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Valorization of *Vicia sativa* through the production of bioactive peptides by submerged fermentation and the reduction of non-nutritional factors using Instant Controlled Pressure Drop (DIC) technology

A dissertation presented by

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Declaration of Authorship

I**, Angel Ivan Hernandez Aguirre** declare that this dissertation titled, **Valorization of *Vicia sativa* through the production of bioactive peptides by submerged fermentation and the reduction of non-nutritional factors using instant controlled pressure drop technology** and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
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- Where I have consulted the published work of others, this is always clearly attributed.
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- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

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Angel Ivan Hernandez Aguirre

Santiago de Queretaro, Queretaro, Mexico, January 31st, 2020

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Abbreviations and units

Symbol	Meaning
°C	Celsius degrees
µg	Micrograms
µL	Microliters
µM	Micromolar
ABTS	2,2'-azinobis (3-ethylbenzothiazolin-6-sulfonic acid)
ACE	Angiotensin Converting Enzyme
ACEI	Angiotensin Converting Enzyme Inhibition
Albs	Albumins
C	Cooking
CT, T	Condensed Tannins
Da	Daltons
DH	Degree of hydrolysis
DIC, D	Instant Controlled Pressure-Drop
DPPH	2,2-Diphenyl-1-picrilhydrazil
DPP-IV	Dipeptidyl peptidase IV
FAO	Food and Agriculture Organization of the United Nations
g	Grams
G	Germination
GIP	Gastric inhibitory peptide
Globs	Globulins
GLP-1	Glucagon like peptide
Glut	Glutelins
h	Hours
HPLC	High Performance Liquid Chromatography
IC50	Inhibitory Concentration
IP6	Phytic acid
kDa	Kilodaltons
L	Liters
M	Molarity
mg	Miligrams
min	Minutes
mL	Mililiters

mM	Milimolar
MPa	Megapascals
MW	Molecular Weight
NNF	Non-nutritional factors
Pa	Pascals
PPH	Pulse protein hydrolyzate
Prols	Prolamins
R	Rafinose
s	seconds
S	Stachyose
SmF	Submerged Fermentation
Symbol	Meaning
TEAC	Trolox equivalents
TFC, F	Total flavonoid compounds
TPC, P	Total phenolic compounds
Trolox	Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

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Abstract

In this work, different contributions have been made to promote vetches as a viable option for human consumption, due to their potential as a source of peptides with antioxidant activity and inhibition of the angiotensin-converting enzyme, as well to explore novel technologies to reduce non-nutritional factors which could limit their use.

Chapter one provides useful information about the uses of pulses as a source of bioactive peptides, as well as the enzymes used. This chapter is intended as an introduction to study the possible use of vetch as a source of bioactive peptides.

Chapter number two was conducted to review one of the pulses whose peptides have various biological activities: common beans. Common bean proteins were hydrolyzed with different enzymes such as pepsin, pancreatin, bromelain, papain, Alcalase®, Flavourzyme®, Corolase®, and Thermolysin®. Diverse conditions for the optimal proteolysis procedure were analyzed in chapter two. There is a wide variety of peptides with biological activity obtained from common beans, for instance, anti-inflammatory, anticancer, angiotensin-converting enzyme inhibition, and antioxidant activity. The activities achieved are also reviewed in this chapter.

Chapter three collects information on one of the most frequently found activities in bioactive peptides in pulses: the inhibition of the angiotensin-converting enzyme. In this chapter, information about the angiotensin-converting enzyme and its mechanism of action was provided. The aim of this chapter was to describe the structure/activity relationship of ACE-inhibitory peptides, as well as their bioavailability, physiological effects on hypertension demonstrated by both *in vitro* and *in vivo* assays and the main legumes used to obtain them. Finally, current reported strategies for the incorporation of antihypertensive peptides into foods and their effects on both availability and activity are also discussed.

Chapter four was developed to test innovative technologies to eliminate non-nutritional factors in vetches since these can limit their human consumption. The use of instant controlled pressure-drop (DIC), is positioned as a viable option for cooking and germination in the removal of non-nutritional factors. Results showed that compared to raw vetches, DIC treatment reduced total phenolic compounds (48%), condensed tannins (28%), flavonoids (65%), IP6 (92%), raffinose (77%), and stachyose (92%). These results are very similar to the ones achieved by traditional ways of removing non-nutritional factors. High pressure/short time treatments could be a different way to remove non-nutritional factors.

Chapter five covers the study of the antioxidant and inhibitory activity of the angiotensin-converting enzyme of the peptides obtained from the submerged fermentation of vetch protein isolate. The fungal strains isolated and characterized for this study were obtained from vetch protein isolate. The fungal strains isolated were identified as *Aspergillus flavus, Rhizopus oryzae* and *Trichoderma asperellum*. *A. flavus* was the strain that achieved the higher degree of hydrolysis, ABTS scavenging, and angiotensin-converting enzyme inhibitory activity.

Finally, a general conclusion, as well as recommendations and aims for the future of this work, are presented in Chapter six.

CHAPTER 1: Pulses as a source of bioactive peptides

1.1 Legumes used as peptides sources

Milk has been the primary source of peptides with biological activity; however, peptides from vegetable sources such as soybean, rice, wheat, and sunflower, have been also isolated (Carbonaro, Maselli, and Nucara 2015). Since pulses contain high protein amounts, they represent a promising source of peptides that could exert different physiological benefits to human health.

Plant-derived food, such as pulses, represent a promising source of nutrients of interest due to its availability, low cost, and variability. In this respect, pulses are one of the most consumed vegetable foods, due to their nutritional benefits (proteins, energy, and essential amino acids) as well as their techno-functional roles (formation and stability of foams, emulsions, and gels). Although there is a wide diversity of pulses, there is only a small number of varieties that are intended for human consumption. In this respect, their value can be increased by the extraction of the components that exert biological activity. At present, due to the enormous potential in health improvement, there is a rising interest (scientific and industrial) in the bioprospection and production of bioactive peptides (Tovar-Pérez, Lugo-Radillo, and Aguilera-Aguirre 2019).

Pulse proteins can be classified according to their function in structure or implicated in storage or defense. Their solubility can also group pulse proteins in albumins, globulins, prolamins, and glutelins. The albumins fraction (Albs) is composed of two types of albumin: albumin 1 (Alb-1), which is extracted by solubility in water or in low ionic strength solutions and albumin 2 (Alb-2), which is extracted with water after Alb-1 and globulin removal (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

Globulins (Globs) are storage protein of dicotyledonous seeds; they are mainly composed of two fractions: 7S and 11S. The 7S globulin is composed of three subunits: α (57-68 kDa), α′ (57-72 kDa), and β (42-45 kDa), all of which form a trimer. The 11S fraction

has a hexameric structure with a molecular weight ranging from 343 to 398 kDa and a high content of lysine and valine (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

Several studies have reported that glutelins (Gluts) are another dominant individual protein group in legume grains (reaching a concentration of up to 49.5 %). This fraction comprises five polypeptide subunits with Molecular Weight (MW) of 20, 22, 35, 50 y 65 kDa, and it has a high content of leucine, threonine, and histidine (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

Prolamins (Prols) are the less abundant protein fraction in the legume grains; they are characterized by its abundance of sulfur-containing amino acids and phenylalanine. Prols are composed of three types of peptide units: α, β, and γ. The α and β-Prols are subunits with high MW (more than 94 kDa), while γ-Prols have a molecular weight ranging from 60 to 67 kDa. Moreover, low MW polypeptides, named Prols-3 (19–26 kDa), have also been identified (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

According to the Food and Agricultural Organization (FAO), pulses are dry seeds of plants belonging to the *Leguminosae* family. Within the most common pulses, stand out beans, broad beans, peas, chickpeas, cowpeas, pigeon peas, lentils, Bambara beans, lupins, vetches, and other minor legumes (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

It is known that pulses represent an important source of proteins and nutrients necessary for human feeding (FAO 2016a). Numerous studies suggest that the consumption of legumes may have potential health benefits as reducing the risk of cardiovascular diseases (Padhi and Ramdath 2017), cancer (Mathers 2007), diabetes (Ramdath, Renwick, and Duncan 2016), hypertension (Jayalath et al. 2014), among others.

In México, according to FAO, the most consumed legume are common beans (*Phaseolus vulgaris*); nevertheless, they present a low crop yield (FAOSTAT 2017).

On the other hand, *Vicia sativa spp.* (common vetch or Ebo in Spanish) are widely cultivated as soil improvement and as feed for livestock due to their high content of proteins

and other nutrients (Ribeiro, Teixeira, and Ferreira 2004). Moreover, vetches have a better crop yield than common beans (FAOSTAT 2017).

1.2 *Vicia sativa*

Common vetch (*Vicia sativa*) is widely cultivated worldwide due to its capabilities for soil improvement, growth with lower water quantities, and moderated resistance to drought conditions. Although vetches constitute a source of proteins, oil, and other nutrients for the human diet, they are usually intended for livestock (Ribeiro, Teixeira, and Ferreira 2004). This can be attributed to non-nutritional factors, such as phenolics compounds, condensed tannins, flavonoids, phytic acid, raffinose, and stachyose. It is known that these compounds can cause undesirable physiological reactions such as flatulence, inhibition of digestive enzymes, and vitamin absorption among others (Sadeghi et al. 2009). Usually, the non-nutritional factors on pulses are removed by cooking or germinating; however, germination is a process that requires extended time, and cooking may compromise the viability of some nutrients. Novel technologies such as Instant Controlled Pressure-Drop (DIC), represent an innovative way to dispose of non-nutritional factors.

In Mexico, vetch is usually known as ebo (Figure 1.1). It is cultivated in the regions of Queretaro, San Luis Potosi, Estado de Mexico, Hidalgo, and Puebla. Ebo is mainly used for livestock despite having better crop yields than common beans (FAOSTAT 2017).

Figure 1.1 Common vetch (*Vicia sativa*)

1.3 Bioactive peptides

According to Segura-Campos et al. (2013), proteins can generate peptides with biological activity, regardless of their source or nutritional quality. Zambrowicz et al. (2013) stated that peptides released can exhibit different bio-functionalities that have many benefits to human health; even they can exert improved activity compared to the original protein, usually called parent protein (Udenigwe and Aluko 2012).

Since activity is hindered within the parent protein, peptides should be released in different ways, for instance, manufacture of protein hydrolysates, food fermentation, and proteolysis during *in vitro* and *in vivo* digestion (Korhonen and Pihlanto 2006).

Bioactive peptides obtained from pulses have exerted a wide variety of activities, such as hypoglycemic (Oseguera Toledo et al. 2016), hypocholesterolemic (Ramírez-Jiménez et al. 2015), anti-inflammatory (Garcia-Mora, Frias, et al. 2015a), and anticancer activity (Luna-Vital, González de Mejía, and Loarca-Piña 2016); however, most of the peptides also exert antioxidant activity (Durak et al. 2013, Evangelho et al. 2017, Valdez-Ortiz et al. 2012, Wongekalak et al. 2011), and anti-hypertensive activity (expressed as inhibition of the angiotensin-converting enzyme) (Barbana and Boye 2011, Ajibola et al. 2013, Betancur-Ancona et al. 2014, Limón et al. 2015), as the most reported activities.

1.4 Production of bioactive peptides

Proteins should be hydrolyzed to release peptides with biologic activity (Korhonen and Pihlanto 2006). There are many enzymes and microorganisms used for protein cleavage in *in vitro* studies. Its source can classify proteases, for instance, gastric enzymes, plant-derived enzymes, enzymatic preparations (trademark enzymes) and microorganisms.

1.4.1 Gastric enzymes

Pepsin, pancreatin, and trypsin are usually applied to simulate gastric or intestinal digestion. Either way, they can be used *in tandem* as sequential hydrolysis or alone. Some of the optimal conditions for pepsin such as the enzyme-substrate ratio (1:10 to 1:25), hydrolysis time (1 to 8 hours), pH (2.0 to 2.5), may vary, but the temperature of 37 °C is stated as the most common optimal condition for pepsin activity. Bioactive peptides from pulse proteins have been obtained hydrolyzing protein isolate with pepsin. In this regard, pulses used include common beans such as black (Evangelho et al. 2017), Negro 8025 (Oseguera-Toledo et al. 2011, Luna Vital et al. 2014), Azufrado Higuera (Luna Vital et al. 2014), Pinto Durango (Luna Vital et al. 2014, Oseguera-Toledo et al. 2011), Jamapa (Carrasco-Castilla et al. 2012b, a), adzuki (Durak et al. 2013) and Bayo Madero (Luna Vital et al. 2014); lentils (Barbana and Boye 2011);r chickpea (Torres-Fuentes et al. 2015, Xue et al. 2015, Girón-Calle, Alaiz, and Vioque 2010); for soybean (Capriotti et al. 2015, Roblet et al. 2014, Puchalska, Concepción García, and Luisa Marina 2014, Puchalska, Marina, and García 2014); lupine (Lammi et al. 2016) and cowpea (Marques, Fontanari, et al. 2015, Marques, Soares Freitas, et al. 2015)

Pancreatin, a pancreatic mixture of enzymes, has been used to hydrolyze pulse proteins under similar conditions than pepsin, (temperature, hydrolysis time and enzyme-substrate ratio); the only difference is their optimal pH. Studies using Pancreatin to produce

bioactive peptides from protein isolates of common beans are extensive (Carrasco-Castilla et al. 2012b, a, Durak et al. 2013, Luna Vital et al. 2014, Oseguera-Toledo et al. 2011, Valdez-Ortiz et al. 2012) Other pulse protein hydrolyzed with pancreatin are cowpea (Marques, Fontanari, et al. 2015, Marques, Soares Freitas, et al. 2015) and soybean (Capriotti et al. 2015, Puchalska, Concepción García, and Luisa Marina 2014, Puchalska, Marina, and García 2014).

Trypsin, another digestive enzyme, shares the optimal temperature 37 °C with the previously mentioned enzymes, but its optimal pH ranges from 6.0 to 8.0 to obtain bioactive peptides from lentils (Barbana and Boye 2011), lupine (Lammi et al. 2016) and chickpea (Xue et al. 2015).

1.4.2 Plant-derived enzymes

According to Meshram et al. (2019), plant-derived enzymes represent about 60 % of the total enzymes market, however, proteases or peptidases, comprise the major group of plant-derived enzymes in the bioindustry. Bromelain and papains are the most used plant-derived proteases used to produce peptides with biological activity.

Bromelain, an enzyme found in the stems of pineapples (*Ananas comosus*), is used to hydrolyze proteins from pulses, resulting in the production of different peptide sizes with various physiological activities. Optimal conditions state temperature of 37 °C and pH 8.0 to obtain peptides from lentils and common beans (Barbana and Boye 2011, Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015, Oseguera Toledo et al. 2016).

Papain is a proteolytic enzyme obtained from papaya (*Carica papaya*) that is also used to produce bioactive peptides from pulses. Studies by Rui et al. (2012) and Rui et al. (2013) have reported an optimal temperature and pH of 60 °C and 6.5, respectively, to hydrolyze common bean proteins. However, studies by Barbana and Boye (2011) on lentil

proteins, have reported a different temperature for papain activity (37 °C) while pH remained at 6.5.

1.4.3 Trademark enzymes

There are trademark enzymes (Table 1.1) obtained from diverse microorganisms (*Bacillus subtilis*, *Geobacillus stearothermophilus*, *Bacillus thermoproteolyticus*, among others) that are used to produce bioactive peptides.

Table 1.1 Trademark Enzymes

Enzyme	Source	Cleavage	Reference
Alcalase®	*Bacillus licheniformis*	Cuts before Gln, Pro, Leu, Asp, or Glu	de Souza Rocha et al. (2015)
Flavourzyme®	*Bacillus subtilis*	Ala, Leu, Lys, or Asn	Kou et al. (2013)
Corolase®	*Aspergillus spp.*	Phe, Leu, Pro, Ile, or Val	Coscueta et al. (2016)
Thermolysin®	*Geobacillus stearothermophilus*	Cuts before Ile, Met, Phe, Trp, Tyr, or Val	Valdez-Ortiz et al. (2012)

Alcalase®, the most common trademark enzyme, has been used to hydrolyze different legume protein isolates at 40 to 50 °C and pH at 7.0 to 9.0 such as soybean (Vernaza et al. 2012), lentils (Barbana and Boye 2011, Garcia-Mora, Peñas, et al. 2015), chickpea (Zhang et al. 2012, Ghribi et al. 2015, Mokni Ghribi et al. 2015, Kou et al. 2013) and common bean cultivars (Evangelho et al. 2017), (Ajibola et al. 2013, de Souza Rocha et al. 2014, de Souza Rocha et al. 2015, Garcia-Mora, Frias, et al. 2015a, Oseguera Toledo et al. 2016, Oseguera-

Toledo et al. 2011, Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015, Rui et al. 2012, Rui et al. 2013, Valdez-Ortiz et al. 2012).

Flavourzyme® can also produce bioactive peptides hydrolyzing protein isolates from pulses at 50 °C and pH 7.0 to 8.0 in lentils (Barbana and Boye 2011), common beans (Rui et al. 2012), chickpea (Kou et al. 2013) and cowpea (Segura-Campos, Chel-Guerrero, and Betancur-Ancona 2011).

There are other less used trademark enzymes such as Protamex® and Savinase®, that have an optimal temperature of 40 °C and pH of 8 to produce bioactive peptides from lentil protein isolate (Garcia-Mora et al. 2014, Garcia-Mora, Peñas, et al. 2015) as well as Corolase® used to hydrolyze soybean protein isolate (Coscueta et al. 2016), common bean (Garcia-Mora, Frias, et al. 2015a) and lentil (Garcia-Mora et al. 2014).

On the other hand, Thermolysin® best reaction conditions used by Valdez-Ortiz et al. (2012) to produce peptides from common beans are 55 °C and pH of 9.0.

Most of the enzymes used to produce bioactive peptides are pepsin and pancreatin; however, these enzymes are also used to test the activity of peptides obtained with trademark and vegetable-derived enzymes under simulated gastrointestinal conditions.

1.4.4 Submerged fermentation

Fermentation is a biological conversion of complex substrates into simple compounds mediated by microorganisms and enzymes. Technologies such as Solid-State Fermentation (SSF) and Submerged Fermentation (SmF) lead the industrial-level production of bioactive peptides. These techniques have been further optimized based on various process conditions, substrates, and organisms used during the fermentation (Subramaniyam and Vimala 2012a).

Submerged fermentation is defined as the growth of microorganisms in a nutrient broth, in which the major constituent is water. This fermentation system is used to obtain a wide diversity of secondary metabolites and products. Better control of the process (pH,

dissolved oxygen, temperature, time), higher yields, good reproducibility, short time periods, low costs, well-defined media, and easier purification of products, are the highlights of SmF. According to Martínez-Medina et al. (2019), different factors, such as type of fermenter, inoculum, temperature, and pH, are the main factors influencing the production of various products through this process.

SmF nutrient-rich uses liquid substrates (molasses and broths) and is best suited for microorganisms that require high moisture. According to Subramaniyam and Vimala (2012a), the fermentation products are secreted into the fermentation broth.

Diverse filamentous fungi were used in SmF, for instance, *Aspergillus* spp. (Boer and Peralta 2000, Wang, Law, and Webb 2005, Hossain et al. 2006, Samarntarn, Cheevadhanarak, and Tanticharoen 1999), *Trichoderma* spp (Kredics et al. 2005, Elad and Kapat 1999), and *Rhizopus* spp (Wang et al. 2019, da Silva et al. 2019), due to its high proteolytic activity.

There is information about *Lactobacilli* exerting proteolytic activity in SmF for the production of bioactive peptides in the studies by Torino et al. (2013), Subrota et al. (2013), Kobayashi et al. (2012), Limón et al. (2015), and Wu et al. (2015).

1.4.5 Germination and sprouting

Germination is the process where the embryonic axis of a seed returns to its development after being interrupted by maturity (Paucar-Menacho et al. 2010). Germination improves protein digestibility and protein efficiency ratio in germinated Brazilian soybean cultivar BRS 258 (Paucar-Menacho et al. 2010) and increases soluble protein due to the production of peptides. This peptides have health benefit such as the reduction of some inflammation markers, improvement in the antioxidant capacity of Brazilian soybean flours (Vernaza et al. 2012), green pea, lentil, and young mung bean sprouts (Świeca and Gawlik-Dziki 2015), inhibitory effect of dipeptidyl peptidase IV by cowpea peptides (de Souza

Rocha et al. 2014), reduction of the risk of development of type-2 diabetes due to common bean peptides (de Souza Rocha et al. 2015) as well as changes in anti-inflammatory and antioxidant activities of germinated beans hydrolyzed with Alcalase® (López-Barrios, Antunes-Ricardo, and Gutiérrez-Uribe 2016).

1.5 Instant Controlled Pressure-Drop (DIC)

Instant Controlled Pressure-Drop is also known by its French acronym: Détente Instantanée Contrôlée (DIC) and it consists in a short time process (< 10 min) combined with high-temperature/high-pressure (0.05 to 1 MPa) system, followed to an instant pressure drop towards the vacuum. This thermo-hydro-mechanical process induces instant auto vaporization of a quantity of the product water, which provokes a controlled expansion and an immediate cooling of treated products, which stops thermal degradation (Mounir and Allaf 2008). DIC has been used in many fields and various industrial applications have shown that DIC provides high product quality and high process performance, thus, preserving the environment and saving energy as an alternative to other technologies (Allaf and Allaf 2014).

The effect of DIC treatment has been previously evaluated by Haddad and Allaf (2007) and Pedrosa et al. (2012) on several pulses such as lupin, chickpea, and lentil, as well as other crops, for instance, soybean and peanuts to reduce non-nutritional factors.

DIC had become a strategic technology for treating different types of functional foods, thanks to its capacity to change the structures on the food products into a porous one; hence, DIC technology could improve the processing time. DIC treatment allows to improve the availability of antioxidants, to reduce anti-nutritional, and allergic factors of foodstuffs (Téllez-Pérez, 2018).

1.4 Justification

Currently, pulses are a fundamental food source for humans, not only for their high nutritional value but also for their large number of molecules with biological activity. Although there is a great diversity of pulses, only a small group is intended for human consumption and research.

Pulses stand out among other grains due to their high protein content, which has been shown to provide different benefits to human health. These benefits could be exerted after a process of protein hydrolysis, either using proteases or microorganisms with high proteolytic activity. One of the most used exponents of pulses is the common bean, which has been extensively investigated for the production of peptides with biological activity, however, vetch is a pulse that is not intended for human consumption and is rarely used in research, despite being highly cultivated and having high amounts of protein.

Further investigation is necessary to promote vetch for human consumption and research purposes as well. The use of vetch protein isolate into fermentation systems could be an alternative way to produce peptides with attractive benefits to human health.

It is known that pulses contain diverse non-nutritional factors that cause undesirable effects on human health. There are traditional treatments such as soaking, cooking and germination that reduces non-nutritional factors. However, novel technologies such as Instant Controlled Pressure-Drop could represent a novel way to reduce non-nutritional factors.

1.5 Hypotheses

Instant Controlled Pressure-Drop represents an alternative to cooking and germination to reduce non-nutritional factors on vetches.
If a fungal strain is capable of growth in vetch protein isolate, then it is possible to obtain peptides with biological activity.

1.6 Main objective

To evaluate the production of bioactive peptides by submerged fermentation using fungi isolated from vetch protein isolate and to reduce the vetch nutritional factors by using instant controlled pressure-drop technology.

1.7 Specific objectives

To perform a physicochemical characterization of vetch.

To isolate a fungal strain capable of growing into vetch protein isolate.

To identify the fungi isolated by microscopy and by genomic DNA analysis.

To perform a fermentation kinetic of vetch protein isolate by the selected fungi.

To evaluate the biological antioxidant and ACE inhibitory activities in the protein hydrolysate.

To reduce non-nutritional factors using instant controlled pressure-drop technology.

1.8 Concluding remarks

Proteolytic enzymes have been widely studied to produce peptides that can exert biological activity; however, there are proteolytic sources, such as fungi and fungal enzymes, that can produce peptides with a different activity, or even improve any biological activity. Although most of the peptides studied come from animal sources, vegetable proteins represent a viable option to produce peptides with biological activity. It is known that high protein foods, soybean, and pulses, such as common beans, have been widely studied. Little is known about the production of bioactive peptides from less consumed pulses, for instance, common vetches, as well as the effect of other proteolytic sources such as fungi. The importance of this study is to promote vetches as a new source of peptides with biological activity, produced by fungal strains.

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CHAPTER 2: *Phaseolus vulgaris* as a source of biologically active peptides

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PHASEOLUS VULGARIS PROTEINS AS A SOURCE OF BIOLOGICALLY ACTIVE PEPTIDES

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ABSTRACT

Common beans (Phaseolus vulgaris) are one of the world's most consumed crops with around 150 cultivars spread over American, European, and Asian regions. Being a good source of proteins, complex carbohydrates, vitamins, and minerals, it is known that common beans contribute to diverse health benefits. In recent years, it has been recognized that proteins from vegetable sources, such as common beans, represent a very important source of biologically active peptides. These peptides are encrypted in proteins and are released by in vivo and in vitro digestion. The use of digestive enzymes (pepsin, pancreatin, and trypsin), plant-derived proteases such as bromelain and papain, commercial enzymatic preparations such as Alcalase®, Flavourzyme®, Corolase®, Thermolysin® on Phaseolus protein isolates releases bioactive peptides. Also, microorganisms used in solid or liquid fermentation can produce bioactive peptides due to the enzymes excreted to the culture media. Peptides obtained from the Phaseolus genus tend to have different sizes, sequences, and unique activities, for instance, antihypertensive (due to inhibition of angiotensin I-converting enzyme), antioxidant, anti-inflammatory, anti-diabetic, hypocholesterolemic, antiproliferative and anticancer. This chapter provides information about the optimal in vitro conditions to produce peptides from common bean proteins, as well as the

documented biological activities that exhibit the potential use of common beans as nutraceuticals to prevent chronic diseases.

Keywords: Common beans, peptides, biological activities, peptides production

2.1 Introduction

The Food and Agriculture Organization of the United Nations (FAO) declared 2016 as the international year of pulses. The term "pulses" applies only to leguminous crops of dry grains excluding crops used mainly for oil extraction (e.g. soybean and groundnut). Among pulses, common bean (*Phaseolus vulgaris*) is widely consumed around American, Asian, and European regions with around 150 cultivars. It is well known, that consumption of common beans could contribute to multiple health benefits and could prevent chronic diseases due to bioactive peptides encrypted within beans proteins (FAO 2016b).

Bioactive peptides are usually inactive until they are released in three ways: food fermentation using proteolytic starter cultures, during the manufacture of protein hydrolysates and during *in vivo* and *in vitro* enzyme digestion (Korhonen and Pihlanto 2006).

Proteins regardless of their source or nutritional quality can generate bioactive peptides (Segura-Campos, Salazar-Vega, Chel-Guerrero, & Betancur-Ancona, 2013). These peptides, once released, exhibit several bio-functionalities that have many therapeutic roles and physiological benefits (Zambrowicz et al. 2013) and demonstrate superior activity compared to the parent proteins (Udenigwe and Aluko 2012).

2.2 Production of Bioactive Peptides

2.2.1 Digestive Enzymes

Several enzymes (table 2.1) are used for protein cleavage in *in vitro* studies using common bean protein hydrolysate or flour as a substrate under optimal conditions. Pepsin, pancreatin and trypsin are usually applied to simulate gastric or intestinal digestion. Either way, they can be used *in tandem* as sequential hydrolysis or alone. Some of the optimal conditions for pepsin may vary, such as the enzyme-substrate ratio (1:10 - 1:25), hydrolysis time (1 - 8 h), pH (2.0 - 2.5) but 37 °C is the most common optimal temperature condition for pepsin activity. Pepsin has preferential cleavage site over hydrophobic aromatic residues and cleaves F-/-V, Q-/-H, G-/-A, A-/-L, L-/-Y, Y-/-L, G-/-V, V-/-V, and V-/-L, while trypsin and chymotrypsin cleave R-/-, K-/- and Y-/-, W-/-, V-/- and L-/-, respectively. Bioactive peptides have been obtained by protein isolate hydrolysis with pepsin from proteins of pulses such as common beans, for instance, black (Evangelho et al. 2017), Negro 8025 (Luna Vital et al. 2014, Oseguera-Toledo et al. 2011), Azufrado Higuera (Luna Vital et al. 2014), Pinto Durango (Luna Vital et al. 2014, Oseguera-Toledo et al. 2011), Jamapa (Carrasco-Castilla et al. 2012b, a), adzuki (Durak et al. 2013) and Bayo Madero (Luna Vital et al. 2014). The optimal conditions for pancreatin, a pancreatic mixture of enzymes, are similar in temperature, hydrolysis time and enzyme-substrate ratio to pepsin but different only in pH (7.0 to 7.5). There is evidence of pancreatin being used to produce bioactive peptides from protein isolates of common beans (Carrasco-Castilla et al. 2012b, a, Durak et al. 2013, Luna Vital et al. 2014, Oseguera-Toledo et al. 2011, Valdez-Ortiz et al. 2012).

Table 2.1. Enzymes and substrates used to produce bioactive peptides from several varieties of common beans.

Enzyme	Process parameters	Cultivar	Reference
Pepsin/Gastric enzyme (E.C. 3.4.23.1)	37 °C, pH 2.0-2.5	Black, jamapa, adzuki, Azufrado Higuera, Bayo Madero, Pinto Durango, Negro 8025, Great Northern, navy	Evangelho et al. (2017), Carrasco-Castilla et al. (2012b), Carrasco-Castilla et al. (2012a), Durak et al. (2013), Luna Vital et al. (2014), Oseguera-Toledo et al. (2011), Mojica, Chen, and Mejía (2015)
Pancreatin/Pancreatic enzyme (3.4.21.4)	37 °C, pH 7.0-7.5	Jamapa, adzuki, Azufrado Higuera, Bayo Madero, Pinto Durango, Negro 8025, Azufrado Noroeste, Azufrado Regional, navy, Great Northern	Carrasco-Castilla et al. (2012b), Carrasco-Castilla et al. (2012a), Durak et al. (2013), Luna Vital et al. (2014), Oseguera-Toledo et al. (2011), Valdez-Ortiz et al. (2012), Mojica, Chen, and Mejía (2015)
Bromelain/Plant-derived enzyme (E.C. 3.3.22.33)	37 °C, pH 8.0	Negro 8025, Pinto Durango	Oseguera Toledo et al. (2016), Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano (2015)
Papain/Plant-derived enzyme (E.C. 3.4.22.2)	37 to 60 °C, pH 6.5	Navy, black, small red	Rui et al. (2012), Rui et al. (2013)
Enzymatic preparations			
Alcalase® (E.C. 3.4.21.62)	40-50 °C, pH 7.0-9.0	African yam, black, Pinto Durango, Negro 8025, Azufrado Higuera, Azufrado Noroeste, Azufrado Regional, navy, small red	Evangelho et al. (2017), Ajibola et al. (2013), de Souza Rocha et al. (2014), de Souza Rocha et al. (2015), Garcia-Mora, Frias, et al. (2015a), Oseguera Toledo et al. (2016), Oseguera-Toledo et al. (2011), Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano (2015), Rui et al. (2012), Rui et al. (2013), Valdez-Ortiz et al. (2012)
Flavourzyme® (3.4.21.62)	50 °C, pH 7.0-8.0	Common bean	Rui et al. (2012)
Corolase® (E.C. 3.4.21.62)	40-50 °C, pH 8.0	Common bean	Garcia-Mora, Frias, et al. (2015a)

Plant-derived enzymes such as bromelain and papain are also used to produce bioactive peptides from common beans. Bromelain is a proteolytic enzyme found in the stems of pineapples (*Ananas comosus*), that hydrolyzes proteins at 37 °C and pH 8.0. The preferential cleavage site for bromelain is Bz-F-V-R-/-NHMec. resulting in the production of different peptide sizes with physiological activities (Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015, Oseguera Toledo et al. 2016). On the other hand, papain is obtained from papaya (*Carica papaya*) and has broad specificity for peptide bonds, but preference of an amino acid bearing a large hydrophobic side chain, however, hydrolysis temperature rises to 65 °C while pH states at 6.5 to produce peptides from beans proteins (Rui et al. 2012, Rui et al. 2013)

Enzymatic preparations that are obtained from diverse microorganisms (*Bacillus subtilis*, *Geobacillus stearothermophilus*, *Bacillus thermoproteolyticus*, among others) can also be used to produce bioactive peptides, with a broad peptidic bond specificity, but showing a preference for a large uncharged residue.

Alcalase® is a subtilisin that has been used to hydrolyze protein isolates from common bean cultivars (Ajibola et al. 2013, Evangelho et al. 2017, de Souza Rocha et al. 2014, de Souza Rocha et al. 2015, Garcia-Mora et al. 2014, Garcia-Mora, Peñas, et al. 2015, Oseguera-Toledo et al. 2011, Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015, Oseguera Toledo et al. 2016, Rui et al. 2012, Rui et al. 2013, Valdez-Ortiz et al. 2012) has its optimal conditions varying at 40 – 50 °C and pH 7.0 – 9.0. Flavourzyme®, another subtilisin, shares optimal conditions with Alcalase® (50 °C and pH 7.0 - 8.0), demonstrating that this enzyme can also be used to produce peptides from common beans (Rui et al. 2012).

There are other infrequently used commercial enzymes such as Thermolysin®, whose best reaction conditions (55 °C and pH 9) were used to produce peptides with biological activity (Valdez-Ortiz et al. 2012). Most of the enzymes used for bioactive peptides production are pepsin and pancreatin; however, these enzymes are also used to test the

activity of peptides obtained with commercial and plant-based enzymes under simulated gastrointestinal conditions.

2.2.2 Microorganisms

The conversion of proteins into bioactive peptides is usually called fermentation, where complex substrates are made into simple compounds, in a process mediated by microorganisms or enzymes. Technologies such as Solid-State Fermentation (SSF) and Submerged Fermentation (SmF) lead the industrial-level production of bioactive peptides. These techniques have been further optimized based on various process conditions, substrates and organisms used during the fermentation (Subramaniyam and Vimala 2012b).

SmF uses liquid substrates (molasses and broths) and is best suited for microorganisms that require high moisture. The fermentation products are secreted into the fermentation broth (Subramaniyam and Vimala 2012b).

Proteolytic microorganisms have also demonstrated that they can be used to produce bioactive peptides in fermentative processes. For instance, a strain of *B. subtilis* was used to obtain peptides with antioxidant and antihypertensive activity from kidney bean (Limón et al. 2015).

Lactobacilli are microorganisms with a proteolytic activity that has been used in SmF for the production of bioactive peptides from kidney bean (Limón et al. 2015), mungbean (Wu et al. 2015) and pinto bean (Jakubczyk et al. 2017).

The studies involving SSF are performed with *B. subtilis* strains, while *Lactobacillus spp.* are used in SmF, in both cases to produce bioactive peptides.

2.2.3 Germination

Germination is the process where the embryonic axis of a seed returns to its development after being interrupted by maturity (Paucar-Menacho et al. 2010). Germination not only improves protein digestibility and protein efficiency ratio but also increases soluble protein due to peptide production. These peptides exert health benefits such as the reduction of some inflammation markers, improvement in the antioxidant capacity of young mung bean sprouts (Świeca and Gawlik-Dziki 2015), risk reduction in the development of type-2 diabetes (de Souza Rocha et al. 2015), as well as changes in anti-inflammatory and antioxidant activities of germinated beans hydrolyzed followed by enzymatic hydrolysis (López-Barrios, Antunes-Ricardo, and Gutiérrez-Uribe 2016).

2.3 Documented Biological Activities of peptides

Beans are a rich source of peptides that have demonstrated diverse biological activities as well as beneficial effects on the consumer's health (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014). These peptides often exhibit antioxidant and angiotensin I-converting enzyme inhibition. Other infrequent activities, for instance, hypoglycaemic, hypocholesterolemic, anti-inflammatory, have also been reported from protein hydrolysates of common beans. Table 2.2 shows the documented activities of common beans from the last 6 years up to date.

Table 2.2. Sources and activity of bioactive peptides obtained from common bean proteins**.**

Activity	Source	Reference
Angiotensin I-Converting Enzyme (ACE) inhibitory activity	African yam bean	Ajibola et al. (2013)
	Pinto bean	Betancur-Ancona et al. (2014); Garcia-Mora, Frias, et al. (2015a)
	Kidney bean	Limón et al. (2015)
	Small red bean	Rui et al. (2012); Rui et al. (2013)
	Sulphur yellow bean	Valdez-Ortiz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy (2012)
	Adzuki bean	Durak, Baraniak, Jakubczyk, & Świeca, 2013
Antioxidant activity	Jamapa	Carrasco-Castilla et al. (2012b); Carrasco-Castilla et al. (2012a)
	Adzuki	Durak et al. (2013)
	Black	Evangelho et al. (2017); López-Barrios, Antunes-Ricardo, and Gutiérrez-Uribe (2016)
Antioxidant	Pinto	Garcia-Mora, Frias, et al. (2015a); Ngoh and Gan (2016)
	Mung	Wongekalak et al. (2011)
	Sulphur yellow	Valdez-Ortiz et al. (2012)
Hypoglycaemic activity	Common beans	Oseguera Toledo et al. (2016); Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano (2015)
Hypocholesterolaemic and fat-lowering	Common beans	Ramírez-Jiménez et al. (2015)
Anti-inflammatory activity	Common beans	Garcia-Mora, Frias, et al. (2015a); Oseguera-Toledo et al. (2011); López-Barrios, Antunes-Ricardo, and Gutiérrez-Uribe (2016)
Anticancer and antiproliferative activity	Common beans	Chan, Zhang, and Ng (2013); Luna Vital et al. (2014); Luna-Vital, González de Mejía, and Loarca-Piña (2016)
	Mung bean	Wongekalak et al. (2011)
	Chickpea	Girón-Calle, Alaiz, and Vioque (2010); Xue et al. (2015)
	Soybean	Krishnan and Wang (2015)

2.3.1 Antioxidant and Angiotensin I-converting Enzyme (ACE) Inhibitory Activity

In vitro antihypertensive activity is usually reported as angiotensin I-converting enzyme (ACE) inhibition and expressed as half-maximal inhibitory concentration (IC_{50}). Several methods are used to measure *in vitro* antioxidant activity, such as free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, Trolox equivalent antioxidant capacity (TEAC) or the scavenging activity of 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic-acid) (ABTS).

Evangelho et al. (2017) analyzed the enzymatic (Alcalase® and pepsin) hydrolysis of black bean protein isolates and evaluated the degree of hydrolysis and antioxidant activity by ABTS and DPPH scavenging. Pepsin hydrolysates showed a higher degree of hydrolysis (27 %) and DPPH scavenging activity than the black bean protein concentrate and Alcalase® hydrolysates. Conversely, Alcalase® hydrolysates showed higher ABTS scavenging at 30 min than the pepsin treated hydrolysates. The lower degree of hydrolysis of Alcalase® hydrolysates (23 %) may favor the antioxidant activity of the hydrolysate.

The antioxidant and ACE inhibitory activities of peptides obtained with Alcalase® hydrolysis of African yam bean protein isolate was reported by Ajibola et al. (2013). After hydrolysis, hydrolysates were separated into six fractions with ACE inhibitory activities ranging from 54.86 % to 83.03 % achieving values of antioxidant activity such as 40 % DPPH, 20 % hydroxyl radical, 65 % superoxide radical scavenging activities and higher ACE inhibitory activity (60 % inhibition) than non-hydrolyzed proteins (Ajibola et al. 2013).

Adzuki bean protein fractions (albumins, globulins, prolamins, and glutelins) have shown ACE inhibitory and antioxidant activities after a simulated *in vitro* pepsin digestion during 2 hours at 37 °C and pH 2.5, followed by pancreatin and bile extract during 1 hour at 37 °C and pH 7.0. After digestion, the prolamin fraction had the highest ACE inhibitory activity (IC_{50} of 0.17 mg/ml), ABTS antioxidant activity (IC_{50} of 0.06 mg/ml) and an IC_{50} of 0.02 mg/ml by DPPH method (Durak et al. 2013).

Protein extract of navy, black and small red beans hydrolyzed with Alcalase®/Flavourzyme® and Alcalase®/papain demonstrated ACE inhibitory activity with an IC_{50} of 68 µg, 83 µg and 78 µg protein/ml, respectively (Rui et al. 2012). A three-step purification process of small red bean including ultrafiltration, gel-filtration and preparative reverse phase high-performance chromatography (RP-HPLC), lead to an octapeptide (PVNNPQIH) that yielded an IC_{50} of 206 µM, however, further studies are required to investigate the *in vivo* behavior of this fractions (Rui et al. 2013). Conversely, peptides obtained from African yam bean proteins, after enzymatic hydrolysis with Alcalase®, 50 °C, pH 9, 4 h displayed ACE inhibitory (IC_{50} 0.1 mg/ml) and antioxidant properties; 35% scavenging DPPH (at 1 mg/ml concentration), a similar scavenging superoxide radicals and up to 70% of Fe^{2+} chelating activities. RP-HPLC purification increased all activities (Ajibola et al. 2013).

Protein enzymatic hydrolysis of pinto bean with Alcalase® and Savinase® at 50 °C and pH 8.0 released bioactive peptides with an ACE IC_{50} of 0.22-0.26 mg/ml and 326-345 mmol TEAC/g of radical scavenging activity (Garcia-Mora, Frias, et al. 2015a). On the other hand, SSF and SmF (48 and 96 h) has been applied to the production of water-soluble extracts containing hydrolyzed proteins and phenolic compounds from the pinto bean. Both, SSF and SmF extracts showed antioxidant activity (508-541 µg Trolox equivalent/g) but only SmF extract exhibited potential antihypertensive activity (>90% ACE inhibition), although phenolic compounds may contribute to these activities. These results lead to promote pinto bean proteins as potential functional ingredients in the design of healthy foods (Garcia-Mora, Frias, et al. 2015a).

A detailed study by Valdez-Ortiz et al. (2012) evaluated the antioxidant and ACE inhibitory activities of Azufrado beans (Azufrado Higuera, Azufrado Noroeste and Azufrado Regional) protein isolates, hydrolyzed with Alcalase® (50°C, pH 8.0), Thermolysin® (55 °C, pH 9.0) or pancreatin (39 °C, pH 8.0) during two h. The resulting peptides of the three cultivars exhibited ACE inhibitory activity reported as IC_{50} for each enzyme as follows:

Alcalase® (0.19, 0.45 and1.30 µg/ml); Thermolysin® (0.11, 0.35 and 2.78 µg/ml) and pancreatin (60.36, 204, and 320 µg/ml for Higuera, Noroeste and Regional, respectively). Although these beans exerted ACE inhibitory activity, these values are lower than other sources of protein hydrolysates, for instance, an IC_{50} value of 291.4 µg/ml from fermented soybean products (Pyo and Lee 2007) or IC_{50} of 0.011 to 0.021 mg/ml from storage protein from chickpea (Yust et al. 2003). Antioxidant activity by free radical DPPH scavenging activity ranged from 24 to 44 % with the Azufrado Higuera/Alcalase® as the best combination, while the antioxidant activity by the ABTS method exhibited values ranging from 52.2 to 99.9 % for Azufrado Regional/Alcalase® as the best combination to obtain these activities. These results lead the authors to conclude that the ABTS method is more sensitive to determine antioxidant activity of protein hydrolysates due to its scavenging capacity at lower concentrations, four times less than the protein concentration used in DPPH determination (Valdez-Ortiz et al. 2012).

Inadequate postharvest handling and storage under high temperature and relative humidity conditions produce hard-to-cook beans (HTC) which can be used as raw material to produce hydrolysates with functional activities such as ACE inhibitory and antioxidant activities. HTC beans were hydrolyzed extensively with Alcalase®/Flavourzyme® (pH 8.0 at 50°C for Alcalase® and pH 7.0 at 50 °C for Flavourzyme®) and pepsin-pancreatin (pH 2.0 at 37 °C for pepsin and pH 7.5 at 37 °C for pancreatin) during 90 min. The non-hydrolyzed HTC beans showed no ACE inhibitory activity or antioxidant activity, while the IC_{50} value for Alcalase®/Flavourzyme® hydrolysate was 4.5 mg/ml and a TEAC of 8.1 mM/mg and the pepsin-pancreatin treatment achieved an IC_{50} value of 6.5 mg/ml and TEAC of 6.4 mM/mg. These TEAC values are higher than those reported for enzymatic hydrolysates from mungbean (Li et al. 2005). The peptides obtained from HTC beans by Alcalase® and Flavourzyme® hydrolysis can potentially compete with those available in the market-place due to the low cost of HTC beans as a substrate (Betancur-Ancona et al. 2014).

Conversely, if beans are handled with adequate temperature and postharvest conditions, sprouting will occur. Sprouting is a cheap, effective, and simple tool to improve the nutritional and nutraceutical quality of beans. Black beans were germinated in the dark at 26 °C with nebulization of water and sampling was done every 24 hours for five days to produce cotyledon protein hydrolysates followed by enzymatic digestion according to Oseguera-Toledo et al. (2011). Oxygen radical absorbance capacity (ORAC) can also be used to determine antioxidant activity. The antioxidant activity was highest after 1 h hydrolysis at 1 day germination time and raw black beans (1604 and 1769 μmol TE/g, respectively); however, further hydrolysis (2 h) of these two protein hydrolysates reduced the antioxidant activity to 1353 and 1451 μmol TE/g, respectively (López-Barrios, Antunes-Ricardo, and Gutiérrez-Uribe 2016).

In a study by Ngoh and Gan (2016), antioxidant and α-amylase inhibitory peptides were successfully extracted from pinto bean protein isolate using Protamex® at pH 7.5 and 50 °C during 1 h. The low molecular weight (<3 kDa) peptide fraction exhibited the highest ABTS scavenging activity (42%), ferric reducing power assay (FRAP) (expressed as 3.71 mM $FeSO_4$) and α-amylase inhibitory activity (~62 %). In this fraction, 7 peptides (6 to 16 amino acids) were sequenced by mass spectrometry, suggesting that these peptides may potentially contribute to antioxidant and α-amylase inhibitory activities. Five peptides predicted by bioinformatics approaches showed potential ACE inhibitory activity (9.66 to 10.67 %) convincing to pronounce that pinto beans derived peptides have ACE inhibitory activity (Ngoh and Gan 2017).

Peptides generated by enzymatic hydrolysis (37 °C with pepsin [pH 2.5, 90 min] followed by pancreatin [pH 7.5 120 min]) of Jamapa bean whole protein isolate, phaseolin, and lectin fractions were used in Caco-2 cells to evaluate antioxidant activity, obtaining a 71 % inhibition of fluorescein production. The protein hydrolysates showed higher copper and iron-chelating activities for lectin (53 %) and phaseolin (83 %) hydrolysates, respectively

compared to whole protein isolate hydrolysate (40 % for copper chelating activity and 20 % for iron-chelating activity) (Carrasco-Castilla et al. 2012b).

A further study focused on obtaining different molecular weight fractions of Jamapa bean protein isolate and phaseolin was performed. Jamapa bean protein isolate and phaseolin were hydrolyzed with pepsin and pancreatin as described by Megías et al. (2008) and filtered through a 1 kDa cut-off membrane before fractionation by size exclusion chromatography resulting in three fractions (MW 0.43 to 1 kDa) for both protein isolate and phaseolin. The antioxidant activity of peptide fractions was reported as the scavenging ABTS radical cation, β-carotene bleaching method, and iron and copper chelating activities. The highest MW fractions showed the highest copper chelating activity (78 % and 82 % for hydrolyzed bean protein isolate and hydrolyzed phaseolin); while the highest and lowest iron-chelating activity (36 % and 16 %, respectively) was displayed by the high molecular weight protein isolate fraction and the lowest molecular weight for hydrolyzed phaseolin, respectively. The lower molecular weight fractions of hydrolyzed phaseolin showed the highest ABTS radical scavenging activity (999 mM TEAC/mg protein) and inhibition of β-carotene bleaching (80 %), while the heaviest molecular weight hydrolyzed protein isolate fraction, showed the highest ABTS radical scavenging activity (934 mM TEAC/mg protein). These authors concluded that small molecular weight fractions of Jamapa bean protein hydrolysates and phaseolin hydrolysates exerted higher antioxidant activities than the whole protein hydrolysates (Carrasco-Castilla et al. 2012a).

2.3.2 Anticancer and Antiproliferative Activities

Enzymatic digestion of common bean (Azufrado Higuera, Negro 8025, Bayo Madero, and Pinto Durango) non-digestible fraction performed by pepsin followed by pancreatin, resulted in the identification of five peptides sequenced as GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY, which represented 70 % of total protein (Luna-Vital et al., 2014).

Hydrolyzed non-digestible protein fractions showed antiproliferative activity in human colorectal cancer cell lines (HCT-116, RKO and KM12L4). HCT-116 was the most sensitive to Azufrado Higuera bean extract (IC_{50} = 0.53 mg/ml) and RKO to Bayo Madero extract (IC_{50} = 0.51 mg/ml); all four bean extracts had effect on KM12L4 cells. Results suggest that peptides present in common bean non-digestible fraction contributed to antiproliferative effect on human colorectal cancer cells by modifying the expression of markers associated with cell cycle arrest/apoptosis, or markers related to mitochondrial activated apoptosis (Luna Vital et al. 2014). The peptides mentioned above were purified to test the cytotoxic effect of HTC116 cancer cell line and CCD-33Co (human normal colon cells). The peptides were toxic to normal cells. GLTSK and GEGSGA exhibited antiproliferative effects on HTC116 (IC_{50} 134 μM and 156 μM, respectively); however, LSGNK, MTEEY, and MPACGSS were less potent but exerted a cytoprotective effect on CCD-33Co human colon normal cells against peroxide-induced oxidative stress (Luna-Vital, González de Mejía, and Loarca-Piña 2016).

2.3.3 Hypoglycaemic Activity

Hypoglycaemic activity occurs as inhibition of α-amylase, an enzyme that hydrolyzes α-linked polysaccharides such as glycogen and starch, yielding maltose and glucose. On the other hand, hypoglycaemic activity is often reported as inhibition of dipeptidyl peptidase IV, an enzyme that degrades incretin hormones such as glucagon-like peptide (GLP-1) or gastric inhibitory polypeptide (GIP).

Common beans germinated for 24, 48 and 72 h at 25 °C and subjected to gastrointestinal digestion (sequential hydrolysis 37 °C, pH 2, 2 h for pepsin and pH 7.5, 2 h for pancreatin) after Alcalase® hydrolysis produced bioactive peptides that inhibited DPP-IV (IC_{50} 1.2 mg soluble protein/ml). Peptide RGPLVNPDPKPFL obtained at 48 h

germination, one h Alcalase® hydrolysis, and simulated gastrointestinal digestion inhibited DPP-IV by competitive inhibition of the active site (de Souza Rocha et al. 2015).

Pinto Durango bean protein isolate Alcalase® hydrolysate (<1 kDa fraction) inhibited DPP-IV (55.3 %) and α-amylase (76.4 %) activities, while the <1 kDa protein isolate hydrolyzed with bromelain showed higher α-amylase inhibitory activity (50 %) (Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015). Seven peptides (3 to 6 amino acids) were further analyzed by computational modeling to study their interaction with DPP-IV and α-amylase, showing interaction with at least one of the active sites of each enzyme. On the other hand, protein isolates from hard-to-cook bean flour (Pinto Durango and Negro 8025) were hydrolyzed by Alcalase® or bromelain before hydrolysis with pepsin-pancreatin. The hydrolysates obtained increased glucose-stimulated insulin secretion, reduced DPP-IV expression and increased the glucose uptake by *in vitro* insulin-resistant 3T3-L1 adipocytes (Oseguera Toledo et al. 2016).

Raw and precooked protein from five common bean cultivars (Navy, Great Northern, Pinto, Black, and Red) was previously hydrolyzed sequentially with pepsin and pancreatin to generate peptides that inhibit DPP-IV, α-amylase, and α-glucosidase. Peptides obtained from raw and precooked bean hydrolysates showed DPP-IV inhibitory activity (IC_{50} 0.1-1.0 mg protein/ml), with navy beans hydrolysates exhibiting the most potent inhibitors of DPP-IV (IC_{50} 0.093 mg protein/ml). The difference in α-glucosidase inhibitory activity (40-70 %) was not significant among the bean cultivars or processes (raw or pre-cooked) (Mojica, Chen, and Mejía 2015).

The hypoglycaemic activity has also been demonstrated in black bean by studies using in silico, in vitro and in vivo approaches, blocking glucose transporters GLUT2 and SGLT1, reducing intracellular oxygen reactive species about 70 %. Also, in Caco-2 cell lines, glucose absorption decreased by 21.5 % (Mojica et al. 2017).

Peptides released from pinto bean protein isolate hydrolyzed with Protamex® elicited α-amylase inhibitory activity. The effect of pH, hydrolysis time and substrate/enzyme ratio

were also studied. The peptides were fractionated into 6 fractions with the lowest the molecular weight (< 3 kDa) fraction exhibiting the highest α-amylase inhibitory activity (62 %). Sequences of these peptides ranged from 6 to 12 amino acids (690-1128 Da) obtained at pH 6.5, 1 h and substrate/Protamex® ratio of 10 (Ngoh, Lim, and Gan 2016). In a further study, five peptides were selected as potential inhibitors α-amylase using web servers such as Pepsite2 and Peptide Ranker, resulting in PPLHMLP, PLPWGAGF, PLPLHMLP, PPHMGGP, LSSLGMGSLGALFVCM (Ngoh and Gan 2017). These peptides were tested in *in vivo* studies using AR42J cells. The concentrations of peptides with 7 or 8 amino acids ranged from 5.5 to 10.1 mM, but a lower concentration was used for the peptide with 16 amino acids (0.21 to 0.36 mM). The results achieved by Ngoh, Tye, and Gan (2017) shown that the peptide with most amino acids has higher α-amylase inhibitory activity (90 %) at lower concentration (0.21 mM), however, α-amylase inhibitory activity decreased (60 %) with a higher concentration (0.36 mM). These results showed no significative difference between peptides with 7 or 8 amino acids. All peptides tested, showed inhibition of α-amylase secreted by AR42J cells, demonstrated by Michaelis-Menten and Lineweaver-Burk plots, resulting the peptide LSSLGMGSLGALFVCM as the most potent (IC_{50} 0.31 mM) (Ngoh, Tye, and Gan 2017).

2.4 Concluding Remarks

Most reported activities of peptides from pulses are mainly antioxidant and ACE inhibitory, followed by other less-occurring activities such as anticancer and hypoglycaemic; however, there is evidence of hypocholesterolemic activity and anti-inflammatory activity in other pulses such as lupin, groundnuts, and soybeans. Although these activities are attributed to peptides, there is some information about the presence of other compounds in beans that could contribute to these biological activities. There is plenty of information on biologically active peptides mostly obtained from animal sources than vegetables, specifically, common

beans. Most studies regarding bioactive peptides from pulses are made on *in vitro* assays. Therefore, further studies are needed to explore the mechanisms for their *in vivo* action. There is vast information about the peptides obtained used commercially available enzymes and enzymatic preparations, also bacteria with proteolytic activity, however, there is no information on microorganisms such as fungi being used over common bean protein isolates to produce bioactive peptides. There are evidence of peptides regulating some physiological functions, for instance, increase of protease activating (333 to 400 %), inhibiting lipase activity (23 to 87 %), and bile acid-binding activities (18 to 71%) (Ngoh, Choi, and Gan 2017) suggesting that there are another benefits to human health left to explore and to demonstrate. Also, enzymes related to metabolic syndrome can be inhibited by peptides obtained by simulated gastrointestinal digestion of fermented bean seeds (Jakubczyk et al. 2017). About 6 to 9 cultivars of common beans have studies around bioactive peptides; it is well known that there are more than 150 *Phaseolus* genus species that have not been tested as well. This chapter provides useful information to demonstrate the overall potential of common beans as a source of novel ways to access health benefits, as well as research opportunities to find non-documented activities of common bean proteins.

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CHAPTER 3: Angiotensin inhibitory peptides derived from legume seeds proteins

ANGIOTENSIN INHIBITORY PEPTIDES DERIVED FROM LEGUME SEEDS PROTEINS

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ABSTRACT

High blood pressure is considered as a significant health problem worldwide. In addition to numerous preventive and therapeutic drug treatments, important advances have been achieved in the identification of dietary compounds that may contribute to cardiovascular health. Bioactive food peptides are encrypted within the sequence of food proteins but can be released during food processing (by enzymatic hydrolysis or fermentation) or during gastrointestinal transit. Among bioactive food peptides, those with antihypertensive activity are receiving special attention due to the high prevalence of hypertension. These angiotensin-converting enzyme (ACE)-inhibitory peptides are derived from many food proteins, especially legume seeds proteins.

The aim of this work is to describe the structure/activity relationship of ACE-inhibitory peptides, as well as their bioavailability, physiological effects on hypertension demonstrated by both *in vitro* and *in vivo* assays and the main legumes used to obtain them. Finally, current reported strategies for the incorporation of antihypertensive peptides into foods and their effects on both availability and activity are also discussed.

Keywords: Bioactive peptides; Legume proteins; Hypertension; Bioavailability

3.1 Introduction

Cardiovascular diseases, such as atherosclerosis, coronary heart disease, stroke, and heart failure, are a major health concern because they are one of the leading causes of death in most industrialized countries. One of the main risk factors is hypertension, defined by the World Health Organization (WHO) as the exceeding of 90 mmHg for the diastolic arterial pressure, and 140 mmHg for the systolic pressure. Hypertension is a complex and multifactorial cardiovascular disorder. With different mechanisms contributing to a different extent to an individual's blood pressure, the discovery of novel pathogenetic principles of hypertension is challenging (Boschin et al. 2014). However, there is an urgent and unmet clinical need to improve the prevention, detection, and therapy of hypertension in order to reduce the global burden associated with hypertension-related cardiovascular diseases (Delles et al. 2018).

Hypertension is commonly treated with blood pressure lowering drugs, such as the inhibitors of the angiotensin-I converting enzyme (ACE; EC 3.4.15.1), which plays an important role in regulating blood pressure in the renin-angiotensin system. This enzyme catalyzes the conversion of the biologically inactive angiotensin I to the potent vasoconstrictor angiotensin II and inactivates the potent vasodilator bradykinin. Inhibitors bind tightly to the ACE active site, competing with angiotensin I for occupancy; as a consequence, ACE cannot convert angiotensin I to angiotensin II (Rudolph et al. 2017).

The body secretes a proteolytic enzyme called renin from the kidneys as a defense mechanism to decrease blood pressure. The secreted renin catalyzes the conversion of angiotensinogen, which is continuously secreted from the liver to an inactive decapeptide called angiotensin I. The inactive angiotensin-I is then converted to active octapeptide angiotensin II by an ACE enzyme found in plasma (Daskaya-Dikmen et al. 2017).

ACE is a Zn-metallopeptidase synthesized in the lung but can be expressed as an ectoenzyme bound to the membrane in endothelial cells, epithelial or neuroepithelial cells,

in the brain and in soluble form in the blood and different body fluids. The angiotensin I-converting enzyme (ACE) is important in the renin-angiotensin system (RAS) of the human body. This enzyme catalyzes the cleavage of the C-terminal dipeptide from inactive angiotensin I (DRVYIHPFHL) to become active angiotensin II (DRVYIHPF) and inhibits the activity of the vasodilator bradykinin. Consequently, this concerted action endows ACE with a crucial role in controlling blood pressure. (Torruco-Uco et al. 2009).

Hypertension may be controlled by dietary modifications, exercise, calcium channel agonists, angiotensin II receptor blockers, diuretics and ACE inhibitors (Barbana and Boye 2010). Considering that synthetic ACE inhibitors, such as captopril, lisinopril and enalapril have been widely used for the effective clinical treatment of hypertension and heart failure in humans. These synthetic drugs, however, may produce several side effects including diarrhea, coughing, allergies, taste disturbances, skin rashes, impaired renal function, and in some cases excessively low blood pressure, i.e. hypotension (Karami and Akbari-adergani 2019). Therefore, search for natural ACE inhibitors as alternatives to synthetic ones is one of the great interests for safe and economical use of them as pharmaceuticals. Although the effectiveness of the ACE-inhibitory activity may not be as high as those of synthetic drugs, many natural ACE-inhibitory peptides isolated from different food proteins could be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals. There is interest in natural inhibitors, and numerous studies are focused on the production and isolation of ACE-inhibitory peptides from different food proteins. Food derived ACE-inhibitory peptides can be introduced into functional foods or dietary supplements. An alternative solution may be the incorporation into functional foods of specific proteins that can release ACE-inhibitory peptides during digestion. In both cases, their integrity, absorption and bioavailability are relevant issues (Martinez-Maqueda et al. 2012).

Proteins are macronutrients which maintain body functions as well as the structural and functional integrity of the organism. The nutritional quality of proteins depends on the

amino acid composition. Nowadays, there is an increased interest in health-promoting functional foods, whereby consumers hold higher expectations of health-promoting benefits beyond basic nutrition. Dietary proteins provide a rich source of bioactive peptides, which are hidden in a latent state within the native protein. Enzymatic proteolysis can be done by vegetable proteases such as serine proteases, Alcalase®, Flavourzyme®, papain and Bromelain. Protein concentrates or isolates are treated with exogenous proteases in reactors such as batch reactor system (Saavedra et al. 2013). Bioactive or functional peptides are food derived peptides that exert a beneficial effect on body functions and/or positively impact human health, beyond its known nutritional value. These peptides can regulate important bodily functions through their activities, including antihypertensive, antimicrobial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding functions (Chakrabarti, Guha, and Majumder 2018).

3.2 Release and identification of antihypertensive peptides

As aforementioned, bioactive peptides are encrypted in the primary structure of legume seeds proteins, but they can be released by proteolysis *in vitro*, *in vivo* or a combination of both. The *in vivo* release of bioactive peptides involves the gastrointestinal digestion as well as enzymes derived from the human microbiota. On the other hand, the *in vitro* production of bioactive peptides includes the enzymatic hydrolysis of the food protein by endogenous enzymes present in the food matrix as well as proteolysis occurring during food processing or ripening by the action of starter cultures or by enzymes isolated from proteolytic microorganisms. If the peptide sequence is known, it is also possible to synthesize the peptide by chemical or enzymatic synthesis or by recombinant DNA technology (Hernández-Ledesma, del Mar Contreras, and Recio 2011).

3.2.2 Gastrointestinal digestion

It has been recognized that dietary proteins and peptides are susceptible to hydrolysis during the different stages of gastrointestinal digestion, ingestion, and absorption. Once ingested, these proteins and peptides are subjected to hydrolysis by different enzymes present in the gastrointestinal tract such as pepsin, trypsin, chymotrypsin and peptidases at the surface of epithelial cells to release peptides of different sizes. Some of these peptides may exert a direct function in the gastrointestinal tract. However, other peptides can be absorbed to reach target organs and tissues through systemic circulation (Vermeirssen, Van Camp, and Verstraete 2004).

To examine the effect of gastrointestinal proteases on the release and breakdown of ACE-inhibitory peptides from food proteins, simulated gastrointestinal digestion processes have been carried out on various protein sources, as well as vegetal proteins (Shimizu 2004).

Food processing, such as thermal treatment and high hydrostatic pressures, may enhance protein digestibility and peptide release (Lee et al. 2018).

3.2.3 Enzymatic hydrolysis

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. Many studies have demonstrated the release of ACE-inhibitory and/or antihypertensive peptides from food proteins, by hydrolysis with gastrointestinal enzymes, such as pepsin, trypsin, and chymotrypsin.

Besides, to live microorganisms, proteolytic enzymes from bacterial and fungal sources have been also used to generate bioactive peptides from various proteins. The use of commercially available microbial-derived food-grade proteinases to hydrolyze food proteins is advantageous as these enzymes are low-cost and safe, and the product yields are very high.

Recently, the interest of food technologists has turned to the use of different

techniques, such as high pressure and heat-denaturing and power ultrasound, to modify protein structure and increase enzymatic hydrolysis. As compared to the proteolysis at atmospheric pressure, qualitative and quantitative differences were detected in the hydrolysis pattern when enzymatic proteolysis was carried out under high-pressure treatments. Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of several food enzymes (Kadkhodaee and Povey 2008). However, in some cases, enzymes have been found to increase activity following short exposures to ultrasound.

3.2.4 Genetic recombination in bacteria

During the last years, several techniques based on genetic engineering are being developed. One of the challenges of these approaches is the susceptibility of short antihypertensive peptides to degradation by proteases or peptidases. Moreover, the expression products may be harmful to the host, impacting the high-level expression of the gene. This shortcoming has been conquered by the expression of antihypertensive peptides in the forms of a fusion protein or a tandem gene. Although special proteases are needed to release the target active protein, thus increasing the cost of separation and purification after enzymatic hydrolysis. Though promising results are being obtained, to date, the use of genetically modified microorganisms in food products is controversial (Rao et al. 2009).

3.3 In vitro and in vivo assays for inhibition of ace

The angiotensin-converting-enzyme catalyzes the conversion of the biologically inactive angiotensin I to the potent vasoconstrictor angiotensin II. It also inactivates vasodilator bradykinin. The inhibitors bind to the active site of the enzyme, so angiotensin-I is blocked; therefore, ACE will not convert angiotensin I to angiotensin II (Boschin et al.

2014). The nature of this phenomenon leads to the development of different methods using different analytical procedures, substrates and media to measure ACE-inhibitory activity. The most used synthetic inhibitors of ACE are captopril, enalapril, zofenopril, ramipril, lisinopril and fosinopril for effective clinical treatment of hypertension and heart failure in humans (Cushman et al. 1989). These synthetic drugs, however, have several side effects including diarrhea, coughing, allergies, taste disturbances, skin rashes, impaired renal function, and in some cases hypotension. Therefore, the search for natural ACE inhibitors as alternatives to ACE inhibitors is one of the main interests for safe and economical using them as pharmaceuticals. Although the effectiveness of the ACE-inhibitory activity may not be as high as those of synthetic drugs, many natural ACE-inhibitory peptides isolated from legume seeds proteins could be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals.

The search for *in vitro* ACE inhibitors is the most usual strategy followed in the tracking of potential ACE-inhibitory peptides derived from legume seed proteins. *In vitro* ACE-inhibitory activity is generally measured by monitoring the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors (figure 3.1). There are several methods, but those based on spectrophotometric and high-performance liquid chromatography (HPLC) assays are most commonly utilized (Lam et al. 2008). Hippuryl-his-leu (HHL) and furanocryloyl tri-peptide (FAPGG) have been employed as substrates, among others, such as o-aminobenzoylglycyl-p-nitrophenylalanylproline, which has been designed to perform fluorimetric assays (Vermeirssen, Van Camp, and Verstraete 2002). The broadly used spectrophotometric method developed by Cushman and Cheung (1971), based on the determination of the concentration of hippuric acid (HA), by spectrophotometry, at 228 nm, after ethyl acetate extraction, which is formed from HHL by the action of ACE. Although this method is simple and economical, it has some limitations when it is applied to the complex peptide mixtures derived from the hydrolysis of plant proteins, due to different molecules that can be interfering with the method. For this reason, a chromatographic method

based on HPLC coupled with a diode-arrange detector (DAD) is reported following published procedures, with some modifications (Boschin et al. 2014).

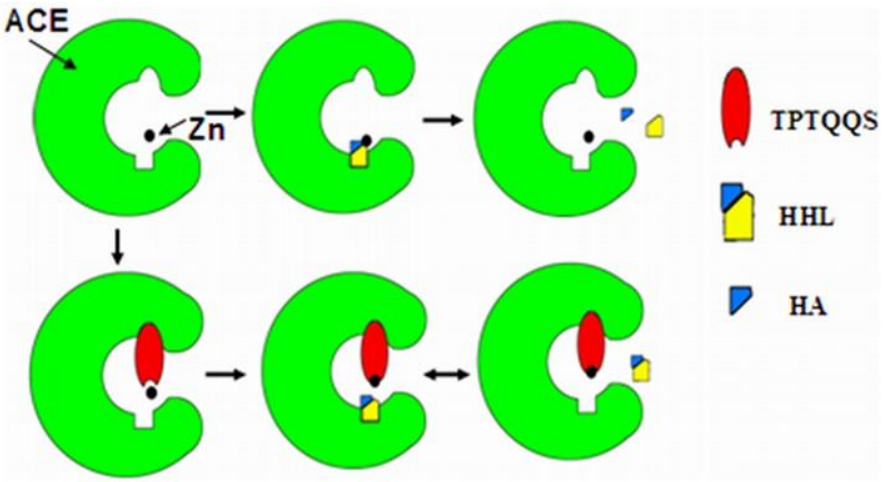

Figure 3.1. Inhibition mechanism of ACE (Ni, 2012).

For the bioactive peptides to have the potential of use as ACE-inhibitors agents, confirmation of *in vivo* activity across an animal or human intervention studies is required. This is because *in vitro* activity does not always reflect *in vivo* effects since the bioactive peptides may be inactivated through structural degradation by gastrointestinal enzymes or during interaction with the target enzymes (ACE). Most of the studies involving *in vivo* inhibition of ACE are performed using normosensitive rats (NMR) or spontaneous hypertensive rats (SHR), the later constitute an accepted animal model to study human hypertension, following intravenous injection and intraperitoneal or oral administration of the synthesized bioactive peptide, prior to its determination by the above-described procedures (Wu, Aluko, and Muir 2002).

Moreover, *in vivo* studies include the evaluation of the effect of ACE inhibitory peptides on arterial blood pressure of SHR. Hydrolysates and peptide fractions obtained from

Mucuna pruriens protein concentrate were studied for their ACE inhibitory activities. The hydrolysate (peptide mixtures) obtained by pepsin-pancreatin (HPP) was the most active with an ACE IC_{50} value of 19.5 μg/mL. At a dose of 5 mg/kg HPP decrease systolic (32.2%) and diastolic (37%) blood pressure in SHR more pronounced than captopril. The peptide fraction <1 kDa from HPP was the most active with an ACE inhibitory of 10.2 μg/mL (IC_{50}). These results indicate that the hydrolysates and peptide fractions of *M. pruriens* would be used as nutraceuticals ingredients for preventing and providing therapy against hypertension (Chalé, 2014).

3.4 Structure-activity relationship

Several structural features that influence the potency of ACE-inhibitory peptides derived from legume seeds proteins have been identified. The activities or functions of the bioactive peptides depend on a set of structure features, such as the amino acid composition, the type of amino acid in C- and N-terminal, the length and weight of the peptide chain, the hydrophobic property, charge character of amino acid, and spatial structure. Recently, it has been reported that artificial neural networks (ANN) and quantitative structure-activity relationship (QSAR) modeling may be used to develop computer models potentially capable of identifying ACE-inhibitory peptides based on structure-activity data (Kamran and Reddy 2018). Besides comparing characterized peptide sequences in databases, bioactive peptide QSAR models could also be used in peptide bioinformatics study. QSAR models are mathematical functions that describe the relationship between activity and chemical structure expressed by variables (Wu, Aluko, and Nakai 2006, Hua, Bo, and Shouzhuo 2006). Such models are applied both to predict the activity of untested chemical structures and to predict the chemical structure of compounds with specific activity (He et al. 2012). Several descriptor variables such as molecular mass and shape, hydrophobicity, charge and electronic

properties have been recognized as critical in this QSAR modeling. The majority of ACE-inhibitory peptides are relatively short sequences containing from 2 to 12 amino acids, which demonstrated from crystallography studies, that the active site of ACE could not accommodate large peptide molecules. Of many ACE-inhibitory peptides identified from different food sources, structure-activity studies indicated that C-terminal tripeptide residues play a predominant role in competitive binding to the active site of ACE. It has been reported that this enzyme prefers substrates or inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions. The most effective ACE-inhibitory peptides identified contain Y, F, W, and/or P at the C-terminal.

The study of García-Mora et al. (2017), helps to clarify the relationship between structure and ACE inhibitory activities of fragments from lentil storage proteins vicilin, convicilin, and legumin, peptides LLSGTQNQPSFLSGF, NSLTLPILRYL, TLEPNSVFLPVLLH, were the most abundant peptides identified. In the C-terminal region positively charged amino acids and hydrophobic amino acid residues are determinant for high ACE inhibitory potency. In addition, molecular docking studies have revealed that C-terminal residues of lentil-derived peptides interact by hydrogen bonds with three ACE residues of the catalytic site, also involved in the binding of Angiotensin II (AngII) and bradikinin (BPPb), (Tyr520 and Lys511 and Gln281). Also, it has been suggested that amino acid L may contribute significantly to increase ACE-inhibitory potential. Furthermore, other branched-chain aliphatic amino acids such as I and V are predominant in highly peptide inhibitors. In addition, structure-activity data suggest that the positive charge of K (ε-amino group) and R (guanidine group) as the C-terminal residue may contribute to the inhibitory potency.

It has been recognized that ACE-inhibitory peptides possess a characteristic pattern (i.e. a similar positive potential located at the C-terminal end) different from that of inactive peptide molecules. For long-chain peptides, it is expected that peptide conformation, i.e. the structure adopted in the specific environment of the binding site, will influence the binding

to ACE. It was also demonstrated that ACE has a requirement for the L-configuration of the amino acid at position three from the C-terminal. Moreover, changes in cis-trans conformations of P at the C-terminal position of an ACE-inhibitory peptide may cause significant changes in its interaction with the enzyme (Segura-Campos, Chel-Guerrero, and Betancur-Ancona 2011, Hernández-Ledesma, del Mar Contreras, and Recio 2011, Martinez-Maqueda et al. 2012). Moreover, no relationship between N-terminal structure and inhibition activity was found.

In recent years, some progress has been made in the bioinformatics study of peptide potential bioactivity. According to bioinformatics prediction, such as comparing active peptide sequences in a database (He et al. 2012). However, these studies were all based on a known sequence of a protein. In fact, the bioinformatics application on peptide is still difficult because most proteins have complicated components or unknown sequences. Therefore, BIOPEP is an open-access database that permits to hypothesize potential biological activities of peptides depending on the presence of some short amino acid sequences. The software proposed one or more activities for each peptide, mostly related to cardiovascular disease or diabetes prevention (Minkiewicz et al. 2012). Lammi et al. (2016) detected peptides that are potentially ACE inhibitors. The active domains LTF, ILP, LLP, and ALEP occur within proteins from seeds of *Lupinus albus* (cultivar Ares). These data are in agreement with the ACE inhibitory activity observed in previous studies (Boschin et al. 2014).

3.5 Bioavailability and clinical studies

The physiological effects of ACE inhibitory bioactive peptides depend on the ability to reach in an active form their target organs; this implies resistance to gastrointestinal enzymes and brushes border membrane peptidases and absorption through the intestinal epithelium. Although peptides were thought to be rapidly metabolized to constituent amino

acids, these studies have demonstrated that several peptides are resistant to these physiological processes and can reach the circulation (Chakrabarti, Guha, and Majumder 2018). Therefore, the different aspects of bioavailability of antihypertensive peptide sequences have attracted a growing interest in the last years. The possibility of modification or breakdown of peptides during gastrointestinal digestion is one of the most important factors to be considered when evaluating potential food-derived peptides for the promotion of human health.

Study of intestinal absorption *in vitro* is another common aim when elucidating the bioavailability. Although only a few legume protein-derived antihypertensive peptides have been detected in the bloodstream of animals and human subjects, bioavailability is central to understand their efficacy and mechanisms of *in vivo* action. Bioavailability is affected by an array of gastrointestinal barriers, such as digestive enzymes, intestinal epithelium, and enterocyte peptidases, which makes very few peptides reach the circulation as an effective form. Peptidase-resistant bioactive peptides can be transported into the bloodstream at concentrations in the micromolar range and remain intact for several minutes to hours to exert bioactivities. It is easy to predict the cleavage of peptides by the primary digestive enzymes using in silico method, whereas transport and stability are hard to elucidate. Molecular size, charge and structural properties, such as hydrophobicity, amino acid composition, and side-chain flexibility, affect the major transport route for peptides. It has been generally claimed that small peptides (< 6 residues) can pass the enterocyte membrane, with the absorption efficiency decreasing as peptide length prolongs (Fan, Liao, and Wu 2019). Peptides are transported by both passive (paracellular and passive transcellular diffusion) and active (transporter and transcytosis) routes. The absorption studies are commonly performed with the monolayer of intestinal cell lines, such as Caco-2 cells, simulating intestinal epithelium, and analysis of peptides and metabolites in serum after *in vivo* and clinical studies (Lin et al. 2017).

Most of the studies investigating the bioavailability of bioactive peptides have used *in vitro* cell models. However, there is still a lack of ex vivo or *in vivo* experiments to determine the effects, bioavailability, plasma concentrations, and pharmacokinetics of bioactive peptides, although studies in humans, rats (including spontaneously hypertensive rats, SHRs), provide evidence that these peptides are absorbed *in vivo*. It should be emphasized that the bioavailability in humans is the ultimate test for a bioactive peptide and should be strengthened in future analyses. In a study conducted by Li et al. (2011), pea protein hydrolysate (PPH) that contained < 3 kDa peptides can effectively lower blood pressure in rat models of hypertension as well as in human subjects. Oral administration of the PPH to SHR at doses of 100 and 200 mg/kg body weight led to a lowering of hourly systolic blood pressure (SBP), with a maximum reduction of 19 mmHg at four h. Whereas, in a 3-week randomized, double-blind placebo-controlled crossover human intervention trial (7 volunteers), significant ($p < 0.05$) reductions (over placebo) in SBP of 5 and 6 mmHg were obtained in the second and third weeks, respectively, for the PPH group. Therefore, Thermolysin® derived bioactive peptides from PPH reduced blood pressure in hypertensive rats and human subjects, likely via effects on the renal angiotensin system.

Using cell-based experiments to examine the kinetics and activities of bioactive peptides is inherently flawed because *in vitro* studies do not take into account the digestive and metabolic processes that occur before peptides reach their targets, and the doses used in such studies are usually higher than those used in human studies (Xu et al. 2019). Due to the activities of peptidases in the human gastrointestinal tract and plasma, many peptides cannot reach their targets to exert their bioactivities. However, most *in vitro* studies examining bioactive peptides transport routes have used concentrations of peptides that are much higher than normal physiological levels. Therefore, further studies are required to elucidate the kinetics of bioactive peptides in the human circulation, human studies and more accurate techniques to examine the amounts, stabilities, kinetics, and bioavailability of bioactive

peptides in the human gastrointestinal tract and plasma are necessary for the future (Matsui 2018).

3.6 Legumes usually used as peptides sources

The first active peptides were obtained from milk proteins, but some have been isolated from plant seeds, such as soybean, pea, rice, sunflower, and wheat (Carbonaro, Maselli, and Nucara 2015). Vegetable-origin proteins are of interest, and legumes are especially promising due to their high protein content and diverse physiological activities in the human organism, including an antihypertensive effect.

Plant legume-derived food ingredients are an important source of nutrients of interest due to its variety, availability, and cost, having an important role in the population diet. In this respect, proteins are one of the main food components due to their nutritional benefits (nitrogen, energy, and essential amino acids) and to their techno-functional roles (formation and stability of foams, emulsions, and gels) in several food systems. However, despite the diversity of available legume sources, only a small number of varieties are intensively cultivated; in this respect, their aggregated value can be increased by the extraction of their components with biological effect. Thus, during the last decades, numerous studies have shown antihypertensive effects, which are caused by bioactive peptides. At present, due to the enormous potential in health improvement, there is a rising interest (scientific and industrial) in the bioprospection and production of bioactive peptides (Tovar-Pérez, Lugo-Radillo, and Aguilera-Aguirre 2019).

Plant proteins have been classified and grouped according to their solubility in albumins, globulins, prolamins, and glutelins. Another way to classify them is according to their function in structural or implicated in defense or storage. The albumins fraction (Albs) is composed of two types of albumin: albumin 1 (Alb-1), which is extracted by solubility in water or in low ionic strength solutions and albumin 2 (Alb-2), which is extracted with water

after Alb-1 and globulin removal. It has been shown that Alb-1 shows a composition of polypeptides with molecular weights (MW) of 18 and 34 kDa; the latter, was identified as a 2S polypeptide, it is particularly high in methionine, lysine, and valine. Also, 22-56 kDa subunits have been identified in Alb-2. From the sedimentation behavior of globulins (Globs), which are storage protein of dicotyledonous seeds, it is known that they are mainly composed of two fractions: 7S and 11S. However, it has been recently reported that 7S globulin is composed instead by three subunits: α (57-68 kDa), α′ (57-72 kDa) and β (42-45 kDa), all of which form a trimer. While the 11S fraction has a hexameric structure with a molecular weight ranging from 343 to 398 kDa and a high content of lysine and valine. Several studies have reported that glutelins (Gluts) are another predominant individual protein group in legume grains (reaching a concentration of up to 49.5 %). This fraction comprises five polypeptide subunits with MW of 20, 22, 35, 50 y 65 kDa, and it has a high content of leucine, threonine, and histidine. Prolamins (Prols) are the less abundant protein fraction found in the legume grains and have been considered as reserve proteins; they are characterized by its abundance of sulfur-containing amino acids and phenylalanine. Prols are composed of three types of peptide units: α, β and γ. The α and β-Prols are subunits with high MW (bigger than 94 kDa), while γ-Prols have a molecular weight ranging from 60 to 67 kDa. Moreover, low MW polypeptides, named Prols-3 (19–26 kDa), have also been identified (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014).

ACE inhibitory bioactive peptides are probably the most studied from food sources since hypertension has become a serious problem due to its close relation with cardiovascular diseases (CVDs). Many legume seeds proteins derived peptides with ACE inhibitory activity released by enzymatic hydrolysis *in vitro* have been discovered. However, to date a comparison of the ACE inhibitory properties of peptides derived from pea, chickpea and lentil to that of dairy proteins has not been adequately investigated, and further research is warranted. Investigation of legumes proteins as a potential source of ACE inhibitory peptides

has primarily focused on soybean (*Glycine max*), there are diverse studies on common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and chickpea (*Cicer arietinum*), to a lesser extent.

In general, most ACE inhibitory peptides described from all kinds of food sources are relatively short sequences containing from 2 to 20 amino acids. Also, several publications, compiled by Norris and FitzGerald (2013), have identified structural features from the C-terminal tripeptide residue that play a predominant role in competitive binding to the active site of ACE. Such features are the presence of bulky hydrophobic residues, aromatic or branched side chains, P at 1 or more positions, positively charged R and K residues in position 2, Y, F, and W residues, and L-configured residues in position 3.

Diverse enzymes and treatments have been used to obtain ACE inhibitory hydrolysates (bioactive peptides mixtures) from common bean protein. The reported IC_{50} of ACE ranged between 0.001 μg/mL and 6.5 mg/mL of common bean protein hydrolysate (Luna-Vital, 2015). Also, there are some factors to consider when comparing the effectiveness of treatments obtaining ACE inhibitor hydrolysates. For instance, seeds pre-treatment prior to hydrolysis, enzymatic treatment, cultivar, seeds integrity and protein fractionation. Regarding enzymatic reactions, it has been reported that the action of two or more enzymes can improve the ACE inhibitory effect of the hydrolysates compared to those hydrolysates obtained with one single enzyme (Boye, 2010).

Moreover, the fractionation of the legume protein hydrolysates is also an important factor that can contribute with the effectiveness inhibiting ACE activity. Correspondingly, in publications where hydrolysates were fractioned by ultrafiltration, the lowest IC_{50} values were found in the lower molecular weight fractions (<1 kDa). The use of several enzymes and the fractionation of hydrolysates on improving the potential of ACE inhibitor are related to the active sites containing the sequence His-Glu-X-X-His, which is located within the cleft of the 2 ACE domains (C- and N-). The active sites are protected by an N-terminal lid that blocks the access of large polypeptides. Thus, small peptides are more effective inhibiting ACE activity (Barbana and Boye 2010, Bhaskar, Ananthanarayan, and Jamdar 2019).

QSAR modeling and substrate docking can be used to asses *in silico* numerous peptide structures for their bioactivity potential. Wu, Aluko, and Nakai (2006) reported a QSAR of ACE inhibitory di- and tripeptides. Based on a 168 dipeptide and 140 tripeptide databases constructed from published literature, two models were computed using partial least-squares regression. The dipeptide model indicated that amino acids with bulky and hydrophobic side chains were preferred by ACE. The tripeptide model suggested that C-terminal aromatic residues, positively charged residues in position 2, and hydrophobic residues at the amino terminus were preferred excepting the positively charged residues in position 2 (or the middle of the chain).

The potency of ACE inhibitory activity is normally measured as an IC_{50} value, which indicates the concentration of inhibitory peptide or hydrolysates needed for 50% inhibition of ACE activity. The next phase to establish if a bioactive peptide is hypotensive is through trials with spontaneously hypertensive rats (SHRs). Finally, to reach the market bearing a health claim, bioactive peptides must be subjected to clinical trials.

Hydrolysates themselves could be used as commercial sources of ACE inhibitory compounds, but it has been noted that inhibitory activity is higher in the purified peptides. So, further research is necessary to identify specific active sequences from legume seeds hydrolysates. There are reports detailing the specific sequences or the molar composition of bioactive peptides inhibitors of ACE, derived from legume seeds. The mentioned peptides from legume seeds account for some of the characteristics described as relevant for the ACE inhibitory activity, such as peptide length (2 to 12 amino acid residues) and the bulky hydrophobic residues in the C-terminal position.

Proteins, regardless of their source or nutritional quality, can generate bioactive peptides (Segura-Campos, Salazar-Vega, Chel-Guerrero, & Betancur-Ancona, 2013). These peptides once released, exhibit several bio-functionalities that have many therapeutic roles and physiological benefits (Zambrowicz et al. (2013) and demonstrate superior activity compared to the parent proteins (Udenigwe and Aluko 2012).

Antihypertensive activity is usually reported as angiotensin I-converting enzyme (ACE) inhibition and expressed as a percentage (%) or half-maximal inhibitory concentration (IC_{50}).

3.6.1 Common beans (*Phaseolus vulgaris*)

Protein extract of navy, black and small red beans hydrolyzed with Alcalase®/Flavourzyme®, and Alcalase®/papain demonstrated ACE inhibitory activity with an IC_{50} of 68 µg, 83 µg and 78 µg protein/mL, respectively (Rui et al. 2012). A three-step purification process of small red bean including ultrafiltration, gel-filtration and preparative reverse-phase high-performance chromatography (RP-HPLC), leads to an octapeptide (PVNNPQIH) that yielded an IC_{50} of 206 µM. However, further studies are required to investigate the *in vivo* behavior of these fractions (Rui et al. 2013).

Protein enzymatic hydrolysis of pinto bean with Alcalase® and Savinase® at 50 °C and pH 8.0 released bioactive peptides with an ACE IC_{50} of 0.22-0.26 mg/mL and 326-345 (Garcia-Mora, Frias, et al. 2015b).

On the other hand, solid-state fermentation (SSF) and submerged fermentation (SmF) (48 and 96 h) have been applied to the production of water-soluble extracts containing hydrolyzed proteins and phenolic compounds from the pinto bean. Both, SSF and SmF extracts showed antioxidant activity (508-541 µg Trolox equivalent/g), but only SmF extract exhibited potential antihypertensive activity (>90% ACE inhibition), although phenolic compounds may contribute to these activities. These results lead to promote pinto bean proteins as potential functional ingredients in the design of healthy foods (Limón et al. 2015).

A detailed study by Valdez-Ortiz et al. (2012) evaluated the antioxidant and ACE inhibitory activities of Azufrado beans (Azufrado Higuera, Azufrado Noroeste, and Azufrado Regional) protein isolates, hydrolyzed with Alcalase® (50°C, pH 8.0), Thermolysin® (55 °C, pH 9.0) or pancreatin (39 °C, pH 8.0) during 2 h. The resulting peptides of the three

cultivars exhibited ACE inhibitory activity reported as IC_{50} for each enzyme as follows: Alcalase® (0.19, 0.45 and1.30 µg/mL); Thermolysin® (0.11, 0.35 and 2.78 µg/mL) and pancreatin (60.36, 204, and 320 µg/mL for Higuera, Noroeste and Regional, respectively). Although these beans exerted ACE inhibitory activity, these values are lower than other sources of protein hydrolysates, for instance, an IC_{50} value of 291.4 µg/mL from fermented soybean products (Pyo and Lee 2007) or IC_{50} of 0.011 to 0.021 mg/mL from storage protein from chickpea (Yust et al. 2003).

Inadequate postharvest handling and storage under high temperature and relative humidity conditions produce hard-to-cook beans (HTC), which can be used as raw material to produce hydrolysates with functional activities such as ACE inhibitory and antioxidant activities. HTC beans were hydrolyzed extensively with Alcalase®/Flavourzyme® (pH 8.0 at 50°C for Alcalase® and pH 7.0 at 50 °C for Flavourzyme®) and pepsin-pancreatin (pH 2.0 at 37 °C for pepsin and pH 7.5 at 37 °C for pancreatin) during 90 min. The non-hydrolyzed HTC beans showed no ACE inhibitory activity or antioxidant activity, while the IC_{50} value for Alcalase®/Flavourzyme® hydrolysate was 4.5 mg/mL and the pepsin-pancreatin treatment achieved an IC_{50} value of 6.5 mg/mL. The peptides obtained from HTC beans by Alcalase® and Flavourzyme® hydrolysis can potentially compete with those available in the market-place due to the low cost of HTC beans as a substrate (Betancur-Ancona et al. 2014).

3.6.2 Lentils (Lens culinaris)

The effect of enzymatic hydrolysis of Alcalase®/Flavourzyme® (pH 7, 50 °C, 1 h for Alcalase®, then pH 8, 50 °C, 1.5 h for Flavourzyme®), bromelain (pH 8, 8 h) and papain (pH 6.5, 4 h) has been studied in two lentils (green and red). Hydrolyzed lentils showed IC_{50} ACE values ranging from 0.053 to 0.190 mg/mL and a degree of hydrolysis of 27 % for papain, 28 % bromelain, and 64 % for Alcalase®/Flavourzyme. Simulated gastrointestinal

digestion (37 °C, pH 2.0 for pepsin and pH 6.5 for trypsin) was applied on lentil proteins obtaining a degree of hydrolysis like bromelain and papain (~27 %), but almost half that of the mixture of Alcalase®/Flavourzyme® for both lentil proteins. Overall, the highest ACE inhibitory activity was obtained with gastrointestinal enzymes hydrolysis on green lentil (IC_{50} 0.053 mg/mL) followed by papain hydrolysis on red lentil (IC_{50} 0.086 mg/mL). Alcalase®/Flavourzyme had the highest degree of hydrolysis but the lowest ACE inhibitory activity (Barbana and Boye 2011).

Lentils were also fermented by SmF either spontaneously or inoculated with *Lactobacillus plantarum*, or by SSF using *Bacillus subtilis* to produce peptides with antihypertensive activity. SSF extracts displayed lower ACE inhibitory activity (39% at 96 h) and lower than SmF inoculated with *L. plantarum*. In contrast, SmF spontaneously fermented lentils improved the ACE inhibitory activity (from 67.5% to 92% at 96 h) probably due to differences involved in the activity/specificity of microorganisms. Although SSF and liquid fermentation provided aqueous functional extracts with potential antihypertensive compounds, those obtained in liquid fermentation have higher health-promoting the potential that could extend the value-added application for lentils (Torino et al. 2013).

The effect of high-pressure treatments has been studied for peptide release from lentil proteins with ACE inhibitory activity. High pressure (300 MPa) enhanced the hydrolytic efficiency (four to five times) of Protamex®, Savinase®, Corolase® 7089, and Alcalase® compared to non-treated protein. However, Corolase® 7089 achieved the highest ACE inhibitory activity (70 %) at 300 MPa, Protamex® at 200 MPa (65 %), Savinase® at 100 MPa (46 %) and Alcalase® at 0.1 MPa (57 %). According to these authors, Savinase® combined with 300 MPa pressure, gave the best ACE inhibitory activity suggesting that this technology can be applied to increase biological activities of lentil hydrolysates through the appropriate protease and pressure level (Garcia-Mora, Frias, et al. 2015b). Moreover, Savinase® without pressure treatment exhibited higher ACE inhibitory activity regardless of

hydrolysis time (56-63 % ACE inhibition) than Corolase® 7089 (28-51 %) and Protamex® (31-46 %).

So far, only three ACE inhibitory peptides (KLRT, TLGHMV, VNRLM) have been characterized from pepsin-pancreatin hydrolysis of lentil protein; however, none were identified when lentil protein was hydrolyzed with Savinase® (Garcia-Mora et al. 2014).

Hydrolysis of red lentil protein fractions such as albumin, legumin, and vicilin with trypsin (24 h, 37 °C, pH 6.5) achieved IC_{50} values of ACE inhibitory expressed as 4476, 2509 and 539 μg/mL for each protein (Boye et al. (2010).

3.6.3 Chickpea (*Cicer arietinum*)

Conditions for Alcalase®/Flavourzyme®, papain, and *in vitro* gastrointestinal simulation were studied to produce bioactive peptides from two types of chickpea (*kabuli and desi*). Desi chickpea proteins hydrolyzed by *in vitro* gastrointestinal simulation showed higher ACE inhibitory activity (IC_{50} of 140 μg/mL) compared to those obtained by digestion with Alcalase®/Flavourzyme® (IC_{50} of 228 μg/mL) or papain (IC_{50} of 180 μg/mL) and *kabuli* chickpea hydrolyzed by gastrointestinal simulation (IC_{50} of 229 μg/mL) and yellow pea (159 μg/mL). Although the study clearly showed that chickpea and pea digested proteins have ACE inhibitory activity, the IC_{50} values obtained for chickpea and pea at the conditions studied are relatively low indicating high ACE inhibitory activity comparable to milk peptides. After ingestion, gastrointestinal enzymes may break down the bioactive peptides, thereby, increasing or decreasing their activity. Resistance to digestive enzymes is desired for effective ACE inhibitors before their entry into the circulatory system and entering the target cells (Barbana and Boye 2010).

3.6.4 Pea (Pisum sativum)

In a study performed by Jamdar, Deshpande, and Marathe (2017), different process conditions were studied to demonstrate the bioactive potential of common legumes. For ACE inhibition, black pea and white pea showed values of ACE inhibitory activity of IC_{50} in the range of 1 – 5 mg/mL. The hypotensive effect of legumes is facilitated by proteolytic enzyme hydrolysis. These values are in the range reported earlier using gastrointestinal enzymes (Barbana and Boye 2010, Jakubczyk et al. 2017).

3.6.5 Soybean (*Glycine max*)

Peptides from Argentinian defatted soy flour protein hydrolyzed with Corolase® (pH 8.0, 50 °C, 24 h) demonstrated to have ACE inhibitory activity. Corolase® is an enzyme mixture consisting of chymotrypsin, elastase, dipeptidase, tryptic and aminopeptidase activities, carboxypeptidase A1, A2 and B exopeptidase activities that achieved a 20 % degree of hydrolysis at 10 h. ACE inhibitory activity measured at 8% of hydrolysis had an IC_{50} value of 225 µg/mL; nine peptides were reported with sequences ranging from 14 to 30 amino acids with non-polar carboxyl-end due to their content of hydrophobic amino acids. Hydrophobic structural characteristics have been related to the high ACE inhibitory activity (Coscueta et al. 2016).

A similar study demonstrated the presence of naturally occurring peptides with ACE inhibitory activity in five different commercially available soy-based infant formulae (90% protein isolate) (Puchalska, Marina, & García (2014). Suspensions from these formulae were prepared as suggested by manufacturers for infant ingestion followed by fractionation by ultrafiltration, obtaining a peptide fraction with a molecular weight below 3 kDa which showed ACE inhibitory activity for all formulae (IC_{50} 0.57-18.03 µg/mL). Gastrointestinal digestion with pepsin (pH 2.0, 37 °C, 1 h) and pancreatin (pH 7.0-8.0, 37 °C, 2 h) of the

formulae fractions decreased ACE inhibitory activity (IC_{50} 4.87-59.03 µg/mL). A total of 278 different peptides were identified in the five formulae, 42 common to all of them, highlighting the presence of peptide RPSYT which, although showing moderate ACE inhibitory activity, survives gastrointestinal digestion (Puchalska, Concepción García, and Luisa Marina 2014).

3.6.7 Non-conventional legumes

In a study by Boschin et al. (2014), lupin protein isolate was hydrolyzed with pepsin (37 °C, 18 h, pH 2). Lupin hydrolysates achieved an IC_{50} value of 226 µg/mL and were the highest among other legumes tested, such as pea, soybean, common bean, and lentil. With these results in hand, industrial lupin protein isolate was tested to determine its possible ACE inhibitory activity. The highest ACE inhibitory activity among industrial samples resulted in an IC_5 value of 165 µg/mL (91 % max ACE inhibition).

ACE inhibitory activities of peptides obtained with Alcalase® hydrolysis of African yam bean (*Vigna unguiculata*) protein isolate was reported. After hydrolysis, protein hydrolysates, the highest ACE-inhibitory ($p<0.05$) activity of 58.28% (at 0.1 mg/mL) was reported (Ajibola et al. 2013).

Adzuki bean protein fractions (albumins, globulins, prolamins, and glutelins) have shown ACE inhibitory activity after pepsin digestion for 2 hours at 37 °C and pH 2.5, followed by pancreatin and bile extract during 1 hour at 37 °C and pH 7.0. After digestion, the prolamin fraction had the highest ACE inhibitory activity (IC_{50} of 0.17 mg/mL) (Durak et al. 2013).

Peptides with reported ACE inhibitory activity also obtained from non-conventional legumes and non-leguminous sources can be observed in Table 3.1.

Table 3.1. ACE inhibitory biopeptides released from Non-conventional legume seeds proteins

Legume	Bioactive peptide sequence	Hydrolysis method	Biological activity	IC_{50} (μM)	Molecular weight (Da)	Reference
Vigna radiata	KDYRL	Alcalase	ACE inhibition	26.5	693.35	(Li et al. 2006)
	VTPALR			82.4	655.40	
	KLPAGTLF			13.4	845.68	
Psophorcarpus tetragonolobus	YPNQKV	Papain	ACE inhibition & Antioxidant	78.5%	-	(Yea et al. 2014)
	FDIRA			63.8%		
Cajanus cajan	VVSLSIPR	Fermented with *Aspergillus niger*, a proteolytic fungus isolated from spoiled sweet milk	ACE inhibition	85% was inhibited with 30 μg/ml of the peptide	869.53	(Nawaz et al. 2017)
Parkia speciosa	VLNSNAAPLPN	Alcalase	ACE inhibition & Antioxidant	80.2% was inhibited	< 10,000	(Siow and Gan 2013)
	High amount of hydrophobic AA including, G, V, A, P y L.					
Macrotyloma uniflorum	TVGMTAKF	Alcalase	ACE inhibition	30.3	< 3	(Bhaskar, Ananthanarayan, and Jamdar 2019)
	QLLLQQ			75.0		

3.7 Incorporation of bioactive peptides into food products

Nowadays, there is an increasing demand for healthy and high-quality products with nutraceutical features. Not only leguminous crops provide peptides with ACE inhibitory activity, but also grains, cereals, meat, fish and milk. For instance, brown rice (BR) was fermented (F) before testing and into different yogurt-like products that exerted ACE inhibitory activity. Formulae were coded as follows: crude-BR (F-CBR), soaked-BR (F-SBR), germinated (48 h)-BR (F-GBR48) and germinated (96 h)-BR (F-GBR96). Although fermentation did not modify the proximate composition, it improved the ACE inhibitory activity, among other properties (antioxidant activity, consistency, γ-aminobutyric acid (GABA), and phenolic compounds). ACE inhibitory activity was expressed as a percentage (%) and was improved from 10 to 26 % in non-fermented products to 16 to 61 % in fermented products. F-GBR96 showed the highest ACE inhibition (61 %) and acceptance among panelist (Cáceres et al. 2019).

Soybean yogurt is a functional food made with fermented soymilk with high protein content. In the study performed by Hermanto, Hatiningsih, and Putera (2018), *soyghurt* proteins were hydrolyzed with pepsin (37 °C, pH 4.5) during 2, 4, 8 and 16 h. The highest ACE inhibitory activity was achieved at 16 h hydrolysis (90.2 %) and the highest degree of hydrolysis (51.6 %). Soy foods, such as soy sauce (Nakahara et al. 2009), natto (Gibbs et al. 2004) and miso (Rho et al. 2009) have previously demonstrated ACE inhibitory activity. In a study by Shimakage, Shinbo, and Yamada (2012), 13 peptides were identified with ACE inhibitory activity. Five of the peptides contain Ile-Ile while the other eight contains Phe-Phe-Tyr-Tyr and Trp-Hys-Pro. These peptides are also present in soy-derived foods so that they can be added to other products. The authors conclude that soy-based food provides antihypertensive peptides that could be used in hypertension treatments.

Dry-fermented sausages have exerted ACE inhibitory activity. These dry-fermented sausages are produced in Europe and are considered highly valuable traditional products. In

a study by Gallego et al. (2018) evaluated the difference in three European dry sausages (from Spain, Italy, and Belgium). In their study, Spanish and Belgian dry sausages showed the highest ACE inhibition (around 85 %).

Milk is considered one of the complete food since it contains almost all the essential nutrients necessary for human health and growth. The bioavailability of milk proteins is very high within the gastrointestinal tract, so bioactive peptides are released during the digestion process (Korhonen 2009). Among the commercial dairy products enriched with bioactive peptides are Calpis®, Evolus®, and BioZate®. Calpis® is sour milk related to lowering blood pressure containing peptides IPP and VPP, which are responsible for antihypertensive activity (Singh et al. 2018).

On the other hand, Evolus® has the same peptides with hypotensive activity. However, Evolus® is calcium-enriched fermented milk (produced by Valio Oy, Finland). BioZate® is and hydrolyzed whey protein isolate, manufactured by Davisco, USA. It has different β-lactoglobulins responsible for blood pressure reduction (Singh et al. 2018).

Many of these food products have exerted ACE inhibitory activity in *in vitro* assays. However, it is important to perform *in vivo* studies to analyze if the activity is viable after gastrointestinal digestion. Most of the foods with ACE inhibitory activity are milk-derived or meat-derived. However, little is known about legume-derived meals with ACE inhibitory activity since most of the time, legumes are eaten raw or cooked.

3.8 Future perspectives

Among the different groups of bioactive peptides defined, ACE inhibitory peptides have received special attention, their activity has been tested *in vitro,* animal models and humans, and they have been incorporated into different food products. Controversial results

on clinical studies and the different health claim legislation will contribute to increasing research in this area. In this sense, different studies have been performed to demonstrate the stability of the peptide, absorption, and identification of the active form in the organism. It has been postulated that physiologically relevant concentrations and elimination kinetics will be mandatory for food-derived bioactive components as it is now for pharmaceutical compounds. At the same time, the recent advances in specific analytical techniques able to follow small amounts of the peptides or derivatives from them in complex matrices and biological fluids will allow performing these kinetic studies in model animals and humans. Similarly, advances in disciplines such as nutrigenomic and nutrigenetic will open new ways to follow bioactivity in the organism by identifying novel and more complex biomarkers of exposure and/or of activity. All these advances will be made simultaneously with the knowledge of food technologists since the final formulation of the food product is crucial to ensure activity and bioavailability of bioactive peptides.

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CHAPTER 4: Effect of Instant Controlled Pressure-Drop (DIC), Cooking and Germination on Non-nutritional Factors of Common Vetch (*Vicia sativa spp*)

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Article

Effect of Instant Controlled Pressure-Drop (DIC), Cooking and Germination on Non-nutritional Factors of Common Vetch (*Vicia sativa spp*)

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Abstract: Legumes are widely consumed by humans, being an important source of nutrients; however, they contain non-nutritional factors (NNFs), such as phytic acid (IP_6), raffinose, stachyose, total phenolic compounds, condensed tannins, and flavonoids, that have negative effects on human health. Although vetches (*Vicia sativa*) are widely cultivated, they are not intended for human feeding due to their contents of NNF. Usually, the NNF are removed by cooking or germinating; however, germination is a process that requires extended time, and cooking may compromise the viability of some nutrients. To promote vetches for human consumption, the effect of the Instant Controlled Pressure Drop (DIC) process was studied as an alternative to cooking and germinating to decrease NNF contents. Results showed that compared to raw vetches, DIC treatment reduced total phenolic compounds (48%), condensed tannins (28%), flavonoids (65%), IP6 (92%), raffinose (77%), and stachyose (92%). These results are very similar to the ones achieved by traditional ways of removing NNF.

Keywords: non-nutritional factors (NNFs); vetches (*Vicia sativa* spp.); instant controlled pressure drop (DIC); cooking; germination; total phenolic compounds; tannins; oligosaccharides; phytic acid

4.1 Introduction

According to the Food and Agricultural Organization (FAO), pulses are dry seeds of plants belonging to the *Leguminosae* family. Within the most common pulses, stand out beans, broad beans, peas, chickpeas, cowpeas, pigeon peas, lentils, Bambara beans, lupins, vetches, and other minor legumes (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014). It is known that pulses represent an important source of proteins and nutrients necessary for human feeding (FAO 2016b). Moreover, numerous studies suggest that consumption of legumes may have potential health benefits as reducing the risk of cardiovascular diseases (Padhi and Ramdath 2017), cancer (Mathers 2007), diabetes (Ramdath, Renwick, and Duncan 2016), hypertension (Jayalath et al. 2014), among others. In México, according to FAO, the most consumed legume are common beans (*Phaseolus vulgaris*); nevertheless, they present a low crop yield (FAOSTAT 2017). On the other hand, *Vicia sativa spp.* (common vetch) is not commonly used for human feeding, but is widely cultivated as soil improvement and as feed for livestock due to their high content of proteins and other nutrients (Ribeiro, Teixeira, and Ferreira 2004). Moreover, by comparing to common beans, vetches have a better crop yield (FAOSTAT 2017). However, the use of pulses such as vetches is reduced due to the presence of compounds known as Non-nutritional factors (NNF). NNF are defined as compounds that reduce the nutrient utilization and/or food intake of plants or plant products used as human foods or animal feeds (O Soetan and Oyewole 2009). Among the NNF identified on pulses, phytic acid, oligosaccharides, and phenolic compounds are the most commonly linked to the most undesirable physiological reactions such as flatulence, inhibition of enzymes, or vitamin absorption and low digestibility (Ribeiro, Teixeira, and Ferreira 2004). Phytic acid (IP_6) is one of the most

common heat resistant NNF in plants. IP_6 chelates micronutrients and reduces their bioavailability for monogastric animals, including humans, because of the lack of phytase enzyme in their digestive tract (Gupta 2015). Oligosaccharides, such as raffinose and stachyose, are well known to cause flatulence in mammals that have no α-galactosidase (Pedrosa et al. 2012). These saccharides contain one, two or three galactose units joined to α-1-6 galactosidic linkages. The lack of α-galactosidase leads to the production of flatus gases (H_2, CO_2 and small amounts of CH_4), diarrhea, and discomfort (Pugalenthi, Siddhuraju, and Vadivel 2006). Phenols bind to positively charged proteins, amino acids, and/or multivalent cations or minerals such as Fe, Ca, and Zn in foods, and decrease their digestibility (Suneja et al. 2011).

However, NNF have also shown health benefits as the improving of essential minerals bioavailability (Roberfroid 1997), the reduction of the blood glucose and insulin responses to starchy foods and/or the plasma cholesterol and triglycerides (Thompson 1993), the prevention of kidney stone formation, caries, atherosclerosis, and coronary heart disease as well as against of cancer (Gemede 2014). According to Muzquiz et al. (2007), exposure time, biochemistry, concentration, and interaction with other dietary components, may influence the balance between beneficial or deleterious effects of NNF consumption. Therefore, a relevant challenge is to determine the adequate processing conditions to preserve the adequate amount of NNF in legumes to make the most of the positive effects in human health and to reduce as far the negative effects (Cuadrado et al. 2018)–(Champ 2002, Campos-Vega, Loarca-Piña, and Oomah 2010).

To reduce the NNF of pulses, various treatments such as soaking, fermentation, germination, washing, heating, among others, have been applied (Avilés-Gaxiola, Chuck-Hernández, and Serna Saldívar 2017, Ibrahim et al. 2002, Khandelwal, Udipi, and Ghugre 2010, Vidal-Valverde et al. 1994, Sadeghi et al. 2009, Silva and Braga 1982, Kon 1979). Among all these treatments, heat is the most used, with cooking times between 20 to 120 min. However, the final nutritional value of legumes can be damaged in the function of the

type and intensity of heating (Walker and Kochhar 1982, Xu and Chang 2008, Wang et al. 2010). Then the use of HTST (High Temperature/Short Time) treatments becomes promising to reduce the non-nutritional factors (Pedrosa et al. 2012).

The instant controlled pressure drop technique, better-known by its French acronym Détente Instantanée Contrôlée (DIC) is an HTST treatment. It consists of subjecting a product to a high pressure saturated dry steam (almost between 100 and 1000 kPa according to the product and the objectives) for a short period (seconds), followed by an abrupt pressure drop towards a vacuum (about 5 kPa). This thermo-hydro-mechanical process induces instant auto vaporization of a quantity of the product water, which provokes a controlled expansion and an immediate cooling of treated products, which stops thermal degradation (Mounir and Allaf 2008). The effect of DIC treatment has been previously evaluated by Haddad and Allaf (2007) and Pedrosa et al. (2012) on several NNF of soybean, lupin, lentil, chickpea, and roasted peanut. Their results showed an important reduction of the NNF. Figure 4.1 shows the schematic time-pressure profiles of a DIC processing cycle.

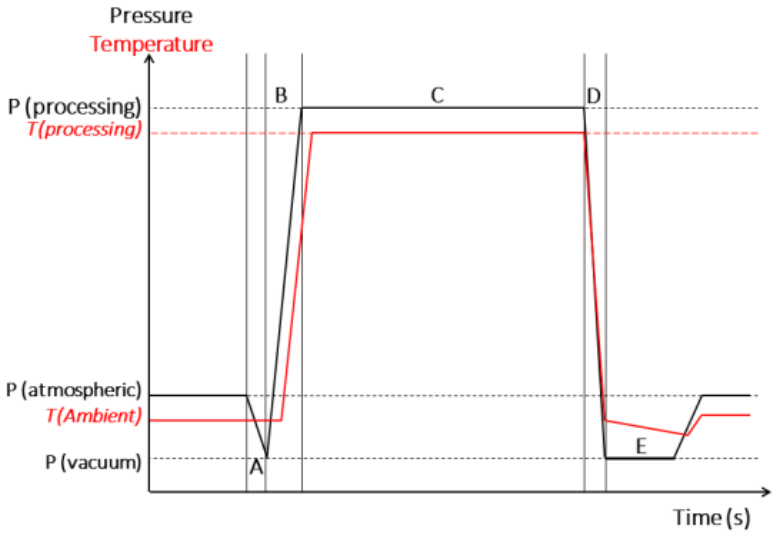


Figure 4.1. Schematic time-pressure profiles of a DIC processing cycle.

On the other hand, germination has also been applied to decrease the NNF of pulses (Jiménez Martínez et al. 2012, Singh et al. 2014, Savelkoul, Van Der Poel, and Tamminga 1992, Sampath et al. 2008). Germination starts when the dry seed begins to take up water and is completed when the embryonic axis elongates (López-Martínez et al. 2017). However, the effect of germination depends on the type of legume and the conditions and duration of the germination process (Savelkoul, Van Der Poel, and Tamminga 1992, Liang et al. 2008, Sampath et al. 2008).

Therefore, this work aims to compare the impact of the instant controlled pressure drop (DIC) technique to cooking, and germination, on the reduction of NNF of vetches, to lay the basis for possible future use as human food.

4.2 Results and Discussion

4.2.1 Chemical Proximate Analysis

Table 4.1 shows the average contents of moisture, ashes, crude fiber, total nitrogen, and ethereal extract of non-treated flours. These results are similar compared to the study of bitter vetch seeds performed by Sadeghi et al. (2009); the slight differences can be attributed to the growing conditions and postharvest handling. Proximate analysis performed on others legume flours (chickpea, pea, common bean and lentils), showed that by comparing these legumes to vetches, the latter presented higher contents of crude fiber (5.69 to 10.4 g/100 g dry matter), ashes (3.12 to 4 g/100 g dry matter) and lipids content (2.34 to 6.73 g/100 g dry matter), and lower protein content (18.5 to 23.7 g/100 g dry matter) (de Almeida Costa et al. 2006).

Table 4.1. Chemical proximate analysis of non-treated vetch flour.

Moisture (%)	15.0 ± 0.5
Ashes (%)	2.5 ± 0.1
Crude fiber (%)	12.1 ± 0.6
Total nitrogen (%)	16.1 ± 0.9
Ethereal extract (%)	5.0 ± 0.9
Nitrogen-free extract (%)[1]	49.3

Values are expressed as means of three replicates ± SD. Calculated by difference.

4.2.2. Effect of Instant Controlled Pressure Drop, Cooking and Germination on Non-nutritional factors of vetches

Total phenolics content (TPC), condensed tannins content (CT), total flavonoids content (TFC), phytic acid content (IP_6), raffinose content, and stachyose content of DIC, cooked, germinated and raw vetches are shown on Table 4.2. Raw vetch flour have shown total phenolics compounds of 191.4 mg eq. of gallic acid/g dry basis; condensed tannins of 12.31 mg eq. of catechin/g dry basis; flavonoids of 16.55 mg eq. of rutin/g dry basis; phytic acid (expressed as mg/g dry basis) of 13.5; raffinose (expressed as mg/g dry basis) of 7.86; and stachyose of 24.67 mg/g dry basis.

4.2.2.1. Effect of Instant Controlled Pressure Drop Treatment on NNF of Vetches

To better evaluate the effect of DIC treatment on the total phenolic compounds, condensed tannins, flavonoids, phytic acid, raffinose, and stachyose of vetch flours, obtained results were expressed as percentage respect to NNF of non-treated vetch flour (Table 4.3).

The most significant reductions for total phenolics (48%), condensed tannins (28%), phytic acid (92%), raffinose (77%), and stachyose (92%) are observed at 0.41 MPa. In the case of flavonoids, the most significant reduction (67.43%) was found between 0.3 to 0.33

MPa and 195 s. For raffinose, the highest reduction (77.42%) was obtained under 0.41 MPa and 78 s.

Table 4.2. Total phenolics content, condensed tannins content, total flavonoids content, phytic acid content, raffinose and Stachyose content of DIC, cooked, germinated and raw vetches.

Sample	TPC [1] (mg/g)	CT [2] (mg/g)	TFC [3] (mg/m)	IP_6 [4] (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)
DIC1	150.25 ± 0.22	10.16 ± 0.90	12.13 ± 0.22	10.40 ± 0.62	4.32 ± 0.03	47.20 ± 1.10
DIC2	129.20 ± 0.45	9.10 ± 0.70	12.35 ± 0.45	8.48 ± 0.71	4.98 ± 0.08	38.50 ± 0.60
DIC3	112.54 ± 0.41	9.41 ± 0.90	5.68 ± 0.04	11.87 ± 0.80	6.67 ± 0.11	53.82 ± 1.40
DIC4	178.96 ± 0.46	11.54 ± 0.70	6.45 ± 0.03	10.04 ± 0.39	4.38 ± 0.07	45.73 ± 1.12
DIC5	99.53 ± 0.17	8.83 ± 0.80	11.17 ± 0.24	0.99 ± 0.11	2.25 ± 0.01	4.41 ± 0.22
DIC6	147.38 ± 0.22	9.96 ± 1.02	11.78 ± 0.14	1.02 ± 0.32	1.78 ± 0.00	4.66 ± 0.33
DIC7	98.38 ± 0.27	10.16 ± 0.81	14.38 ± 0.17	10.38 ± 1.17	4.77 ± 0.45	47.69 ± 0.77
DIC8	164.60 ± 0.22	11.43 ± 0.83	6.01 ± 0.05	5.09 ± 0.72	2.00 ± 0.04	23.05 ± 0.56
DIC9	126.13 ± 0.12	10.64 ± 1.17	5.83 ± 0.03	5.08 ± 0.47	1.89 ± 0.05	23.11 ± 0.14
DIC10	152.74 ± 0.10	10.30 ± 0.66	5.40 ± 0.07	10.41 ± 1.25	4.76 ± 0.03	47.51 ± 0.36
DIC11	161.16 ± 0.54	11.05 ± 0.87	15.11 ± 0.77	6.28 ± 0.16	4.68 ± 0.01	53.15 ± 0.14
DIC12	185.47 ± 0.45	12.79 ± 0.92	13.98 ± 0.87	11.53 ± 2.16	6.89 ± 0.44	28.26 ± 0.22
DIC13	147.76 ± 0.44	10.18 ± 0.60	5.89 ± 0.11	10.49 ± 0.43	2.92 ± 0.01	47.57 ± 0.47

C45	132.45 ± 0.13	10.67 ± 0.33	11.68 ± 1.01	1.65 ± 0.91	0.68 ± 0.02	7.54 ± 0.50
C60	115.80 ± 0.36	10.12 ± 1.81	8.06 ± 0.23	0.55 ± 0.12	0.49 ± 0.02	2.57 ± 0.07
C90	212.26 ± 0.98	10.02 ± 1.27	8.47 ± 0.11	0.59 ± 0.07	0.46 ± 0.05	2.64 ± 0.01
C120	173.22 ± 0.78	9.80 ± 1.39	8.75 ± 0.19	0.63 ± 0.08	0.31 ± 0.06	3.07 ± 0.00
Germinated	136.66 ± 0.78	11.76 ± 0.70	11.68 ± 0.53	0.57 ± 0.08	0.31 ± 0.04	2.51 ± 0.17

[1] Total Phenolics Content; [2] Condensed Tannins Content; [3] Total Flavonoids Content and [4] Phytic Acid Content Values are expressed as means of three replicates. DIC= Instant Controlled Pressure Drop treatments, C= Cooking treatments.

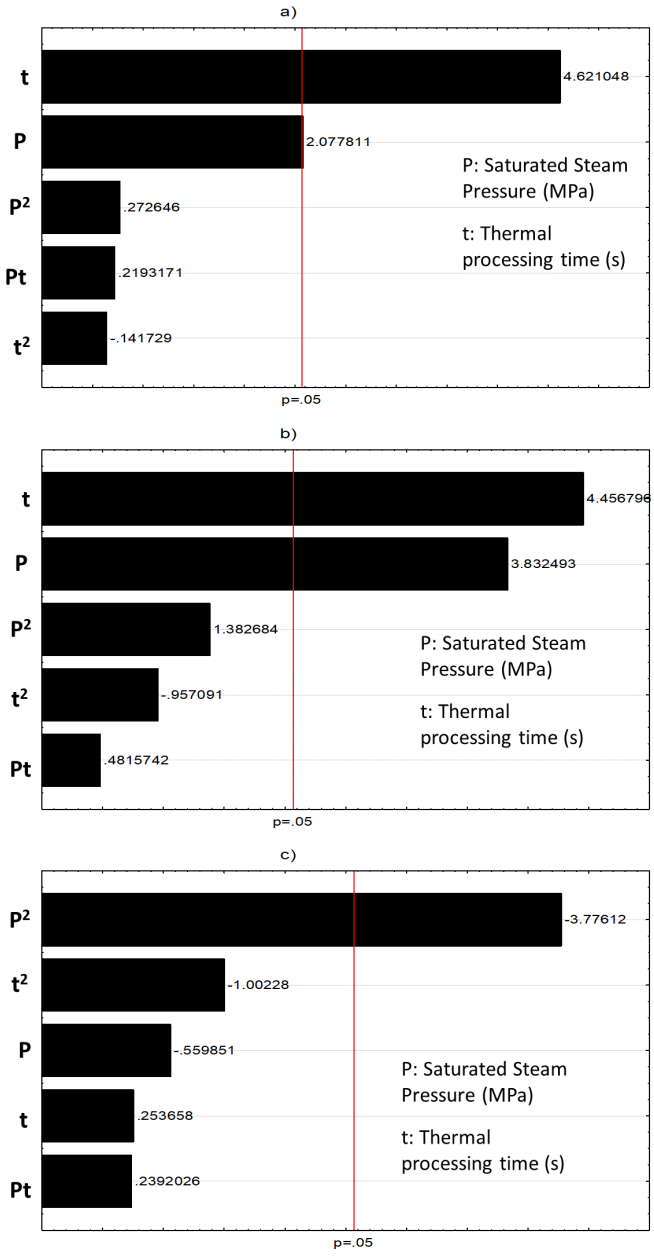
Table 4.3. Effect of DIC over total phenolics, condensed tannins, and flavonoids.

Sample	Total Phenolics Reduction (%)	Tannins Reduction (%)	Flavonoids Reduction (%)	Phytates Reduction (%)	Raffinose Reduction (%)	Stachyose Reduction (%)
DIC1	21.5^{cd}	17.4^{b}	26.7^{abc}	23.0^{c}	45.0^{a}	23.0^{bc}
DIC2	32.5^{de}	26.0^{b}	25.4^{abc}	37.2^{e}	36.6^{c}	37.2^{d}
DIC3	41.2^{ef}	23.5^{b}	65.7^{c}	12.1^{a}	15.1^{b}	12.2^{a}
DIC4	5.6^{ab}	6.2^{ab}	61.0^{abc}	25.6^{d}	44.3^{e}	25.4^{c}
DIC5	48.0^{ef}	28.2^{b}	32.5^{abc}	92.7^{h}	71.4^{g}	92.8^{g}
DIC6	23.9^{cd}	19.0^{ab}	28.8^{abc}	92.4^{h}	77.4^{i}	92.4^{g}
DIC7	48.6^{f}	17.4^{b}	13.1^{a}	23.1^{c}	39.3^{d}	22.2^{b}
DIC8	14.0^{abc}	7.1^{ab}	63.7^{bc}	62.3^{g}	74.5^{h}	62.4^{f}
DIC9	34.1^{de}	13.5^{ab}	64.8^{bc}	62.4^{g}	75.9^{hi}	62.3^{f}

DIC10	20.2^{abc}	16.3^{ab}	67.4^{bc}	22.9^{c}	39.4^{d}	22.5^{bc}
DIC11	15.8^{abc}	10.2^{ab}	8.7^{a}	53.5^{f}	40.5^{d}	13.3^{a}
DIC12	3.1^{a}	-4.0^{a}	15.5^{ab}	14.6^{b}	12.3^{a}	53.9^{e}
DIC13	22.8^{cd}	17.2^{ab}	64.4^{bc}	22.3^{c}	62.9^{f}	22.4^{bc}

Values are expressed as means of three replicates. Letters in the same column indicate significative differences. DIC= Instant Controlled Pressure Drop treatments, C= Cooking treatments.

Figure 4.2 shows the Pareto charts of the significative effects of DIC treatments, and Figure 4.3 shows the surface response graphs of NNFs reduction caused by DIC treatment. Pareto charts (Figure 4.2), shows the impact of DIC operating parameters (saturated steam pressure and treatment time) on the reduction percentage of NNFs. In the case of total phenolics (Figure 4.2a) and condensed tannins (Figure 4.2b), it can be observed that both pressure and time have a significant effect on the reduction of these NNFs. In fact, the higher the steam pressure and treatment time, the higher the reduction of total phenolics and condensed tannins (Figures 4.3a,b, respectively). Figure 2c illustrates the significant quadratic effect of saturated steam pressure on IP_6. By exploring the response surface graph (Figure 4.3c), it can be observed that under the higher and lower values of saturated steam pressure, a significant IP_6 reduction can be obtained. With respect to stachyose, Figure 4.2d shows that both the quadratic effect of saturated steam pressure and the quadratic effect of treatment time have a significant impact on the reduction of stachyose. Moreover, Figure 4.3d shows that an important reduction of stachyose can be achieved under high values of the saturated steam pressure and low values of treatment time. In the case of flavonoids and raffinose reduction, it can be observed that under the selected operating conditions of steam pressure and time, neither P nor t presents a significant effect on both response variables. Then, the best DIC treatment condition to reduce the most NNF of vetches was DIC 5 (0.41 MPa and 312 s).


a)
t
P
P²
Pt
t²
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2.077811
.272646
.2193171
-.141729
P: Saturated Steam Pressure (MPa)
t: Thermal processing time (s)
p=.05
b)
t
P
P²
t²
Pt
4.456796
3.832493
1.382684
-.957091
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P: Saturated Steam Pressure (MPa)
t: Thermal processing time (s)
p=.05
c)
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t²
P
t
Pt
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-.559851
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P: Saturated Steam Pressure (MPa)
t: Thermal processing time (s)
p=.05

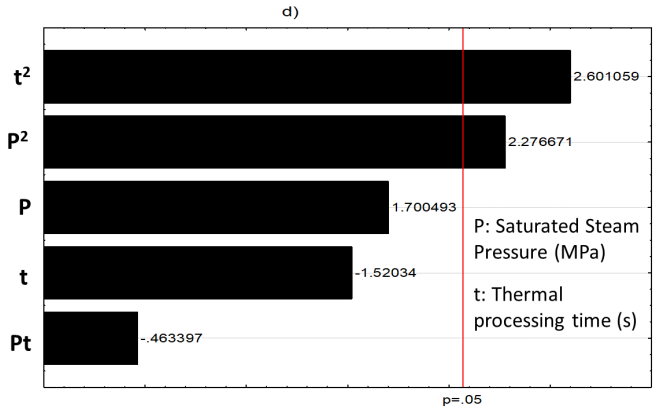
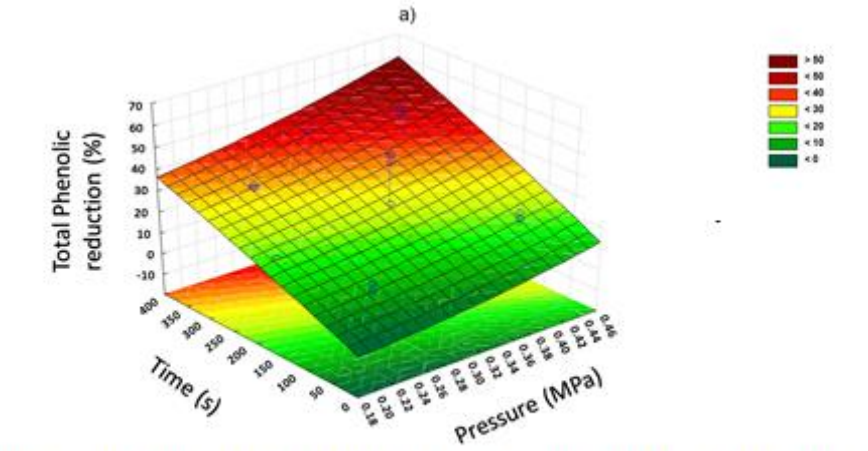
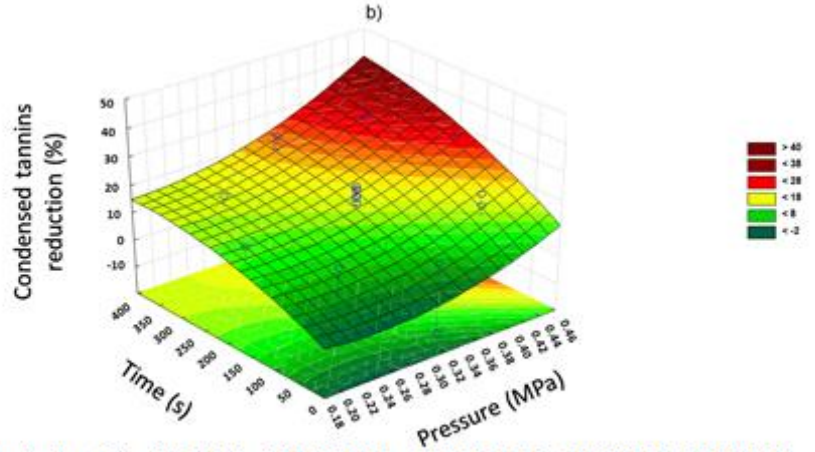


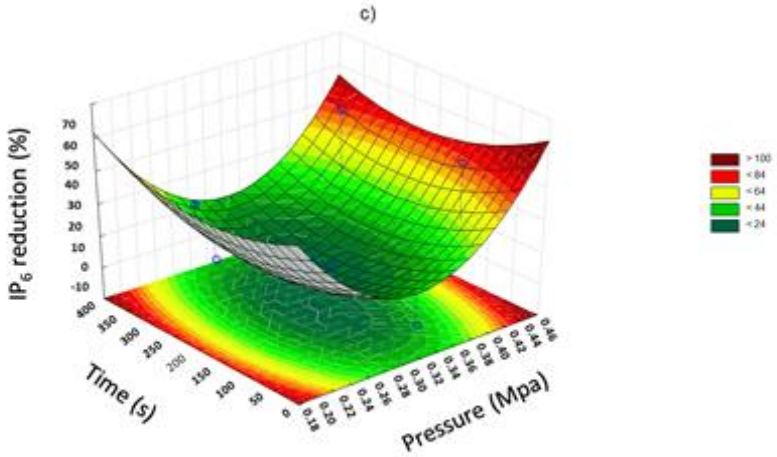
Figure 4.2. Pareto charts of significative effects on DIC treatments. (**a**) Total phenolics reduction, (**b**) Condensed tannins reduction, (**c**) IP_6 reduction, (**d**) Stachyose reduction.



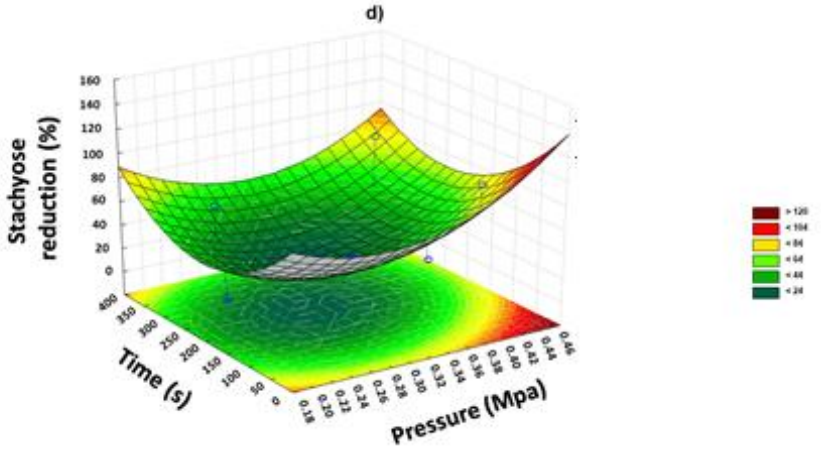
TPC reduction (%) = -0.55881 - 18.69485*P + 105.73812*P² + 0.08891*T - 0.00003*T² + 0.08380*T*P



CT reduction (%) = 15.31835 - 152.82595*P + 312.7827*P^2 + 0.06946*T -0.00012*T^2 + 0.10733*T*P



IP6 reduction (%) = 336.83231 - 1884.76670*P+3012.65222*P^2 - 0.11876*T + 0.00043 *T^2 - 0.18803*T*P



Stachyose reduction (%) = 213.19943 - 1004.39673*P + 1828.27101*P² -0.39109 *T + 0.00113T² - 0.36664*T*P

Figure 4.3. Surface response plot of NNFs reduction by DIC treatment. **a**) Total phenolics, **b**) Condensed tannins, **c**) IP_6, **d**) Stachyose. P = pressure; T = Time.

In the study of Pedrosa et al. (2012), the optimal DIC conditions to achieve a global reduction of NNFs of different legumes were 0.6 MPa and 60 s. In the specific case of oligosaccharides, DIC reduced the raffinose concentration for all legumes in 5%, 7%, 9%, 18%, and 24% for soybeans, lupins, roasted peanuts, chickpea, and lentils respectively. Not the same behavior for stachyose, where for roasted peanuts, lupin, and chickpea DIC allowed a concentration reduction of 11%, 13%, and 14% respectively; however, for lentils and soybean, the concentration of this sugar was increased on 12% and 3% respectively. By comparing to our results, it can be observed that there is a considerable difference between the oligosaccharides reduction of those legumes and vetches (77% for raffinose and 92% for stachyose). This difference could be linked to the particle size of the raw material and the DIC treatment time (1 min vs. 5 min). Whole meals of legumes were submitted to DIC treatment in the study previously described, however, in this work we used vetches flour. Besides, the longer treatment time could allow the increase of sugar leaching on the steam.

According to Haddad and Allaf (2007), their results showed that their applied operating parameters, DIC treatment could achieve different reduction ratio of NNF, being remarked that under high pressure treatment (0.70 MPa) and short time (60 s) the NNF of soybeans and lupins seeds (*L. albus* and *L. mutabilis)* could be reduced to 94%, 16%, and 19% respectively

Furthermore, respect to IP_6 content, the study of Pedrosa et al. (2012) showed that DIC treatment allowed its reduction for all legumes: lupin (91%), lentil (51%), chickpea (45%), soybean, and roasted peanut (34%). By comparing to our results, it could be observed that according to the DIC treatment conditions, IP_6 reduction could vary from 12% to 92%. IP_6 reduction of vetches could be linked to the thermal degradation of these molecules, the formation of insoluble complexes and the changes in their chemical reactivity during the DIC treatment (Rathod and Annapure 2016).

On the other hand, according to Yağcı and Evci (2015), the DIC process increased the total phenolic content of chickpea varying according to the treatment conditions. As higher the processing pressure and treatment time, the polyphenols content was increased, achieving the highest content (twofold respect to the control) at 0.5 MPa and 10 min. By comparing to our results, it could be observed an opposite behavior; this difference could be linked to the intrinsic chemical composition of each legume and their initial moisture content (30% in chickpea and 15% in vetches). Depending on the achieved temperature during DIC treatment, and the initial water content of food polymers, the rate of vapor generated by auto vaporization during the DIC treatment starts acting as a swelling gas on the concerned polymer. Then according to the initial moisture content, DIC treatment can generate structural expansion, which increases the extraction of biomolecules, such as polyphenols and tannins (Allaf and Allaf 2013). Moreover, in this study, phenols reduction could be also linked to its decarboxylation during heating of DIC treatment. The reduction percentage of phenolics compounds achieved by DIC treatments, are accordingly to those reported by Xu and Chang (2008), where not only a reduction of phenolic compounds was reported, but an increase of antioxidant activity in steamed legumes. Since DIC is a HTST treatment that

consist in high pressure steam, it could be that vetches show improved antioxidant activity after DIC treatment.

4.2.2.2. Effect of Cooking on NNF of Vetches

The effect of cooking on the NNF of vetches respect to RM is shown in Table 4.4. The cooking time of 45 min showed the lowest raffinose (91.3%), stachyose (81.7%), and phytates (87.78%) reductions. Phytates reduction showed no significant difference in times above 45 min. The reduction of phytic acid is probably due to hydrolysis during cooking and also caused by the formation of insoluble complexes (Lee et al. 2018).

Table 4.4. Effect of cooking over total phenolics, condensed tannins, and flavonoids.

Sample	Total Phenolics Reduction (%)	Tannins Reduction (%)	Flavonoids Reduction (%)	Phytates Reduction (%)	Raffinose Reduction (%)	Stachyose Reduction (%)
C45	30.8^{a}	13.2^{a}	29.4^{b}	87.8^{b}	91.3^{b}	87.7^{b}
C60	39.5^{a}	17.7^{a}	51.2^{a}	95.9^{a}	93.8^{a}	95.8^{a}
C90	-10.9^{b}	18.5^{a}	48.8^{a}	95.6^{a}	94.2^{a}	95.7^{a}
C120	9.5^{c}	20.3^{a}	47.1^{a}	95.3^{a}	96.1^{c}	95.0^{c}

Values are expressed as means of three replicates. Letters in the same column indicate significative differences.

Raffinose showed higher reduction after 120 min of cooking time (96%), while stachyose showed their highest reduction (95%) with no significative differences among 60–90 min. Ibrahim et al. (2002), reported reductions around 100 % of raffinose and stachyose of chickpea soaked in 0.03 % sodium bicarbonate solution (16 h) followed by two heating methods: cooking at 100 °C for 45 minutes and pressure cooked (1 kg/cm^2) at 120 °C during

20 min. Since oligosaccharides are relatively heated stable, their reduction is mainly attributed to their diffusion into the water during soaking and cooking (Hefnawy 2011, Onyenekwe, Njoku, and Ameh 2000). Moreover, according to the solubility and diffusion rate of each oligosaccharide, sugar losses could be enhanced by increasing the soaking and cooking time and employing different soaking media (Onyenekwe, Njoku, and Ameh 2000).

The highest flavonoids reduction was found at 60 min of cooking (51.17%), with no significant difference among other treatments with high flavonoids reduction. In the case of condensed tannins, no significant differences were found among the four different cooking treatments, showing reductions from 13% up to 20%. Total phenolics reduction showed no significant differences between C45 and C60; moreover, after 60 min of cooking the highest reduction of total phenolics was achieved (39.52%). The reductions are in agreement with those reported for colored beans and chickpeas (Wang et al. 2010). Khandelwal, Udipi, and Ghugre (2010) also reported reductions of total polyphenol and tannins of four legumes cooked until softness (121 °C, 15 psi): lentil (41% and 36%), green gram (41% and 45%), Bengal gram (35% and 28%), and red gram (45% and 34%), respectively. Similar results are reported by Xu and Chang (2008), which showed that boiling legumes reduced phenolic compounds up to 50%, however, for steamed legumes, the reductions were lower (up to 28%), but they also reported an increase of the antioxidant activity due to polyphenols. Then, the reduction of polyphenols and tannins could be attributed to both, its diffusion to the water during soaking and cooking (Kataria, Chauhan, and Punia 1989), and the destruction or transformation of its chemical structures during heat treatment (Burgos et al. 2018).

4.2.2.3. Effect of Germination on NNF of Vetches

The effect of germination on the NNF of vetches respect to RM is shown in Table 4.5. As can be observed, germination boosted the reduction of raffinose (96%), stachyose (95%)

and IP_6 (95%), however, not the same behavior was found for total phenolics, flavonoids, and condensed tannins which increased its concentration on 28%, 27%, and 4% respectively.

Table 4.5. Effect of germination on NNF.

NNF	Reduction (%)	Increase (%)
Total phenolics	-	28.6 ± 3.1
Flavonoids	-	27.2 ± 2.8
Condensed Tannins	-	4.4 ± 0.0
IP6	95.8 ± 0.2	-
Raffinose	96.1 ± 0.6	-
Stachyose	95.9 ± 0.0	-

Values are expressed as means of three replicates ± SD.

Respect to oligosaccharides, similar results were founding on cowpeas, where 85% and 95% stachyose reduction occurred after germination at 35 °C for 48 h and 72 h respectively; moreover, after 48 h of germination at 30 °C stachyose was disappeared (Nnanna and Phillips 1988). According to Martín-Cabrejas et al. (2008) germination increase the activity of the enzyme α-galactosidase, which hydrolyzes the α-1-6-galactosidic linkages, thereby causing an efficient reduction of the α-galactosides content.

On the other hand, phytic acid reduction on germinated vetches could be linked to an increase in phytase activities. In fact, this enzyme makes the phytates soluble to support seedling growth (Cominelli et al. 2018). Similar results were found on chickpea after 48 h of germination, where phytic acid content was reduced on 59% (Khattak et al. 2007). Our results are in agreement with those reported by Mohammed et al. (2017), where the germination significantly decreased the content of phytic acid of lupin (*Lupinus albus*) due to phytase activity, that increases over time.

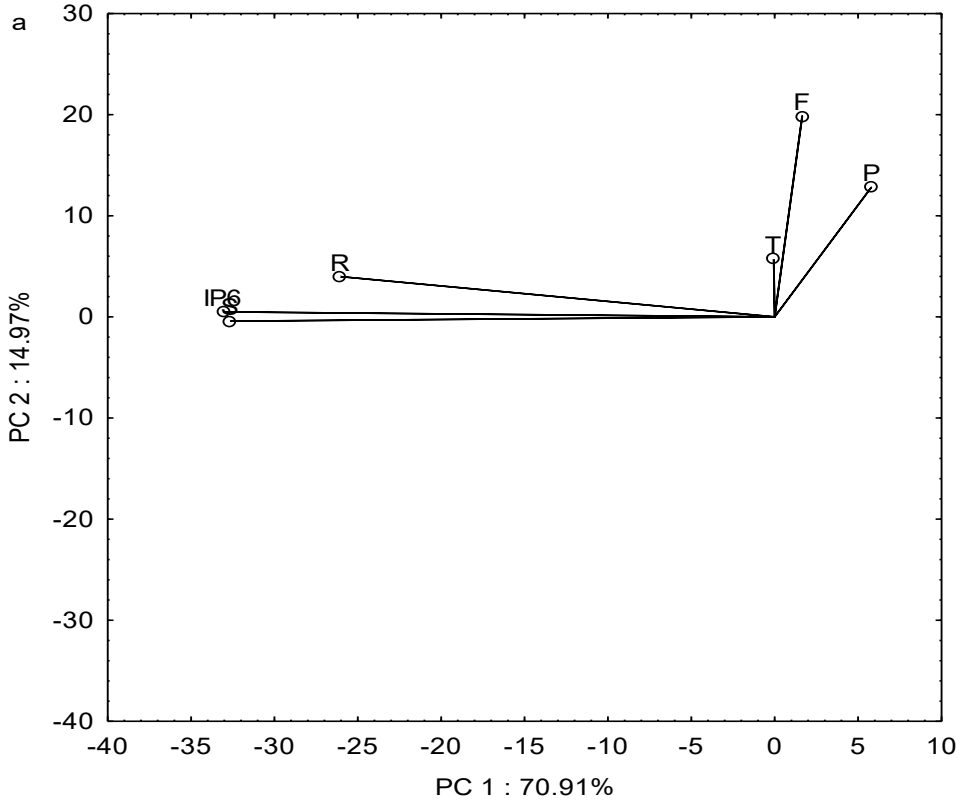
Respect to total phenolics, flavonoids, and condensed tannins content, most studies found that germinated seeds presented a higher content of these molecules than raw seed. For

example, Dicko et al. (2005) reported an increase of total phenolics and flavonoids in different sorghum varieties, due to the activity of enzymes stimulated during germination. Xu et al. (2018) found a significant increase in the total soluble phenolic compounds (free + soluble bound phenolics) of germinated chickpea and yellow pea. Lin and Lai (2006) reported that after long-term germination, the contents of bioactive compounds (total phenolics and flavonoids) significantly increased in black soybeans. According to López-Martínez et al. (2017), glucose is the original precursor for the synthesis of phenolic compounds, and several crucial molecular signaling pathways, including hydrolyzable tannin pathway. Duenas et al. (2009) also reported an increase of total phenolics and flavonoids, on the germination of lupin seeds, however, the authors reported an increase of 84% of phenolics compounds, after 9 days of germination. Then in function of the kind of legume and the germination process (seeds water content, temperature, lightness or darkness, days of germination, oxygen concentration, among others) the final content of polyphenols on germinated legumes could varied significantly. Although germination significantly reduce IP_6, raffinose, and stachyose within our germination time, these reductions can be improved if germination is extended to 9 days or more. This also could lead to higher increase of total phenolics, flavonoids, and tannins, that could improve some beneficial aspects to human health, such as antioxidant activity.

4.2.3. Principal Component Analysis

To reduce the number of response variables, a principal component analysis (PCA) was done. Six components were obtained, where principal component 1 (PC1) and principal component 2 (PC2) described 85% of the variability. Figure 4.4 shows the classification of the three different treatments (cooking, germination, and DIC) applied to vetches in function to PC1 and PC2. Figure 4a shows that IP_6, stachyose, and raffinose reduction have a higher contribution to PC1 construction, while total phenolics, condensed tannins, and flavonoids

have a higher contribution to PC2. The resultant biplot (figure 4b) shows three different groups respect to PC1. The first is formed by cooking treatments (C45, C60, C90, and C120) and DIC treatments (D5 and D6) characterized by the highest IP_6, stachyose, and raffinose reduction. Although D5 and D6 were performed at the same pressure (0.41 MPa), they differ in process time (312 and 78 s, respectively). A second group is only formed by D8 and D9, which is characterized by sharing the same pressure tested (0.24 MPa). These treatments have shown intermediate values of NNF reductions. The third group is formed by D1, D2, D4, D7, D10, D11, D12, and D13, with the lowest NNF reduction with different pressure-time combinations. Germination showed high reduction percentages on IP_6, raffinose, and stachyose, but an increase total phenolics, condensed tannins, and flavonoid content.



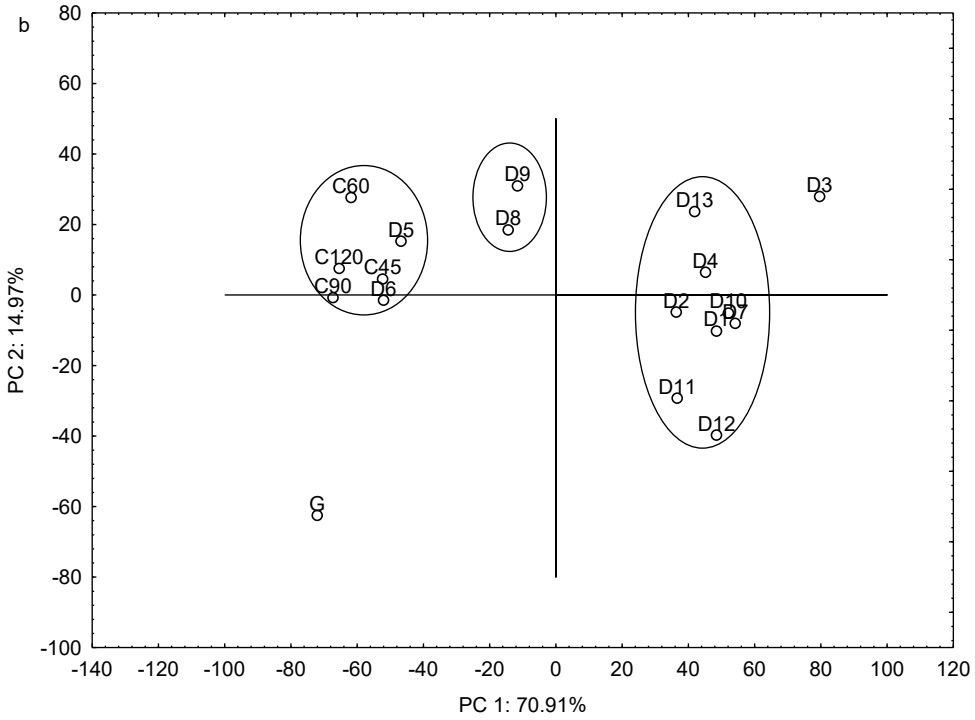


Figure 4.4. Principal component analysis. **a**) Projection of the variables on the factor-plane, **b**) Projection of the cases on the factor-plane. P = Total phenolics, T = condensed tannins, F = flavonoids, R = raffinose, S = stachyose, G = germination, C = cooking, D = DIC treatments.

Since our investigation tried to reduce NNF content on vetches, germination may not be a suitable method to reduce all NNF, however, germination could benefit human health due to the increase of phenolic compounds. On the other hand, D3 has the lowest reduction of IP_6 and raffinose reduction, despite having high flavonoid reduction among DIC treatments, however,IP_6 have been demonstrated beneficial effects to human health such as reduced bioavailability of heavy metals (lead and cadmium), antioxidant activity, among others (Muzquiz et al. 2012).

4.3 Materials and Methods

4.3.1. Seeds

Common vetch (*Vicia sativa*) was kindly provided by Campo Agropecuario Experimental of Tecnologico de Monterrey (Queretaro, Mexico). Seeds were kept under dark and dry conditions until their use.

4.3.1.1. Chemical Proximate Analysis

Moisture (925.10), ashes (942.05), total nitrogen (920.87), ethereal extract (945.39), and crude fiber (962.60E) were carried out in non-treated vetch flour according to AOAC official methods (AOAC 1990). Results were expressed as a percentage (wet basis).

4.3.2. Vetches Treatments

Before any treatment, vetches were divided into four groups of samples: instant controlled pressure drop, cooking, germination and raw material.

4.3.2.1. Instant Controlled Pressure Drop

An Instant Controlled Pressure Drop treatment (DIC) was carried out according to Pedrosa et al. (2012) with slight modifications. One hunderd g of non-treated grounded seeds

were put into the LABIC 0.1 DIC equipment (ABCAR-DIC Process, La Rochelle, France) following a central composite rotatable experimental design (Allaf et al. 2014). Saturated steam pressure (P) and thermal treatment time (t) were the independent variables. The design yielded 13 experiments with four (2^2) factorial points, four-star points ($-\alpha$, -1, 0, $+1$ and $+\alpha$) and five central points (0,0). Pressure values ranged from 0.20 to 0.45 MPa and treatment time from 30 to 360 s. Run experimental values are shown in Table 4.6. After DIC treatment, obtained flours were kept under dry conditions and darkness until their use.

Table 4.6. Vetch flour DIC processing conditions.

Muestra	Presión (MPa)	Tiempo (s)
DIC1	0.33	195
DIC2	0.45	195
DIC3	0.33	360
DIC4	0.33	195
DIC5	0.41	312
DIC6	0.41	78
DIC7	0.33	195
DIC8	0.24	78
DIC9	0.24	312
DIC10	0.33	195
DIC11	0.20	195
DIC12	0.33	30
DIC13	0.33	195

4.3.2.2. Cooking Treatment

Cooking was carried out according to de Almeida Costa et al. (2006) and Barampama and Simard (1995) with slight modifications. First, vetch seeds were soaked in sterile water during 12 h (10% *w/v*), at 25 °C. Then, soaking water was discarded and replaced with fresh water. Seeds were cooked with boiling water (10% *w/v*) for 45, 60, 90, and 120 min, (C45, C60, C90, and C120, respectively). After cooking, seeds were dried at 45 °C until a final

moisture content of 15–20 % dry basis. Finally, all samples were ground and passed through 60 mesh sieve.

4.3.2.3. Germination

Germination was carried out according to de Souza Rocha et al. (2015). Seeds were rinsed with sterile water and kept at 25 °C under light/dark cycles (12 h). Once cotyledons sprouted about 10 cm (5 to 6 days), germinated seeds were also dried at 45 °C until a final moisture content of 15–20 %. Dried samples were milled to pass 60 mesh sieves.

4.3.3. Non-Nutritional Factors Evaluation

4.3.3.1. Methanolic Extracts Preparation

1 g of grounded seeds (treated and not treated) were extracted with 20 mL of methanol with HCl (1%) in agitation (130 rpm) during 2 h under dark conditions at 25 °C. Methanolic extracts were filtered and stored in the dark at -20 °C until analysis (Cardador-Martínez, Loarca-Piña, and Oomah 2002).

4.3.3.2. Total Phenolics Quantification

Total phenolics were estimated according to Singleton, Orthofer, and Lamuela-Raventós (1999) with slight modifications. 150 µL of water was added to 20 µL of methanolic extract and oxidized with 50 µL of Folin-Ciocalteu reagent (0.5 N), then, neutralized with 50 µL of sodium carbonate solution (20% *w/v*). The mixture was incubated for 2 h at 25 °C. After the incubation time, absorbance was measured at 760 nm using an xMark Microplate

Spectrophotometer (Bio-Rad, Hercules, CA, USA). A standard curve of gallic acid (0 to 300 µM) was done. Results were expressed as mg of gallic acid equivalent per g of vetch flour dry weight. Samples were analyzed in triplicates.

4.3.3.3. Flavonoids Quantification

Quantitative determination of flavonoid content was performed by mixing 50 µl of a methanolic extract with 180 µL of distilled water and 20 µL of 2-aminoethyldiphenyl borate solution (10 g/L) (Oomah, Cardador-Martínez, and Loarca-Piña 2005). After 10 min, absorbance was measured at 404 nm using a 96 micro well flat-bottom plate using an xMark Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA). Extracts absorbance was compared with a standard curve of rutin (0 to 0.05 g/mL). Flavonoid content was expressed as mg rutin equivalent per g of vetch flour dry weight. Samples were analyzed in triplicate.

4.3.3.4. Tannins Quantification

Tannins were quantified by spectrophotometric vanillin assay (Deshpande and Cheryan 1985, 1987) adapted to microplates. 20 µL extract were mixed with 100 µL vanillin reagent (vanillin 1% plus 8% HCl 1:1 *v/v*). The mixture was incubated at 30 °C for 30 min. Absorbance was measured at 500 nm (xMark Microplate Spectrophotometer). Results were expressed as mg equivalent of catechin (compared with the standard curve from 0.25 to 1 mg/mL) per g of vetch flour dry weight. Samples were analyzed in triplicate.

4.3.3.5. Oligosaccharides Quantification

5 mL of ethanol (50 % *v/v*) was added to 0.5 g of vetch flour and was homogenized for 1 min in Ultra-turrax (IKA Works Inc., Wilmington, DE, USA). Afterward, centrifugation (HERMLE Z383K, Wehingen, Germany) was carried out at 2 817 g (4 °C, 10 min) recovering supernatant. This process was carried out three times. The supernatants were collected and passed through a solid phase extraction (SPE) SampliQ C-18 column (200 mg bonded silica, 3 mL, 45 µm, 1200 series, Agilent Technologies, , Santa Clara, CA, USA) previously activated with 3 mL distilled water and 3 mL of methanol. Oligosaccharides collected were vacuum-dried in a rotary evaporator and resuspended with 1 mL of distilled water, then filtered through 0.45 µm membrane (Burbano et al. 1999). Extracts were analyzed with a liquid chromatographer (Agilent Technologies 1200 series) with a refraction index detector. A 20 µL injection of oligosaccharides was passed through a Zorbax Carbohydrate column (4.6 × 150 mm, 3.5 µm, Agilent Technologies). The mobile phase contained acetonitrile-water (60:40 *v/v*) and set to a flow rate of 1 mL/min. The concentration was calculated using a calibration curve of raffinose and stachyose (0–2 mg/mL) (Da Costa Leite et al. 2000). Results were expressed as mg per g of vetch flour dry weight.

4.3.3.6. Phytates Quantification

For phytates determination, 10 mL of HCl 0.5 M was added to 0.5 g of vetch flour and homogenized in Ultra-Turrax (IKA Works Inc.) during 1 min; then, mixtures were centrifugated at 2 817 g (4 °C, 10 min) recovering supernatant. This procedure was done three times. The total supernatants collected were passed through a strong anionic exchange (SAX) column (100 mg bonded silica, 1 mL, 45 µm) (Agilent Technologies 1200 series) activated with 5 mL methanol, then 5 mL HCl 0.5 M. The filtrate was discarded, and the phytates retained in column were eluted with 2 mL HCl 2 M, vacuum-dried in a rotary evaporator and resuspended with 2 mL of a solution containing 51.5 mL methanol, 48.5 mL distilled water, 1.6 mL of tert-butyl ammonium hydroxide (TBNOH) (Fluka Analytical,

Charlotte, NC, USA), 0.2 mL sulfuric acid 5 M and 1 mL formic acid (91%) and filtered through a 0.45 µm membrane prior to vial incorporation. A 20 µL injection of the filtered solution was passed through a reverse phase (Zorbax Eclipse XDB C-18 column, 4.6 × 150 mm, 5 µm) for HPLC analysis using a diode array detector. The mobile phase was the same solution used to resuspend phytates, with a flow rate of 1 mL/min. The identification of phytic acid was done by comparing the retention time of sodium phytate (IP_6) standard (Graf and Dintzis 1982). IP6 was expressed as phytic acid mg per g of vetch flour dry weight.

4.3.3.7. Statistical Analysis

Statistical Analysis was performed using the Statistica software (TIBCO Software Inc., Palo Alto, CA, USA). For each treatment analysis of variance (ANOVA) and multiple comparisons by Tukey's honest significant test ($\alpha < 0.05$) was applied to evaluate any significant difference. In the case of the experimental design of DIC treatment, statistical analysis was also performed by the surface response methodology. Finally, for all the responses, principal component analysis (PCA) was also performed to visualize the distance and relatedness between treatments.

4.4. Conclusions

To improve the nutritional profiles of pulses, this study focused on the effect of Instant Controlled Pressure Drop technology (DIC), cooking and germination on the reduction of IP_6, raffinose, stachyose, total phenolics, flavonoids and condensed tannins of vetches. By cooking, it was possible to reduce significantly the NNFs of vetches, however long thermal treatments could cause adverse effects on nutrients such as lipids degradation or vitamin loss. Germination also removed IP_6 and oligosaccharides (raffinose and stachyose) well and, it

also may lead to an increase of total phenolics, flavonoids, and condensed tannins. This could be beneficial since total phenolics, flavonoids, and condensed tannins have been demonstrated to increase some important bioactivities for human health, for instance, antioxidant activity. Moreover, according to the literature, the selected operation conditions during germination directly impact the NNFs content. Therefore, further studies are needed to evaluate the effect of the main variables during germination such as seed water content, lightness or darkness, temperature, and days of germination. The instant controlled pressure drop (DIC) method was an effective technology to reduce NNFs of vetches in only 5 minutes, getting similar results to cooking, which exerted the highest reductions. Further studies must be performed to evaluate the effect of the initial moisture content of seeds, the particle size, and the possibility to couple DIC process in tandem with soaking and germination, as well as other novel technologies such as ultrasound and microwaves to achieve optimal NNFs levels. Moreover, DIC can be easily adapted at an industrial scale. Nevertheless, in all the cases, more studies are needed to evaluate the effect of cooking, germination and DIC treatment on antinutritional factors with protein nature, and the effect of these treatments over the bioactive compounds present in vetches.

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Sample Availability: Samples are available from the authors.

CHAPTER 5: Determination of the degree of hydrolysis, antioxidant activity and angiotensin-converting enzyme inhibitory activity of vetch (*Vicia sativa*) protein isolate fermented with three fungal strains

5.1. Introduction

Bioactive peptides can be defined as amino acid sequences that are encrypted and inactive in parent proteins. According to Korhonen and Pihlanto (2006), the original protein should be hydrolyzed to release peptides that can exert biological activity, for instance, antioxidant activity and inhibition of the angiotensin-converting enzyme (ACE). This hydrolysis occurs in different ways such as food manufacturing, *in vitro* proteolysis and food fermentation.

In *in vitro* studies, protein hydrolysis is carried out by different proteases, for instance, gastric enzymes, plant-derived proteases and commercial enzymatic preparations. Gastric enzymes such as pepsin (Evangelho et al. 2017, Oseguera-Toledo et al. 2014, Luna Vital et al. 2014); plant-derived proteases, for instance, bromelain and papain (Barbana and Boye 2011), as well as commercial enzymatic preparations such as Alcalase® (Najafian and Babji 2014, Oseguera-Toledo et al. 2014, Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015); Flavourzyme® (Oseguera-Toledo, Gonzalez de Mejia, and Amaya-Llano 2015); Corolase® (Coscueta et al. 2016), and Thermolysin® (de Souza Rocha et al. 2015), have been widely used for their ability to release peptides from pulses, showing diverse activities, being antioxidant activity and inhibition of the angiotensin-converting enzyme the most occurring.

The use of microorganisms during submerged fermentation could represent a more advantageous way to produce bioactive peptides, due to minimal nutrient requirements, as well as the possibility to produce peptides with more accentuated properties (Martínez-Medina et al. 2019). There are several studies involving submerged fermentation and proteolytic filamentous fungi in food (Hossain et al. 2006, Samarntarn, Cheevadhanarak, and Tanticharoen 1999, Wang et al. 2019, Wang, Law, and Webb 2005).

Pulses have gained attention as a source that provide a wide variety of functional molecules (Martínez-Medina et al. 2019). Common beans (*Phaseolus vulgaris*), lentils (*Lens culinari*), cowpea (*Cicer arietinum),* and chickpea (*Vigna unguiculata*), have exerted antioxidant and antihypertensive activity, due to the hydrolysis of their respective protein isolates, however, little is known about the potential of other pulses, for instance, vetches (Vicia sativa), to produce peptides with biological activity. According to Ribeiro, Teixeira, and Ferreira (2004), vetch is widely cultivated, particularly, for soil improvement and dryland farming, however, vetches are usually intended to livestock rather than human consumption.

This study aims to produce peptides with antioxidant and angiotensin-converting enzyme inhibition, obtained by submerged fermentation with filamentous fungi isolated from vetch protein isolate.

5.2 Materials and methods

5.2.1 Raw materials

Common vetch (*Vicia sativa*) was kindly provided by Campo Agropecuario Experimental del Tecnologico de Monterrey (CAETEC). Seeds were ground until the flour passed through #60 mesh. Vetch flour was kept in dark and dry conditions until analysis.

5.2.2 Protein isolate preparation

Protein isolate was obtained according to Chavan, McKenzie, and Shahidi (2001) with slight modifications. Vetch flour was mixed with distilled water (10 % w/v), and pH was adjusted to 9.0 using sodium hydroxide 1M solution; the mixture was stirred for 45 minutes at 200 rpm; afterward, the mixture was filtered through Whatman paper #1.

Dissolved protein was precipitated at a pH of 4.3 using hydrochloric acid solution 1 M. The obtained protein was washed with distilled water before centrifugation (HERMLE Z383K, Wehingen, Germany) at 4000 g for 10 min. Then, the supernatant was discarded; this process was repeated two times. Washed protein isolate was kept at -40 °C for 12 h. Then, it was freeze-dried (0.54 mbar vacuum level; -40 °C for 24 h, -10 °C for 12 h, and 5 °C for 12 h) in a Labconco Triad freeze-dryer (LABCONCO, Kansas City, MO, USA). Freeze-dried vetch protein isolate was kept in dry and dark conditions until analysis.

5.2.3 Fungal strains

5.2.3.1 Isolation and identification

Fungal strain isolation was performed according to Ortíz et al. (2011). Freeze-dried protein was mixed with distilled and sterilized water until a moisture level of 75 % (w/w); then, the mixture was incubated at 28 °C for seven days. After the incubation time, three fungi strains were identified; then, strains were purified by sowing into Potato Dextrose Agar (PDA). Purified strains were kept at 28 °C.

5.2.3.2 Microscopic characterization

The isolated fungi were characterized microscopically according to the microculture methodology described by Rodríguez Dos Santos and del Pozo Núñez (2003). An L-shaped glass rod was placed into a Petri dish with a sterile glycerol solution (30 % v/v). A cube of PDA (1 cm x 1 cm x 1 cm) was placed on top of a slide and it was covered with a coverslip. The agar cube was inoculated by sowing with the mycelium previously isolated in each of the corners. This preparation was placed on the L-shaped glass rod and incubated at 28 °C

for 5 days. The coverslip was removed and stained with a drop of lactophenol blue for observation under an optical microscope.

5.2.3.3 Genetic identification

Genetic identification was performed according to Centro de Investigacion y de Estudios Avanzados (CINVESTAV) Unidad Irapuato.

Prior to extracting DNA, fungi were cultivated in Luria-Bertani (LB) medium, at 37 ° C for five days. Extracted genomic DNA was amplified by PCR with oligonucleotides of conserved regions of the forward and reverse ITS (Internal Transcribed Spacer of nuclear ribosomal RNA) by Sanger sequencing (Sequencer 454, Roche).

The electropherograms resulting from the sequencer were manually verified using the 4peaks (MAC) program to determine the quality and to delimit the reliable regions obtained by sequencing.

The assembling of the reliable regions (forward and reverse) was done in CodonCode Alignear. Finally, the microorganisms were identified using BLAST search and sequence comparison functions provided by NCBI.

5.2.4 Submerged fermentation

5.4.4.1 Culture media

1 L of culture media containing 5 g of vetch protein isolate in distilled water was prepared. Sodium hydroxide 1 M solution (1 % v/v) was added to improve vetch protein isolate solubility; subsequently, a sterilization process was carried out (121 °C, 15 lb/in^2, 15 min).

5.4.4.2 Media inocululation and fermentation kinetics

Media inoculum and sampling were done according to Limón et al. (2015) with modifications. The media was inoculated with 1 % spore suspension (10^6 UFC/mL). Fermentation was carried out for 96 h and samples were withdrawn at 0, 12, 24, 48, 60, 72, 84 and 96 h to determinate the degree of hydrolysis, antioxidant activity, and ACE inhibition

5.2.5 Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was done by the o-phtaldehyde method described by Nielsen, Petersen, and Dambmann (2001) adapted to the microplate. Briefly, 40 µL of the sample with 150 µL o-phtaldehyde reagent were mixed in a 96-well plate. Samples were analyzed in triplicate.

5.2.6 Antioxidant activity determination

5.2.6.1 Determination of DPPH scavenging activity

Antioxidant activity was determined by the 2,2-Diphenyl-1-picrilhydrazil (DPPH) method, according to Fukumoto and Mazza (2000) adapted to a 96-well plate. 20 µL of the sample (or standard) was mixed with 200 µL solution of 125 DPPH µM. Subsequently, the plate was covered and incubated at 25 °C in darkness for 90 min. Then, absorbance was measured at 520 nm using an xMark Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA). Antioxidant activity was expressed as percent DPPH discoloration.

5.2.5.2 Determination of ABTS scavenging activity

The scavenging of the ABTS radical was performed according to the methodology described by Re et al. (1999). The reagent was prepared by combining 7 mM solution of 2,2'-azinobis (3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) and 2.45 mM ammonium persulfate. The mixture was kept in dark conditions at room temperature (25 °C) for 16 h; subsequently, a 1:25 dilution was made before to the use of the reagent. 20 µL of the sample was mixed with 200 µL of ABTS solution. The absorbance was read at 734 nm after 6 minutes; the antioxidant capacity was expressed as µg equivalent of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)/g dry basis, using a standard Trolox curve (0-700 µM).

5.2.7 Inhibition of Angiotensin-Converting Enzyme (ACE) activity

The inhibition of ACE was performed using hippuryl-histidyl-leucine (HHL) as a substrate. ACE, HHL, and samples were prepared in 100 mM borate buffer, 300mM of sodium chloride at pH 8.3. The reaction was done by mixing 10 µL ACE, 50 µL of 2.17 mM HHL and 10 µL borate buffer for blank or 10 µL of the sample. The mixture was incubated at 37 °C for 30 min. Then, the reaction was stopped by adding 85 µL of hydrochloric acid 1M solution. 10 µL of each sample was eluted into a reverse-phase C-18 column (Zorbax Eclipse XDB C-18 column, 4.6 × 150 mm, 5 µm, Agilent Technologies) using 0.05 % trifluoracetic acid in water (A) and 0.05 trifluoroacetic acid in acetonitrile (B) as solvents with the following gradient: 0 min 5 % B, 10 min 60 % B, 12 min 60 % B, and 15 min, 5 % B. Detection of the hippuric acid was done using a diode-array detector set at 200 nm. Percent inhibition was calculated according to Boschin et al. (2014).

5.2.8 Statistical analysis

Statistical Analysis was performed using the Statistica software (TIBCO Software Inc., Palo Alto, CA, USA). For each treatment analysis of variance (ANOVA) and multiple comparisons by Tukey's honest significant test ($\alpha < 0.05$) was applied to evaluate any significant difference. Finally, for all the variables, principal component analysis (PCA) was also performed to visualize the distance and relatedness between samples.

5.3 Results and Discussion

5.3.1 Identification of fungal strains

Figure 5.1 shows the growth in PDA of the purified fungi isolated from the vetch protein isolate. 5.1a (Strain 1) shows the mycelium appears like fine, fluffy white threads over the surface; the growth of the mycelium is homogeneous. According to Bullerman (2003), *Rhizopus* genus tend to have young white sporangia before turning black. Figure 5.1b (Strain 2), presented a fine fluffy shape in olive-green color with irregular growing on the surface. In the study by Bullerman (2003), *Aspergilli* are described as fungi of fast conidia production, that can be distinguished by its olive-green color. While the fungi in Figure 5.1c (Strain 3) showed a two-color mycelium (white and green). Some fungi can have dual color if a sporulation phase has begun (Moya et al. 2020, Talavera-Ortiz et al. 2020). According to Bullerman (2003), *Trichoderma* colonies are bright green.

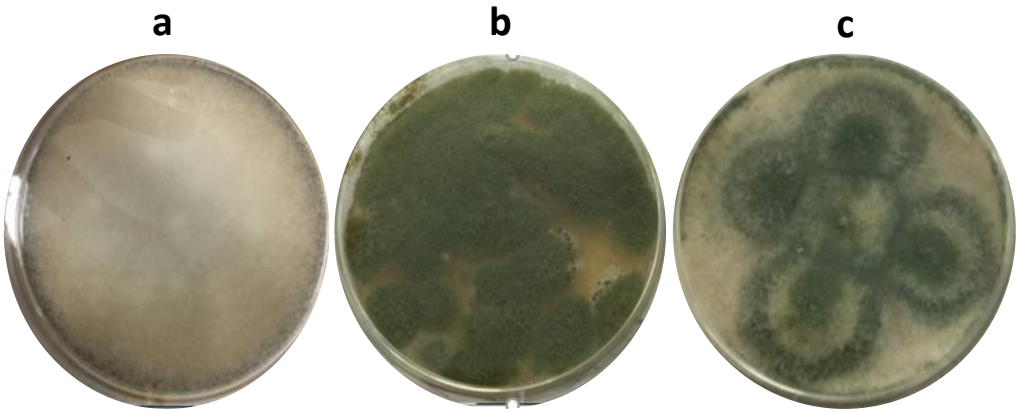

Figure 5.1. Macroscopic fungal growth on PDA plates. a) Strain 1, b) Strain 2, c) Strain 3

The results of the microscopic analysis of the isolated strains are shown in Figure 5.2. Strain 1 (Figure 5.2a) presented both mycelium and reproduction structures. The hyphae are filamentous, hyaline, and present black spores, suggesting that this strain corresponded to *Rhizopus* genus (Wang et al. 2019, da Silva et al. 2019). Rhizopus is a very-fast growing fungus that forms rhizoids at the base of sporangiophores, and columella in the sporangium (Bullerman 2003).

Strain 2 had a filamentous, coenocytic and hyaline hypha (Figure 5.2b). Its conidiophore was typical of *Aspergillus* genus (Bennett 2010, Schuster et al. 2002). According to Bullerman (2003), *Aspergilli* reproduce by producing conidia on a conodiophore. This conidiophore is a single cell that grows upright and has structures called sterigma where conidia are produced.

In Figure 5.2c (Strain 3) an irregular septate hypha is observed, with a branching aggregation of conidiophores into fascicles. Conidial shapes vary from ellipsoidal to globose, both with a smooth surface. Conidial pigments also are characteristic ranging from white to

yellow with light whitish pigments, and small round columns can be observed (Bissett et al. 2015, Moya et al. 2020). These characteristics can be attributed to *Trichoderma* species.

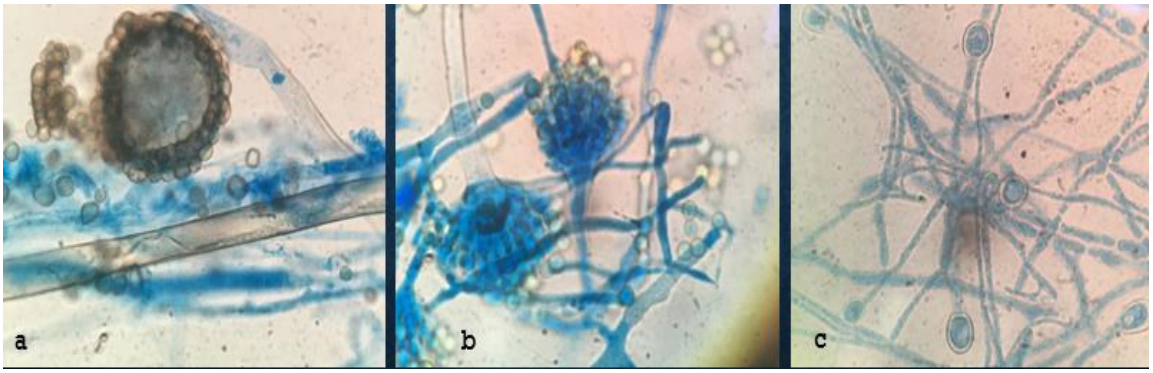

Figure 5.2. Microscopic fungi observations a) Strain 1 = *Rhizopus*; b) Strain 2 = *Aspergillus*; c) Strain 3 = *Tricodherma*

For Strain 1, ribosomal RNA genes 18S, 5.8S and 28S were sequenced with 99 % accuracy resulting in *Rhizopus oryzae*. For strain 2, the 5.8S ribosomal RNA gene was sequenced with 100 % accuracy resulting in *Aspergillus flavus*. For Strain 3, the ribosomal RNA gene 5.8S was also sequenced with 100 % accuracy resulting in *Trichoderma asperellus* (*Trichoderma koningiopsis*). Full sequences can be consulted in Appendix 1.

5.3.2 Degree of hydrolysis (DH)

A similar behavior of microbial growth kinetic can be observed in Figure 5.3. *A. flavus* is the strain that takes less time to adapt to the medium, while *R. oryzae* is the strain that takes longer to adapt to the medium. No significant difference among the DH values could be observed until 48 h. This may be due to the media conditions where *Aspergilli* are more tolerant of alkaline conditions than any other fungi (Abubakar et al. 2013).

For *R. oryzae* the highest value was 21.86 %, at 96 h, as well as *T. asperellum* who reached it in only 60 h. The *A. flavus* DH was double than that of *R. oryzae* in less time (47.76 % at 84 h, respectively). The DH of vetch protein isolate hydrolyzed by *R. oryzae* and *T. asperellum* are lower to those of pepsin in soymilk reported by Hermanto, Hatiningsih, and Putera (2018) but are similar to those reported by Evangelho et al. (2017) in black beans hydrolyzed with Alcalase®. The DH of *A. flavus* is higher compared to enzymatic preparations such as Corolase® in soybean (Coscueta et al. 2016), and lentil protein isolates hydrolyzed by bromelain and papain (Barbana and Boye 2011). Differences in DH could be attributed not only to the enzyme but also to the time of proteolysis. Microbial fermentation requires longer times to achieve high DH (60-84 h), while *in vitro* enzymatic assays require lower times (16 h).

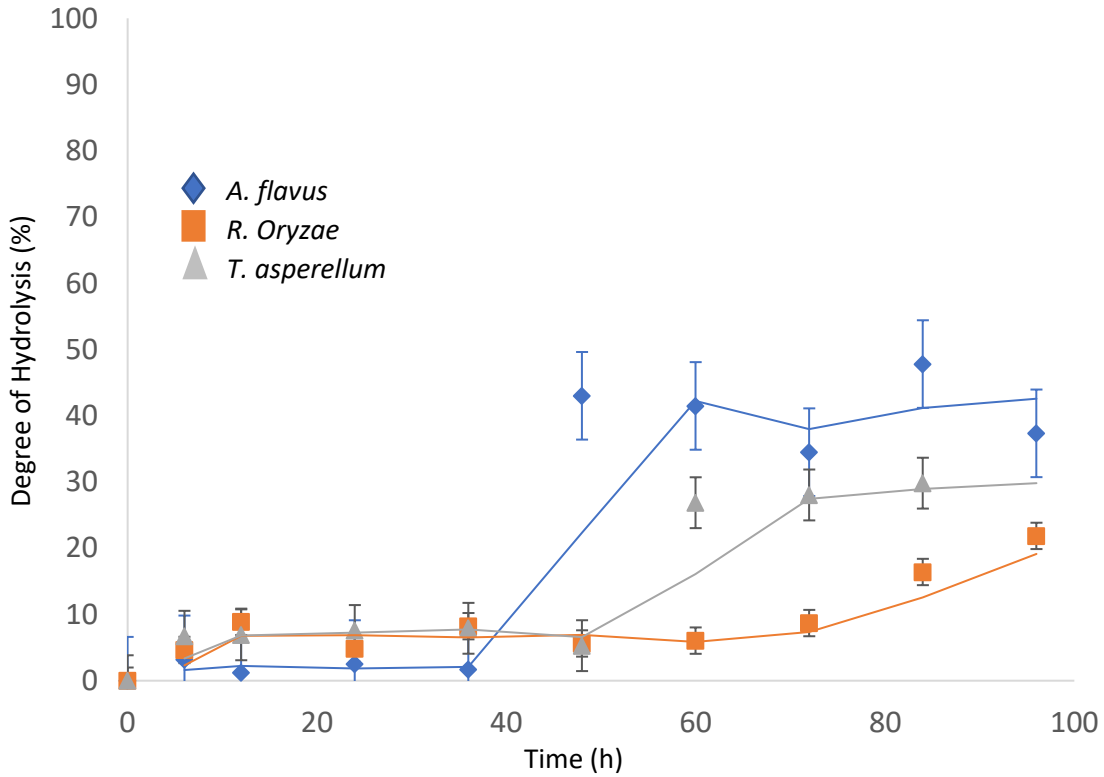

Figure 5.3 Degree of hydrolysis of vetch protein isolates fermented with fungal strains

5.3.3 DPPH Scavenging activity

Figure 5.4 shows the DPPH scavenging activity. There were no differences in DPPH activity neither by time nor by strain; however, these results are higher than the ones reported by Ajibola et al. (2013) in African yam bean (40 %) and by Valdez-Ortiz et al. (2012) in yellow beans (44 %). Differences may be attributed to the different enzymes used, as well as the hydrolysis time. DH has an inversely proportional relationship with DPPH scavenge. Larger peptides could contribute to improving the antioxidant activity by DPPH scavenging. According to López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar (2014), DPPH scavenging is preferred by hydrophobic amino acid residues. It is possible that the proteolytic activity of the fungi isolated, cleavages in hydrophilic amino acids.

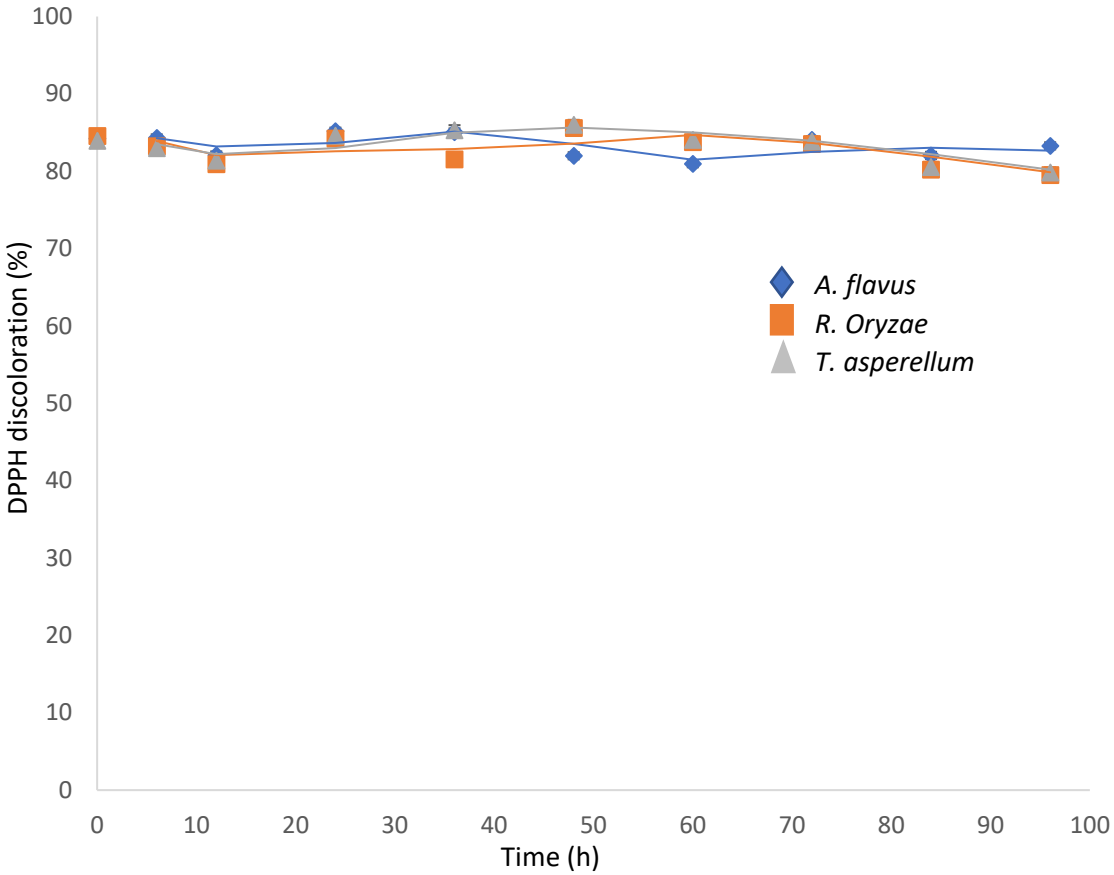

Figure 5.4 DPPH scavenging activity. Results are expressed as DPPH discoloration

5.3.4 ABTS Scavenging capacity

Figure 5.5 shows the ABTS scavenging activity. For *R. oryzae*, there was no significant difference among the hydrolysis time. For *A. flavus*, the highest value was 545.60 µg Trolox equivalent/g dry matter, achieved at 72 h. Although the highest value for *T. asperellum* was 545.26 (12 h), lower values are not significantly different at any other tested times (Figure 5.4). The results shown for *A. flavus* and *T. asperellum* are similar to those reported by Garcia-Mora, Frias, et al. (2015); they used Alcalase® and Savinase® to produce peptides from pinto bean protein isolate. Other studies involving ABTS scavenging are the

ones performed by Durak et al. (2013) in adzuki beans, by (Carrasco-Castilla et al. 2012) in jamapa beans, and by Ngoh and Gan (2016) in pinto beans, which reported higher ABTS scavenging activity. According to Valdez-Ortiz et al. (2012), ABTS method is very sensitive to determine the antioxidant activity at low concentrations. For *A. flavus* and *T. asperellum*, ABTS scavenging is proportional to DH; however, the opposite occurred for *R. oryzae*. According to Wongekalak et al. (2011), the same peptides can exert different antioxidant activities due to the method conditions, which could explain the unusual behavior between DPPH and ABTS values obtained in this study.

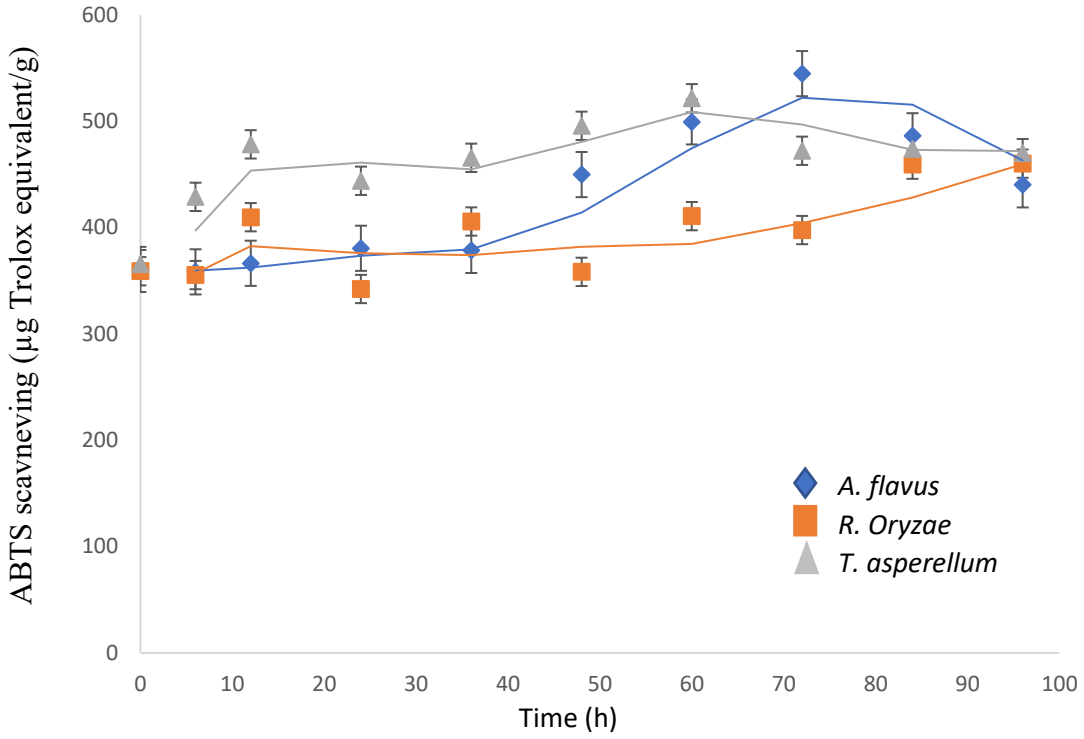

Figure 5.5 ABTS Scavenging activity of vetch protein isolate. Results expressed as µg Trolox equivalent/g dry matter

5.3.5 ACE activity

Figure 5.5 shows the ACE inhibitory (ACEI) activity of hydrolyzed vetch protein isolate. For *R. oryzae* the maximum value at 72 h. Protein isolate hydrolyzed by *A. flavus* and *T. asperellum* were reached at 60 h. These results are higher than the ones reported in lentils by Torino et al. (2013) fermented by *B. subtilis* (36 % at 96 h) and similar to the ones fermented by *L. plantarum* (62 to 92 %). On the other hand, ACE inhibition was higher than the reported Garcia-Mora, Frias, et al. (2015) in pinto beans and in lentil protein isolate hydrolyzed with Corolase® (70 %), Protamex® (47 %) and Savinase® (63 %) (Garcia-Mora, Peñas, et al. 2015); even in fermented rice products according to Cáceres et al. (2019) and higher than adzuki beans protein isolate according to Ajibola et al. (2013), however, they are lower to the results achieved by Limón et al. (2015) in kidney bean (> 90 % inhibition), and in fermented soybean yogurt (Hermanto, Hatiningsih, and Putera 2018). Further information is needed to compare the effect of ACEI of fermented vetch protein isolate with synthetic ACE inhibitors such as captopril and enalapril, as well as the IC_{50}. In this study, the degree of hydrolysis has a directly proportional relationship with ACE inhibitory activity.

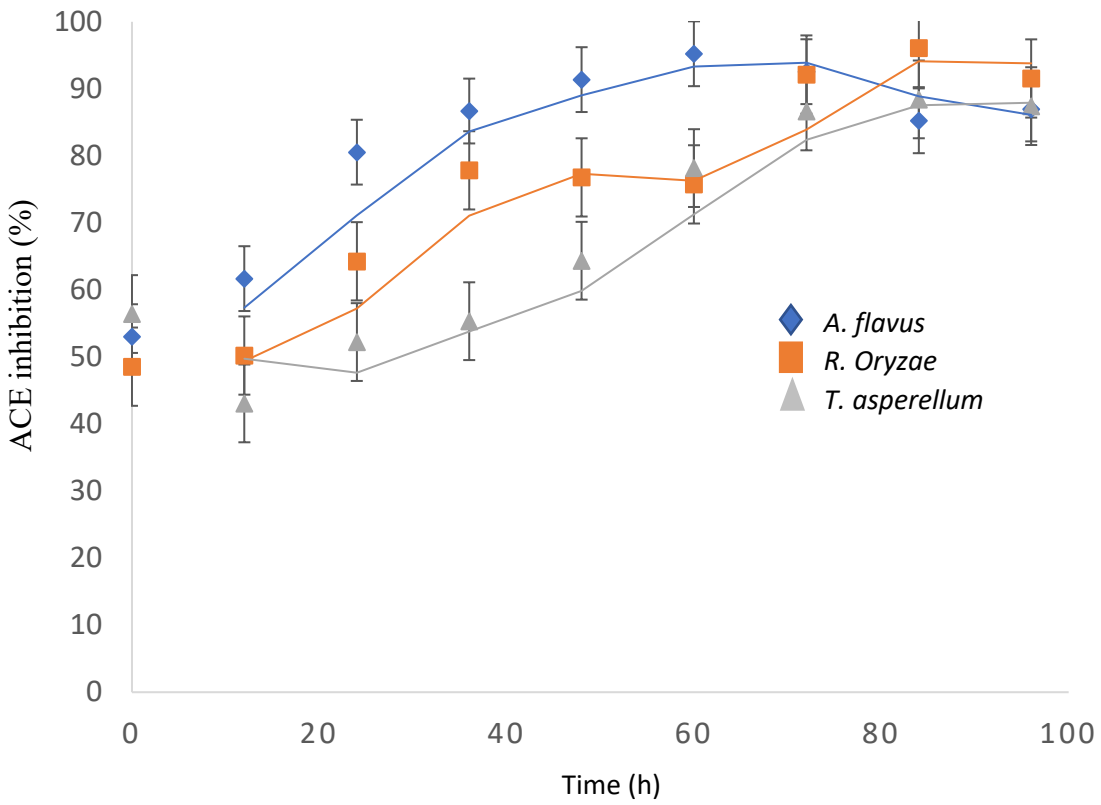

Figure 5.6 ACE inhibitory activity of vetch protein isolate. Results expressed as ACE inhibition

5.3.6 Principal component analysis (PCA)

To reduce the number of response variables, a principal component analysis (PCA) was done. Figure 5.7 shows the variables influence in the construction of PC1, being ABTS A, DH A, ACEI A, ACEI T and ACEI R the most influential for PC1, while ABTS A, ABTS T and ABTS R the most influent for PC2.

The resultant biplot (Figure 5.8) PC1 and PC2 contribute to 88 % of the total variability of the experiments. It also shows 3 different groups: a. The first group (0 to 36 h) does not excel at any of the variables studied; this may be attributed to lag phase of microbial

growth, in which the microorganisms start to assimilate the nutrients in the broth and starting to exert the proteases needed for growing; b. The second group is formed by 48, 60 and 72 hours, this group is characterized for having some of the highest degree of hydrolysis, as well as the highest ABTS scavenging; this may be attributed to the start of the exponential phase for microbial growth, in which fungal strains start to grow and reproduce; c. The third group is formed by 84 and 96 h, since some of the highest ACE inhibitory activity, DH, DPPH scavenging and ABTS scavenging occurs at this time, this grouping could be attributed to a continuation of the exponential phase of microbial growth and maybe the start of the stationary phase. In the latter, the strain stops to grow, and the nutrients are only used to cellular maintenance.

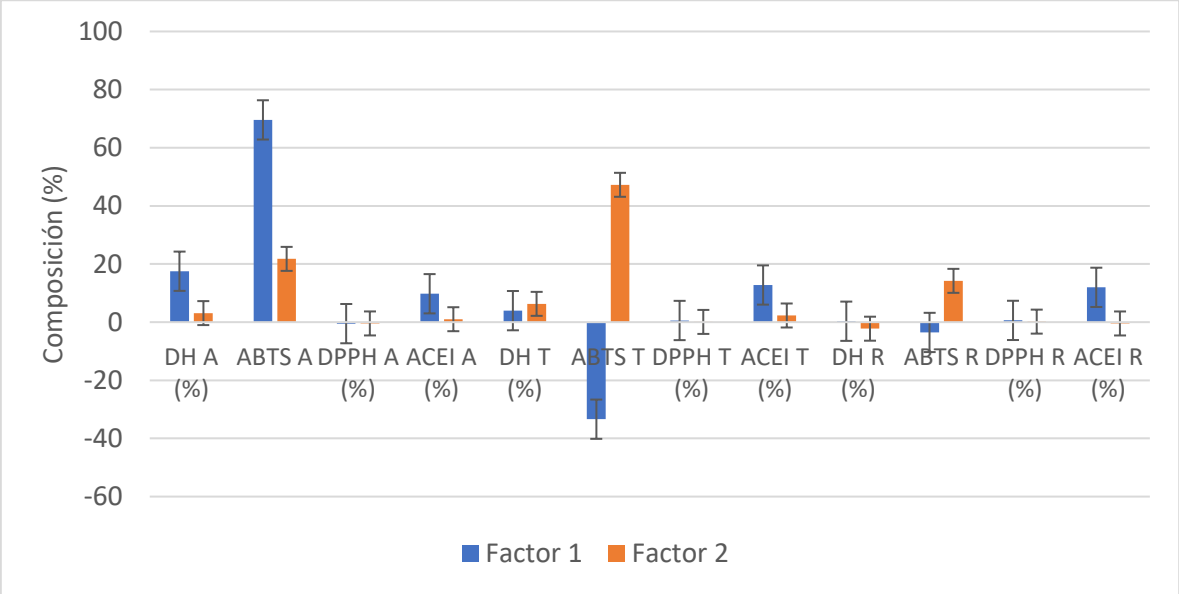

Figure 5.7 PCA based in correlations. DH A: degree of hydrolysis of *A. flavus*; ABTS A: ABTS scavenging of *A. flavus*; DPPH A: DPPH scavenge of *A. flavus;* ACEI A: ACE inhibitory activity of *A. flavus*; DH T: degree of hydrolysis of *T. asperellum*; ABTS T: ABTS scavenging of *T. asperellum*; DPPH T: DPPH scavenge of *T. asperellum*; ACEI T: ACE inhibitory activity of *T. asperellum*; DH R: degree of hydrolysis of *R. oryzae*; ABTS R: ABTS scavenging of *R. oryzae*; DPPH R: DPPH scavenge of *R. oryzae;* ACEI R: ACE inhibitory activity of *R. oryzae.*

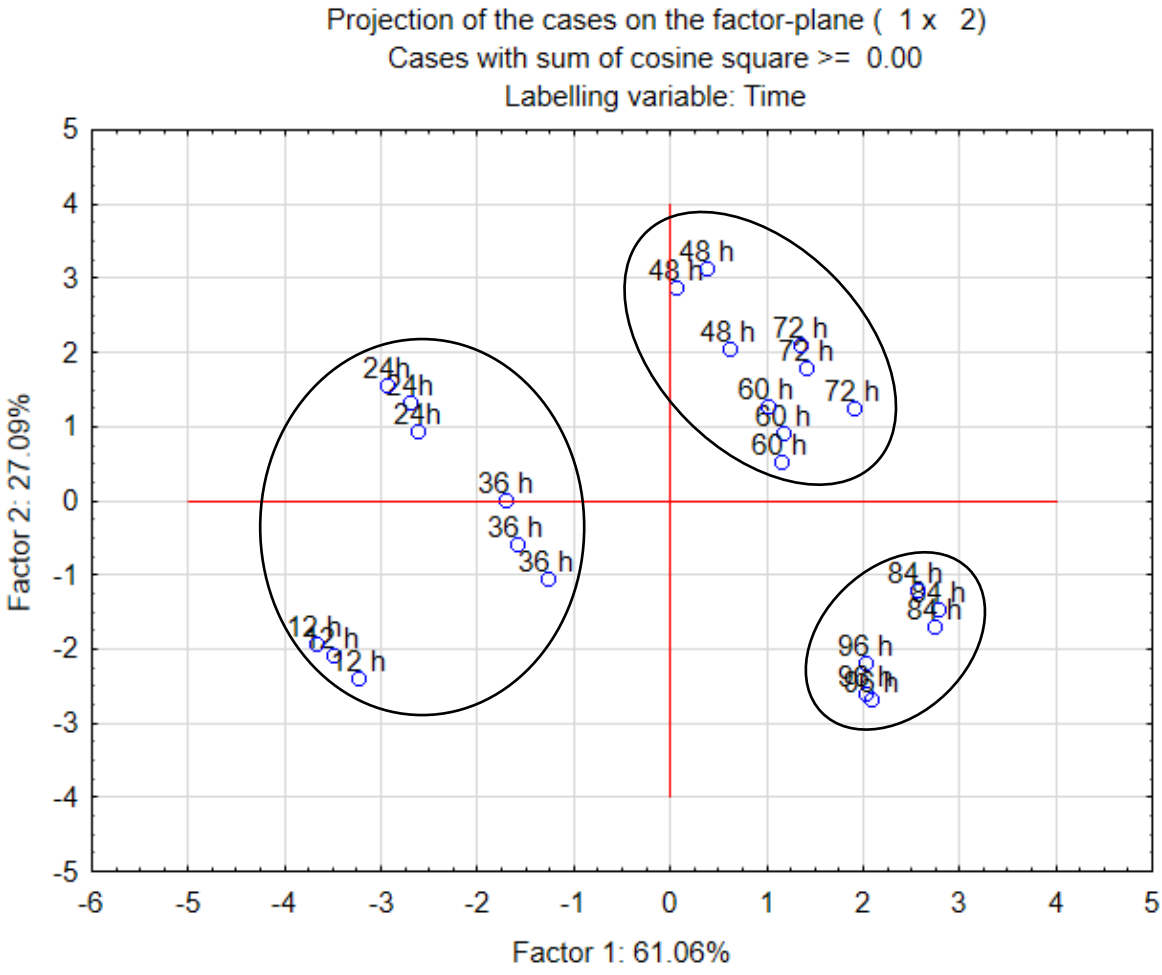

Figure 5.8 PCA resultant biplot

5.4 Conclusion

The vetch protein isolate, after fermentation with fungal strains, showed significant antioxidant and ACE inhibitory activities. These activities were, in most cases, better than those reported for different legume proteins isolates hydrolyzed with gastric, enzymatic preparations or plant-derived proteases.

Vetch protein isolate hydrolyzed by *A. flavus* could achieve high ABTS scavenging capacity at mid-time fermentation (48-60 h), however, the best ACEI values were observed at longer times (84 to 96 h). Moreover, *A flavus* was the strain that better adapted to the culture media by achieving high degree of hydrolysis in less than 48 h.

On the other hand, vetch protein isolate hydrolyzed by *T. asperellum* showed better ABTS scavenge values at the earlier fermentation stages (24 to 48 h). Moreover, the best results for ACE inhibition, were showed at the final stages of fermentation (84 to 96 h).

Although vetch protein isolate hydrolyzed by *R. oryzae* did not excel at any activity studied, it could be observed that it required longer times to achieve high DH, ACEI and ABTS scavenging activities, due to their less adaptation to the culture media.

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CHAPTER 6: Conclusions, future perspectives and recommendations

6.1 Conclusions

- Vetches showed a protein content similar to other legumes such as common beans, lentils, and cowpea. Although the protein content of vetches is lower compared to soybean, it is higher to protein content from animal sources such as milk and egg white.
- Three fungal strains were capable of growth on vetch protein isolate. The macroscopic and microscopic characterization of these strains allowed to identify genus fungi as *Rhizopus*, *Aspergillus*, and *Trichoderma*, moreover, the identification was completed by genomic analysis of the 5.8S ribosomal unit. The strains were fully identified as *Rhizopus oryzae, Aspergillus flavus*, and *Trichoderma asperellum* with 99 % confidence.
- The only source of carbon and nitrogen for fungal development for fungal strain, was vetch protein isolate, during isolation and fermentation process.
- *A. flavus* was the strain that showed the best degree of hydrolysis, ABTS scavenging and ACE inhibition during the fermentation process. Although the degree of hydrolysis, ABTS scavenging and ACE inhibition showed by *T. asperellum* and *R. oryzae* were lower than *A. flavus*, these results are higher than the achieved by other proteases in other legumes.
- Instant controlled pressure-drop (DIC) represented an alternative to the removal of non-nutritional factors on vetches, similar to cooking and germination. High pressure and times up to 312 s, are the optimal conditions to reduce most of the non-nutritional factors.

6.2 Future perspectives and recommendations

- A complementary amino acid composition of vetch protein could show the quality of the protein of vetches.
- Perform other experiments to address different bioactivities than the ones studied.
- Evaluate the bioactivity of vetch peptides after simulated intestinal digestion conditions.
- Apply the isolated strains in other pulse proteins to study their hydrolysis capacity.
- Evaluate the yield of protein extraction after DIC treatment.

APPENDIX

Ingeniería Biológica

Cinvestav
Unidad Irapuato

Reporte de servicio de identificación molecular de cepas de hongos
Solicitante: MC. Ángel Iván Hernández Aguirre

Breve descripción de la Metodología empleada:

1. Recepción y cultivo de las bacterias en medio LB, a 37ºC.
2. Extracción de ADN genómico de cada cepa.
3. Amplificación por PCR y con oligonucleótidos de regiones conservadas de los ITS forward y reverse (Internal Transcribed Spacer of nuclear ribosomal RNA)
4. Envío a secuenciación, por Sanger (secuenciador 454, Roche) de los productos obtenidos por PCR usando los ADN genómicos y los oligos Forward y Reverse de los ITS.
5. Verificación manual de los electroferogramas resultantes del secuenciador con el programa 4peaks (MAC), para determinar su calidad y para delimitar las regiones confiables obtenidas por secuenciación.
6. Ensamblado de las regiones confiables (forward y reverse) en CodonCode Alignear, para tener una región de secuencia confiable.
7. La región del alineamiento se ingresó al servidor de NCBI para realizar los blastn y obtener los resultados de los genomas a los que tiene identidad.

1. SECUENCIAS CEPA 2

Alineamiento de las secuencias resultado de la secuenciación con los ITS forward y reverse

Range 1: 29 to 540Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
888 bits(984)	0.0	508/514(99%)	6/514(1%)	Plus/P

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Query  1    AGGGT--CTAGCGAGCC--ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG   56
            |||||  ||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  29   AGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG   88

Query  57   GCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACC   116
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  89   GCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACC  148

Query  117  ACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTT  176
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  149  ACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTT  208

Query  177  CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGT  236
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGT  268

Query  237  GTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT  296
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Sbjct  269  GTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT  328

Query  297  TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGG  356
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Query  357  GTCGTCGTCCCCTCTCCgggggggACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT  416
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Sbjct  389  GTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT  448

Query  417  CCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGC  476
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Sbjct  449  CCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGC  508

Query  477  AAATCAATCTTTTTCCAGGTTGACCTCGGATCAG  510
            ||||||||| ||||||||| ||||||||||||||
Sbjct  509  AAATCAATC-TTTTCCAGG-TGACCTCGGATCAG  540
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SEGMENTO DE SECUENCIAS QUE ALINEAN ENTRE EL RESULTADO DE LA SECUENCIACIÓN FORWARD Y REVERSE COMPLEMENT

>FRAGMENTO LENTEJA QUE ALINEA ENTRE LA SECUENCIA FORWARD Y REVERSE 470 pb

ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATT
CATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGT
CTGATCTAGTGAAGTCTGA
GTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGAT
GAAGAACGCAGCGAAATGC
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CCCTGGTATTCCGGGGGGC
ATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCC
CTCTCCGGGGGGGACGGGC

CCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTC
TGTAGGCCCGGCCGGCGCT
TGCCGAACGCAAATCAATC

Resultado de alineamiento en la base de datos del NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Aspergillus flavus isolate 6412 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. Fungal endophyte.
Sequence ID: MG437409.1Length: 574Number of Matches: 1
Related Information
Range 1: 45 to 514GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	S
869 bits(470)	0.0	470/470(100%)	0/470(0%)	P

```
Query  1    ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  45   ACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGC  104

Query  61   CGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105  CGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTA  164

Query  121  GTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165  GTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGT  224

Query  181  TCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225  TCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGT  284

Query  241  GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285  GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC  344

Query  301  CGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCgg  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345  CGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGG  404
```

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Query  361  gggggACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405  GGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTT  464

Query  421  TGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATC  470
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465  TGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATC  514
```

2. SECUENCIAS CEPA 1

Alineamiento de las secuencias resultado de la secuenciación con los ITS forward y reverse

Range 1: 34 to 553Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
944 bits(511)	0.0	519/522(99%)	3/522(0%)	Plus/P

```
Query  1    CTCCC-AACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTG  59
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34   CTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTG  93

Query  60   CGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94   CGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCC  153

Query  120  CCTCGCGGACGTATTTCTTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  154  CCTCGCGGACGTATTTCTTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCA  213

Query  180  ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  214  ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT  273

Query  240  GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTC  299
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Sbjct  274  GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTC  333

Query  300  TGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGATCGGCGT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334  TGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGATCGGCGT  393

Query  360  TGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCTAAATACAGTGGCGGTCTCGCCGCA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  TGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCTAAATACAGTGGCGGTCTCGCCGCA  453

Query  420  GCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGT  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  GCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGT  513

Query  480  AAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAG  521
            |||||||||||||||||||||||||||| ||| |||||||||
Sbjct  514  AAAACACCCAACTTTCTGAAATGTTGAC-TCG-ATCAGGTAG  553
```

> SECUENCIA COMPLEMENTARIA HABA 502 PB

AACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCG

CAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTTACAGC

TCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA

TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG

GGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGG

TGCCGGCCCCTAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGG

CGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGAC

Trichoderma asperellum (también aparece como T. koningiopsis) isolate T14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MG266013.1Length: 579Number of Matches: 1

Related Information

Range 1: 25 to 526GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Stran
928 bits(502)	0.0	502/502(100%)	0/502(0%)	Plus/P

```
Query  1    AACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGC   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   AACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGC   84

Query  61   AGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGC   120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   AGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGC   144

Query  121  GGACGTATTTCTTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACG   180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GGACGTATTTCTTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACG   204

Query  181  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG   240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG   264

Query  241  CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG   300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG   324

Query  301  GCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGA   360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  GCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGA   384

Query  361  TCGGGACCCCTCACACGGGTGCCGGCCCCTAAATACAGTGGCGGTCTCGCCGCAGCCTCT   420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  TCGGGACCCCTCACACGGGTGCCGGCCCCTAAATACAGTGGCGGTCTCGCCGCAGCCTCT   444

Query  421  CCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACA   480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  CCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACA   504

Query  481  CCCAACTTTCTGAAATGTTGAC  502
            |||||||||||||||||||||
Sbjct  505  CCCAACTTTCTGAAATGTTGAC  526
```

3. SECUENCIAS CEPA 3

Alineamiento de las secuencias resultado de la secuenciación con los ITS forward y reverse

Range 1: 35 to 576Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Stran
1029 bits(535)	0.0	542/543(99%)	1/543(0%)	Plus/P

```
Query  5    CTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGA  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35   CTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGA  94

Query  65   GAGACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTT  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   GAGACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTT  154

Query  125  CAGGAGTACCCTTCATAATAAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAA  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  155  CAGGAGTACCCTTCATAATAAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAA  214

Query  185  CTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAAC  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215  CTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAAC  274

Query  245  TAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGG  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275  TAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGG  334

Query  305  TTTTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTA  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  TTTTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTA  394

Query  365  TGTGGTGATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTT  424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395  TGTGGTGATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTT  454

Query  425  CTGTCTGGCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCtttttttttGGTTCGC  484
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455  CTGTCTGGCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCTTTTTTTTTGGTTCGC   514

Query  485  CCAGGAAGTAAAGTACAAGAGTATAATCCAGTAACTTTCAAACTATGATCTGAAGTCAGG   544
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  515  CCAGGAAGTAAAGTACAAGAGTATAATCCAGTAAC-TTCAAACTATGATCTGAAGTCAGG   573

Query  545  TGG  547
            |||
Sbjct  574  TGG  576
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> SECUENCIA COMPLEMENTARIA EBO 515PB
CTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAG
A
GACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAG
GAGTACCCTTCATAATAAA
CCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAACAATGGATCTCTTGGT
TCTCGCATCGATGAAGAAC
GTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACG
CAGCTTGCACTCTATGGTT
TTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTATGT
GGTGATGGGTCGCATCGCT
GTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTGGCTTGCTAGGCAGGAA
TATTACGCTGGTCTCAGGA
TCTTTTTTTTTGGTTCGCCCAGGAAGTAAAGTACAAGAGTATAATCCAGTAAC

Rhizopus oryzae strain SW135 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: KY260672.1Length: 631Number of Matches: 1
Related Information
Range 1: 47 to 561GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Stran
952 bits(515)	0.0	515/515(100%)	0/515(0%)	Plus/P

```
Query  1    CTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGA   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47   CTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGA   106
```