated hyperglycemia by regulating key enzymes related to carbohydrate metabolism and exerted significant antioxidant effect [36]. *Talinum triangulare* polysaccharides reduced fasting blood glucose in STZ male mice [39]. On the other hand, common bean aqueous extract, formed primarily by phenolic compounds, had a positive effect on glucose levels and lipid metabolism parameters, and anti-inflammatory markers in STZ diabetic rats [40]. Similarly, Hernandez-Saavedra et al. [41] observed a 20% reduction in blood glucose levels compared to the control of a supplemented diet of 25% cooked common beans by maintaining the β-cell mass. PF did not show a negative effect on lipid metabolism, liver and kidney function. Besides, that hyperglycemic groups receiving PF and insulin presented lower glucose levels and maintain body weight compared to hyperglycemic receiving metformin, glibenclamide or no treatment. The insulin concentration in hyperglycemic groups was lower on all hyperglycemic groups except insulin group. Moverover, fasting glucose levels were lower on hyperglycemic groups receiving PF, indicating that their action takes place at gastrointestinal level.

In vitro and *in vivo* results suggest that PF and its pure peptides exert a hypoglycemic effect at GI level, potentially through inhibition of starch degrading enzymes, blocking glucose transporters and reducing of protein expression and translocation of GLUT2 and SGLT1 glucose transporters to the apical membrane. This may be related to the energy sensing pathway in enterocytes and the depolarization of microtubule in the cytoplasm. Moreover, the mechanism of action of peptides may be also linked to the decrease mRNA expression of glucose transporters in the long term.

10.5 Conclusions

Black common bean PF and its pure peptides presented significant hypoglycemic potential mainly by blocking the glucose transporters and decreasing the expression and translocation to the apical membrane of GLUT2 and SGLT1. Furthermore, protein fractions

showed outstanding antioxidant potential that can contribute to the decrease of ROS and protect tissues of oxidative stress. To the best of our knowledge, this is the first report showing the anti-diabetes potential of black common bean protein fractions and pure peptides. Black bean protein fractions are an inexpensive alternative food source of bioactive compounds to be used in the management of glucose levels in diabetic patients with the advantage of no known side effects.

10.6 References

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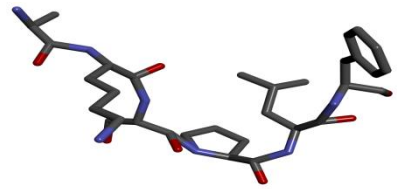
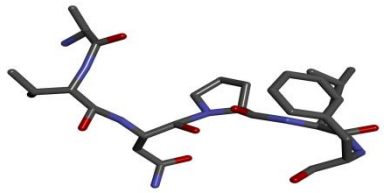
10.7 Tables and figures

Table 21. Peptide profile from black bean protein fraction, structure and physicochemical properties of four peptides with the highest anti-diabetes potential.

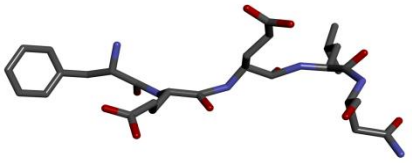
MW range	Peptide sequence
400-500	LLPK, QTPF
501-600	FFQS, GSLGGH, GSRAH, YVFLS, LALVL, WEVM
601-700	FEELN, LKEGGK, LSKSVL, LYELN, ATNPLF, AKSPLF, SGPFGPK, GSPVSSR, TTNPLF
701-900	TTGGKGGK, ASATTGVL, SKGSGGGKL
901-1000	SAKGPPMGAK, SAKGPPTSAK, SARVLAAGAK, SANRLPSAGS, RKLKMRQ
1001-1100	SLKWWDLGS
1101-1200	SLPAGGNRYGK
1201-1300	SRSPAGPPPTEK, ALMLEEYLLE, QQRRRLRLK, EGLELLLLLLAG, DLALLLLLAELG
1301-1400	LPPSPERTAAPPF



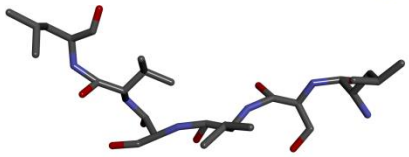
Sequence:	**AKSPLF**
Length:	6
Mass:	661.3787
Isoelectric point (pI):	9.93
Net charge:	+1
Hydrophobicity:	+8.84 Kcal * mol $^{-1}$



Sequence:	**ATNPLF**
Length:	6
Mass:	661.3424
Isoelectric point (pI):	5.53
Net charge:	0
Hydrophobicity:	+6.68 Kcal * mol $^{-1}$



Sequence:	**FEELN**
Length:	5
Mass:	650.2901
Isoelectric point (pI):	2.97
Net charge:	-2
Hydrophobicity:	+13.05 Kcal * mol $^{-1}$



Sequence:	**LSKSVL**
Length:	6
Mass:	645.4048
Isoelectric point (pI):	10.14
Net charge:	+1
Hydrophobicity:	+8.66 Kcal * mol $^{-1}$

Peptides obtained from the LC-ESI-MSMS elution profile with intensity of at least 70%; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

Table 22. mRNA relative expression and function

Gene symbol	Gene name	Cell action	Time	Fold-change relative to untreated control					
				PHL 100 µM*	AKSPL F 100 µM*	ATNPL F100 µM*	FEELN 100 µM*	LSVSV L100 µM*	P FRA 10 mg/mL *
SLC2A2	Solute carrier family 2 member 2	The encoded protein mediates facilitated bidirectional glucose transport.	30 min	1.79±0.08	0.19±0.09	2.33±0.11	0.81±0.05	0.49±0.07	0.39±0.17
			24 h	1.16±0.06	-0.62±0.04	0.56±0.10	-0.51±0.09	-0.56±0.05	-1.20±0.51
SLC5A1	Solute carrier family 5 member 1	The encoded integral membrane protein is the primary mediator of dietary glucose and galactose uptake from the intestinal lumen.	30 min	1.32±0.06	0.15±0.04	2.17±0.24	0.76±0.09	0.38±0.04	0.26±0.13
			24 h	1.05±0.12	-0.9±0.13	0.13±0.13	-0.32±0.20	-0.44±0.11	-0.78±0.13
GCG	Glucagon	The proteins encoded by this gene are peptides secreted from gut endocrine cells and promote nutrient absorption through distinct mechanisms	30 min	1.33±0.15	-0.4±0.05	2.54±0.35	0.80±0.07	0.49±0.19	-0.22±0.11
			24 h	1.39±0.31	-1.7±0.37	-0.70±0.52	-0.85±0.10	-2.39±0.38	-1.83±0.16
DPP4	Dipeptidyl peptidase 4	The protein encoded by this gene is a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides.	30 min	1.55±0.06	0.24±0.06	2.28±0.05	0.67±0.14	-0.14±0.11	0.30±0.02
			24 h	1.17±0.12	-0.07±0.02	0.67±0.05	0.15±0.04	-0.26±0.04	-0.8±0.10

*: Fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are expressed as the mean ± SD compared to untreated control.

Table 23. Molecular docking free energy, inhibition constant and amino acid interaction of peptides with glucose transporters GLUT2 and SGLT1.

Trans-porter	Peptide	Free energy	Ki (μM)	Hydrogen bonding [A°]	Hydrophobic [A°]	Polar [A°]	Cation pi [A°]	pi-pi [A°]
GLUT2	AKSPLF	-0.62	-	GLY29 [2.74] GLN312 [3.04] GLN313 [3.37] THR340 [2.55] GLU410 [2.68]	ILE28 [2.98] ILE28 [3.06] ILE28 [3.90] VAL30 [3.87] VAL30 [3.22] VAL30 [3.41] VAL30 [3.47] ILE31 [3.05] ILE31 [3.81] ILE317 [2.64] ILE317 [3.81] TRP442 [3.08]	GLN202 [3.42] GLN312 [3.40] GLN312 [2.50] GLN312 [3.85] GLN313 [3.53] GLN313 [2.69] ASN347 [3.19] ASN347 [2.98] ASN347 [2.11] ASN347 [2.96] ASN347 [3.64] GLU410 [2.14] GLU410 [2.43] GLU410 [3.04]	PHE409 [2.50] PHE409 [3.30] PHE409 [3.43]	TRP95 [3.78] PHE102 [3.34] PHE102 [2.76] PHE102 [3.45] TRP442 [3.41] TRP442 [3.34]
	ATNPLF	-3.3	3420	GLY29 [2.44] GLN312 [3.11] GLN313 [2.70] GLN313 [3.05]	ILE28 [2.90] ILE28 [3.64] ILE28 [3.63] ILE31 [3.65] PHE102 [3.82] PHE102 [2.85] ILE317 [3.34] ILE317 [2.95] ILE317 [3.54]	GLU36 [3.70] GLN312 [2.08] GLN313 [1.69]		TRP95 [3.29] TRP95 [3.42] PHE102 [3.56] PHE102 [3.18] TRP442 [3.17]
	FEELN	-7.9	1.5	TYR26 [2.71] GLY29 [2.54] GLN191 [2.93] GLN312 [3.17] GLN313 [3.21]	ILE28 [3.86] ILE28 [3.17] ILE28 [3.65] ILE28 [3.90] VAL30 [3.71] VAL30 [3.12] VAL30 [2.84] VAL30 [3.31] LEU194 [3.03] ILE198 [3.00] ILE198 [3.45] ILE198 [3.51] VAL343 [3.40]	SER167 [3.15] ASN347 [3.71] GLU410 [3.42]	TRP418 [3.04]	HIS309 [3.44] HIS309 [3.59] TRP442 [3.73]
	LSKSVL	-5.2	144.4	GLY29 [3.11]	VAL30 [3.14] VAL30 [3.29] VAL30 [3.82] VAL30 [3.53] VAL30 [3.45] VAL30 [3.31] ILE31 [3.01] VAL99 [3.76] PHE102 [3.10] PHE102 [3.30] PHE102 [3.35] PHE102 [3.28] ILE317 [3.17] ILE317 [3.46] ILE317 [3.43] ILE317 [3.68] ALA449 [3.16]	GLN313 [3.66] GLN313 [3.57] TRP442 [3.83]	TRP442 [2.18] TRP442 [3.64] TRP442 [3.39]	
	PHL	-4.3	648.6		VAL195 [3.20] VAL195 [3.70] ILE198 [3.58] ILE198 [3.59] ILE198 [3.72] LEU199 [3.72]	GLN312 [3.69] ASN347 [3.32] ASN347 [3.60] GLU410 [3.04] GLU410 [2.09]		
SGLT1	AKSPLF	-8.66	0.5	GLU108 [3.46] SER453 [2.96] ASP507 [2.51]	ILE107 [3.39] ILE107 [3.31] ILE503 [3.36] ILE503 [3.82] ILE503 [3.44] PRO508 [3.50]	GLU108 [3.30] GLU108 [2.61] SER453 [2.05] SER453 [3.69] SER453 [3.34] ASN50 [3.74] ASN504 [3.11] ASN504 [2.94] ASN504 [2.54] ASN504 [2.93] ASP507 [3.21] ASP507 [1.95] ASP507 [1.77] ASP507 [3.66]		
	ATNPLF	-7.2	5.3	GLU108 [2.58]	ILE503 [3.26] ILE503 [3.22] ILE503 [3.19] ILE503 [3.27] ILE503 [3.35] PRO508 [3.22]	GLU108 [1.89] GLU108 [2.81] GLU108 [2.58] GLU108 [3.42] ASP507 [1.96] ASP507 [3.21] ASP507 [2.93]		
	FEELN	-6.4	19.1	GLY110 [2.52] ASP507 [2.51]	GLU108 [3.86] ILE503 [3.85] ILE503 [3.63] ILE503 [3.18] PRO508 [3.10] PRO508 [3.47] PRO508 [3.81]	GLU108 [3.78] GLU108 [3.49] GLU108 [2.66] TYR112 [3.37] GLU116 [3.53] ASP507 [2.31] ASP507 [1.98] ASP507 [2.87] ASP507 [3.44]		TYR112 [3.36]
	LSKSVL	-4.4	588.2	TYR112 [2.52] THR501 [3.19] SER516 [3.48]	ILE503 [3.04] ILE503 [3.57] ILE503 [3.36] VAL519 [3.56] VAL519 [3.47]	TYR112 [2.11] TYR112 [2.06] TYR112 [3.24]	TYR112 [2.33] TYR112 [3.26]	
	PHL	-4.44	556.4		ILE107 [3.31] VAL280 [3.67] ILE503 [3.24] ILE503 [3.84] ILE503 [3.37] ILE503 [3.50] PRO508 [3.49]	GLU108 [3.11] GLU108 [2.27] THR501 [3.69] ASN504 [3.47] ASP507 [2.93] ASP507 [1.98]		

AKSPLF, ATNPLF, FEELN and LSKSVL peptides were selected based on outstanding inhibitory potential; free energy expressed as kcal mol-¹; K_i: inhibition constant expressed in μM; Å: interaction length in angstroms; aa: amino acids; amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine, W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine.

Table 24. Weight of the organs of hyperglycemic rats relative to the body weight (BW) after 10 days of treatment per group.

Treatments	ΔBody weight (g)	Liver (mg/kg BW)	Pancreas (mg/kg BW)	Heart (mg/kg BW)	Kidney (mg/kg BW)
HP200	61 ± 9^{a}	32 ± 5^{ab}	7.3 ± 0.8^{ab}	3.8 ± 0.3^{a}	2.8 ± 0.7^{a}
HP150	45 ± 4^{ab}	35 ± 3^{ab}	6.6 ± 0.4^{ab}	3.2 ± 0.2^{a}	3.5 ± 0.1^{ab}
HC	42 ± 14^{ab}	29 ± 4^{b}	5.7 ± 0.9^{b}	3.2 ± 0.3^{a}	2.3 ± 0.4^{a}
SP200	42 ± 11^{ab}	33 ± 4^{ab}	7.2 ± 0.5^{ab}	3.4 ± 0.6^{a}	3.2 ± 0.4^{a}
HP100	35 ± 3^{ab}	26 ± 2^{b}	5.6 ± 0.2^{b}	3.3 ± 0.6^{a}	2.9 ± 1.4^{a}
INS	34 ± 4^{ab}	30 ± 2^{b}	5.9 ± 0.3^{b}	3.1 ± 0.3^{a}	2.1 ± 0.3^{a}
SP150	22 ± 8^{bc}	30 ± 5^{b}	6.9 ± 0.7^{ab}	3.1 ± 0.5^{a}	3.1 ± 0.7^{a}
SP100	2 ± 16^{d}	34 ± 8^{ab}	7.2 ± 1.3^{ab}	3.3 ± 0.6^{a}	2.7 ± 0.7^{a}
SME	-13 ± 14^{d}	40 ± 3^{a}	8.2 ± 0.8^{a}	3.8 ± 0.4^{a}	4.8 ± 0.6^{b}
STZ	-15 ± 23^{d}	31 ± 3^{b}	7.2 ± 0.7^{ab}	3.2 ± 0.4^{a}	3.1 ± 1.1^{a}
SGI	-20 ± 12^{d}	36 ± 5^{ab}	8.4 ± 0.4^{a}	3.4 ± 0.5^{a}	3.3 ± 0.5^{a}

STZ: streptozotocin group with no treatment; SIN: STZ and 4 U insulin/ kg BW; SGI; STZ and 2.5 mg/kg glibenclamide; SME: STZ and 500 mg metformin /kg BW; SP100, SP150, SP200: STZ and 100, 150 or 200 mg PF/kg BW; HC: healthy control; HP100, HP150, HP200: healthy animals and 100, 150 or 200 mg PF/kg BW. Means with different letters were significantly different ($p < 0.05$) according to Tukey's test. ΔBody weight: difference in rats group weight gain/lost at the end of the experiment, negative values and small values are groups losing weight.

Table 25. Biochemical parameters of hyperglycemic rats measured in serum after 10 days of the respective treatment.

Parameter	SGI	SME	STZ	INS	SP200	SP100	SP150	HC	HP150
Glucose mg/dL	433±93^{a}	406±111^{ab}	384±142^{abc}	251±107^{bcd}	236±37^{cd}	208±42^{cd}	200±50^{d}	126±14^{d}	125±20^{d}
BUN mg/dL	22±4^{ab}	20±5^{b}	28±5^{a}	21±2^{ab}	21±3^{ab}	29±1^{a}	22±4^{ab}	21±3^{ab}	20±1^{ab}
Creatinine mg/dL	0.4±0.1^{a}	0.5±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}	0.4±0.1^{a}
Uric acid mg/dL	1.0±0.6^{a}	1.3±0.8^{a}	1.2±0.2^{a}	1.4±0.4^{a}	1.1±0.3^{a}	1.8±0.4^{a}	1.4±0.4^{a}	1.0±0.3^{a}	1.2±0.1^{a}
Cholesterol mg/dL	64±27^{ab}	61±10^{ab}	65±13^{ab}	43±6^{b}	69±7^{ab}	77±14^{a}	71±9^{ab}	74±27^{ab}	80±8^{ab}
Triglycerides mg/dL	80±49^{a}	141±80^{a}	96±55^{a}	103±36^{a}	73±36^{a}	76±26^{a}	65±27^{a}	110±38^{a}	108±47^{a}
HDL mg/dL	19±3^{ab}	21±3^{a}	23±4^{a}	14±1^{b}	22±4^{a}	23±4^{a}	20±3^{ab}	21±2^{ab}	26±6^{a}
LDL mg/dL	5±3^{ab}	2±1^{ab}	4.4±1^{ab}	2±0^{b}	5±1^{ab}	5±1^{ab}	6±3^{a}	4±1^{ab}	4±1^{ab}
Proteins mg/dL	5±0.5^{b}	5±0.4^{ab}	5±0.4^{ab}	5±0.5^{ab}	5±0.2^{ab}	6±0.3^{a}	5±0.4^{ab}	6±0.1^{ab}	5±0.0^{ab}
Albumins mg/dL	3±0.4^{b}	4±0.3^{ab}	3±0.2^{b}	4±0.3^{ab}	4±0.1^{ab}	4±0.2^{ab}	4±0.2^{ab}	4±0.1^{ab}	4±0.1^{ab}
Globulins mg/dL	2±0.2^{ab}	2±0.1^{ab}	2±0.2^{ab}	2±0.2^{ab}	2±0.1^{ab}	2±0.1^{ab}	2±0.1^{ab}	3±0.1^{a}	2±0.1^{ab}
AST U/L	158±90^{a}	177±136^{a}	117±59^{a}	136±68^{7a}	113±30^{a}	151±58^{a}	157±89^{a}	90±44^{a}	225±163^{a}
ALT U/L	61±29^{a}	71±47^{a}	77±40^{a}	52±14^{a}	62±17^{a}	65±17^{a}	70±43^{a}	66±19^{a}	88±27^{a}
ALP U/L	446±82^{a}	317±168^{ab}	366±194^{ab}	148±31^{b}	269±72^{ab}	141±33^{b}	221±120^{b}	159±44^{b}	266±55^{ab}
LDH U/L	705.±318^{ab}	932±472^{ab}	231±168^{b}	638±248^{ab}	523±235^{ab}	984±490^{ab}	878±433^{ab}	992±181^{ab}	1084±273^{ab}
Insulin ng/ml	0.5±0.1^{c}	0.7±0.3^{bc}	0.7±0.6^{bc}	2.1±1.2^{abc}	0.5±0.1^{c}	0.9±0.4^{bc}	0.5±0.2^{bc}	3.0±1.6^{a}	2.9±0.5^{ab}
GLP-1 pcm/L	2.2±0.2^{ab}	2.2±0.3^{ab}	1.4±0.9^{bcd}	1.3±0.9^{bcd}	2.0±0.3^{abc}	0.8±0.3^{d}	2.6±0.9^{a}	0.7±0.7^{cd}	2.2±0.2^{abcd}

STZ: streptozotocin group with no treatment; SIN: STZ and 4 U insulin/ kg BW; SGI; STZ and 2.5 mg/kg glibenclamide; SME: STZ and 500 mg metformin /kg BW; SP100, SP150, SP200: STZ and 100, 150 or 200 mg PF/kg BW; HC: healthy control; HP100, HP150, HP200: healthy animals and 100, 150 or 200 mg PF/kg BW. Means with different letters were significantly different ($p < 0.05$) according to Tukey's test.

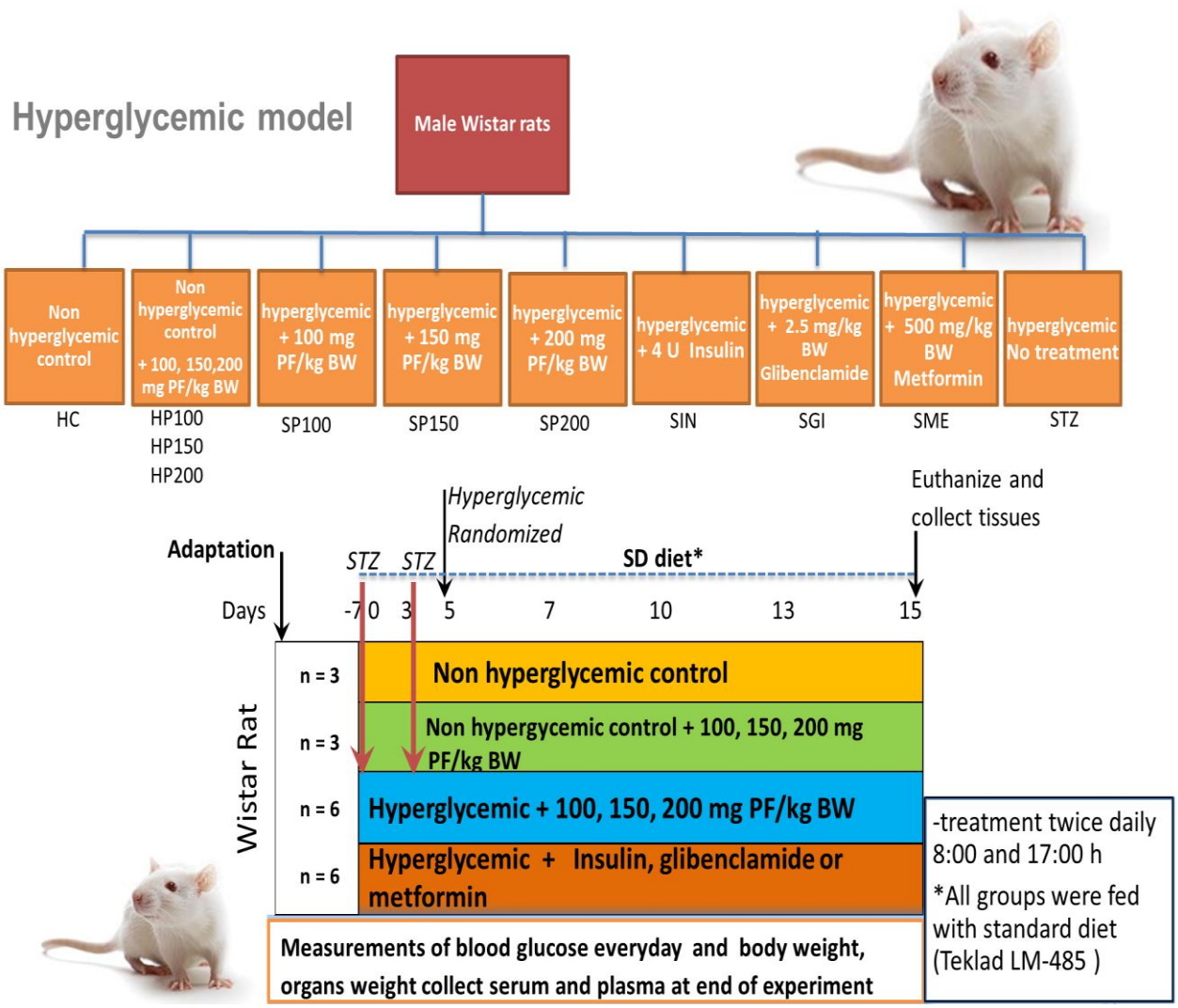


Figure 32. Hyperglycemic animal model experimental design.

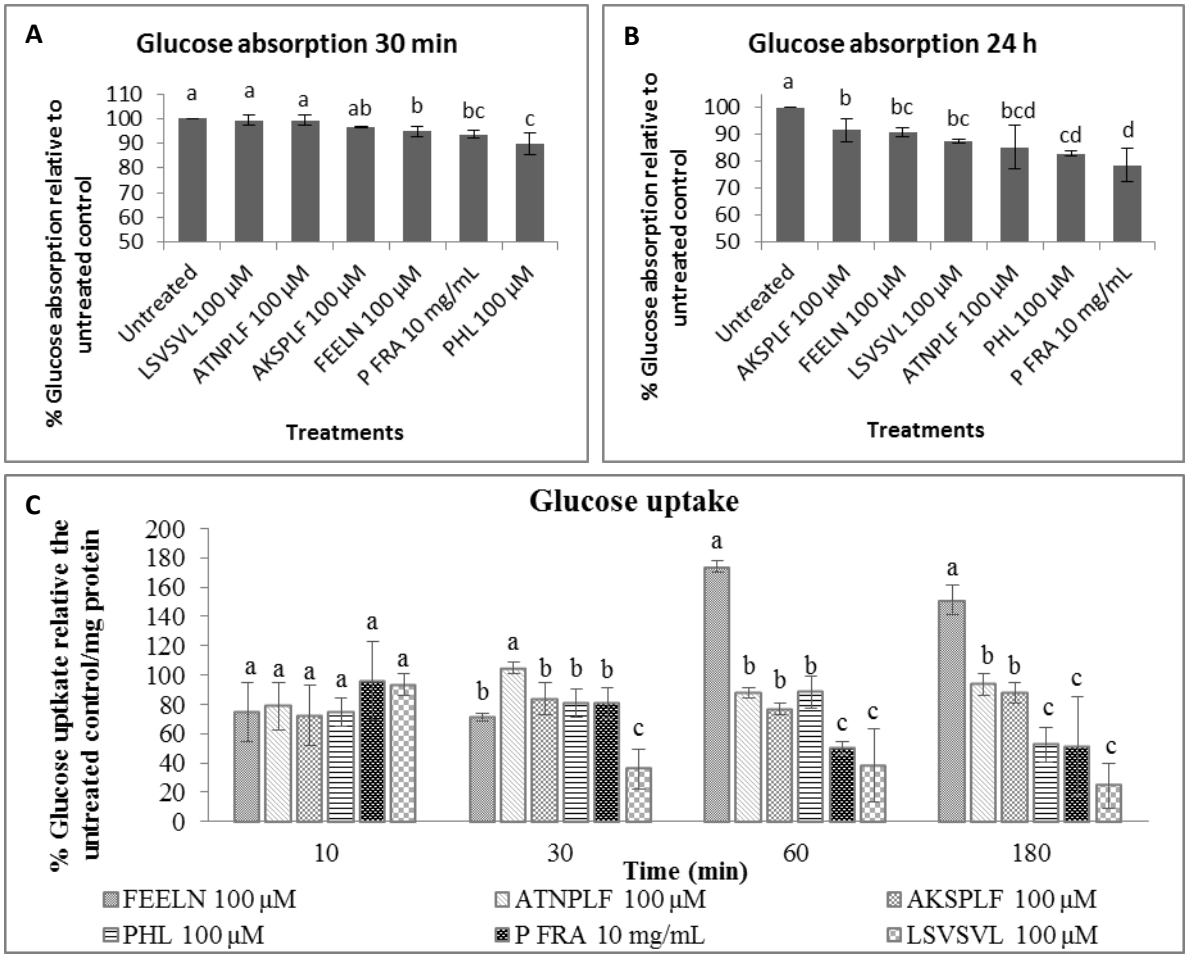
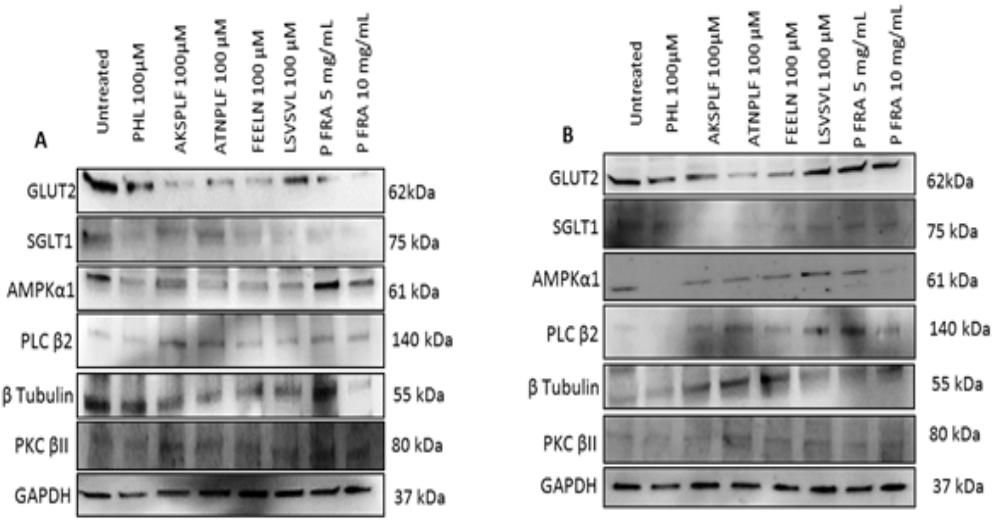


Figure 33. Percentage of glucose absorption in Caco-2 cells A) Glucose absorption after 30 min glucose stimulation in monolayer inserts. B) Glucose absorption after 24 h glucose stimulation in monolayer inserts. C) Glucose uptake kinetic from 10 min to 180 min using fluorescent glucose DCFDA.



			Percentage of protein expression relative to the untreated control						
Protein	Metabolic function	Time	PHL 100 µM	AKSPLF 100 µM	ATNPLF 100µM	FEELN 100 µM	LSKSVL 100 µM	P FRA 5 mg/mL	P FRA10 mg/mL
GLUT2	High capacity, low affinity transmembrane carrier protein that enables protein facilitated glucose movement across cell membranes .	30 min	53.8±1c	87.8±9b	62.7±14bc	89.6±3b	116.7±19a	70.1±1bc	71.8±2bc
		24 h	65.8±24ab	72.5±4ab	62.7±5ab	57.1±15b	73.1±11ab	92.1±5a	78.2±10ab
SGLT1	Sodium-glucose cotransporter 1 (SGLT1) is a secondary active symporter located in the apical membrane of the enterocyte.	30 min	69.9±14cd	78.0±4c	57.9±2d	75.8±12c	95.2±2b	124.4±2a	74.8±11c
		24 h	68.1±10a	39.0±9bc	35.6±10c	46.0±7bc	50.1±2b	67.6±11a	49.4±5bc
AMPKα1	AMP-activated protein kinase (AMPK) plays a key role as a master regulator of cellular energy homeostasis	30 min	84.0±9bc	101.4±4b	138.6±2a	67.8±17cd	75.2±15bc	73.8±18bc	40.2±6d
		24 h	72.1±5abcd	66.9±4cd	95.1±9ab	98.6±14a	53.5±14d	68.0±30bcd	85.5±3abc
PLC β2	Sweet taste receptor initiates β2 isoform of phospholipase C intracellular signal necessary to activates PKCβII	30 min	82.3±11ab	108.3±46ab	106.2±18ab	131±5a	87.2±24ab	86.9±25ab	58.0±20b
		24 h	47.5±14c	52.8±21c	114.8±1ab	132.3±29a	92.7±2b	101.0±1ab	127.2±8ab
β Tubulin	β-tubulins polymerize into microtubules, a major component of the eukaryotic cytoskeleton	30 min	81.0±11ab	83.8±13ab	94.9±5a	81.8±10ab	48.7±4c	73.8±6b	76.4±2ab
		24 h	59.0±17a	56.5±3a	58.5±13a	69.2±12a	59.6±4a	49.7±17a	50.8±15a
PKC βII	Main effector of GLUT2 trafficking, causes GLUT2 to be targeted and inserted into the apical membrane from a cytosolic pool of preformed GLUT2.	30 min	138.9±23a	118.86a	126.5±3a	131.5±12a	134.8±24a	139.3±5a	118.0±1a
		24 h	95.5±1ab	106.7±1a	93.5±12ab	83.6±15ab	78.2±13b	98.2±11ab	74.5±12b

Figure 34. Effect of the protein fractions and pure peptides originally identified in black bean on GLUT2, SGLT1, AMPKα1, PLCβ2, β Tubulin, PKC βII AND GAPDH protein expression. A. Protein expression after 30 min treatment. B. Protein expression after 24 h treatment. The data represents the mean ± SD of at least two independent replicates with consistent results. Means with different letters were significantly different ($p < 0.05$) according to Tukey's test.

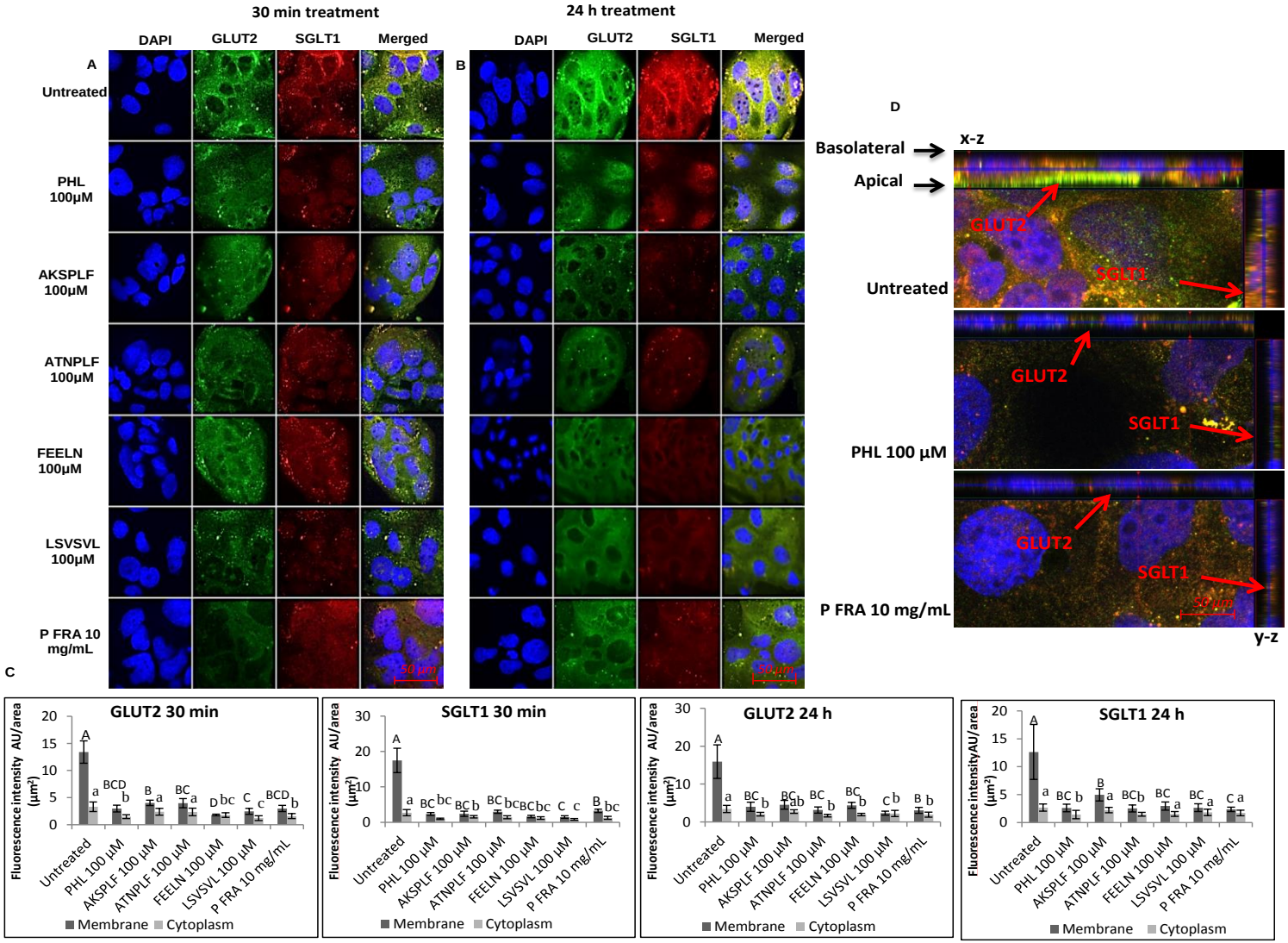


Figure 35. Confocal laser scanning microscopy of Caco-2 cells glucose transporters depicting two-dimensional fluorescence detection and quantification. A-B. The nucleus (blue); GLUT2 (green) and SGLT1 (red) expression and translocation after 30 min and 24 h glucose stimulation and treatment. C. Quantification was determined by the intensity (AU) over area (µm^2) in cell membrane and cytoplasm. D. Transversal view of either cells untreated, phloretin (100 µM) or protein fractions (10 mg/mL) indicating the location of the glucose transporters on the membrane. The data represents the mean ± SD of four independent fields of view from two independent cellular replicates. Means with different letters were significantly different ($p < 0.05$) according to Tukey's test. Uppercase represents measurement in membrane; Lowercase represents measurement in the cytoplasm.

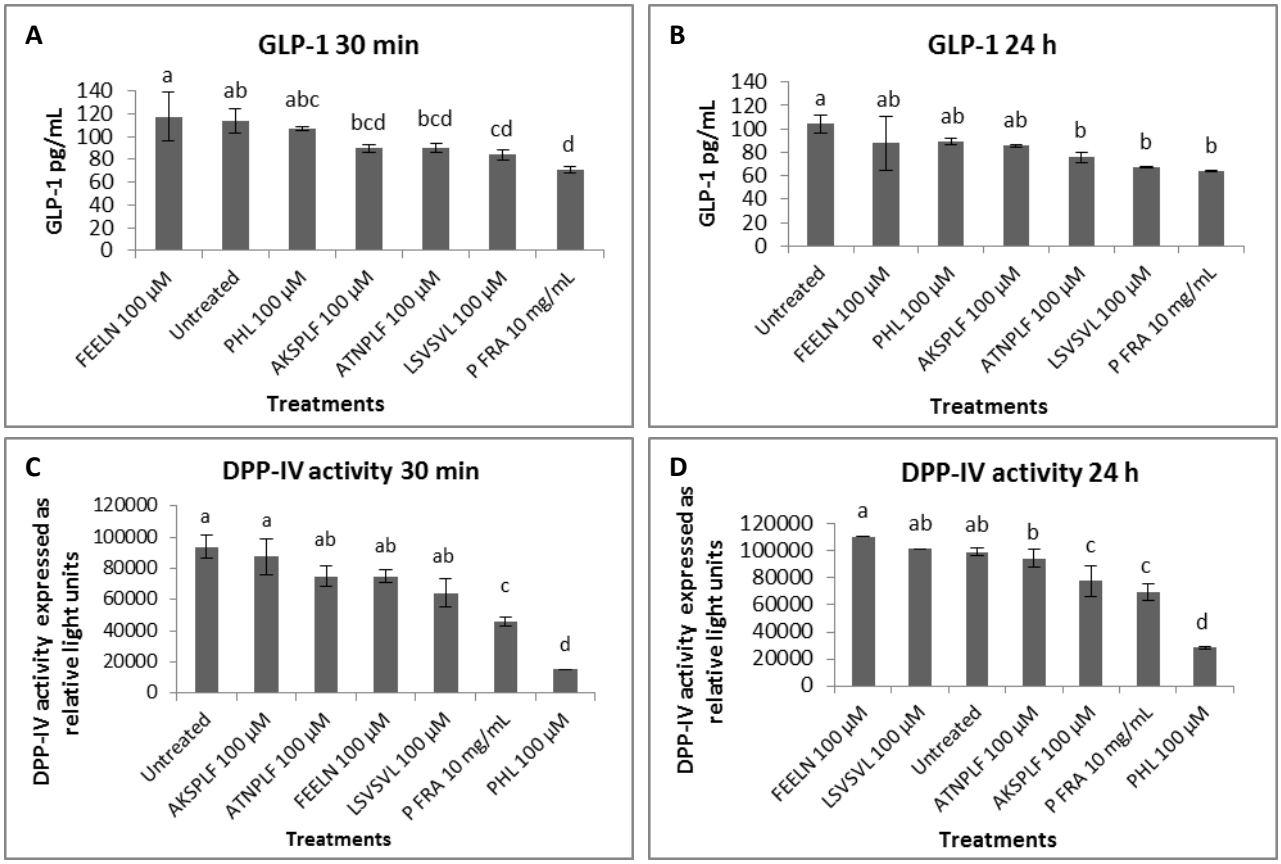


Figure 36. Glucagon like peptide (GLP-1) concentration and dipeptidyl peptidase IV (DPP-IV) activity in Caco-2 cell media after 30 min and 24 h of treatment. A-B. GLP-1 after 30 min and 24 h treatment. C-D. DPP-IV after 30 and 24 h treatment.

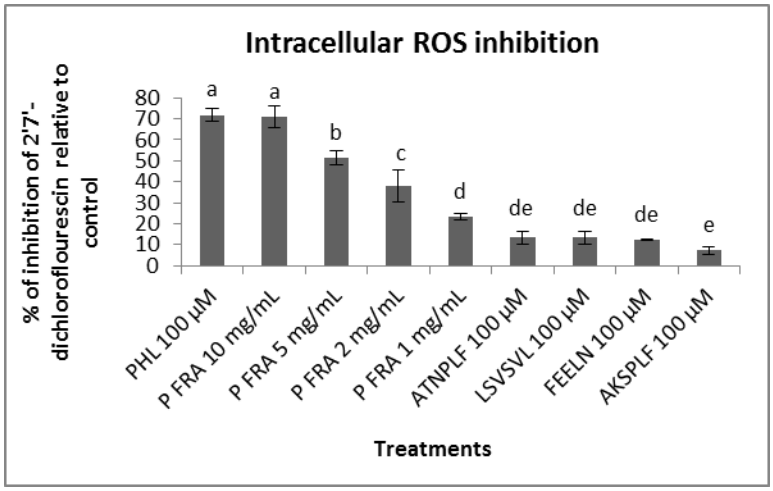


Figure 37. Potential of black bean protein fraction and proteins to inhibit the reactive oxygen species formation.

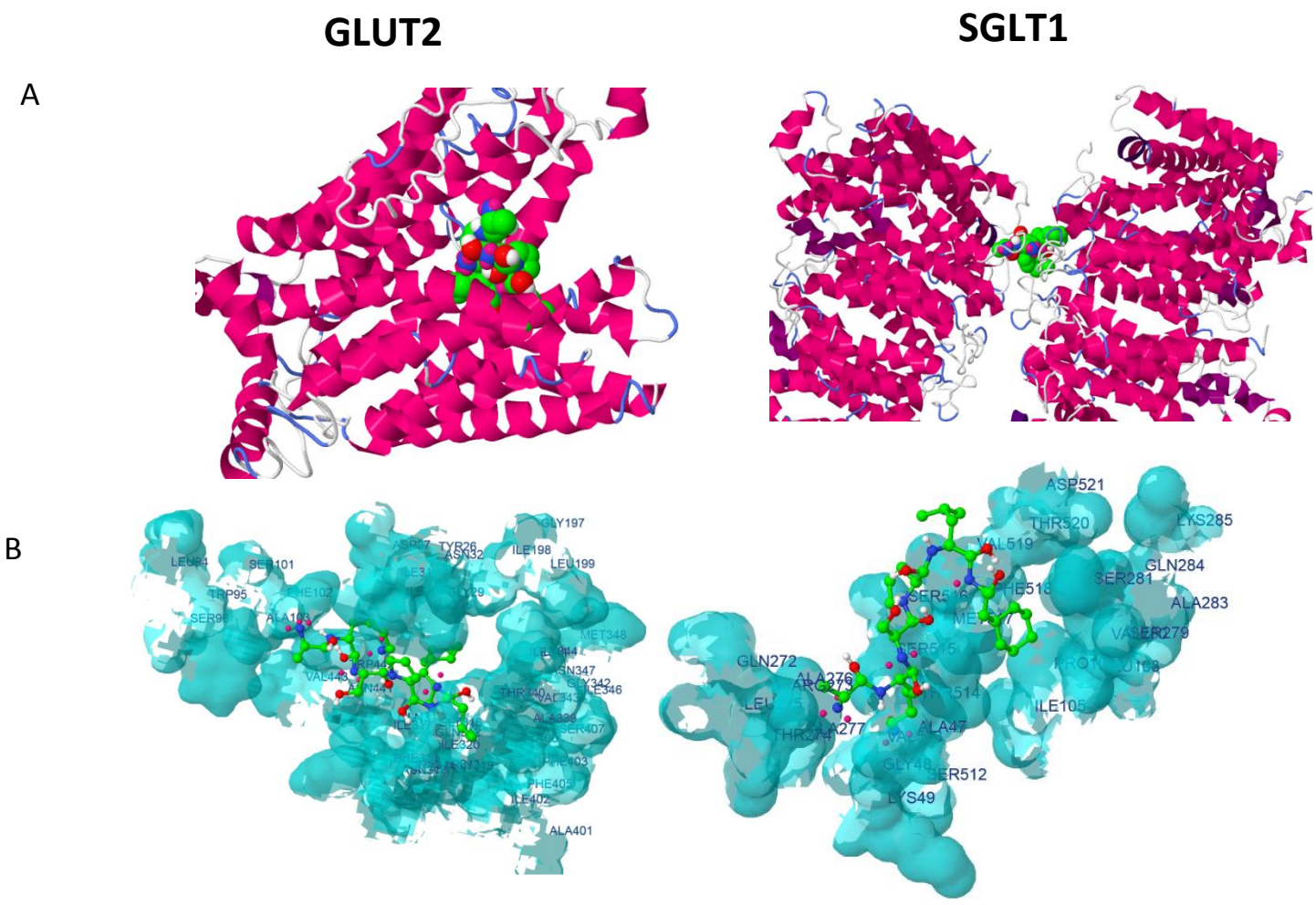


Figure 38. Molecular docking example of peptides AKSPLF interacting in the motif of glucose transporters GLUT2 and SGLT1. A) Representative example of the best pose of GLUT2 and SGLT1 proteins with AKSPLF peptide. B) Representative example of the best pose of AKSPLF interacting in the catalytic site of the glucose transporters GLUT2 and SGLT1.

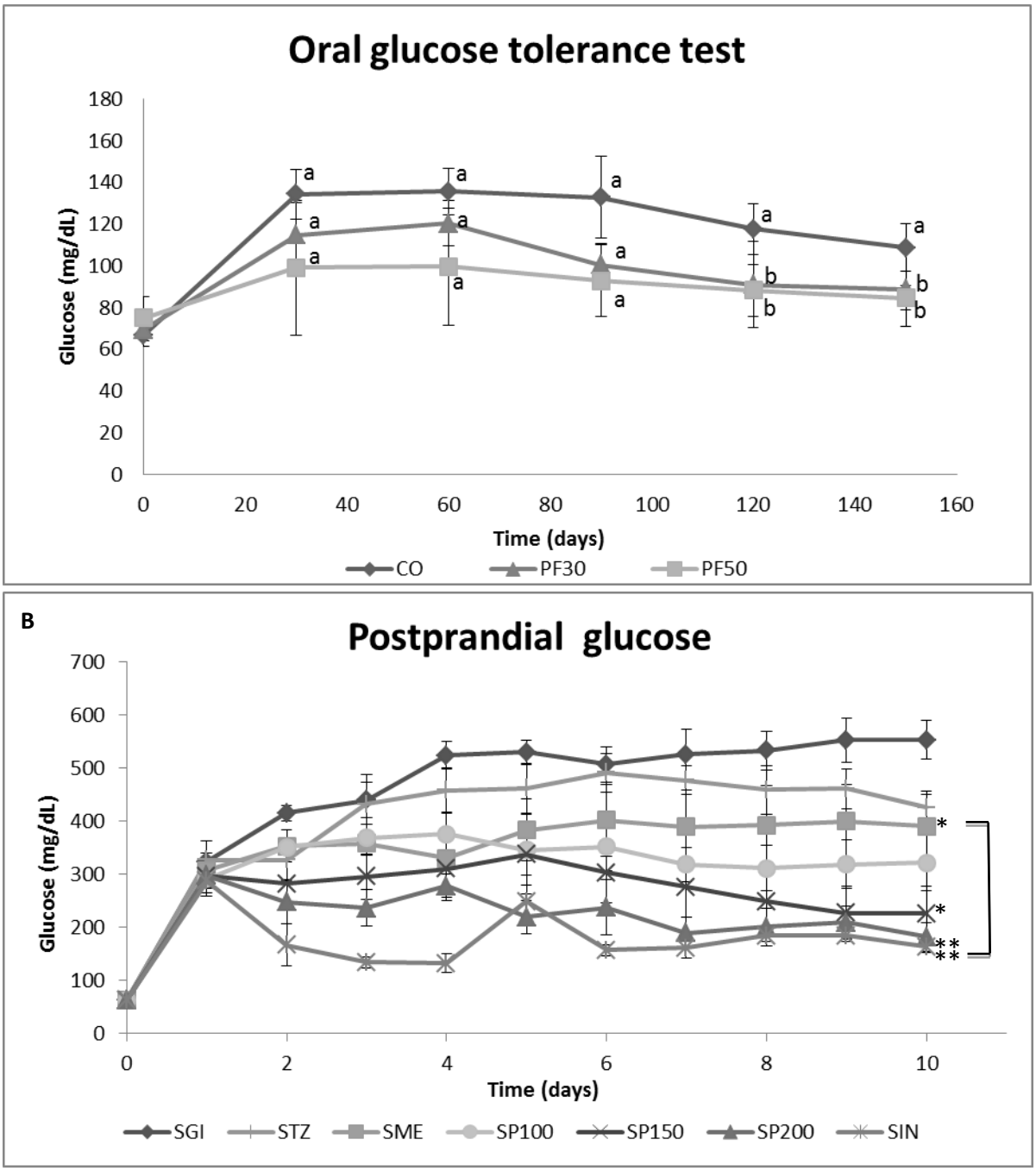


Figure 39. A. Oral glucose tolerance test in healthy rats. B. Postprandial glucose level of hyperglycemic rats exposed to different treatments STZ: streptozotocin group with no treatment; SIN: STZ and 4 U insulin/ kg BW; SGI; STZ and 2.5 mg/kg glibenclamide; SME: STZ and 500mg metformin /kg BW; SP100, SP150, SP200: STZ and 100, 150 or 200 mg PF/kg BW per day. Data were expressed as mean ± standard deviation in A or mean and standard error in B; with at least three independent replications; values within a column followed by different letters are significant at P < 0.05. *: significant p<0.05, **: significant p<0.001.

CHAPTER 11: CONCLUSIONS

- Peptides from common bean protein generated after GI digestion presented outstanding potential biological activities from *in silico* studies, related to lowering markers of hypertension and diabetes. Moreover, black beans contained delphinidin and ferulic acid commonly used as ingredients in functional foods due to their associated health benefits.
- Common bean protein isolate after simulated gastrointestinal digestion, and their pure peptides GGGLHK, CPGNK, KKSSG, KTYGL, demonstrated significant antioxidant, anti-diabetes, and potential antihypertensive properties. Besides, using computational modeling tools we found that common bean peptides were able to interact with amino acid residues in the catalytic site of DPP-IV, ACE, and α-glucosidase enzymes.
- Commercial processing did not affect bioactive properties of common bean protein hydrolysates. Besides, there was a significant increment on protein digestibility, remarkable improvement for low digestibility proteins such as phaseolin in common beans. The consumption of fast cooking common beans has the advantage of reducing cooking time without affecting bioactive properties.
- LC-ESI-MS/MS identified thirty-three peptide sequences from bean protein isolates; among those the ones with outstanding potential to inhibit DPP-IV, α-amylase and α-glucosidase were AKSPLF, ATNPLF, FEELN and LSVSVL.
- *In vitro* assays showed glucose absorption decreased by 6.5 and 24.5% after 30 min and 24 h of treatment, respectively, with 10 mg PF /mL ($p < 0.05$) in Caco-2 cells. This reduction in glucose absorption could be due to a decrease in the translocation of glucose transporter 2 (GLUT2) and sodium-glucose linked transporter 1 (SGLT1) to the cellular membrane, and also lowering their protein expression. Also, PF were able to reduce intracellular oxygen reactive species formation by 70% in the same cell line.
- The potential mechanism of PF involved in the decrease of the translocation of glucose transporters to the membrane could be the energy sensing pathway which affected the phosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPKα1) and cytoplasmic microtubules showing a reduction of protein expression of 59.84 and 23.57 % after 30 min treatment respectively.

- In the oral glucose tolerance test 30 and 50 mg PF/kg BW reduced postprandial glucose by 16.9 and 24.5% respectively, compared to the nontreated control.
- The hyperglycemic animal model showed that 100, 150 and 200 mg of PF/kg BW per day significantly reduced postprandial glucose levels compared to untreated group by 22.1, 35.5 and 47.8% of AUC respectively. Additionally, renal profile, sera proteins profile, hepatic profile, insulin and GLP-1 did not show significant differences among healthy control groups and hyperglycemic groups ($p > 0.05$).
- In general pure peptides initially found in black beans and their protein fractions showed substantial potential to decrease translocation of glucose transporters to the membrane, reduction in their protein expression, and as a consequence reduced glucose absorption in *in vitro* and *in vivo* models.

CHAPTER 12: INTEGRATION AND FUTURE WORK

Diabetes is a chronic noncommunicative disease characterized by high sugar blood levels; the International Diabetes Federation predicts an increase of people living with diabetes in the world to around 55% by 2035. The cost associated with the treatment of diabetes represents billions of dollars around the word. The American Diabetes Association recommends to diabetic patients to increase physical activity, weight control and nutritional education; in combination with drugs in a monotherapy, dual therapy, triple therapy or the combination of injectable treatment. However, the use of anti-diabetes drugs could cause undesirable side effects such as hypoglycemia, weight gain, and discomfort among others.

Bioactive compounds from foods such as phenolic compounds peptides and saponins with potential hypoglycemic present an alternative for people living with diabetes with the advantage of low cost and no known adverse side effects. These bioactive compounds can be obtained directly from foods rich in phytochemicals. The American Diabetes Association present a list of 10 super foods for people with diabetes including beans, dark green leafy vegetables, citrus fruits, sweet potatoes, berries, tomatoes, fish high in omega-3 fatty acids, whole grains, nuts and fat free milk and yogurt. Also could be obtained from food extracts or enriched bioactive components formulations.

Common beans are rich sources of bioactive components such as polyphenols, oligosaccharides, resistant starch and bioactive peptides. Research on common bean peptides has centered primarily in their antihypertensive, anti-inflammatory, anticancer and antioxidant potential. However, few studies recently focused on the anti-diabetes potential of common bean peptides. The long-term goal of this research was to provide an understanding of the role of common bean protein fractions in controlling hyperglycemia and their potential mechanism of action.

To achieve this goal the study was divided in six aims using the common bean as a source of bioactive peptides. In the aim number one, the protein and peptide profile, Bowman-Birk inhibitors, Kunitz inhibitors, lectins, α-amylase inhibitors, phenolic and anthocyanin content of improved common bean cultivars from Mexico and Brazil were determined. As a result of this aim, it was found that improved common bean cultivars presented a high amount of defense-related proteins, peptides with potential do inhibit enzymes associated to diabetes and phenolic

compounds with important biological potential. Moreover, we confirmed that peptides from beans can be used to block enzymes related to diabetes. In aim number two, bean protein isolates were digested using a simulated gastro-intestinal system and the peptides obtained were able to inhibit angiotensin converting enzyme (ACE), dipeptidyl peptidase IV (DPP-IV), and α-glucosidase. It was found that common bean protein hydrolysates and their pure peptides GGGLHK, CPGNK, KKSSG, KTYGL, and AKSPLF demonstrated good potential to inhibit the enzymes mentioned above and significant antioxidant potential. Negro Otomi bean peptides presented outstanding anti-diabetes potential compared to carioca beans because showed the lowest IC_{50} values for DPP-IV and around 50% of capacity to inhibit α-glucosidase. In the aim number three, the impact of commercial precooking of common beans was evaluated, and we compared the bioactive properties of the released peptides. From this aim we found that commercial processing did not have an effect on common bean protein hydrolysates bioactive properties related to anti-diabetes activity such as inhibition of DPP-IV, α-amylase, and α-glucosidase. The consumption of fast cooking common beans has the advantage of reducing cooking time without affecting bioactive properties. Besides, this result suggested that processing does not have an adverse effect on their biological potential, even though increase the digestibility, both important factors to consider in our next objective related processing the black beans to obtain protein fractions with hypoglycemic potential. As aim number four, the conditions needed to generate anti-diabetes peptides from black bean proteins were optimized using commercial enzymes and evaluated their effectiveness using biochemical and *in silico* approaches. From this process protein fractions from black bean proteins were obtained with optimum results using alcalase after two h of hydrolysis and E/S ratio of 1:20. Furthermore, the *in silico* studies of the thirty-three sequenced peptides found showed that bean peptides presented low free energy values to diabetes-related enzymes and their interaction was direct with the protein amino acids in the catalytic site.

Other important bioactive compounds in beans are anthocyanins and these were also optimized from Negro Otomi and evaluated their stability in a food matrix condition in aim five. The result we obtained was an enriched anthocyanins extract that at a concentration of 1 mg/mL was enough to color a beverage showing color and anthocyanin stability.

Finally, in aim six the mechanism of the anti-diabetes action of black bean bioactive peptide fractions and pure peptides were evaluated using, *in vitro* and *in vivo* models. Pure

peptides AKSPLF, ATNPLF, FEELN and LSVSVL and protein fractions presented potential to decrease glucose absorption trough inhibition of the translocation of glucose transporters GLUT2 and SGLT1 to the cellular membrane. Also, the potential mechanism of action of peptides to decrease glucose absorption may be linked to the inhibition of translocation of glucose transporters to the membrane, and also the reduction on their protein expression (Figure 40).
To confirm the hypoglycemic potential of black bean peptides, an oral glucose tolerance test, and a hyperglycemic animal model were performed to investigate the effect *in vivo*. For *in vivo* experiments dose were selected based on literature review and results from *in vitro* assays.

In vivo results offer promissory potential to decrease postprandial glucose, having their main effect at the gastrointestinal level, nowadays there is no approved commercially available anti-diabetes drugs that act at the gastrointestinal level; reducing glucose absorption by blocking translocation and glucose transporters to the apical membrane of GI tract. Besides, common bean peptides also showed potential to block starch degrading enzymes and decreased the available free glucose for absorption. PF were able to downregulate SGLT1 and GLUT2 after 30 min of treatment by 21 and 28% respectively, and reduce glucose absorption and uptake *in vitro* and *in vivo*. This effect can be compared to some drugs used in the treatment of diabetes such as the α-glucosidase inhibitors. Even though bioactive peptides from black bean showed remarkable anti-diabetes potential *in silico*, biochemical, *in vitro* and *in vivo*; further animal studies with the combination of protein fractions and prescribed anti-diabetes drugs are needed to evaluate potential synergistic or antagonistic effects. In fact, the anti-diabetes mechanism of action of bean protein fractions and their interaction with common anti-diabetes drugs will open the window to evaluate their potential as food supplements for diabetes patients.

Future directions of this research should be to perform a clinical evaluation of type 2 diabetes patients in early stages of the disease or pre-diabetes to evaluate the benefits of having bean protein hydrolysates as a supplement to manage glycemia level and can delay the progress of the disease.

Black bean protein fractions significantly reduced glucose absorption in *in vitro* and *in vivo* models and could represent an inexpensive alternative for patients with diabetes or prediabetes to improve glucose levels. Overall results from *in silico*, biochemical, *in vitro* and *in vivo* suggest that protein fractions and their bioactive peptides, and potentially phenolic compounds, from black bean exert significant anti-diabetes potential.

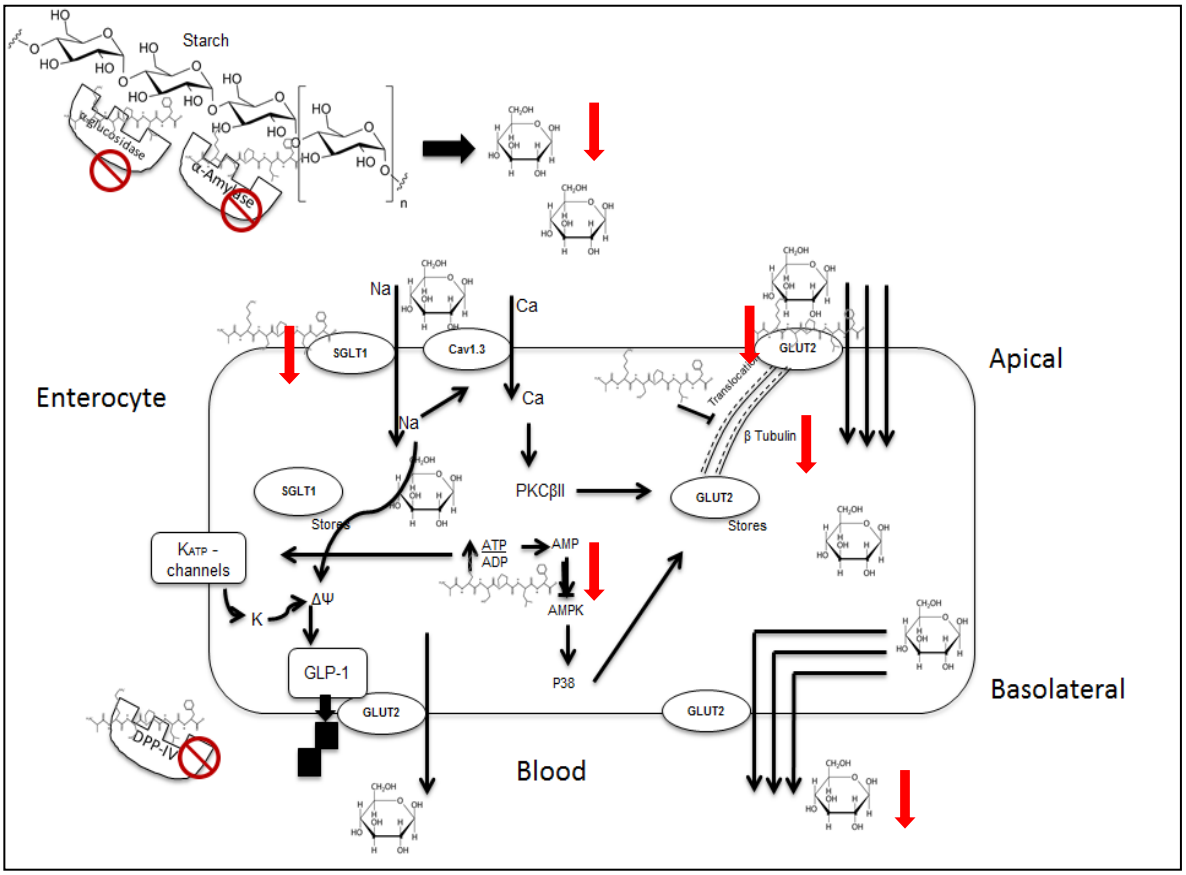


Figure 40. Proposed mechanism of action of black bean protein fractions to decrease glucose absorption in epithelial cells.

APPENDIX

Abreviations

a*	Redness
AAPH	α,α′-azodiisobutyramidine dihydrochloride
AC	Antioxidant capacity
ACE	Angiotensin converting enzyme
ANC	Anthocyanins
b*	Yellowness
BBI	Bowman-Birk inhibitor
BPI	Bean protein isolate
BRS	Brazilian
C*	Chroma
C3GE	Cyaniding 3 glucoside equivalents
CAE	Catechin equivalents
CDV	Cardiovascular disease
DM	Dry matter
DPP-IV	Dipeptidyl peptidase IV
Ea	Arrhenius activation energy
FJ	Flor de Junio
FM	Flor de Mayo
GAE	Gallic acid equivalents
GI	Gastrointestinal Inhibition
GIP	Insulinotropic peptide
GLP-1	Glucagon-like peptide 1
h°	Hue
HDC	High energy collision
IC50	Half maximal inhibitory concentration
K	First order kinetic rate
KTI	Kunitz trypsin inhibitor
L*	Brightness

APPENDIX (cont.)

m/z	Mass/charge
PCA	Principal component analysis
Q_{10}	Change in the reaction rate constant for 10˚C
RE	Rutin equivalents
ROS	Reactive oxygen species
RSM	Response Surface Methodology
$t_{1/2}$	Half-life
TE	Trolox equivalents
TP	Total polyphenols
t_R	Retention time
ΔE*	Change of color

This research was performed under the direction and supervision of Dr. Elvira de Mejia, Professor in the Department of Food Science and Human Nutrition at the University of Illinois at Urbana-Champaign.