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PhD dissertation

Proteins from Legumes as Natural Ingredients to Improve Food Quality and Security: Proteomic Characterization and Digestibility Assay

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XXXI ciclo

Ai miei genitori.

Sicuro approdo nei giorni di burrasca.

A mia madre.

Demiurgo del mio sapere.

Table of contents

Overview of PhD research activities and publications

Alfieri, F. 2016. Politics, Economics, and Demographics of Food Sustainability and Security. In Reference Module in Food Science, Elsevier: Amsterdam, The Netherlands;

Alfieri, F. 2019. The Role of Omic Sciences in Food Security and Sustainability. In: Ferranti, P, Berry, EM, Anderson, JR (Eds.), Encyclopedia of Food Security and Sustainability. Elsevier, vol. 1, pp. 44–49;

Alfieri, F. 2019. Novel Foods: Artificial Meat. In: Ferranti, P, Berry, EM, Anderson, JR (Eds.), Encyclopedia of Food Security and Sustainability. Elsevier, vol. 1, pp. 280–284;

Alfieri, F. Proteomic Methods for the Study of Allergens from Legumes, XXI Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Portici, September 14th-16th, 2016;

Alfieri, F. Proteomic Characterization and Digestion Study of Legumes for Food Technological Applications, XXII Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Bolzano, September 20th-22th, 2017;

Mamone, G, Picariello, G, **Alfieri, F**, Nicolai, MA, Ferraro, I, Ferranti, P. Proteomic Characterization and Digestion Study for the Technological Evaluation of Proteins from Legume Water Extracts, 5th International Conference on Food Digestion, Rennes, April 4th-6th, 2017;

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Chapter 1. General introduction

The agricultural system is currently going through a progressive and rapid evolution. Phenomena such as climate change, world population growing and food unavailability (Matthews *et al*., 2013), will give food technologies a key role in the management of food resources in the next decades. Interestingly the higher protein demand of the increasing world population is focusing the attention on vegetal proteins. As it is known, animal proteins do not represent an inexhaustible and easily accessible source. Moreover, meat consumption negatively affects food sustainability since intensive livestock can be responsible for critical phenomena such as air pollution increase (gas emissions), groundwater contamination and deforestation (Bhat and Fayaz, 2011; Goodwin and Shoulders, 2013; Bhat *et al.,* 2015; Zhi-chang *et al.,* 2015). On the other hand, several plant proteins have shown nutritive and biological properties useful for the food industry (Deshmukh *et al*., 2014; Kim, *et al*., 2014; Ruiz *et al*., 2014). Specifically, legume proteins may have functional (i.e. antioxidant activity and hormone regulation) and stabilizing (i.e. antimicrobial activity) properties (Duranti, 2006; Carbonaro *et al*., 2015; Clemente and Olias, 2017; Roy *et al*., 2010). Undoubtedly, this makes legume proteins a very suitable substitute to improve food quality and security.

Legumes: a staple food for many populations

From a nutritional point of view, legumes are a widespread and accessible worldwide protein source. In particular, in many developing countries, legumes are the main source of proteins and calories due to their great ability to easily adapt and grow in adverse conditions. Furthermore, they are a necessary protein supplement in countries where animal protein consumption is limited by religious and cultural reasons or unavailability (Boye *et al*., 2010). The protein content of legume seeds ranges from 17 to 40% (**Table 1**):

a higher content in comparison with that of cereals, and roughly equal to that of meat (de Almeida Costa *et al.*, 2006). However, vegetal proteins have a biological value lower than those of animal origin. In fact, legume proteins contain few sulphured amino acids. On the contrary, legumes have a high content of lysine, arginine, glutamic and aspartic acid that makes them nutritionally complementary to cereals (Rizzello *et al*., 2015). Unlike animal protein-based foods, legumes have a very low fat content (2-4%), with the exception of soybean and peanut. They also represent an excellent source of carbohydrates (starch and fiber), and provide for essential minerals and vitamins (group B) as well (Roy *et al*., 2010; Zhou *et al*., 2013).

Table 1. Protein content of some legumes per 100g of dry seeds

	Soybean	Pea	Chickpea	Grass pea	Bean	Lentil
Proteins (g)	36.9	21.7	21.8	28.01	23.6	25.0

Legume protein components

Legumes mainly contain storage proteins that are classified as albumins, globulins and glutelins according to their solubility properties (Osborne, 1907). Globulins, soluble in salt-water solutions, are about 70% of the total protein content. These proteins are categorized into two main classes based on their sedimentation coefficient (S): the class of vicilins and convicilins (7S), and the class of legumins (11S). Albumins (soluble in water) and glutelins (soluble in dilute acids and bases) both account for 10-20% of the total proteins (Nwokolo and Smartt, 1996; Duranti, 2006). Legumes contain also minor proteins, such as enzymes, protease inhibitors or lectins, and bioactive peptides. The most part of these proteins and peptides are within the water-soluble albumin class (Nwokolo and Smartt, 1996). Bioactive

peptides from legumes can be present naturally in the matrix or they can be produced by *in vivo* digestion, *in vitro* proteolysis (under controlled conditions) and food processing (i.e. fermentation and germination). According to their amino acid sequences, these peptides can exert different biological activities (i.e. cardiovascular, endocrine, antimicrobial, antimutagenic, immunological and neurological activities) (Carbonaro, *et al*., 2015). The structural properties of proteins with functional activity and the biological activities of some peptides and proteins from legume grains are reported in **Tables 2** and **3**, respectively.

Table 2. Structural properties of legume proteins with functional activity (*source:* Carbonaro *et al*., 2015)

Protein	MW (KDa)	Structure type*	α-Helix (%)	β-Sheet (%)	No. of SS	No. of SH	pI
Kunitz trypsin inhibitor	21.5	Globular, monomeric	6	40-60	2	0	4.5
Bowman-Birk inhibitor	8	Globular, monomeric	0	60	7	0	4.2
α-Amylase inhibitors	12-60	Globular, monomeric/ dimeric/tetrameric	15-30	25-60	2-5	0	4.7-6.7
Concanavalin A	110	Globular, tetrameric	0	47	0	0	5.0
Phaseolin	150	Globular, trimeric	16	37	0	0	5.5
Glycinin	340	Globular, oligomeric	15	36	22	2	4.6
Conglycinin	200	Globular,oligomeric	15	31	2	0	4.6
Conglutin γ	200	Globular,tetrameric	15	35	24	0	4.5

*In phosphate-buffered saline (pH 7.0)

Table 3. Biological activities of some peptides and proteins from legume grains (*adapted from* Carbonaro, *et al*., 2015)

Protein/peptide	Biological activities
Kunitz trypsin inhibitor	Cytotoxic, anticarcinogenic
Bowman-Birk inhibitor	Cytotoxic, anticarcinogenic
α-Amylase inhibitors	Anticarcinogenic
Lectins (i.e. concanavalin A)	Cytotoxic, anticarcinogenic, immunomodulat antibacterial
7S globulins (i.e. phaseolin, conglycinin)	Cholesterol/triglyceride lowering
11S globulins (i.e. LPYPR, VLIVP, hydrophobic peptides)	ACE-inhibitory, hypotensive
Conglutin γ	Hypoglycemic
2S albumins (lunasin)	Anticarcinogenic, anti-inflammatory

Legume bioactive proteins and peptides

Protease inhibitors (PIs). Numerous botanical families accumulate PIs to produce a protective mechanism able to develop plants in adverse conditions. These inhibitors are active on proteolytic enzymes of bacteria, insects and animals (Dunaevsky *et al.,* 2005). The function of PIs is therefore indispensable for plants since exogenous enzymes can reduce the amount of amino acids available for their growth and development (Roy *et al*., 2010). Two well-characterized protease inhibitors of legume seeds are trypsin and chymotrypsin inhibitors that belong to Kunitz and Bowman-Birk families, respectively. Kunitz inhibitors (KIs) are proteins of about 22 kDa able to inhibit trypsin activity through a single active site (Clemente and Olias, 2017). The trypsin binding is due to the sequence Ser-Tyr-Arg-Ile-Arg-Phe. Bowman-Birk inhibitors (BBIs) are proteins of low molecular weight (7-9

kDa) which contain two active sites able to inhibit both trypsin and chymotrypsin enzymes (Clemente and Olias, 2017) (**Figure 1**).

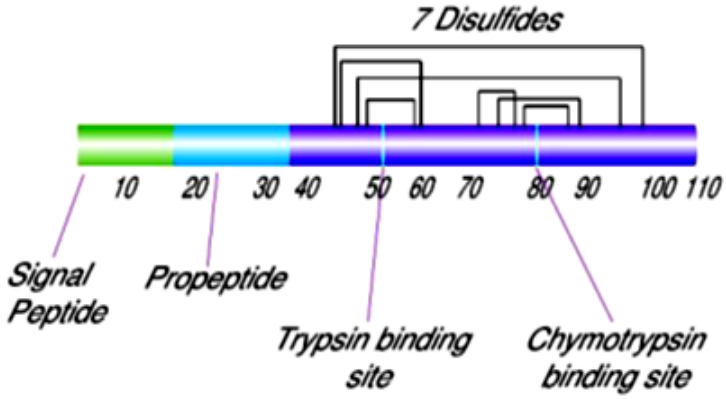

Figure 1. IBB1 soy inhibitor (*source*: Sigma-Aldrich)

The BBI coding genes are also present in corn (*Zea mays L.*), rice (*Oryza sativa L.*) and other botanical families; however, legume seeds contain these inhibitors at higher concentrations (Clemente *et al.,* 2011). Several *in vitro* and *in vivo* studies showed as soybean BBIs can have a protective and suppressive effect against inflammation and cancer development within the gastrointestinal tract (GIT) (Clemente *et al.,* 2011). In fact, these molecules can reach the intestine in active form due to their resistance to the extreme conditions of GIT. The conformational rigidity of BBIs, linked to the network of disulphide bridges, is mostly responsible for their high stability. However, many studies would suggest that BBIs are not the main cancer preventing agents of soybean. These inhibitors are actually able to protect the bioactive peptide lunasin from protease activities, thus preserving its bioavailability (Hsieh *et al.,* 2010). Interestingly the reduction of disulfide bridges, and the subsequent alkylation of cysteine sulfhydryl groups, increases BBI vulnerability to proteases, and decreases their heat stability as well (Clemente *et al*., 2010).

α-amylase-inhibitors (AAis). These inhibitors are active on the exogenous α-amylases, which are a family of endoamylases able to catalyse the hydrolysis of α-D-(1→4) glucan linkages in starch components, glycogen and other carbohydrates. AAIs can be employed in the control of obesity thanks to their antidiabetic properties. To prove their potential therapeutic applications, Barrett and Udani (2011) tested white bean AAIs by clinical trials obtaining significant results. Specifically, they investigated the AAI effect on weight loss and glycemic control through reduction of the post-prandial spike in blood glucose levels.

Lectins. Lectins, or agglutinins, are ubiquitous carbohydrate-binding proteins that bind reversibly to specific mono- or oligosaccharides (Peumans and Van Damme, 1995). They are isolated from a wide variety of important crop plants where their purpose is to protect the plant from predators. Lectins are able to prevent certain type of cancers and to activate certain innate defense mechanisms. They can also be considered therapeutic agents to prevent or control obesity (Pusztai and Bardocz, 1996; Lima *et al.,* 1999; Wang, *et al.,* 2000; Sames *et al.,* 2001; Ewen *et al.,* 2006; Hartmann and Meisel, 2007). The capacity of legume lectins to control obesity is due to their extremely tight β-sandwich structure, which confers them a high resistance to the acidic conditions of gastric digestion. In addition, lectins can be absorbed into the blood stream in active form.These carbohydrate-binding proteins act as immunomodulatory agents able to enhance the immune system stimulating the lymphocyte and cytokine proliferation or the antibody synthesis (Hartmann & Meisel, 2007). For example, concanavalin A, a well-known lectin from jackbean seeds (*Canavalia ensiformis L.*) has been shown to have a very high anti-hepatoma activity (Carbonaro *et al.,* 2015).

Conglutin γ. Conglutin γ is a minor protein component isolated from some legume seeds (i.e. lupin and soybean). It is a tetrameric protein where each monomer contain a 29 and a 17 kDa polypeptides linked by a disulfide

bond. Conglutin γ shows a unique sequence and it is not degraded by proteases during seed germination. This protein can cross the intestinal barrier in an intact form and thus performing its glucose-controlling effect thanks to its ability to bind insulin (Magni *et al.,* 2004)

Lunasin. Lunasin is a naturally-occurring 43-amino acid peptide (5.4 kDa) corresponding to the small subunit of the 2S albumin (Hernandez-Ledesma *et al.,* 2009). This bioactive peptide displays an α-helix structure and contains nine aspartic acid residues at the C-terminal region. It is highly bioavailable and heat stable (100°C, 10 min), and shows anticancer, anti-inflammatory, antioxidant and cholesterol-lowering activities (Lule *et al.,* 2015). Lunasin activity is based on inhibition of histone (H3 and H4) acetylation and repression of cancer cell cycle progression. This peptide also shows hypocholesterolemic activity, reducing the production of 3-hydroxy-3-methylglutaryl-CoA reductase that is necessary for cholesterol biosynthesis (Carbonaro *et al.,* 2015). Cholesterol-lowering food ingredients based on lunasin extracts are already present on the market (Jeong *et al.,* 2003; Udenigwe and Aluko, 2012). Some examples of soybean protein preparations, with the relative lunasin concentrations, are shown in **Table 4**.

Table 4. Soybean protein preparations on the market with the relative lunasin concentrations (*source*: Carbonaro *et al.,* 2015)

Soy preparation*	**mg lunasin/g protein**
Soy protein concentrate, water washed (70% protein)	16.52
Soy protein concentrate, alcohol washed (70% protein)	8.72
Soy protein isolate (90% protein)	6.92
Defatted soy flour (50% protein)	5.48

*Proteins extracted with 0.1 M PBS (pH 7.0)

Angiotensin I-converting enzyme (ACE) inhibitory peptides. ACE inhibitory peptides have been isolated from different legumes like soybean, pea and chickpea (Pedroche *et al*., 2002; Vermeirssen *et al*., 2005). They showed antioxidative properties and proved to be effective in the prevention and treatment of hypertension, heart failure, myocardial infarctions and diabetic nephropathy in human and animal models (Meisel *et al*., 2005).

Peptides from storage protein degradation. Active peptides may result from proteolysis of β-conglycinin, as in the case of those with inhibiting effect on lipid accumulation in adipocytes *in vivo,* or from glycinin, as in the case of thehydrophobic peptide corresponding to residues from 114 to 161 with anticancer properties (Kim *et al*., 2000; Martínez-Villaluenga *et al.,* 2008). Another hydrophobic peptide of 0.75 kDa was produced by *in vitro* gastrointestinal digestion of soybean lipoxygenase. Jiménez-Escrig *et al.* (2010) showed as the bioactivity of this peptide is due to the presence of a Val residue at terminal position.

Protein digestion

Proteolytic activity of gastrointestinal enzymes and their effect on protein digestibility and functionality

Protein digestion essentially takes place along the GIT by means of stomach acidic condition (gastric juices), bile and different digestive enzymes released by exocrine system glands. Peptides and amino acids following protein degradation are absorbed by epithelial cells of intestinal lumen and moved into blood or lymph. Gastrointestinal enzymes can be grouped in endopeptidases and exopeptidases. The enzyme pepsin is the main gastric protease. This enzyme is an endopeptidase secreted by stomach mucosa cells and it is mostly active on the bonds involving phenylalanine, tyrosine and tryptophan amino acids. Trypsin, chymotrypsin and elastase are

endopeptidases that produce oligopeptides from gastric polypeptide degradation along the intestinal tract (Silk *et al*., 2007; Bourlieu *et al*., 2014). The protein degradation products are further digested by carboxypeptidases (A and B), which are exopepithases able to remove amino acids from the carboxy-terminal residual. Brush Border Membrane (BBM) peptidases are involved in the final step of protein digestion. These enzymes, which are mainly placed in jejunum microvilli, are responsible for the production of short oligopeptides (di and tri-peptides) and free amino acids. BBM peptidases include both eso and endopeptidase. Differently from pancreatic proteases, BBM esopeptidases, which are mostly constituted by amino-peptidases, specifically remove amino acids from the amino-terminal residual.

Protein digestion can generate a large variety of peptides and free amino acids. In addition, peptides from food matrices can survive gastrointestinal digestion, being resistant to proteolytic activity of digestive enzymes (i.e. proline-rich sequences). Some of these peptides can either be toxic for human (i.e. allergenic sequences) or can improve the human health, thanks to their biological protperties (bioactive peptides and positively-charged amino acids) (Clemente and Olias, 2017).

In vitro and ex vivo gastrointestinal digestion models

In vitro gastrointestinal digestion models. Non-cellular fluid models are essentially aimed to simulate gastrointestinal digestion by using different experimental conditions (pH, digestion time, enzyme amount, etc.) (Mat *et al*. 2016; Bohn *et al*., 2017). They are classified as dynamic and static models. Dynamic models mimic *in vivo* digestion processes. They are compartmentalised and equipped by human juices. The most complex models have also mechanical devices to mimic food decomposition. These models are more complex and expensive than static ones. In the static models,

chemical and enzymatic digestion are reproduced by using synthetic fluids without reproducing *in vivo* physical processes (cutting, mixing, hydration, etc.). The main advantages of static systems are the relatively low costs and the good adaptability to studied food needs (Wickham *et al*., 2009; Lefebvre *et al*., 2015; Mat *et al.,* 2016). Digestive enzymes (i.e. α-amylase, pepsin, pancreatin and lipase) used in static models have different origin (human, vegetal or animal). They are used in different concentrations according to the matrix type and the experimental conditions. During the simulated digestion, the temperature value is always about 37°C, while the times may vary (Hur *et al*., 2011). Given the high number of proposed protocols, to overcame the problems related to data comparison among different research groups, COST action INFOGEST (a network composed by more than 200 scientists from 32 different countries working on digestion studies) proposed a harmonised *in vitro* protocol with the aim to simulate human digestion in a standard way (Minekus *et al*., 2014). Briefly, the harmonised method consists of three phases: (A) oral, (B) gastric and (C) intestinal (**Figure 2**). For each phase, the concentration of *in vivo* salts and enzymes is reported. Standardised assays, to define the activity of each enzyme, are also indicated. Normally, *in vitro* models, including the harmonised model of Minekus e*t al*. (2014), do not provide for the use of BBM enzymes (jejunum step). However, some studies have emphasised the need to include these enzymes into *in vitro* digestion models because of their intense hydrolytic activity able to produce molecules such as amino acids, glucose or free fatty acids (Picariello *et al*., 2016). BBM enzymes, therefore, would be necessary in those investigations on nutrient bioaccessibility and bioavailability.

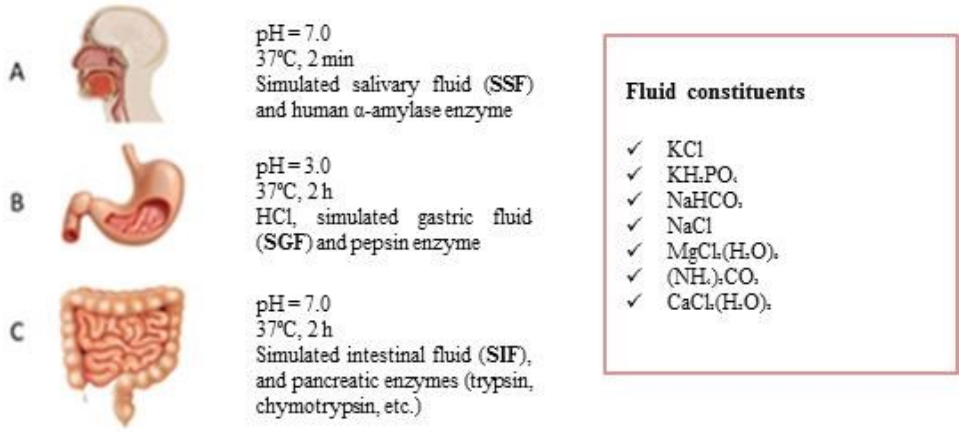

Figure 2. Gastrointestinal conditions of *in vitro* model according to Minekus e*t al*. (2014)

Ex vivo gastrointestinal digestion models. These models use human gastrointestinal juices as digestive fluids in place of commercial enzymes from animal origin. *Ex vivo* models should allow a more realistic simulation of human digestion since the employed juices contain different enzymes, inhibitors and salts. Moreover, juice enzymes are composed by different isoforms, which may differ from commercial enzymes (purified enzymes extracted from animals) in terms of either activity or specificity. The Human Gastric Juice (HGJ) contains pepsin and gastric lipase, while the Human Duodenal Juice (HDJ) consist of proteolytic and lipolytic enzymes, different inhibitors and bile salts (Devle *et al*., 2014). In this kind of models, to significantly reduce the great inter-individual variability related to enzymes and other juice components, the use of gastrointestinal juice pools is suggested (Ulleberg *et al*., 2011).

Effects of germination on legume proteins

The nutritive value of food plants is strictly connected with their protein content. Factors such as anti-nutrients, protein digestibility and amino acid composition can negatively affect nutritional properties of legumes. For instance, protein digestibility may decrease because of the presence of anti-physiological factors or by protein structural properties (Vanucchi *et al.*, 2005). However, legumes have a high nutritional value due to the presence of essential amino acids, such as lysine that is scarce in cereals, and other non-protein components such as fiber, minerals and vitamins.

Food quality and security are worldwide key issues, especially for developing countries where proteins of different vegetable sources (i.e. legumes and cereals) are combined or processed with the aim to increase their nutritional value. In this regard, germination may be an usefull method to modulate vegetal protein content (Fernandez and Berry, 1988). It is a natural plant process necessary for seed growing and development that occurs in specific environmental conditions (i.e. humidity, temperature and nutrient content) (Sangronis and Machado, 2007). During germination, the amount and the biological value of nutrients may change depending on the process conditions and seed variety (Sangronis and Machado, 2007). These changes are due to the hydrolytic activity of proteolytic, amylolytic and lipolytic enzymes (Rahman, *et al.,* 2007). Storage proteins mainly compose the nitrogenous reserves of legume seeds. Germination decomposes storage proteins producing amino acids that are used to synthesize new metabolic proteins by plants. Consequently, the non-protein nitrogen increases (Kumar and Venkataraman, 1978). This natural process is actually an ancient technique employed to improve cereal and legume nutritional value. In fact, germation is able to increase the digestibility of proteins, as well as the bioavailability of certain amino acids and vitamins. This technique is also

used to remove seed flaws such as undesired flavors or trypsin inhibitors (Fernandez and Berry, 1988; Sangronis and Machado, 2007). Trypsin inhibitors prevent trypsin activity reducing both protein degradation and easily absorbed amino acid production. Therefore, the reduction in trypsin inhibitor amount should have to improve protein digestion (El-Adawy, 2002; Zhou *et al*., 2013).

Soybean, chickpea and grass pea crops

Soybean (*Glycine max L.*) is the most important legume crop that provides sources of oil and protein for human and livestock. Soybean seeds are used in Asia and other parts of the world to prepare different fresh, fermented and dried foods (Singh and Hymowitz, 1999). Soy-based products such as tofu, soy milk, soy sauce and miso are addressed to animal feed. Furthermore, this legume contributes to more than 50% of globally consumed edible oil. Besides its domestic use, soy oil is largely employed in industries related to production of pharmaceuticals, plastics, papers, inks, varnishes, pesticides and cosmetics (Song *et al*., 2011; Ko *et al*., 2013). Currently, soy oil also represents a renewable source of energy for industrial uses. As a legume crop, soybean is capable of using atmospheric nitrogen through biological nitrogen fertilization, and it is therefore less dependent on synthetic nitrogen fertilizers. From a nutritional point of view, soy-based products are gaining attention because of their pharmaceutical attributes, such as anti-cancerous properties (Ko *et al*., 2013). A trend that was also confirmed by Food and Drug Administration (FDA), which reported as both total and low-density lipoproteins (LDL) cholesterol decreased significantly following the total or partial substitution of animal proteins with those from soy in human diet. The chemical composition of soybean is shown in **Table 5**.

Chickpea (*Cicer arietinum L.*) is an Old-World legume currently grown in over fifty countries across the Indian subcontinent, North Africa, the Middle East, southern Europe, the Americas and Australia. Globally, *Cicer arietinum L.* is the third most important legume crop in production (FAOSTAT, 2011). India is the largest chickpea-producing country, accounting for 66% of global production (FAOSTAT, 2011), followed by Pakistan, Turkey, Australia, Myanmar, Ethiopia, Iran, Mexico, Canada and the USA. There is a growing demand for this legume due to its nutritional value. If compared with other pulses, chickpea has a higher concentration of globulins that are almost 60% of total proteins. Besides being a protein-rich source, *Cicer arietinum L.* has a high content of carbohydrates, and it represents a good source of dietary fibre, vitamins and minerals (phosphorus, calcium and iron) (**Table 5**) (Agriculture and Agri-Food Canada, 2006; Wood and Grusak, 2007). It is also cholesterol free. Globally, chickpea is mostly consumed as a seed food in several different forms and preparations according to ethnic and regional factors (Muehlbauer and Tullu, 1997; Ibrikci*et al.,* 2003). In the semi-arid tropics, this legume is an important component of the diets of those individuals who cannot afford animal proteins or those who are vegetarian by choice. In the Indian subcontinent, *Cicer arietinum L.* is mostly used to produce flours for the production of bread and other bakery products. In other parts of the world, especially in Asia and Africa, chickpea seeds are used in stews and soups/salads, and consumed in roasted, boiled, salted and fermented forms (Gecit, 1991). Furthermore, this legume is of interest as a functional food with potential beneficial effects on human health.

Table 5. Chemical composition of soybean and chickpea per 100 g of dry grain (*source*: Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, CREA)

Chemical component	**Soybean**	**Chickpea**
Energy (kcal)	398.00	334.00
Water (g)	8.50	13.00
Protein (g)	36.90	21.80
Lipids (g)	18.10	4.90
Fiber (g)	11.90	13.80
Starch (g)	11.10	46.00
Sodium (mg)	4.00	6.00
Potassium (mg)	1740.00	800.00
Iron (mg)	6.90	6.10
Calcium (mg)	257.00	117.00
Phosphorus (mg)	591.00	299.00
Vitamin B1 (mg)	0.99	0.36
Vitamin B2 (mg)	0.52	0.14
Vitamin PP (mg)	2.50	1.70
Vitamin A (mg)	0	30.00
Vitamin C (mg)	0	5.00

Grass pea (*Lathyrus sativus L.*) is an annual leguminous crop addressed to animal and human consumption. It is cultivated in East Africa, Eurasia, North America and in some parts of South America (Smartt, 1990). The plant has a very hardy and penetrating root system that supports growth on a wide range of soil types, including very poor soil and heavy clays. As a legume, it contributes to soil quality through the action of nitrogen-fixing symbiotic bacteria associated with the root system. Grass pea is very resistant to insects and pests, and it is tolerant to extreme environmental conditions, such as flooding, high salinity and low soil fertility (Yan *et al.*, 2006). It is thus a good model for investigating the mechanism of drought resistance and seeking genes associated with drought resistance. In fact, *Lathyrus sativus L.* gained a great popularity in many developing countries in those areas with extreme weather conditions (Yan *et al.*, 2006). However, the prolonged consumption of grass pea can provoke a characteristic motor neuron disease, named neurolathyrism, in both animals and humans. The agent responsible for neurolathyrism is the be b-N-oxalyl-L-a,b-diaminopropionic acid (b-ODAP) b-1, which is present in the seed as free amino acid (Yan *et al.*, 2006). The chemical composition of grass pea is shown in **Table 6**. This legume is a lysine-rich source, but it is deficient in methionine, cysteine and tryptophan amino acids (Ravindran and Blair, 1992; Gatel, 1994). It has also a low amount of polyunsaturated fatty acids and a high starch content, while its mineral amount depends from soil mineral content.

Table 6. Chemical composition of grass pea per 100 g of dry grain (*source*: Yan *et al*., 2006)

Chemical components (g)	Grass pea
Protein	28.07
Ash	3.02
Lipids	1.22
Fiber	5.66

PhD thesis purpose and innovation

Legume proteins have interesting nutritional properties due to their high nutritious value, but they can also exert biological activities useful for human health. In fact, they can have protective or therapeutic effects on chronic diseases such as diabetes, cancer, cardiovascular pathologies, overweight and obesity (Rizzello *et al.,* 2015). Hence, the presence of legumes in human diet has increased considerably, as well as the interest in the formulation of new food products or ingredients based on them. An example can be the addition of legume flours to bread, and other bakery products, with the aim to improve their amino acid profile (nutritional value).

Therefore, this PhD thesis is focused on the characterization of proteins from legumes (soybean, chickpea and grass pea) to assess their potential use as natural ingredients in food preparations. The effect of germination on protein digestibility and allergenicity in legume-based ingredients (chickpea flour) was also investigated.

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Chapter 2. Proteomic characterization of water-soluble extracts from soybean (*Glycine max L.*), chickpea (*Cicer arietinum L.*) and grass pea (*Lathyrus sativus L.*) seeds, and assessment of their resistance to *in vitro* gastrointestinal digestion

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The present chapter is going to be submitted as a research article for publication

Abstract

Legume seeds represent a rich source of storage proteins, but they also contain a water-soluble fraction (WSF) composed by enzymes, protease inhibitors, lectins and bioactive peptides. WSF proteins have an important role in the control of different biological activities. For instance, soybean Kunitz and Bowman-Birk inhibitors are able to inactivate proteolytic enzymes of bacteria, insects and animals. Furthermore, WSF peptides can exert cardiovascular, endocrine, antimicrobial, antimutagenic, immunological and neurological activities. Consequently, legume WSF is currently investigated for potential biological, nutritional and clinical applications. In the present study, water-soluble proteins and peptides from soybean, chickpea and grass pea were extracted using a specific protocol and then subjected to proteomic characterization. The effect of simulated gastrointestinal digestion on protein extracts was also investigated. Briefly, the high number of proteins and peptides from seeds detected in the extracts may suggest their use as stabilizing agents in foods and beverages. Furthermore, several WSF proteins survived to simulated digestion, including BBM enzyme hydrolysis, confirming thus their ability to exert beneficial effects on human health.

Introduction

Legume-based products are one of the most consumed protein source globally. The protein conent of legume seeds ranges from 17 to 40% (Butt and Batool, 2010) and it is mainly composed by storage proteins (Duranti and Gius, 1997). However, legumes also contain enzymes, protease inhibitors, lectins and bioactive peptides (Roy *et al.*, 2010). These minor protein components are mostly water-soluble and show interesting biological activities. In fact, soybean Kunitz and Bowman-Birk inhibitors are able to inactivate proteolytic enzymes of bacteria, insects and animals (Dunaevsky *et al*., 2005), while lunasin peptide shows anticancer, anti-inflammatory, antioxidant and cholesterol-lowering activities (Lule *et al.,* 2015). ACE inhibitory peptides, and those derived from storage protein degradation, can exert cardiovascular, endocrine, antimicrobial, antimutagenic, immunological and neurological activities (Meisel *et al*., 2005; Carbonaro *et al*., 2015). In literature, numerous studies report the great interest of scientific community on legume water-soluble protein fraction. In fact, it is currently investigated for potential biological, nutritional and clinical applications in the control of certain forms of cancer, the activation of innate defence mechanisms and the managing of obesity (Roy *et al*., 2010).

In this perspective, the aim of the study was to assess the ability of the water-soluble protein fraction isolated from legumes to improve food quality and safety (i.e. food healthiness and shelf life). Therefore, water-soluble extracts (WSEs) composed by proteins and peptides were produced from soybean, chickpea and grass pea using a specific protocol, and then subjected to proteomic characterization. The effect of simulated gastrointestinal digestion on WSE proteins was also investigated.

Materials and Methods

Chemicals

Ethanol, trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Carlo Erba Reagents (Milan, Italy). Sodium hypochlorite, acetone, water, Biuret reagent, Folin-Ciocalteau reactive, sodium-phosphate buffer, pepsin, pancreatin, bile salts, sodium thiosulfate, potassium ferrocyanide, acetonitrile (ACN), dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (AMBIC) and MALDI matrices were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for electrophoresis analysis were purchased from Bio-Rad (Milan, Italy). Proteomic grade trypsin was purchased from Promega (Madison, WI, USA).

WSE production

To extract water-soluble proteins and peptides from soybean, chickpea and grass pea seeds was used the protocol of Pavalli *et al.* (2012) with significant changes (**Figure 1**). About 70 dried seeds of soybean, chickpea and grass pea purchased from local retailers were disinfected with 50% ethanol for 5 min, and then with 2.5% sodium hypochlorite for further 5 min. After washing with water, 50 intact seeds were selected and transferred into 250 mL bottle. The seeds were incubated with 100 mL of water and subjected to magnetic stirring (50°C, 4h). After incubation, the extracts were filtered with 10-20 µm filter paper (VWR, Milan, Italy) and then moved to 50 mL plastic tubes. Samples were subjected to centrifugation (4000 g x 10 min) (R-8D Remi centrifuge, Milan, Italy), and the clear supernatants, containing the released proteins from seeds, were transferred into new plastic tubes before to be freeze-dried.

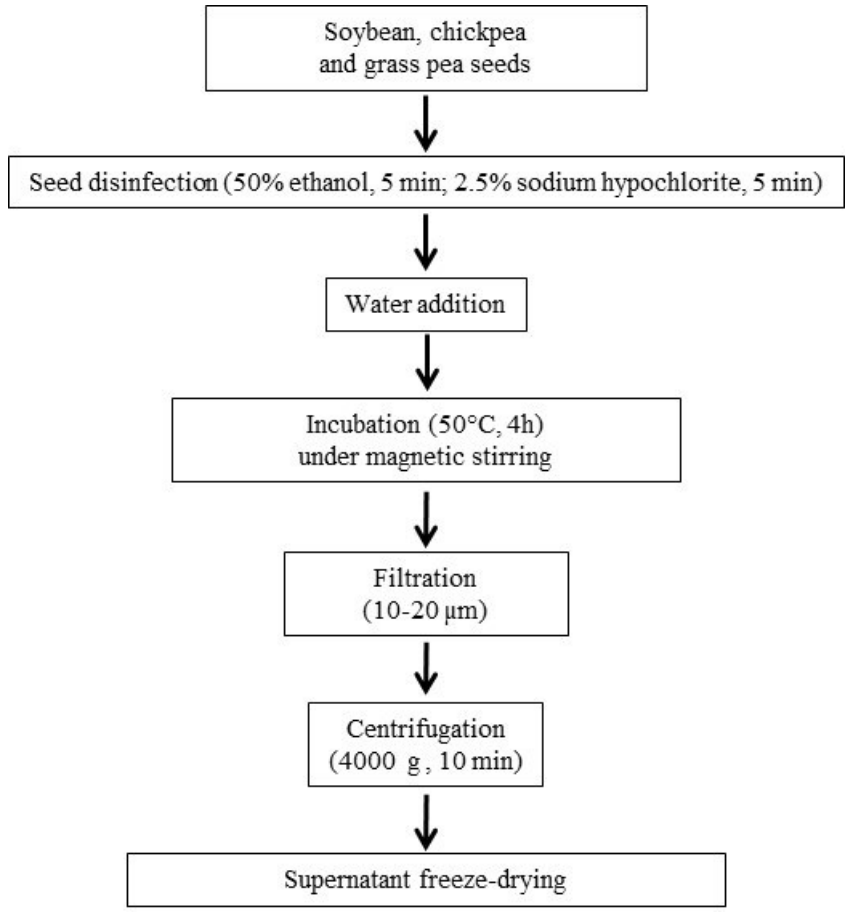

Figure 1. Workflow of soybean, chickpea and grass pea WSE production

WSE protein content determination

WSE protein content was determined by Lowry assay. Samples were suspended in 0.1% TFA (v/v) in water and then subjected to magnetic stirring for 10 min at room temperature. After dilution and Biuret reagent adding, the solution was left to rest for 10 min, in order to allow the cupric compound formation, and then mixed with Folin-Ciocalteau reactive. A blank, in the same conditions, was also carried out. Protein concentration was calculated reading the absorbance at 595 nm using a spectrophotometer mod. UV-1601 (Shimadzu, Milan, Italy).

SDS-PAGE analysis

WSEs were dissolved (1 mg of protein/mL) in sample buffer (0.05 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol (w/v), 5% β-mercaptoethanol (v/v) and trace of bromophenol blue indicator), stirred for 5 min at room temperature and then left to rest overnight to allow the complete protein solubilisation. After heating at 95°C for 10 min, the samples were loaded on 15% polyacrylamide gel. Electrophoretic separation was carried out at constant voltage (100 V) using a Mini-Protean Cells electrophoresis II (Bio-Rad, Hercules, CA, USA). A molecular weight marker was loaded on each gel. After migration, the gel was subjected to silver staining.

Protein in-gel digestion

Electrophoresis-isolated proteins were in-gel digested essentially according to Ferranti *et al.* (2014) with slight changes. Briefly, gel bands from 1-DE gel were manually excised and destained by repeated washing with 0.1 M sodium thiosulfate/0.03 M potassium ferrocyanide (1:1, v/v). Afterwards, gel spots were dehydrated with 500 µL of ACN. Proteins were reduced by incubation with 0.010 M DTT (1 h, 56 °C), and then alkylated with 0.055 M IAA (45 min at room temperature in the dark). Gel spots were further washed with 0.025 M AMBIC, dehydrated with 100 µL of 100% ACN and then dried in a speed-vac. Proteins were digested overnight at 37°C after rehydrating gel pieces with 10–20 µL of a trypsin solution (12.5 ng/mL in 0.025 M AMBIC). The resulting peptides were extracted three times with 40 µL of 50% ACN/5% FA (v/v). Extracts were combined and dried in a vacuum centrifuge prior to mass spectrometry analysis.

In vitro gastrointestinal digestion

WSEs were subjected to *in vitro* gastrointestinal digestion following the protocol of Minekus *et al.* (2014) with some changes. Legume aqueous

extracts were centrifuged (4000 g x 10 min) and the obtained supernatants were freeze-dried. Afterwards, the samples were dissolved in 5% FA (1 mg of protein/mL) and pepsin from porcine gastric mucosa (enzyme/protein 1:100, w/w) was added. After incubation (37°C, 1h) (gastric digestion), sample FA residual was removed by using a speed-vac centrifuge (Termo Fischer, Milan, Italy). Before to perform simulated duodenal digestion, the solutions were freeze-dried and washed with water twice. Samples were thus mixed with 200 µL of 0.1 M sodium-phosphate buffer (pH 7.0), 20 µL of pancreatin (1mg of enzyme/mL) and 80 µL of bile salts. After incubation (37°C, 2h), the digestion was stopped by heating (85°C, 15 min) and the samples were incubated again (37°C, 4h) after adding 10 µL of porcine Brush Border Membrane (BBM) peptidases (650 mU/mg).

HPLC analysis

HPLC analysis was performed both on water-soluble extracts and digests using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) equipped with a reverse phase 218TP Vydac C_{18} column (250 X 4.6 mm, 5µM, Grace, Lokeren, Belgium). HPLC system was connected to Ultraviolet (UV)-Diode Array Detector (DAD) (Agilent Technologies, Waldbron, Germany) setting at 220 and 280 nm. Samples were resuspended in 0.1% (v/v) TFA solution (Eluent A) and centrifuged (4000 *g* x 10 min) before supernatant injection. The injection volume was 50 µL and the flow was set at 0.2 mL/min. Analyte elution was performed with linear gradient from 15 to 100% of 0.1% TFA (v/v) in ACN (eluent B) in 70 min. Each sample was analysed in triplicate.

LC-high resolution (HR)-MS/MS analysis

LC-HR-MS/MS analysis was performed on WSEs using a Q ExactiveOrbitrap mass spectrometer (Thermo Scientific, San Jose, CA,

USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography instrument (Thermo Scientific). Samples were resuspended in 0.1% (v/v) FA solution, loaded through a 5mm long, 300 µm id pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C_{18} column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent C was 0.1% FA (v/v) in water; eluent D was 0.1% FA (v/v) in ACN. The column was equilibrated at 5% D. Peptides were separated applying a 4–40% gradient of D over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/*z scan range of 350 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of $1x10^6$ ions and a maximum ion injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were processed using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were processed using the Proteome Discoverer 2.1 software (Thermo Scientific). Database searching parameters for identification of proteins were the following: Met oxidation and pyroglutamicfor N-terminus Gln as variable protein modifications; carbamidomethyl cysteine as a constant modification; a mass tolerance value of 8 ppm for precursor ion and 0.01 Da for MS/MS fragments; trypsin as the proteolytic enzyme or no enzyme specificity; missed tryptic cleavage up to 2. Database searching parameters for identification of peptides in samples were the same described above, except for no modification of cysteine residues included and no proteolytic enzyme selected. The false discovery rate and protein probabilities were calculated by

Target Decoy PSM Validator working between 0.01 and 0.05 for strict and relaxed searches, respectively. Proteins were considered confidently identified based on at least four sequenced peptides. Each sample was analysed in triplicate.

MALDI-TOF-MS analysis

MALDI-TOF-MS analysis was performed on water-soluble extracts and digests, as well as on in-gel digested proteins. Samples were previously suspended in 0.1% TFA (v/v) in water. MALDI-TOF mass spectra were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA) equipped with a N2 laser (λ = 337 nm), using α-cyano-4-hydroxy-cinnamic acid as matrix (10 mg/mL in 50% ACN, v/v, containing 0.1% TFA). Mass spectra were acquired in the reflector positive ion mode using the Delay Extraction (DE) technology. The accelerating voltage was 20 kV. External mass calibration was performed with a commercial mixture of standard peptides (PerSeptive Biosystems, Framingham, MA). A resolution of $\geq$ 8.000 was calculated in the working mass range. Raw data were analysed using the Data Explorer 4.0 software furnished with the spectrometer. Post-source decay (PSD) MS analysis was carried out after isolation of the precursor ions using a timed ion selector set at an ion gate width of 1 Da. The PSD mass spectra were divided into seven segments; the laser power and the guide wire voltage were varied for each segment to optimize fragmentation and data collection. Approximately 200 laser shots were acquired for each segment. Fragmented ions were refocused onto the final detector by stepping down the voltage applied to the reflector. Finally, the individual segments were stitched together using the software purchased with the instrument. Peptide mass fingerprinting (PMF)-based identifications were carried out interrogating the non-redundant National Center for Biotechnology Information (nrNCBI) and Swiss-Prot/TrEMBL databases

with Mascot (Matrix Science, London, UK) and Protein Prospector MS-FIT (http://prospector.ucsf.edu/) search engines. Mass tolerance of 0.3 Da, fixed carbamidomethylation of cysteines, variable pyro-glutamic acid formation at N-terminal Gln and possible methionine oxidation were set as search parameters. Up to one missed tryptic cleavage was accepted. Probability MOWSE scores were automatically calculated by the search engines; only protein candidates with score higher than the random match region (pb 0.05%) were considered. The identification of top scores of protein candidates was validated by manual peptide mass mapping. Each sample was analysed in triplicate.

Analyte purification for MALDI-TOF-MS analysis

Peptides derived from 1-DE protein spot hydrolysis were analysed afterwards by Zip-Tip purification. C_{18} Zip-Tips were washed twice by methanol and water, respectively, and then equilibrated using eluent A three times. Peptides were eluted by aspirating and dispensing a solution of 50% ACN/0.1% FA three times.

Results and discussion

Proteomic characterization of WSEs and in-gel digested proteins

In **Table 1** are reported the protein concentrations of soybean, chickpea and grass pea WSEs misured by Lowry assay.

Table 1. Protein concentrations of soybean, chickpea and grass pea WSEs by Lowry assay (mg/mL)

WSE	Protein concentration (mg/mL)
Soy	0.31 ± 0.02
Chickpea	0.31 ± 0.01
Grass pea	0.63 ± 0.04

HPLC analysis performed on legume WSEs highlighted the presence of some proteins eluting from 20 to 30 min with molecular weights (MWs) ranged from 7 to 8 kDa. These proteins were mainly identified as Kunitz and BBI inhibitors, and their isoforms, as shown in **Figures 2-4**.

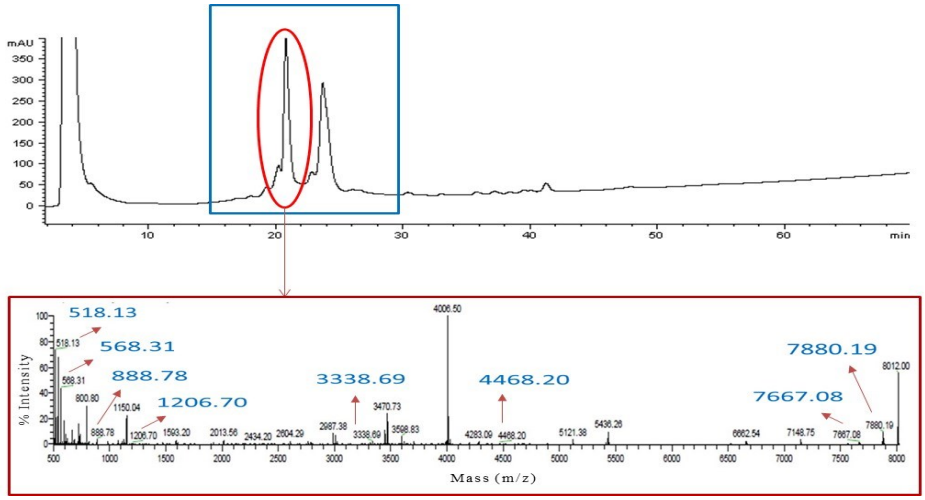

Figure 2. HPLC chromatogram of soybean WSE (220 nm) and ESI-MS spectrum of the protein fraction eluted at 20 min

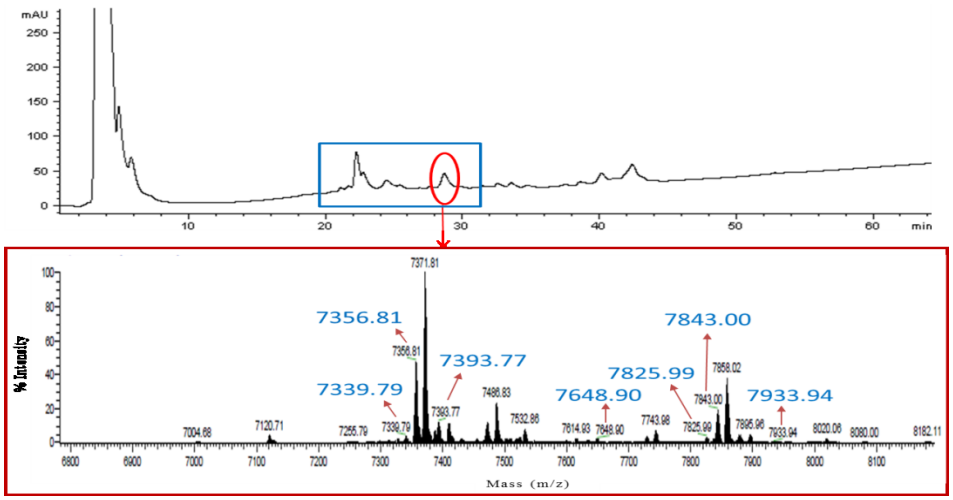

Figure 3. HPLC chromatogram of chickpea WSE (220 nm) and ESI-MS spectrum of the protein fraction eluted at 28 minutes

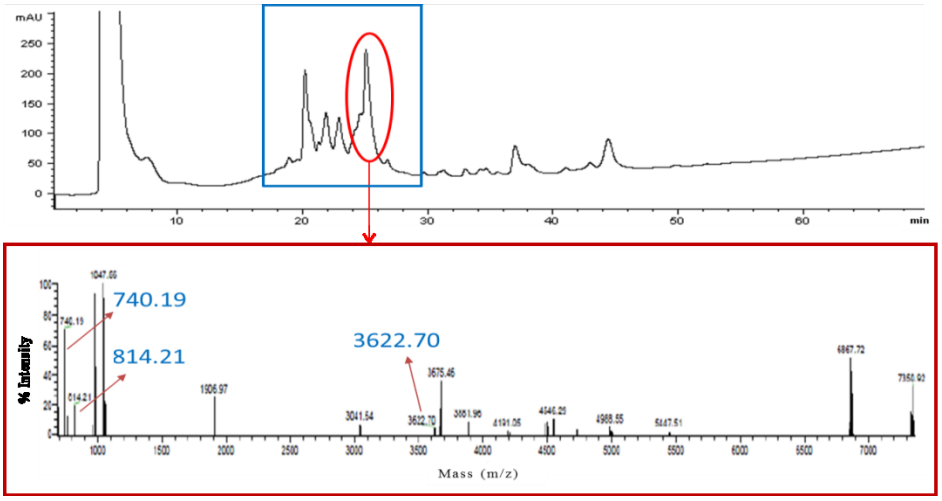

Figure 4. HPLC chromatogram of grass pea WSE (220 nm) and ESI-MS spectrum of the protein fraction eluted at 26 minutes

In soybean extract, a peptide with a mass of 5151.43 Da was revealed. This peptide was identified as the bioactive molecule lunasin, which is normally detected with a mass of 5023.23 Da (43 amino acid form) (**Figure 5**). However, Seber *et al.* (2012), analysing purified samples of lunasin, reported as the most abundant peptide found had a higher mass due to an asparagine residual more.

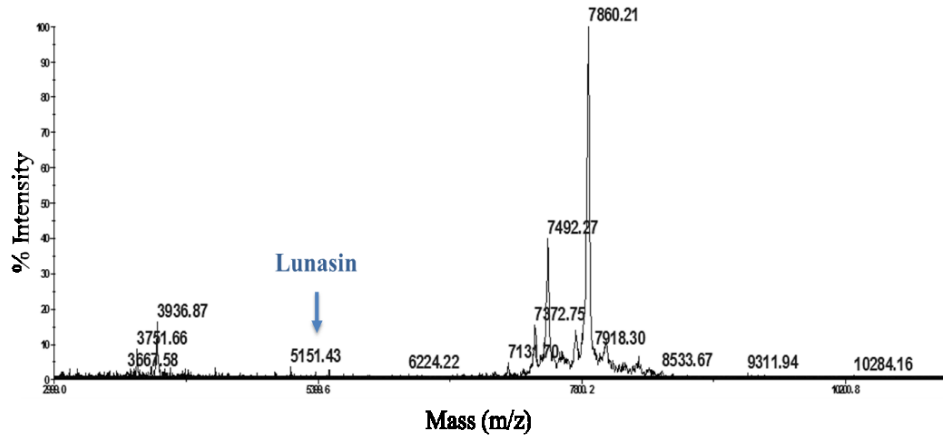

Figure 5. MALDI spectrum section of soybean WSE protein fraction obtained by HPLC in which lunasin is shown

Furthermore, LC-HR-MS/MS analysis confirmed as the chromatographic picks collected at 20.6 and 23.6 min corresponded to IBBD2 (**Figure 6**) and IBB1(**Figure 7**) isoinhibitors, respectively.

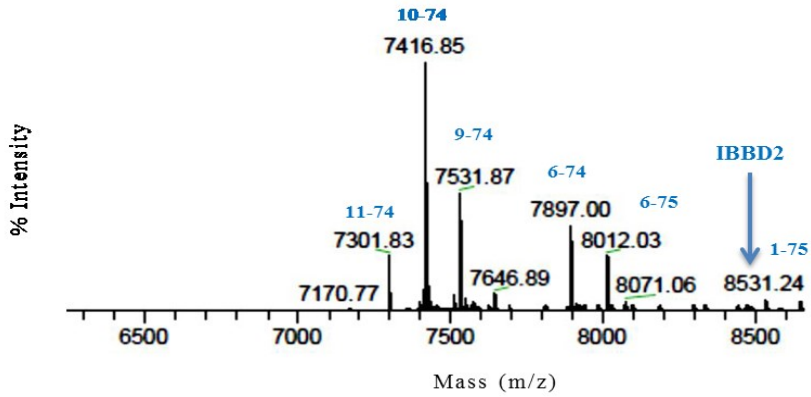

Figure 6. IBBD2 isoinhibitor and its protein fragments in soybean WSE

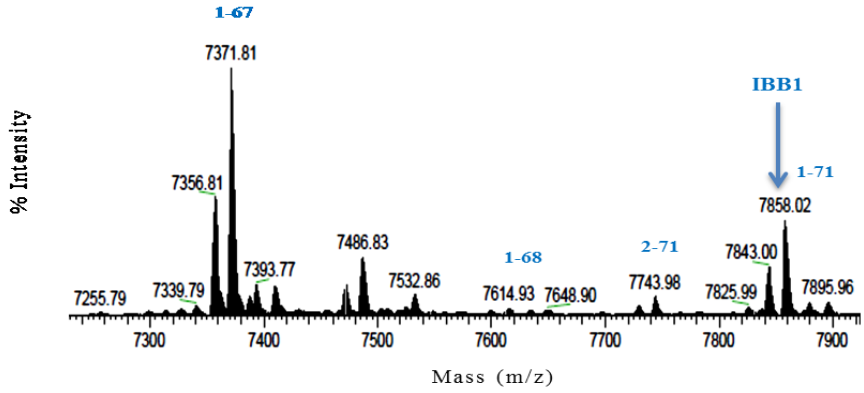

Figure 7. IBB1 isoinhibitor and its protein fragments in soybean WSE

More than 170 peptides were also detected (**Table 2**). Being mostly composed by positively-charged amino acid sequences, most of those peptides might exert biological activities (i.e. antioxidant or antimicrobial activities) (Palmer et al., 1988) potentially useful for the food industry. For instance, thanks to their antimicrobial properties, legume peptides could be

employed as stabilizing agents in food products to increase their shelf life (Amigo-Benavent et al., 2013).

Table 2. Peptides identified in soybean WSE by LC-HR-MS/MS analysis

Sequence	Master Protein Accessions	Theo. MH+ [Da]
DLFSSAHNMPKSGQTTMDSNTSDQSQMQRDTQEGG SKEFTT	Q9XES8	4509.95
TDRQQHGTTGGYAGDTGRQHGNIGGPYYGTN	947090546	3236.45
DKSGEERAQEEAIDIDESKFRTGNNQDKNQNK	255629381	3693.73
TADTGTGPRSGTTGGTGYGGTGGTDYGTTGG TGYGSGTGYGVNTGGAHTE	947090546	4504.93
TDRQQHGTTGGYAGDTGRQHGNIGGPYYG	947090546	3021.36
TDRQQHGTTGGYAGDTGRQHGNIGGPYYGTNTA	947090546	3408.53
TGRQHSSGGYDGDTNKHHGTTGGYNDDTNRHHGTT	947090546	3738.60
EVGQDIQSKAQDTREAAARDARDAREAAARDARD	A0A0R0FZF4	3712.81
GRKGGLSTVEKSGEERAQEE	728048777	2147.07
KLQEKVHDPAGKGGPVFGAGKDEDKQDLGVTGTG	K7L730	3435.75
TDRQQHGTTGGYAGDTGRQHGNIGGPY	947090546	2801.27
GYQEMGRKGGLSTVEKSGEERAQEE	728048777	2755.29
TKEVGQKTKEVGQDIQSK	A0A0R0FZF4	2003.08
ELDEKARQGETVVPGGTGGKSLE	255637579	2357.19
KLQEKVHDPAGKGGPVFGAGKD	K7L730	2235.19
SDEEGFGGVYGGNQSKPEMDPAYDKTQGSEVK	255646215	3406.50
EMGRKGGLSTVEKSGEERAQEE	728048777	2407.15
TDRQQHGTTGGYAGDTGRQHGNIGGPYYGT	947090546	3122.40
EKVHDPAGKGGPVFGAGKDEDKQDLGVTGTG	K7L730	3066.51
TDRQQHGTTGGYAGDTGRQH	947090546	2142.96
GNIGEKAQAAKEKTQEMAQAAKEKTQ	351727184	2788.42
GNIGEKAQAAKEKTQ	351727184	1572.83
DKEGIPPDQQRLIFAGKQLEDGRTL	255632677	2824.49
TRTAAGGYSDDINKQH	947090546	1733.82
GGGGQGRGVGIGSGVGSGTGYGGGGTGIG SGDSSRGGGGR	K7N277	3266.52
TGAAQQKTSEMGQSTKESAQSGKDNTQ	351727184	2798.28
KLQEKVHDPAGKGGPVFGAGKDED	K7L730	2479.26

QNTGAAQQKTSEMGQSTKESAQSGKDNTQ	351727184	3040.39
EYGNVEKQTDEYGNPVHA	947090546	2049.91
YVATRTAAGGYSDDINKQH	947090546	2066.99
AQQHLAEGRSKGGQTRKEQLGTE	728048777	2509.29
RTAAGGYSDDINKQHDTTN	947090546	2063.94
TRTAAGGYSDDINKQHDTTN	947090546	2164.98
DERARQGETVVPGGTGGK	255629381; 728048777	1813.91
TLKAADQIAGQTFNDVGRFDEE	356533407	2425.16
DERARQGETVVPGGTGGKSLE	255629381; 728048777	2143.07
GVVGSHPIGTNRGPGGTATAH	445632; Q541U1	1942.98
DKEGIPPDQQRLIFAGK	255632677	1912.02
QTGEKVKGAAQGATEAVK	351727184	1772.95
GVVGSHPIGTNRGPGGTAT	445632; Q541U1	1734.89
YTERPQNEEPEAYHIR	I1JFL5	2031.95
NTRAGGKPNDYGYGTGGT	Q541U1	1785.81
GGTGYGSGTGYGVNTGGAHTE	947090546	1899.81
YVEATPVEAGRSSAIKEN	I1NGG4	1920.97
EYGNVEKQTDEYGNPVHAA	947090546	2120.95
QTGEKVKGAAQGATEAV	351727184	1644.85
ESKFRTGNNKNQNQNEDQD	728048777	2266.01
GVVGSHPIGTNRGPGGTATA	445632; Q541U1	1805.92
EYGNVERQTDEYGNPVHAT	170022	2178.97
ETAANIGASAKAGMEKT	445632	1649.82
TAAGGYSDDINKQHDTTN	947090546	1907.84
DERARQGETVVPGGTGGKSL	255629381; 728048777	2014.03
EVGQDIQSKAQDTREA	A0A0R0FZF4	1774.86
QTSAMPGHGTGQPTGHVTE	445632; Q541U1	1892.85
FSREEGQQQGEQRL	18536	1691.81
EGVVGSHPIGTNRGPGGTATA	445632; Q541U1	1934.97
GVVGSHPIGTNRGPGGTA	445632; Q541U1	1633.84
QTGEKVKGAAQGATEAVKQTL	351727184	2115.14
GAHQTSAMPGHGTGQPTGHVTE	Q541U1	2157.97
STVEKSGEERAQEEGIGIDESK	728048777	2378.13
STVEKSGEERAQEE	728048777	1578.72
QTGEKVKGAAQGATE	351727184	1474.74
GFLQQTGEKVKGAAQGATEAVK	351727184	2218.18
EGVVGSHPIGTNRGPGGTAT	445632; Q541U1	1863.93
FSREEGQQQGEQRLQES	18536	2035.94

RMEPVTHGAYGGGMYGTE	356508831	1912.83
EVIHDVDIQPDDAAAAR	356533407	1834.89
YEHGSPLEGGKIADSQPV	Q9XES8	1883.91
DKEGIPPDQQRLIF	255632677	1655.87
DKIDGKPSADGVDESK	571563392	1660.80
YVEATPVEAGRSSAIK	I1NGG4	1677.88
YEHGSPLEGGKIADSQPVD	Q9XES8	1998.94
AQQHLAEGRSKGGQTRKE	728048777	1981.03
GFLQQTGEKVKGAAQGATE	351727184	1919.98
VEDLPEGPAVKIGENKDAMD	18770	2127.02
TKEVGQKTKEVGQDIQ	A0A0R0FZF4	1787.95
SAMPGHGTGQPTGHVTE	445632; Q541U1	1663.75
EGVVGSHPIGTNRGPGGTA	445632; Q541U1	1762.88
EVGQDIQSKAQDTREAA	A0A0R0FZF4	1845.89
GFLQQTGEKVKGAAQ	351727184	1561.83
TKEVGQKTKEVGQD	A0A0R0FZF4	1546.81
QATENQALGQTQKGGPA	I1L849	1698.84
EDEQPRPIPFPRPQP	18536	1802.91
FGREEGQQQGEERL	Q4LER6	1662.78
VESSDTIDNVK	255632677	1206.58
MEESRPGAVAD	Q9ZTY1	1161.52
YVATRTAAGGYSDDIN	947090546	1673.78
DELAFKPIAP	I1L849	1100.60
FVDAQPQQKEEGN	Q4LER6	1489.69
DTSNFNNQLDQTPRVF	4249568	1895.89
GQTFNDVGRFDEE	356533407	1513.65
ETATNIGASAKAGME	Q541U1	1450.68
VVSGDLAQKPVAPED	I1NGG4	1524.79
PVQNDGSTGLHWA	18543	1381.65
YEHGSPLEGGKIA	Q9XES8	1357.67
SGDLAQKPVAPEDAA	I1NGG4	1468.73
VVSGDLAQKPVAPE	I1NGG4	1409.76
EVESSDTIDNVK	255632677	1335.63
GEYGQPMGAHQTS	Q541U1	1362.57
GGTGYGSGTGYGINTGGAHTE	170022	1913.82
TAAGGYSDDINKQH	947090546	1476.67
YVEPNPVEAGRTS	I1LE41	1418.69
SGDLAQKPVAPED	I1NGG4	1326.65

RVSDDEFNNY	18770	1258.53
ELAFPGSAKDIE	Q4LER6	1276.64
VTDPTGAGGAEIDITPVEKS	356561627	1956.98
AGGKPNDYGYGTGGT	Q541U1	1414.62
DLSTPDQFDNRY	17467210	1470.65
SGTGYGVNTGGAHTE	947090546	1407.61
DLSTPDQFDNRYYSN	17467210	1834.79
LERVSDDEFN	18770	1223.55
ETAANIGASAKA	445632	1103.57
VLFSREEGQQQGEQ	18536	1634.78
GFLQQTGEKVK	351727184	1234.68
ERVSDDEFN	18770	1110.47
TLTSVLGSEEAAKEA	I1JFL5	1505.77
YVEATPVEAGRSSA	I1NGG4	1436.70
GSGTGYGVNTGGAHTE	947090546	1464.63
RAGGNPNDYGYGTG	445632	1398.60
DGDMEEEGVLHVE	Q9ZTY1	1458.60
RVSDDEFNNYK	18770	1386.63
VEDLPEGPAVKIGEN	18770	1566.80
DLSTPDQFDNRYY	17467210	1633.71
YVEATPVEAGRSS	I1NGG4	1365.66
VLEDDPNSLLQKA	356526401	1441.75
IQIDDDGIR	125722	1044.53
TVEKSGEERAQEE	728048777	1491.69
ANIGASAKAGME	445632	1119.55
FRLPEDANPNQIS	Q9XET1	1500.74
GQTFNDVGRFD	356533407	1255.57
QATENQALGQTQKGGPAS	I1L849	1785.87
GYQEMGRKGGL	255637579; 728048777	1195.59
SVVEDLPEGPAVKIG	18770	1509.81
ILEQPISVSID	1199563	1213.67
DSQIPLTGPNSIIGRA	C6SZ56	1638.89
QLEDGRTLAD	255632677	1117.55
LSEDEAVRVA	Q9XES8	1088.56
GDLAQKPVAPE	I1NGG4	1124.60
TVGQKAVDQSDASAIQ	I1NGG4	1617.81
SDDEFNNYK	18770	1131.46
TVVPGGTGGKSLE	255637579;	1201.64

	255629381; 728048777	
DQHATTGSDPTAPA	I1JFL5	1368.60
EVRATGSNVITPGGL	I1NGG4; I1LE41	1470.79
LSTPDQFDNRY	17467210	1355.62
SDKEDSVFKG	351734522	1111.53
DVARNEGVSVT	I1L849	1146.57
VTDADNVIPKA	I1KMV0	1142.60
SVDLPGLKKED	356501111	1200.65
GLGHAPISLPNQL	18543	1316.73
GYQEMGRKGGLS	255637579; 728048777	1282.62
GLGEHDQDNRRNY	351727184	1573.71
FGREEGQQQGEE	Q4LER6	1393.60
NTRAGGNPNDYGYGTG	445632	1613.69
AGKQLEDGRTL	255632677	1187.64
AANRAMDGDMEE	Q9ZTY1	1309.51
ETATNIGASAKAG	Q541U1	1190.60
VATRTAAGGYSDDIN	947090546	1510.71
VRATGSNVITPGGL	I1NGG4; I1LE41	1341.75
TVVPGGTGGKSL	255637579; 255629381; 728048777	1072.60
IAGASDKPVDESDAAAIQ	I1L849	1757.86
DELAFKPIAPRD	I1L849	1371.73
QLGSEGYHEM	255637579	1150.48
KEAGLSDELGRVSV	356528974	1459.77
DSQIPLTGPNNIIGRA	947120397	1665.89
KIMDNQSEQLE	255630323	1334.62
MEDSPYVKY	955320351	1131.50
QARLSEDEAVRVA	Q9XES8	1443.75
TDEYGNPVHAA	947090546	1173.52
GQTFNDVGRF	356533407	1140.54

In grass pea extract, LSIb-1 (7914 Da), LSIb-2 (6867 Da), LSIb-3 (7341 Da) and LSIb-4 (7460 Da) inhibitors were identified by LC-HR-MS/MS analysis (**Figure 8**). These proteins, which are structurally similar to soybean BBI inhibitors, can be able to exert anti-chymotryptic and antitriptic activities (Rocco *et al.,* 2011).

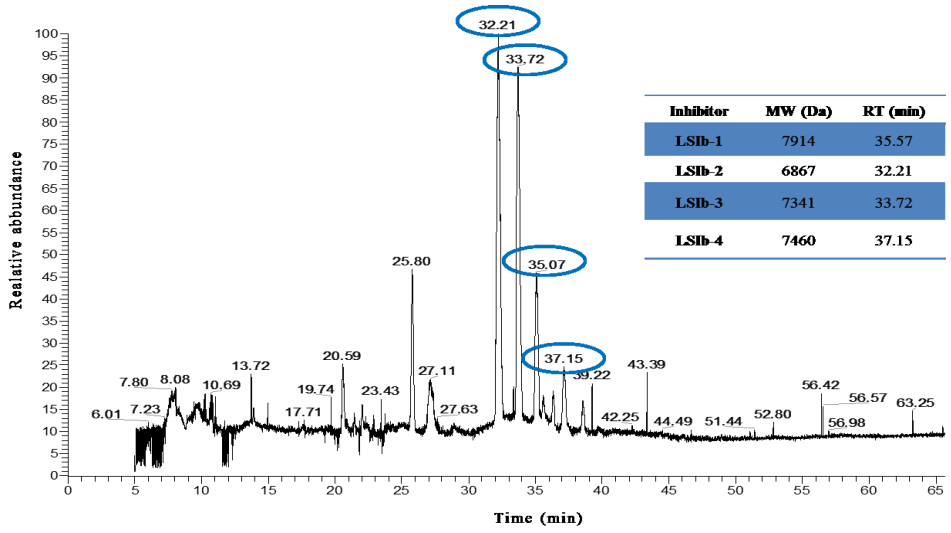

Figure 8. LSIb-1, LSIb-2, LSIb-3 and LSIb-4 inhibitors detected by LC-HR-MS/MS analysis in grass pea WSE

In chickpea extract, some potential isoforms connected with BBI family with MWs from 5.4 to 8 kDa were detected (**Figure 9**).

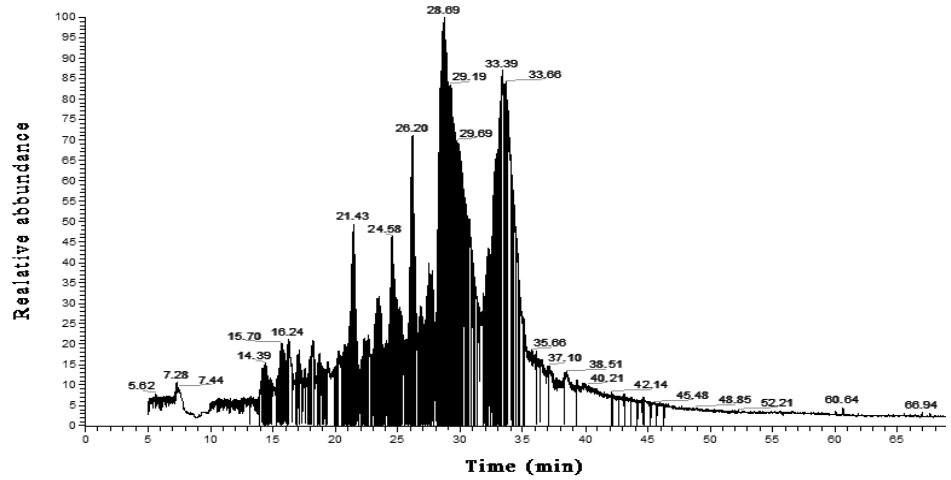

Figure 9. LC-HR-MS/MS chromatogram of chickpea WSE

SDS-PAGE analysis of WSEs highlighted the presence of different water-soluble proteins with MWs ranged from 7 to 100 kDa. Specifically, Kunitz

and BBI inhibitors showed MWs from 6 to 30 kDa (**Figure 10)**. The presence of Kunitz and BBI inhibitors, and that of peptides derived from protein seed degradation, was also confirmed by MALDI-TOF-MS analysis of gel spots digested by trypsin (**Tables 3 and 4**). In soybean WSE, the bioactive peptide lunasin was characterized, and no known allergens were detected (**Table 3**). In addition, in grass pea WSE, the neurotoxin ODAP was completely removed by extraction procedure (**Table 4**).

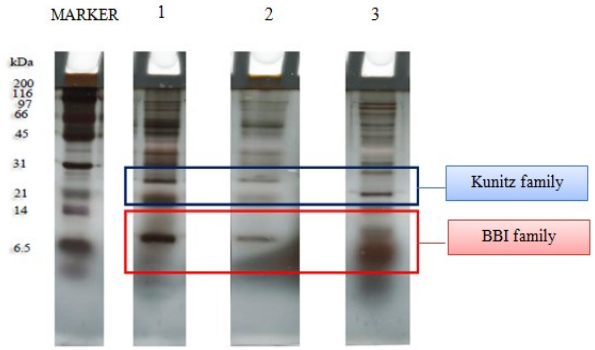

Figure 10. SDS-PAGE of WSEs in reduction condition: line 1, chickpea; line 2, grass pea; line 3, soybean

Table 3. Proteins identified in soybean WSE by MALDI-TOF-MS

Accession	Description	Coverage	Peptides	PSMs	Unique peptides	AAs	MW (kDa)
P08170	Seed linoleate 13S-lipoxygenase-1 [OS=*Glycine max*]	35	29	146	29	839	94.31
P13916	Beta-conglycinin, alpha chain [OS=*Glycine max*]	39	22	159	19	605	70.25

P13916	Beta-conglycinin, alpha chain [OS=*Glycine max*]	22	13	72	11	605	70.25
Q04672	Sucrose-binding protein [OS=*Glycine max*]	12	7	40	7	524	60.48
P10538	beta-amylase [OS=*Glycine max*]	30	13	112	13	496	56.11
P25974	Beta-conglycinin, beta chain [OS=*Glycine max*]	38	16	73	15	439	50.52
P04347	glycinin [OS=*Glycine max*]	16	7	33	7	516	57.92
P04776	Glycinin G1 [OS=*Glycine max*]	18	6	14	3	495	55.67
P04405	Glycinin G2 [OS=*Glycine max*]	11.34	5	12	2	485	54.36
P05046	Lectin [OS=*Glycine max*]	33	9	69	9	285	30.91
P05046	Lectin [OS=*Glycine max*]	25	8	47	8	285	30.91
P04776	Glycinin G1 [OS=*Glycine max*]	7	3	8	1	495	55.67
15216344	Kunitz trypsin inhibitor [OS=*Glycine*	16	4	10	4	217	24.03

	max]						
P04776	Glycinin G1 [OS=*Glycine max*]	15	8	55	7	495	55.67
P04405	Glycinin G2 [OS=*Glycine max*]	10	6	47	5	485	54.36
P02858	Glycinin G4 [OS=*Glycine max*]	6	3	27	3	562	63.55
125722	Kunitz-type trypsin inhibitor KTI1 [OS=*Glycine max*]	24	4	21	3	203	22.53
P13917	Basic 7S globulin [OS=*Glycine max*]	14	4	22	4	427	46.36
18770	trypsin inhibitor subtype A [OS=*Glycine max*]	31	7	24	7	217	24.06
P13917	Basic 7S globulin [OS=*Glycine max*]	11	4	22	2	427	46.36
15216344	Kunitz trypsin inhibitor [OS=*Glycine max*]	13	3	7	3	217	24.03
P13917	Basic 7S globulin [OS=*Glycine max*]	17	4	18	4	427	46.36
I1JLC8	Protein SLE2 [OS=*Glycine*	31	3	11	3	105	11.50

	max]						
15216344	Kunitz trypsin inhibitor [OS=*Glycine max*]	7	1	2	1	217	24.03
157833497	Bowman-Birk Proteinase Inhibitor [OS=*Glycine max*]	44	2	7	1	63	7.20
P19594	2S albumin [OS=*Glycine max*]	15	4	26	4	158	18.45

Table 4. Proteins identified in grass pea WSE by MALDI-TOF-MS

Accession	Description	Coverage	Peptides	PSMs	Unique peptides	AAs	MW (kDa)
P02854	Provicilin [OS=*Pisum sativum*]	19	10	75	8	410	46.36
P15838	legumin A2 [OS=*Pisum sativum*]	19	11	77	11	520	59.23
P05692	legumin J [OS=*Pisum sativum*]	18	7	31	5	503	56.86
P14594	Legumin B [OS=*Pisum sativum*]	15	5	58	5	338	38.97
L7N9M2	BBI inhibitor [OS=*Lathyrus sativus*]	32	3	7	3	114	12.66
L7N9N8	BBI inhibitor [OS=*Lathyrus sativus*]	32	3	28	1	114	12.65
L7N9M2	BBI inhibitor	32	3	16	1	114	12.66

	[OS=*Lathyrus sativus*]						
L7N9M5	BBI inhibitor [OS=*Lathyrus sativus*]	8.77	1	2	1	114	12.69

Proteomic characterization of in vitro protein digests

WSEs were subjected to *in vitro* gastrointestinal digestion and then analysed by HPLC and MALDI-TOF-MS analysis. Interestingly, both in soybean and grass pea WSEs, the persistence of chromatographic picks from 20 to 27 min after simulated gastrointestinal digestion suggests a certain resistance of WSE proteins, including Kunitz and BBI inhibitors (Hsieh *et al*., 2010) (**Figures 11** and **12**).

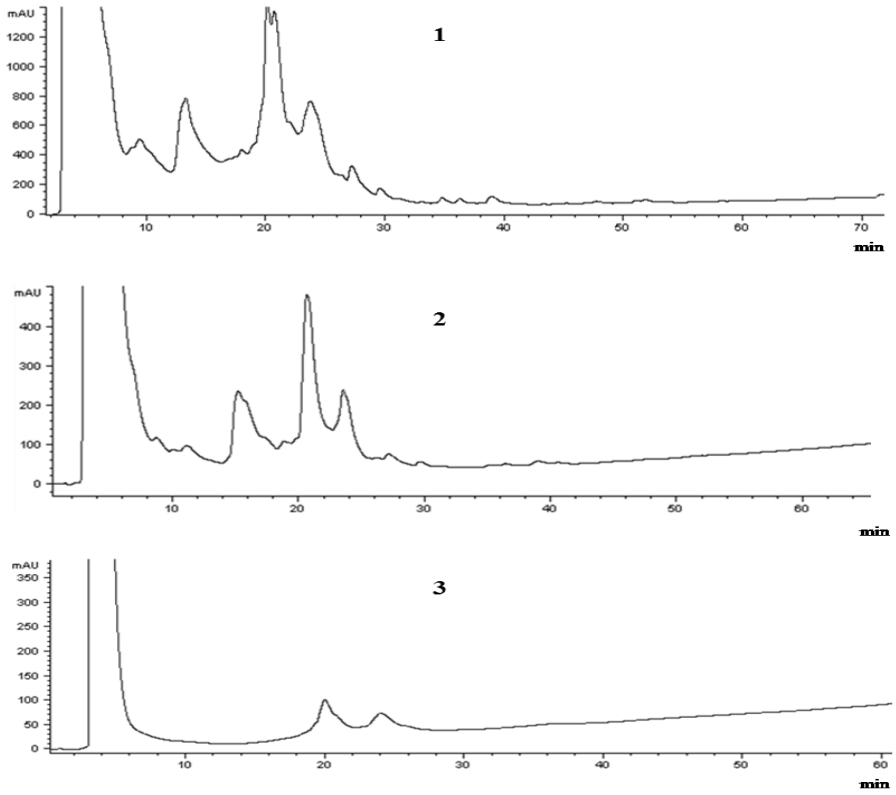

Figure 11. HPLC chromatograms of soybean WSE after (1) peptic, (2) pancreatic and (3) BBM phases of simulated gastrointestinal digestion

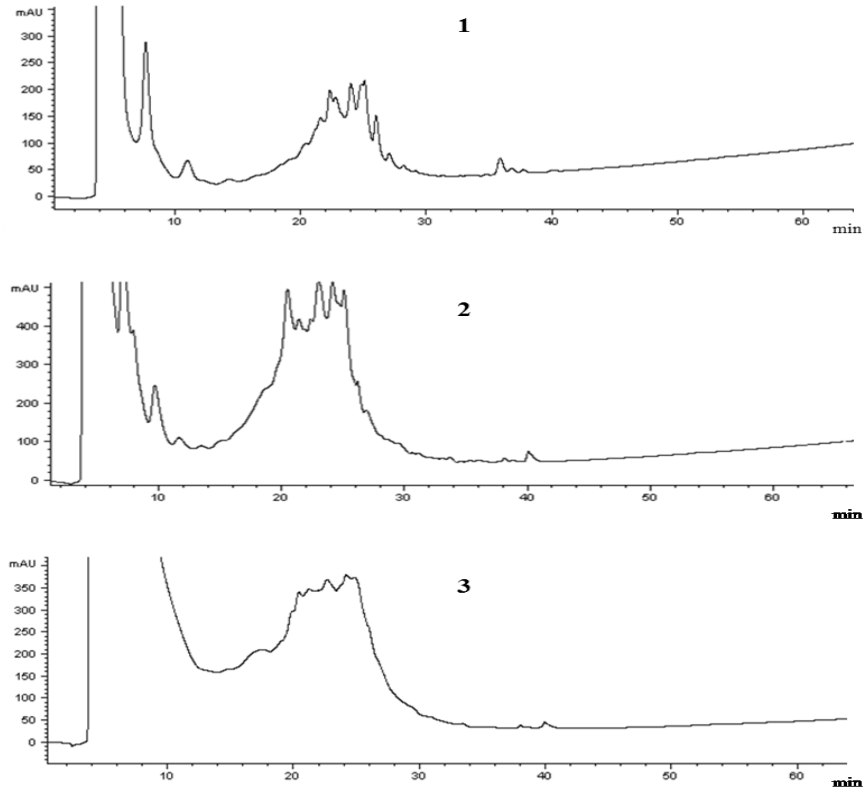

Figure 12. HPLC chromatograms of grass pea WSE after (1) peptic, (2) pancreatic and (3) BBM phases of simulated gastrointestinal digestion

Chickpea extract (**Figure 13**) showed a different behaviour when compared with soybean and grass pea samples. In fact, at the end of *in vitro* gastrointestinal digestion, there was an evident reduction in WSE inhibitor amount, as well as the formation of hydrolysis products with lower MWs. The protein degradation was particularly pronounced following the pancreatic phase. This data highlights the higher digestibility of chickpea proteins in the intestinal tract.

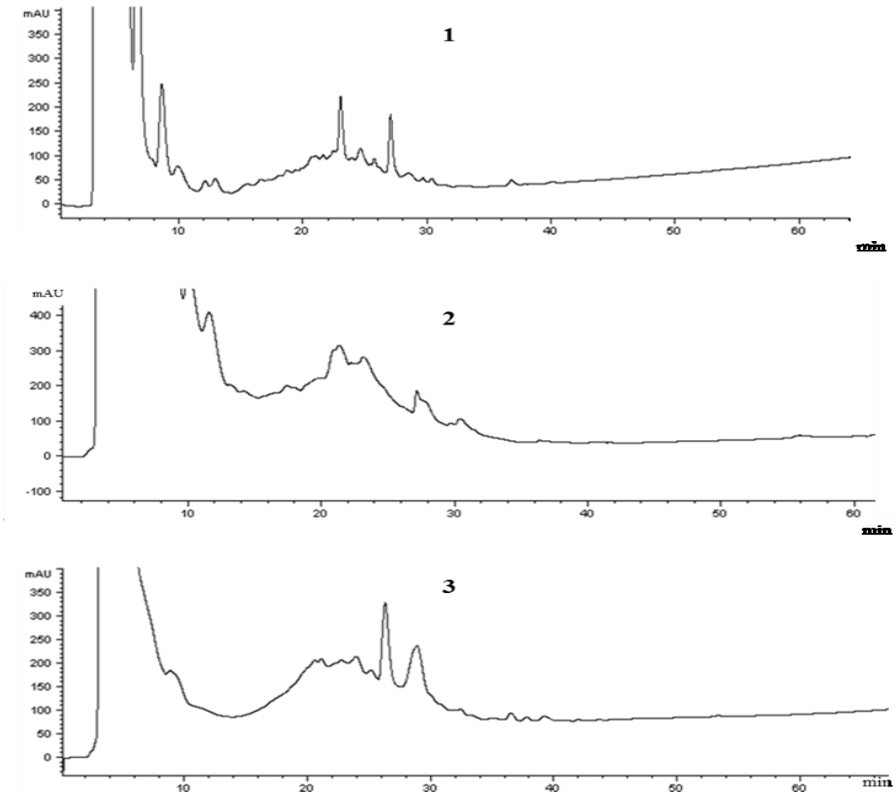

Figure 13. HPLC chromatograms of chickpea WSE after (1) peptic, (2) pancreatic and (3) BBM phases of simulated gastrointestinal digestion

MALDI-TOF-MS analysis of legume extracts confirmed as chickpea proteins were highly digestible unlike those of soybean and grass pea, which offered a great resistance to gastrointestinal enzymes (including BBM peptidases) (**Figures 14-16**).

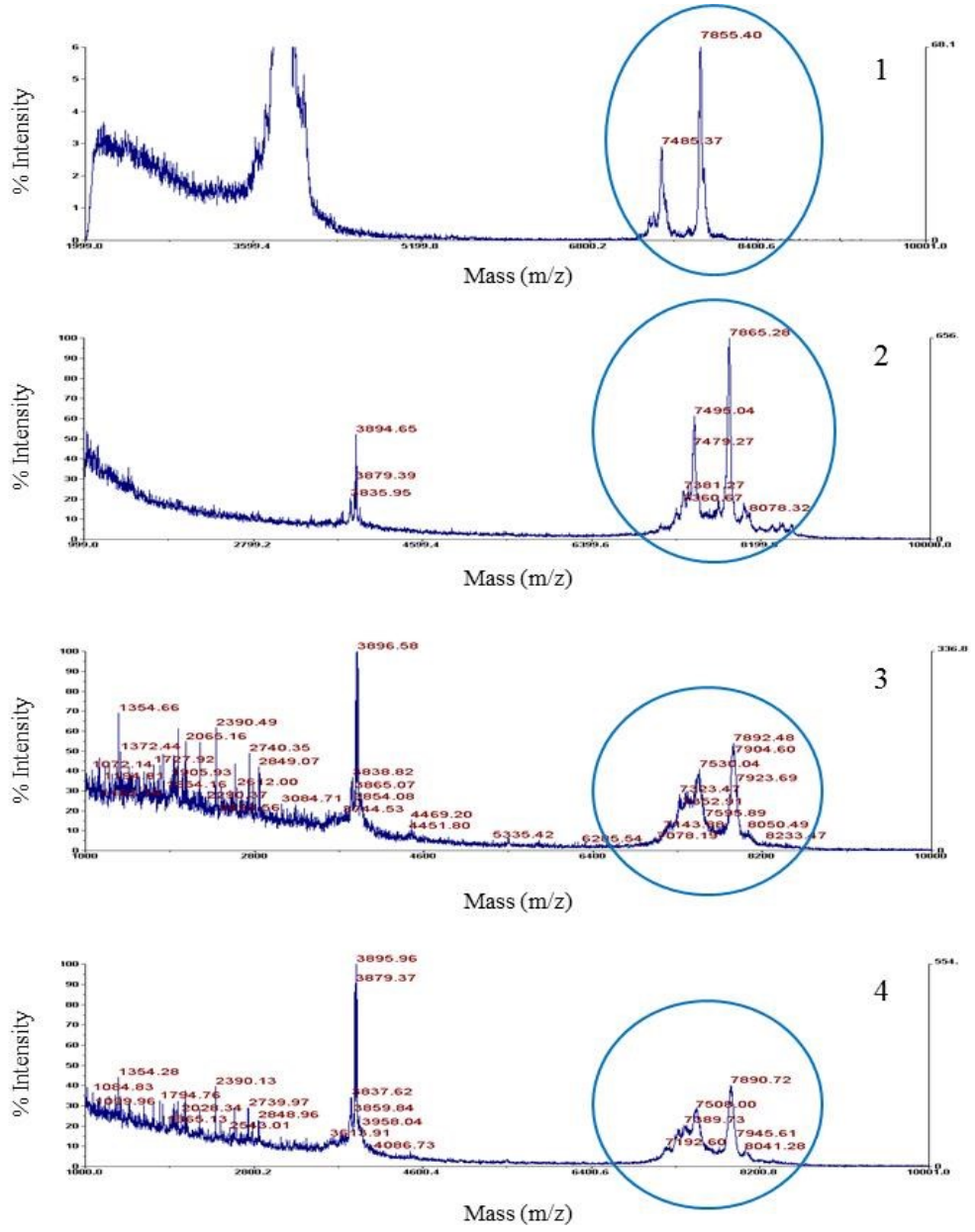

Figure 14. MALDI-TOF-MS spectrums of (1) soybean extract and its (2) peptic, (3) pancreatic and (4) BBM digests

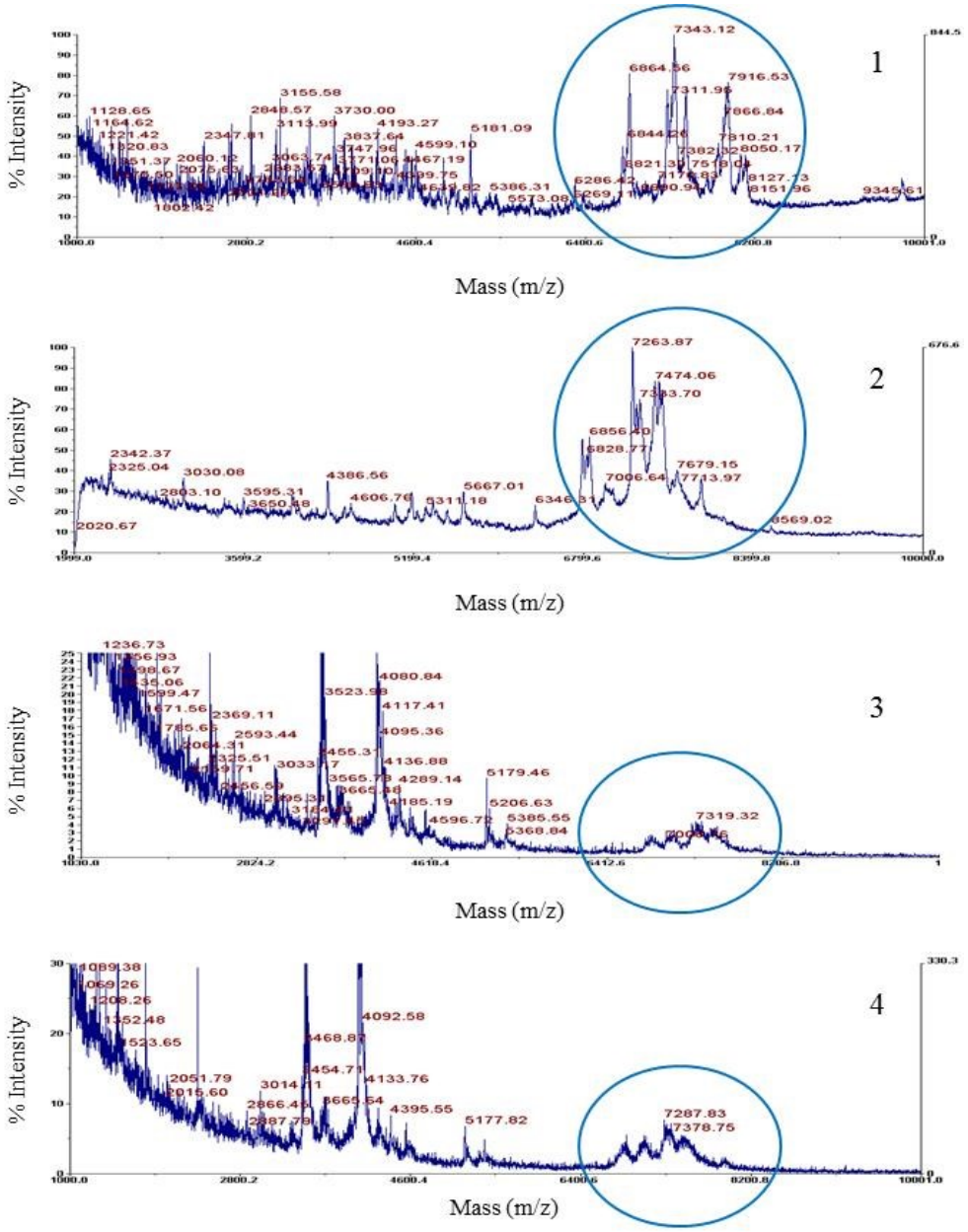

Figure 15. MALDI-TOF-MS spectrums of (1) grass pea extract and its (2) peptic, (3) pancreatic and (4) BBM digests

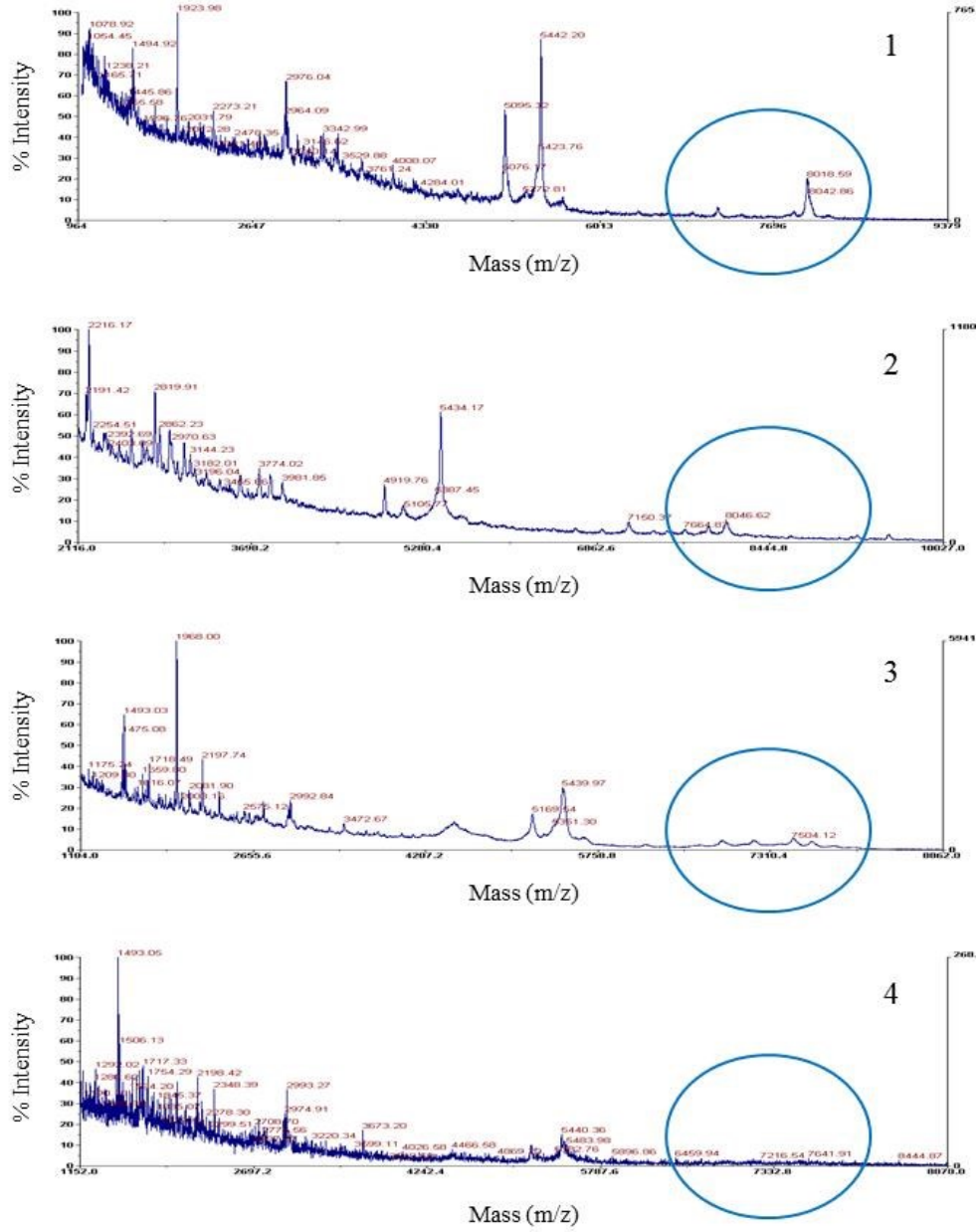

Figure 16. MALDI-TOF-MS spectrums of (1) chickpea extract and its (2) peptic, (3) pancreatic and (4) BBM digests

In soybean extract, two protein fragments with a MW of 3879 and 3895 Da were detected at the end of simulated digestion (**Figure 17**). Also Magee *et al.* (2012) extracted the same proteins from soybean seeds. As their high structural stability suggests, they might be fragments derived from enzyme inhibitor degradation.

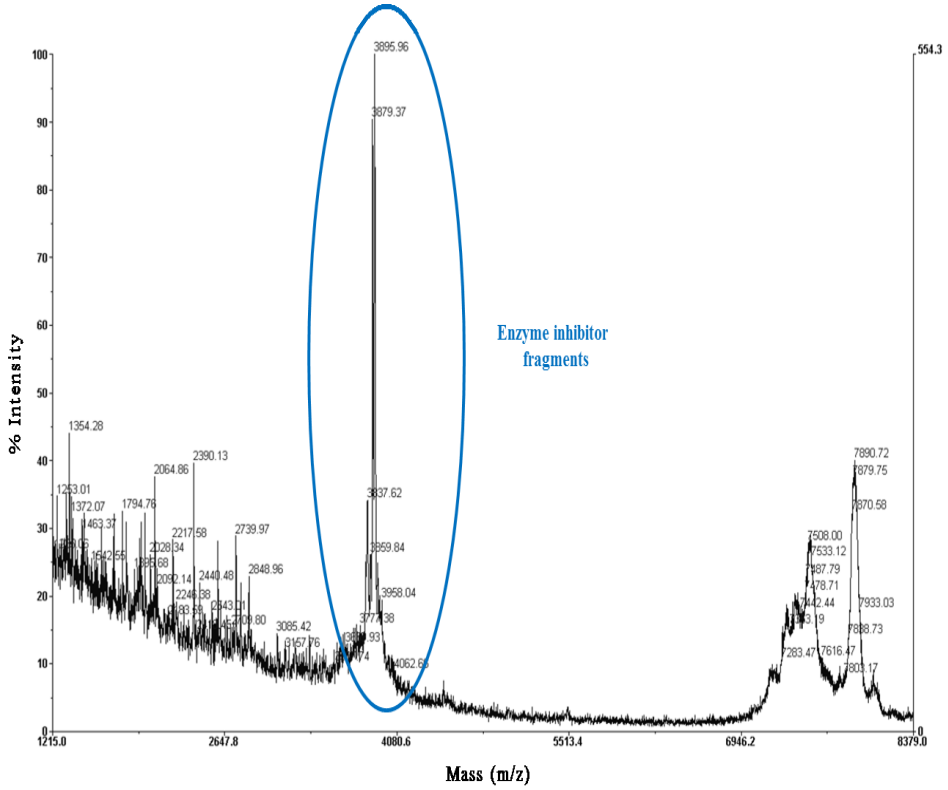

Figure 17. MALDI-TOF-MS spectrum of soybean extract after BBM digestion in which the enzyme inhibitor fragments are shown

In grass pea extract, LSIb-3 was the only enzyme inhibitor detected at the end of simulated digestion (**Figures 18** and **19**). Presumably, the great part of WSE proteins was completely digested.

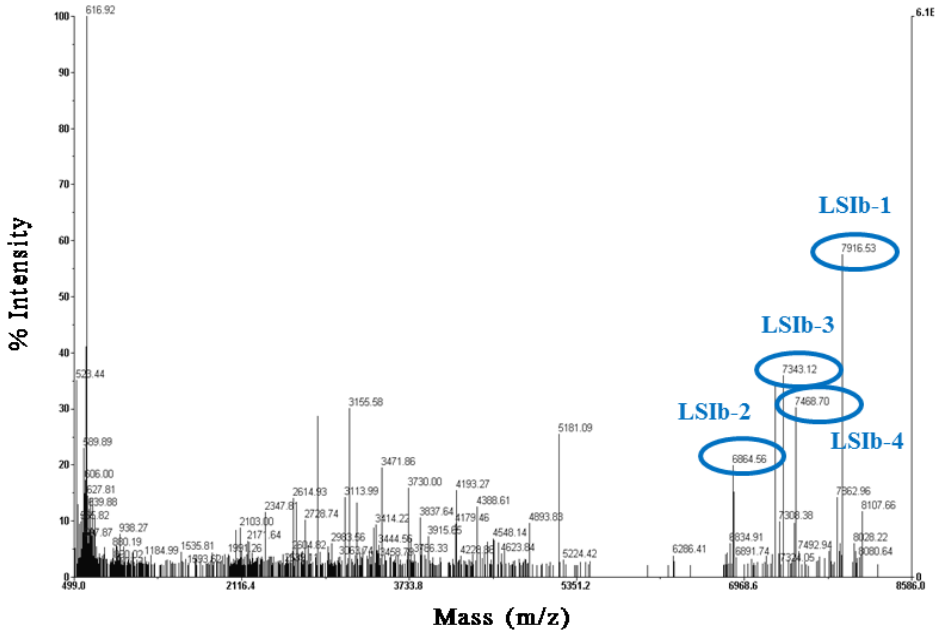

Figure 18. MALDI-TOF-MS spectrum of grass pea WSE

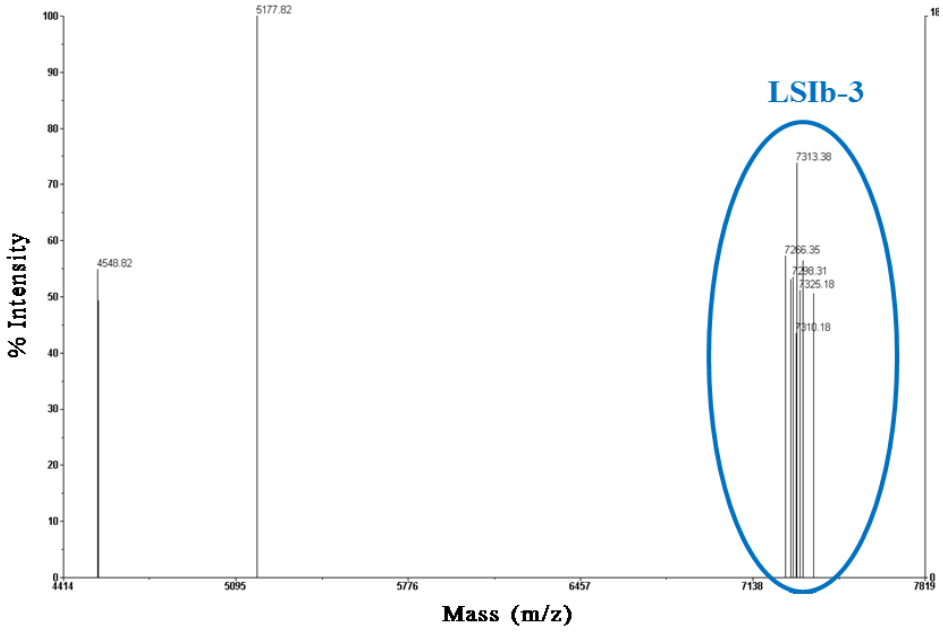

Figure 19. MALDI-TOF-MS spectrum of grass pea WSE at the end of simulated gastrointestinal digestion (including BBM step)

Conversely, in chickpea extract, just few peptide fragments of 7-8 kDa were revealed at the end of pancreatic digestion. However, after BBM digestion, the only protein detected was that with a molecular mass of 5440 Da (**Figure 20**). Once again, this data confirms the higher digestibility of chickpea proteins.

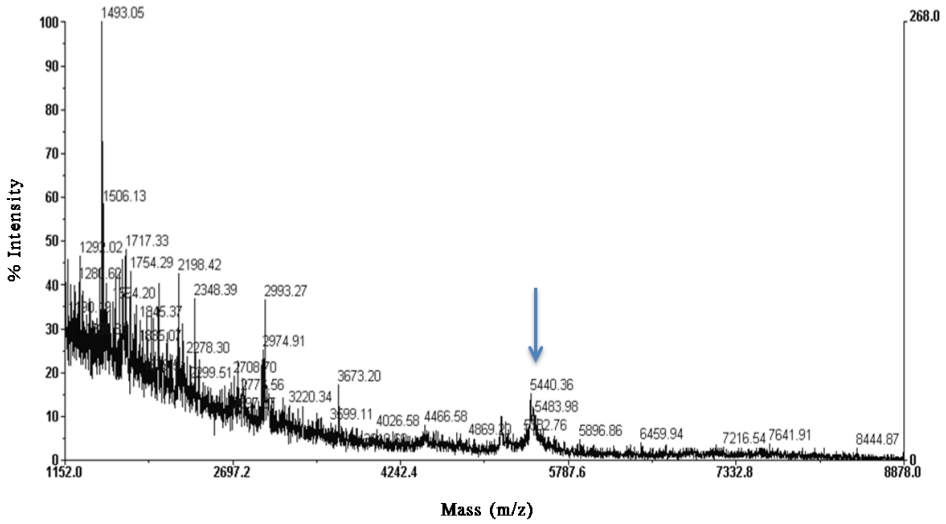

Figure 20. MALDI-TOF-MS spectrum of chickpea extract at the end of simulated gastrointestinal digestion (including BBM step), in which the WSE protein resistant to digestion is evidenced

Conclusions

Legumes are a protein-rich matrix consumed by several world populations. Their suitable nutritional composition and large distribution make legumes an efficient source for the food industry. In fact, in the last years, legume-based ingredients (i.e. flours) have been employed in the formulation of different food preparations (i.e. bakery products), essentially to improve their nutritional properties (protein content). Furthermore, numerous studies have shown that plants can produce different types of water-soluble proteins and

peptides with antimicrobial properties that are involved in their defence mechanisms. In legume seeds, these protein components are mainly composed by protease inhibitors belonging to Kunitz and BBI families. Protein inhibitors can affect the capacity of digestive enzymes to hydrolyse food proteins provoking a reduction in amino acid absorption and eventually in the synthesis of new proteins. However, seeds and flour are subjected to refining processes that are able to reduce the inhibiting effect of these proteins. Recently, some researchers indicated that water-soluble proteins from legumes have the potential to be used as food preservatives on the basis that their antimicrobial activity. In addition, peptides from legume extracts can exert different biological activities (i.e. cardiovascular, endocrine, antimicrobial, antimutagenic, immunological and neurological activities) that may positively affect human health.

In this study, WSEs from soybean, chickpea and grass pea seeds were produced using a specific protocol and then subjected to proteomic characterization. The effect of simulated gastrointestinal digestion on WSE proteins was also investigated. Collected data highlighted the presence of different Kunitz and BBI inhibitors in the analysed extracts. Specifically, in soybean WSE, more than 170 peptides were detected, among which the bioactive peptide lunasin, and no known allergens were revealed as well. In grass pea WSE, the four BBI isoinhibitors characteristic of this legume were detected, while the neurotoxin ODAP was completely removed by extraction procedure. In Chickpea WSE, some potential isoforms connected with BBI family with MWs from 5.4 to 8 kDa were detected. These results, therefore, may suggest a potential use of legume WSEs as stabilizing agents in foods and beverages. Furthermore, soybean and grass pea WSE proteins showed a high resistance to gastrointestinal enzymes (including BBM peptidases) during simulated digestion, confirming the ability to exert their beneficial effects on human health.

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Chapter 3. Food functionality and digestibility of grass pea protein isolate following harmonised *in vitro* gastrointestinal digestion

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The present chapter is going to be submitted as a research article for publication

Abstract

Despite its scarce consumption, grass pea has a high nutritional value with a protein content that range from 26 to 28%. Grass pea proteins and peptides (including peptides from human digestion) may have nutritional and biological properties that are very intriguing for the food industry. In this study, the effect of *in vitro* gastrointestinal digestion on grass pea protein isolate composition and digestibility was investigated by proteomic characterization. Briefly, collected data indicated that the great part of protein degradation took place along the intestinal phase, where a high number of medium/low protein degradation products was realised. Some of those protein components might be allergenic given their size and the presence of negatively-charged amino acids. However, the simulated gastrintestinal digestion also produced high amounts of Lys and Arg. This means that grass pea containing food can be complementary to those matrices poor in positively-charged free amino acids, such as rice and amaranth. In addition, the biological activities (i.e. anti-hypertensive activity) exerted by this type of amino acids may confer to grass pea protein isolate benefical effects for human health.

Introduction

Undoubtedly, grass pea (*Lathyrus sativus L.*) is globally less widespread then other legumes such as soybean or chickpea. In fact, the commercialization and consumption of this minor legume is substantially restricted to some areas of Asia (i.e. India, China, Nepal and Bangladesh), Middle East (i.e. Pakistan) and Africa (i.e. Ethiopia), where it is addressed both to animal and human consumption (Campbell, 1997). However, grass pea is a plant that is adapted to arid conditions and contains high amount of proteins. This crop may be able to grow in drought-stricken and rainfed areas, where soil quality is poor and extreme environmental conditions prevail (Palmer *et al.* 1989). Despite its tolerance to drought, *Lathyrus sativus L.* is not affected by excessive rainfall and can be grown on land subject to flooding (Kaul *et al.*, 1986; Rathod, 1989; Cambpell *et al.*, 1994). It has a very strong and penetrating root system and therefore can be cultivated in a wide range of soil types, including very poor soil and heavy clays. This hardness, together with its ability to fix atmospheric nitrogen, makes grass pea one that seems designed to grow under adverse conditions (Cambpell *et al.*, 1994). Compared with other legumes, *Lathyrus sativus L.* is resistant to many pests including storage insects (Palmer *et al.* 1989). Thus, this crop can be considered perfectly adaptable to ecological sustainability. As regard nutritional composition, grass pea has high amount of proteins, which normally range from 26 to 28%, while its fat content is very low (2.7%). Grass pea proteins and peptides (including peptides from human digestion) may also have nutritional and biological properties very intriguing for the food industry. However, this legume is still inadequately explored as food matrix from a proteomic point of view.

In the present study, therefore, the effect of *in vitro* gastrointestinal digestion on grass pea protein isolate composition (protein functionality) and digestibility was investigated.

Materials and Methods

Chemicals

Water, α-amylase Type IX-A, pepsin, pancreatin, Tris-HCl, β-mercaptoethanol, formic acid (FA), 1,4-dithiothreitol (DTT), acetonitrile (ACN), trifluoracetic acid (TFA), α-Cyano-4-hydroxycinnamic acid and 5-sulfosalicylic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium Dodecyl Sulphate (SDS) and bromophenol blue indicator were purchased from Merck (Darmstadt, Germany). Glycerol was purchased from Panreac Química SAU (Castellar del Vallés, Barcelona, Spain). 12% Bis-Trispolyacrylamide Criterion-XT gel, XT MES running buffer and Precision Plus protein molecular weight (MW) marker were purchased from Bio-Rad (Hercules, CA, USA). Coomasie Blue was purchased from Expedeon (Swavesey, UK).

Production of grass pea protein isolate

Grass pea protein isolate was produced by seeds (Granoro, Italy) according to the protocol of Barac *et al.* (2010) (**Figure 1**). Approximately 155 g of dried grass pea seeds were milling in a food mixer. 13.8 g of the obtained powder were dispersed in 240 mL of water and stirred for 15 min. Then, the solution was brought to pH 9.0, stirred for 60 min at room temperature and centrifuged (4000 g x 15 min) to remove insoluble components like fibers. The pellet was then re-extracted for 30 min at pH 9.0 and centrifuged again. The supernatant was precipitated at pH 4.0 and stored at 4ºC for 120 min.

The pellet was re-dissolved at pH 9.0 for 30 min, precipitate at pH 4.5 and centrifuged (9000 g x 10 min). The sediment (protein isolate) was separated, re-dissolved at pH 7.0 and finally freeze-dried.

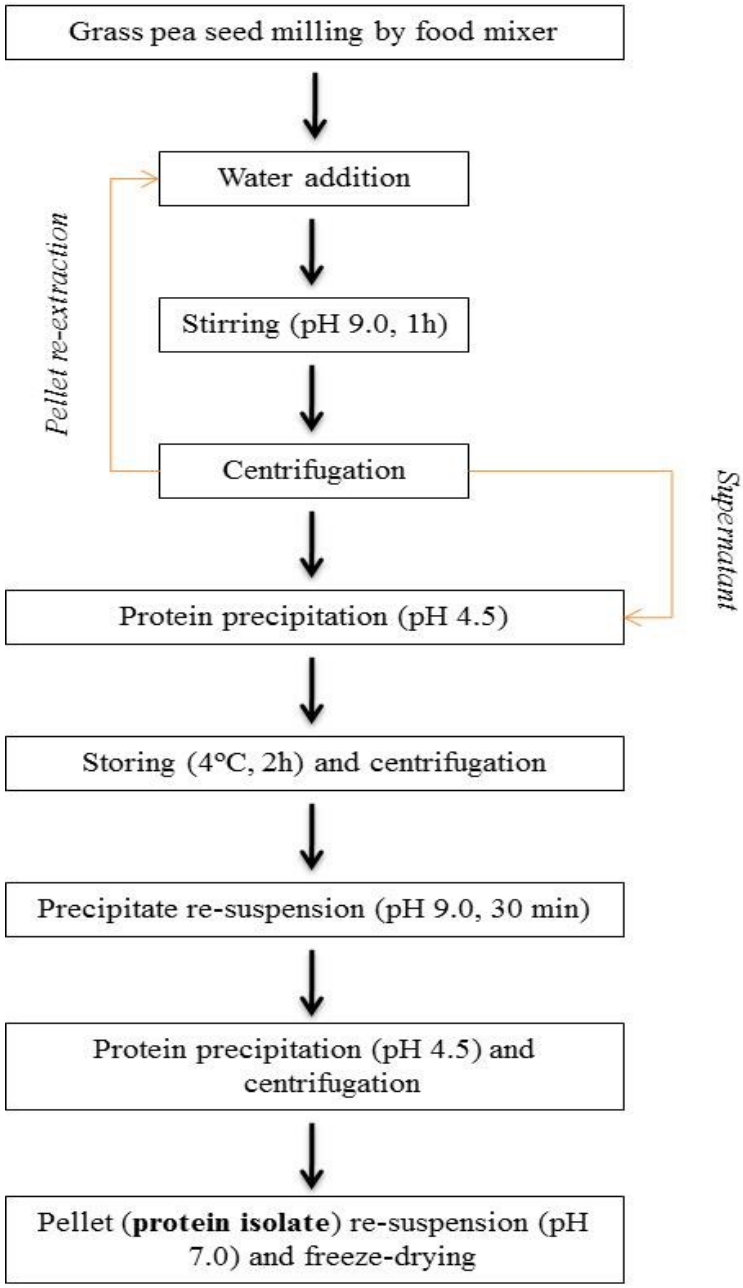

Figure 1. Workflow of grass pea protein isolate production

In vitro simulated gastrointestinal digestion

Grass pea proteins were digested according to the harmonised *in vitro* method of Minekus *et al.* (2014). The freeze-dried sample was dissolved in 25 mL of water (60 mg of protein/mL) and stirred for 20 min. Then, 2.5 mL of the mixture were mixed with 1.5 mL of Simulated Saliva Fluid (SSF) containing α-amylase from human saliva (75 U/mL of final mixture). The solution was then diluted at a ratio of 50:50 (v/v) in Simulated Gastric Fluid (SGF) containing pepsin from porcine gastric mucosa (2000 U/mL of digest). Samples were collected at 30 min (G30) and 120 min (G120) during gastric digestion and the reaction was stopped by adjusting the pH at 7.0 and snap freezing in liquid nitrogen. Intestinal phase was carried out by mixing the gastric phase with the same volume of simulated intestinal fluid containing pancreatin from porcine pancreas (100 U trypsin activity/mL of final mixture). Bile salts were not added to avoid interference both with the subsequent cell-based assays and with the mass spectrometry analysis. All simulated fluids were tempered at 37°C and added of calcium chloride before use. Digestions of each protein powder were performed in duplicate by incubating at 37°C in an orbital shaker at 150 rpm. The samples were collected at 30 min (I30) and 120 min (I120), and the digestion was stopped by heating (85°C, 15 min) and snap freezing. Enzyme activities and fluid concentration were measured according to the assay described in the reference protocol (Minekus *et al.,* 2014).

SDS-PAGE analysis

Protein digests were dissolved (1 mg of protein/mL) in sample buffer (0.05 M Tris-HCl pH 6.8, 1.6% SDS (w/v), 8% glycerol (v/v), 2% β-mercaptoethanol (v/v) and trace of bromophenol blue indicator), heated at 95°C for 5 min and loaded on 12% Bis-Trispolyacrylamide gel. Electrophoretic separation was carried out at 100 V for 5 min and then at 150

V for 60 min, using XT MES running buffer in the criterion cell (Bio-Rad, Hercules, CA, USA). A MW marker was loaded on the gel. After migration, the gel was stained with Coomasie Blue. Gel image was taken with a Molecular Imager®VersaDoc™ MP 5000 system (Bio-Rad, Hercules, CA, USA) and processed with *Quantity One*® 1-D analysis software (Bio-Rad, Hercules, CA, USA).

HPLC-MS/MS analysis

Protein digests were reconstituted in 0.1% FA (v/v) in water (eluent A) and centrifuged (13000 *g* x 10 min) before supernatant injection. In order to improve the identification of disulfide-linked fragments, grass pea samples were reduced with 0.07 M DTT (37°C, 60 min). Samples were analysed by HPLC-MS/MS in duplicate using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) equipped with a Mediterranea Sea C_{18} column (150 × 2.1 mm, Teknokroma, Barcelona, Spain). HPLC system was connected to an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) coupled with an electrospray ionization source. The injection volume was 50 µL and the flow was set at 0.2 mL/min. Peptide elution was performed with a linear gradient from 0 to 45% of 0.1% FA (v/v) in ACN (eluent B) in 115 min. The spectra were recorded over the mass/charge (*m/z*) range 100-600 and 100-2000, selecting 450, 750 and 1200 as target mass, respectively. The results were processed by using Data Analysis (version 4.0, Bruker Daltonics). Homemade database of legume proteins was used for the peptide sequencing in MASCOT v2.4 software (Matrix Science). No specific enzyme cleavage was used. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. Furthermore, Biotools version 3.2 was used for the interpretation of the matched MS/MS spectra. Each sample was analysed in duplicate.

MALDI-TOF/TOF analysis

To perform MALDI-TOF/TOF analysis, protein digests were firstly suspended in 0.1% TFA (v/v) in water and then placed on an Anchorchip plate (set for proteomics II; PAC-II 384well plate; Bruker Daltonics). α-Cyano-4-hydroxycinnamic acid was added as matrix. The mass range was set from 500 to 3500 *m/z* using an exclusion list containing the peaks from the matrix to avoid interferences. Data processing was performed by using Data Analysis^{TM} (version 4.0; Bruker Daltoniks) and the peptide sequencing was done by MASCOT, as previously described by Sanchez-Rivera *et al.* (2015). Each sample was analysed in duplicate.

Cation exchange-HPLC and ninhydrin derivatization analysis

To determine the free amino acid amount of protein digests, the samples were dissolved (4 mg of protein/mL) in water and then 5-sulfosalicylic acid (4ºC, 1h) was added. After centrifugation (15000 g x 15 min, at 4ºC), the supernatants were filtered by 0.45 μm before adjusting pH to 2.2. Finally, the samples were analysed by cation exchange-HPLC and ninhydrin derivatization. Each sample was analysed in duplicate.

Results and Discussion

Protein degradation during in vitro gastrointestinal digestion

As for protein degradation, within the protein patterns of G30 and G120, no significant differences were observed at the two sampling times of simulated gastric phase. A similar behaviour was reported by Laguna *et al.* (2017) in their work on pea protein isolate. In the 1-DE gel shown in **Figure 2,** some fragments at higher MW (70 kDa) corresponding to convicilin fraction are visible, as well as several protein components at lower MW (20-30 kDa) corresponding to legumin fraction. Some protein fragments identified as

legumin A2 α are also present in the 60 kDa region. In addition, 1-DE gel shows various protein components corresponding to vicilin subunits ranging between 37-45 kDa. Vicilin and convicilin fragments are visible at the 15 kDa range as well. Clearly, grass pea proteins appeared quite stable to gastric conditions (pH 3.0 and pepsin activity) with a relative low degradation rate (Nguyen *et al.*, 2015; Laguna *et al.,* 2017). The greater part of protein degradation took place in the intestinal tract, where a large variety of medium-low MW peptides and free amino acids were released. In the soluble fraction, during the intestinal phase, no intact proteins were detected. In the insoluble part of digests, in addition to pancreatic enzymes (identified in **Figure 2**), some slight bands were detected at the end of the intestinal phase below 15 kDa, which were identified as legumin B fragments. Interestingly these protein fragments might be potentially allergenic given their size.

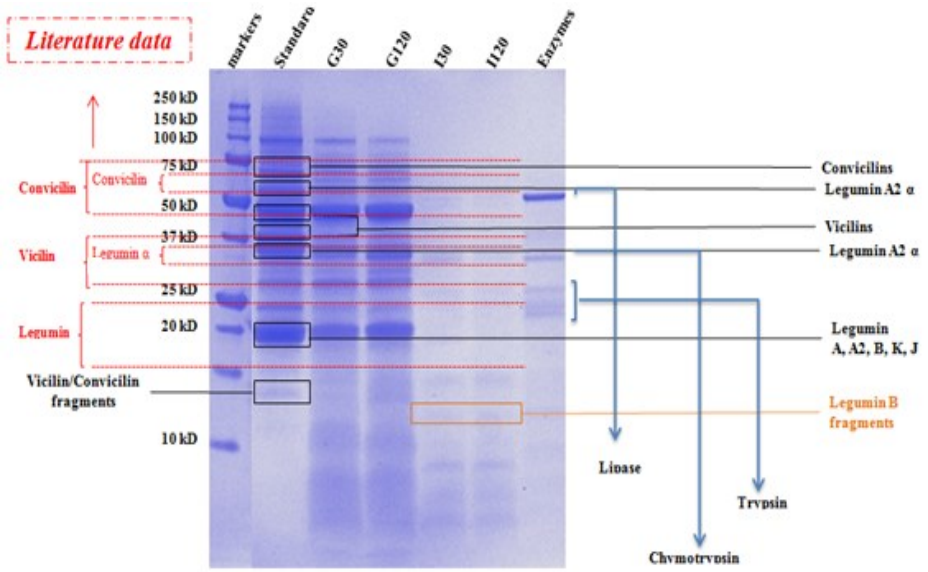

Figure 2. SDS-PAGE protein patterns of digested samples collected during gastric (G30 and G120) and intestinal (I30 and I120) phases of *in vitro* gastrointestinal digestion

Proteomic characterisation of protein digests

The proteomic characterization of protein digests confirmed as protein degradation was quite low during gastric phase (G30 and G120), where an important number of high MW peptides (>2100 Da) was detected. Otherwise, the amount of low MW peptides (<1400 Da) was relatively scarce (**Figure 3**). Protein degradation trend was completely reversed during intestinal phase (I30 and I120), where the number of protein degradation products of medium and low MW (<1400 Da) notably increased (**Figure 3**).

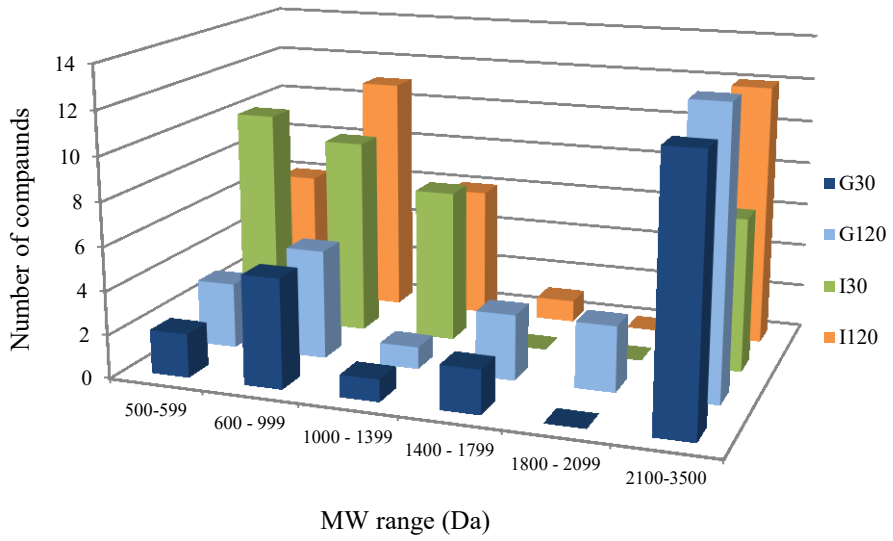

Figure 3. Number of protein components of protein digests grouped according to their MW (Da). Detection by MALDI-TOF/TOF

Collected data also highlighted that protein degradation was not as severe as in the case of other protein sources such as milk. In milk, casein is completely hydrolysed during the gastric phase and whey proteins are rapidly cleaved along the intestinal phase, and no intact proteins were detected after 30 min (Egger *et al.*, 2017; Sanchon *et al.*, 2018). A further difference between milk and grass pea is related to peptides resistant to gastrointestinal

digestion. In grass pea, the identified peptides were rich in negatively-charged amino acids (glutamic and aspartic acid) (**Table 1** and **2**), while, in milk, casein is being reported to be source of proline-rich peptides (Sanchon *et al.*, 2018).

Table 1. Peptides identified in I30 detected by HPLC-MS/MS

Protein	Measured mass	Theoretical mass	Range	Sequence
Provicilin	586.300	586.296	327 - 332	LSPGDV
	571.263	571.333	98 - 103	KLPAGS
	587.248	587.280	244 - 248	EITPE
	507.283	507.218	218 - 222	SVSSE
Convicilin	586.290	586.296	438 - 443	LSPGDV
	570.160	570.301	488 - 492	IENPV
	590.227	590.266	256 - 260	SENKN
	587.248	587.280	360 - 364	EITPE
	572.275	572.281	439 - 444	SPGDVV
	1030.119	1029.545	306 - 314	GEERDAIIK
	742.671	742.434	503 - 508	EVNRLI
	596.340	596.208	1 - 5	NYDEG
	1178.121	1177.562	495 - 505	LTFPGSSQEVN
	1094.031	1093.519	31 - 38	WRPSYEKE
	1121.623	1120.613	266 - 275	FSKNILEASL
Vicilin	586.290	586.296	328 - 333	LSPGDV
	957.683	958.461	190 - 197	EINEENVI
	595.149	595.319	24 - 28	NGHIR
	587.873	587.339	53 - 57	SKPRT
	728.933	729.427	167 - 172	EKVLLE
	587.248	587.280	245 - 249	EITPE
	544.121	544.358	197 - 201	IVKVS
	643.355	643.427	196 - 201	VIVKVS
	592.427	592.286	233 - 237	NPIYS

	570.176	570.374	97 - 101	TIKLP
	507.283	507.218	219 - 223	SVSSE
	592.294	592.282	211 - 216	NAKSSS
	544.213	544.322	374 - 378	VISQV
	457.209	457.254	101 - 105	PAGTI
	585.349	585.323	231 - 235	SRNPI
	994.579	995.565	121 - 129	DLTIPVNKP
	1274.545	1274.526	309 - 318	KEEEDEDEPR
	885.505	885.427	82 - 90	LAVPGCPET
	942.548	942.448	81 - 90	GLAVPGCPET
	685.481	685.365	253 - 258	ISPELQ
	677.321	677.302	64 - 69	YSPSPQ
	802.390	802.372	132 - 138	NHGHEPL
Legumin	860.566	860.366	32 - 38	TETWNPN
	776.638	776.480	120 - 127	IAIPPGIP
	771.414	771.449	241 - 247	GQIVKVE
	772.320	772.343	83 - 90	AVPGCPET
	823.396	823.517	465 - 472	LGQLVVVP
	861.456	861.387	62 - 69	PSYSPSPQ
	760.468	760.372	172 - 177	ETQQKQ
	700.361	700.387	239 - 244	ERGQIV
	646.303	646.317	346 - 350	ELEKE
	586.290	586.296	498 - 503	LSPGDV
	587.248	587.280	420 - 424	EITPE
	572.275	572.281	499 - 504	SPGDVV
CVC protein	570.176	570.374	262 - 266	TIKLP
	596.340	596.208	1 - 5	NYDEG
	1177.123	1177.441	118 - 126	DEEQVDEEW
	1094.031	1093.519	31 - 38	WRPSYEKE

Table 2. Peptides identified in I120 detected by HPLC-MS/MS

Protein	Measured mass	Theoretical mass	Range	Sequence
Provicilin	582.417	582.349	87 - 91	PKKNP
	585.329	585.349	192 - 196	NLNLL
	558.169	558.338	217 - 221	VISQI
	1167.805	1166.666	84 - 93	EITPKKNPQL
	570.204	570.276	252 - 256	AQPQQ
	592.237	592.282	50 - 55	NAKSSS
Vicilin	587.247	587.280	244 - 248	EITPE
	592.604	592.286	232 - 236	NPIYS
	1126.313	1125.614	92 - 102	ERGDAIKLPAG
	587.257	587.303	90 - 94	NLERG
	1060.191	1060.478	300 - 308	ENQGKENDK
	474.921	475.264	144 - 148	SLLSG
	592.237	592.282	210 - 215	NAKSSS
Legumin	857.543	857.461	432 - 439	NINANSLL
	514.243	514.239	65 - 69	SPSPQ
	557.743	557.306	252 - 256	IISPE
	540.297	540.327	469 - 473	VVVPQ
	558.230	558.265	53 - 57	TIDPN
	563.206	563.274	43 - 48	KCAGVS
	592.446	592.358	122 - 127	IPPGIP
	530.260	530.306	15 - 19	TINAL
	529.026	529.311	403 - 407	SLTLP
	1126.313	1126.573	328 - 336	KHTAEKERE
	1274.589	1274.526	309 - 318	KEEEDEDEPR
	885.533	885.427	82 - 90	LAVPGCPET
	662.356	662.239	273 - 277	EEEQE
	776.608	776.480	120 - 127	IAIPPGIP
	662.316	662.276	308 - 312	EKEEE
	1376.723	1377.517	196 - 206	QQEEESEEQNE

As for the free amino acid content of protein digests, at the end of gastric phase, only small chromatographic peaks with the elution time of valine and phenylalanine were detected (**Figure 4**). However, the form of the chromatographic peaks suggests that they could correspond to dipeptides eluting with the same retention time. In addition, the progressive increase in free amino acids of I30 and I120 samples confirmed the progress of protein degradation in the intestinal tract. Serine, glutamic acid, valine, leucine, tyrosine, phenylalanine, lysine, and arginine resulted as the most abundant free amino acids detected at the end of simulated gastrointestinal digestion (**Figure 4**). Interestingly the amount of Lys and Arg resulted quite high. Positive amino acids may be correlated to biological activities. For instance, L-arginine is the physiological precursor for the formation of nitric oxide (NO), which mediates endothelium-dependent relaxation (Palmer *et al*., 1988).

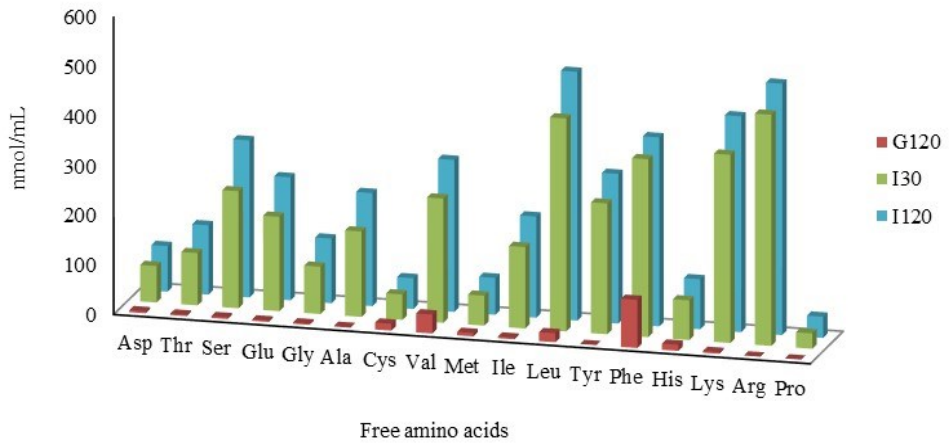

Figure 4. Free amino acid content (nmol/mL) in gastric and intestinal digests quantified by cation exchange-HPLC and ninhydrin derivatization

Conclusions

Although *Lathyrus sativus L*. can be considered a minor legume, its proteins and peptides, including those from human digestion, have shown nutritional and biological properties potentially useful for the food industry. However, this legume is still inadequately explored as food matrix from a proteomic point of view. In particular, very few studies have been carried out on *in vitro* digested proteins from grass pea until now.

In this study, the effect of *in vitro* gastrointestinal digestion on grass pea protein isolate composition and digestibility was investigated by proteomic characterization. First of all, collected data revealed that the most part of storage proteins (legumin, major/minor vicilins and convicilis) are resistant to gastric digestion. These proteins, therefore, can easily reach the duodenum where they are digested by intestinal enzymes (trypsin, chymotrypsin, etc.). Interestingly the greatest part of soluble proteins have been already digested after 30 min of intestinal digestion, producing a high number of medium/low MW (<1400 Da) protein degradation products. However, some insoluble proteins potentially allergic with medium/low MWs were detected at the end of intestinal digestion (I120). Furthermore, free amino acid assay showed as grass pea-based products can be complementary to food matrices poor in positively charged free amino acids, such as rice and amaranth (Gorinstein *et al*., 2002), being this legume a good source of Lys and Arg. The presence of positive amino acids (i.e. L-arginine) may confer to grass pea protein isolate biological activities able to improve human health (i.e. anti-hypertensive activity) as well. As a next step, it would be interesting examine in depth grass pea protein digestion mechanism including jejunum phase. Indeed, the hydrolytic activity of BBM peptidases might further change the peptide patters of protein digests and thus their functionality.

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Chapter 4. Effect of germination in the modulation of protein digestibility and allergenicity in chickpea-based ingredients

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The present chapter is going to be submitted as a research article for publication

Abstract

In the last years, the demand for chickpea-based ingredients has notably increased due to their high protein content. However, chickpea allergens could be a serious problem for consumers especially in developing countries where the demand for protein foods is very high. In this regard, germination may be a suitable solution to reduce the amount of allergens without affecting the nutritive value of chickpea containing food. Thus, some samples from germinated and no-germinated chickpea flour were subjected to proteomic characterization and *ex vivo* gastrointestinal digestion in order to assess the effect of germination on protein digestibility and allergencity. Collected data indicated as germinated sample (S2) was involved by higher hydrolytic activity and protein degradation with the formation of new peptides (potentially bioactive) and essential amino acids. Furthermore, at the end of *ex vivo* gastrointestinal digestion, S2 peptides showed a minor number of putative epitopes. These achivements further confirm the use of germination as a suitable method to increase the biological value and digestibility of proteins in chickpea (legume)-based ingredients, as well as to reduce their allergenicity.

Introduction

Chickpea (*Cicer arietinum L.*) is one of the most worldwide consumed legumes. There is a growing demand for chickpea-based ingredients due to their nutritional value, being that this legume is a protein-rich source with a high concentration of globulins (60% of total proteins). In addition, chickpea has a high content of carbohydrates, and it represents a good source of dietary fibre, vitamins and minerals (phosphorus, calcium and iron) (Agriculture and Agri-Food Canada, 2006; Wood and Grusak, 2007). However, its consumption can be associated to food allergy and cross-reactivity episodes (Bar-El Dadon *et al*., 2013). Cross-reactivity can manifests between chickpea and other legumes (i.e. pea, lentil, soybean and hazelnut) (Bar-El Dadon *et al*., 2014) or pollen. Recently, Kulkarnia *et al*. (2013) identified seven putative and potential cross-reactive chickpea allergens through an *in silico* approach. Some of those putative proteins showed similarity against known legume and pollen allergens (i.e. Ara h 3, Ara h 8, Gly m 4, Vig r 1 and Bet v 1). Certainly, the presence of allergens in chickpea-based ingredients or products can be a serious problem for consumers especially in developing countries where the demand for protein foods is very high (Sasson, 2012; Schönfeldt and Gibson Hall, 2012). In this regard, germination may be a suitable solution to reduce protein allergenicity without affecting the nutritive value of chickpea containing food. In fact, this method has the potential to increase food protein digestibility, as well as the bioavailability of certain amino acids and peptides. Germination is a natural plant process useful for seed sustenance and development that occurs in specific environmental conditions (i.e. humidity, temperature and nutrient content) (Bewleyl, 1997). During germination, the hydrolytic activity of proteolytic, amylolytic and lipolytic enzymes modulates the amount and biological value of nutrients according to their process conditions and seed variety (Rahman *et al.,* 2007;

Sangronis and Machado, 2007). In the food industry, this method is also employed to remove seed flaws such as undesired flavors or trypsin inhibitors (Fernandez and Berry, 1988; Sangronis and Machado, 2007).

In this study, the effect of germination on protein digestibility and allergenicity of chickpea-based ingredients was investigated. To this purpose, samples from germinated and no-germinated chickpea flour were subjected to proteomic characterization and *ex vivo* gastrointestinal digestion.

Materials and Methods

Chemicals

Acetic acid, trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Carlo Erba Reagents (Milan, Italy). Chloroform, methanol, borate buffer, acetone, acetonitrile (ACN) and water were purchased from Sigma Aldrich (St. Louis, MO, USA). Reagents for electrophoresis analysis were purchased from Bio-Rad (Milan, Italy).

Seed germination and flour production

Chickpea seeds from a local retailer were subjected to germination process (16-22°C, 72h). The seeds were suspended in water (22°C, 1 h), in sterile environment, and then stored at room temperature for 20h in sterile containers. Once again, the seeds were suspended in water and stored at 16°C for 48h. Germination was stopped by drying (45 °C) and the seeds were reduced into flour using a homemade mill.

Flour degreasing

Non-germinated (S1) and germinated (S2) chickpea flours were defatted using chloroform/methanol (2:1, v/v). The solution was stirred (30 min) and

centrifuged (4000 g x 20 min). After centrifugation, the supernatants containing fats were discarded and the precipitates air-dried.

Protein extraction

To perform chickpea flour protein extraction was used the protocol of Rubio *et al.* (2014) with slight changes (**Figure 1**). Defatted flours were transferred into a 50 mL tube and about 45 mL of borate buffer (pH 8.0) were added. Samples were stirred (12h) and centrifuged (4000 g x 20 min). After centrifugation, the supernatant (A) was separated and the procedure was repeated on the precipitate. The obtained supernatant (B) was mixed with A and the solution was brought to pH 4.5 by adding 50% acetic acid. Subsequently, the solution was stirred and centrifuged. The supernatant (C) was removed and stored at 4°C, while the precipitate was suspended in borate buffer (pH 8.0) and the procedure was repeated once again. The obtained precipitate was mixed with acetone (1:1, w/v), centrifuged and air-dried (Legumins 11S). The supernatant (D) was mixed with C and dialysed for about 24h. At the end of dialysis, the recovered solution was centrifuged. The supernatant was freeze-dried (Albumins), while the precipitate was suspended in acetone (1:1, w/v), centrifuged and air-dried (Vicilline 7S).

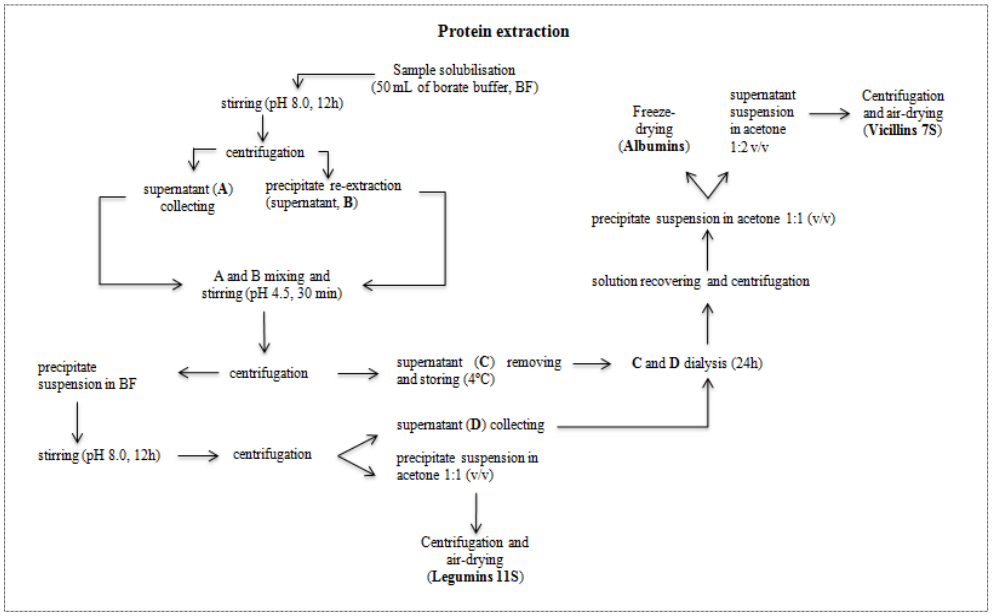

Figure 1. Workflow of chickpea flour protein extraction

Ex vivo gastrointestinal digestion

Ex vivo gastrointestinal digestion was performed on chickpea flours, and related protein fractions, adapting the harmonised *in vitro* protocol of Minekus *et al*. (2014). Gastrointestinal juices were collected by a high number of volunteers at Lovisenberg Diakonale Hospital (Oslo, Norway) and then gathered in a single pool. 20 mg of each flour and fraction were transferred into two 5 mL tubes, respectively, and 1000 µL of water were added (2% protein concentration). The duplicate was used as a control sample. The oral phase was bypassed, starting directly from the gastric phase. In the oral phase, in fact, salivary α-amylase is specifically active on carbohydrates, excluding proteins. To simulate the gastric phase, 0.5 µL of 0.3M $CaCl_2$ and 290 µL of Human Gastric Juice (HGJ) were added to samples. Then, the solution was brought to pH 2.0 with HCl and mixed with Simulated Gastric Fluid (SGF) up to a volume of 2 mL. The samples were incubated (37°C, 1h) under soft stirring. Afterwards, digests from gastric

phase were mixed with 0.5 µL of 0.3M $CaCl_2$ and 1580 µL of Human Duodenal Juice (HDJ). The solution was brought to pH 7.0 with NaOH and Simulated Intestinal Fluid (SIF) was added up to a volume of 4 mL. The samples were incubated (37°C, 2h) under soft stirring. A rate of 2 mL was collected after 1h. The amount of juices to use in simulated gastrointestinal phases was calculated according to the enzymatic activity and the amount of digested proteins. At the end of simulated digestion, samples were immediately stored in freezer at -28 °C.

SDS-PAGE analysis

The samples (both digested and undigested chickpea flours, and relative protein fractions) were suspended (1 mg of protein/mL) in sample buffer (0.050 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol (w/v), 5% β-mercaptoethanol (v/v) and trace of bromophenol blue indicator), stirred for 5 min at room temperature and left to rest overnight to allow the complete protein solubilisation. After heating at 95°C for 10 min, the samples were loaded on 15% polyacrylamide gels. Electrophoretic separations were performed using Mini-Protean Cells electrophoresis II (Bio-Rad, Hercules, CA, USA) at constant voltage (100 V). A molecular weight marker was loaded on each gel. After migration, each gel was stained in a water solution containing 0.05% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 0.7% (v/v) acetic acid, and destained in a water solution containing 10% (v/v) acetic acid and 40% (v/v) methanol.

Preliminary sample preparation for proteomic characterization

Proteomic characterization was carried out both on digested and undigested samples (flours and protein fractions). Before the analysis, all the samples were appropriately diluted to obtain the same protein concentration. To perform HPLC analysis, the samples were suspended in 0.1% TFA (v/v) in

water (eluent A). For LC-high resolution (HR)-MS/MS analysis, the samples were reconstituted in 0.1% FA (v:v) in water (eluent C). Before the supernatant injection, samples were subjected to centrifugation (4000 *g* x 10 min).

HPLC analysis

HPLC analysis was performed by using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) equipped with a reverse phase 218TP Vydac C_{18} column (250 X 4.6 mm, 5µM, Grace, Milan, Italy). HPLC system was connected to an Ultraviolet (UV)-Diode Array Detector (DAD) setting at 220 and 280 nm. The injection volume was 50 µL and the flow was set at 0.2 mL/min. Analyte elution was performed with a linear gradient from 15 to 100% of 0.07% TFA (v/v) in ACN (eluent B) in 70 min. Each sample was analysed in triplicate.

LC-HR-MS/MS analysis

LC-HR-MS/MS analysis was performed by using a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high performance liquid chromatography equipment (Thermo Scientific). Samples were loaded through a 5mm long 300 µm id pre-column (LC Packings, USA) and separated by an EASY-Spray™ PepMap C_{18} column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent C was 0.1% FA (v/v) in water; eluent D was 0.1% FA (v/v) in ACN. The column was equilibrated at 5% D. Peptides were separated applying a 4–40% gradient of D over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/*z scan range of 350 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full

width at half maximum (FWHM), an automatic gain control (AGC) target of $1x10^6$ ions and a maximum ion injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10s was applied. Ions with one or more than six charges were excluded. Spectra were processed using the Xcalibur Software 3.1 version (Thermo Scientific). Mass spectra were processed using the Proteome Discoverer 2.1 software (Thermo Scientific). Database searching parameters for identification of proteins were the following: Met oxidation and pyroglutamic for N-terminus Gln as variable protein modifications; carbamidomethyl cysteine as a constant modification; a mass tolerance value of 8 ppm for precursor ion and 0.01 Da for MS/MS fragments; trypsin as the proteolytic enzyme or no enzyme specificity; missed tryptic cleavage up to 2. Database searching parameters for identification peptides in samples were the same described above, except for no modification of cysteine residues included and no proteolytic enzyme selected. The false discovery rate and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for strict and relaxed searches, respectively. Proteins were considered confidently identified based on at least four sequenced peptides. Each sample was analysed in triplicate.

Results and Discussion

The effect of germination on chickpea flour protein degradation

SDS-PAGE and HPLC analyses showed as S2 was subjected to high hydrolytic activity. After germination, the protein content of chickpea flour decreased, while the degradation rate of high MW proteins increased (**Figure 2**, A and B gel, line 1; **Figure 3**). The same trend was shown by S1 and S2

protein fractions (albunins, legumins 11S and vicilins 7S) (**Figures 4-6**). The data is consistent with those of analogues work carried out on different legumes (i.e. peas, pinto beans, white navy beans, lentils, mung beans and soybeans) where germination was accompanied by turnover of protein and amino acids (Vanderstoep, 1981). Germination, therefore, would be sufficient to significantly improve the nutritive utilisation of chickpea proteins as also indicated by Urbano *et al.* (2005) and Rumiyati *et al.* (2012) in their works on pea and sweet lupin, respectively.

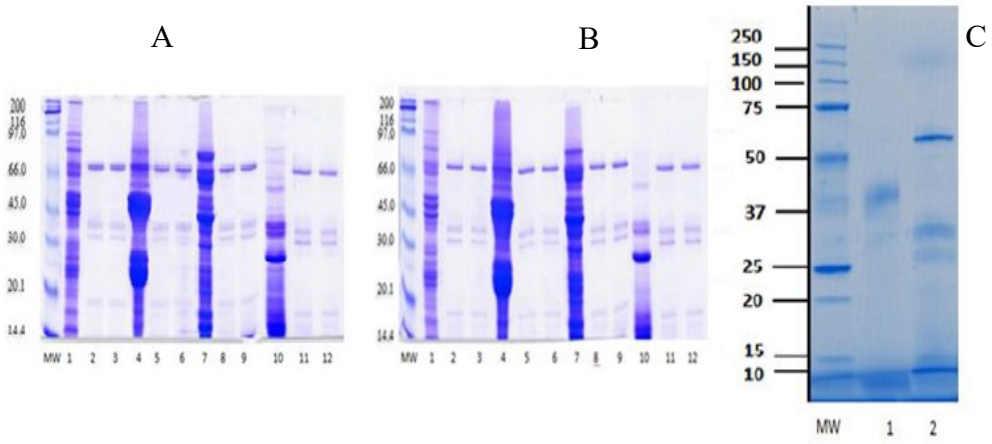

Figure 2. SDS-PAGE of A) S1 and B) S2: 1) chickpea flour; 2) chickpea flour after 60 min of intestinal digestion; 3) chickpea flour after 120 min of intestinal digestion; 4) legumins 11S; 5) legumins 11S digest after 60 min of intestinal digestion; 6) legumins 11S digest after 120 min of intestinal digestion; 7) vicillins 7S; 8) vicilins 7S digest after 60 min of intestinal digestion; 9) vicilins 7S digest after 120 min of intestinal digestion; 10) albumins; 11) albumin digest after 60 min of intestinal digestion; 12) albumin digest after 120 min of intestinal digestion; C) 15% SDS-PAGE in reducing conditions of 1) HGJ and 2) HDJ

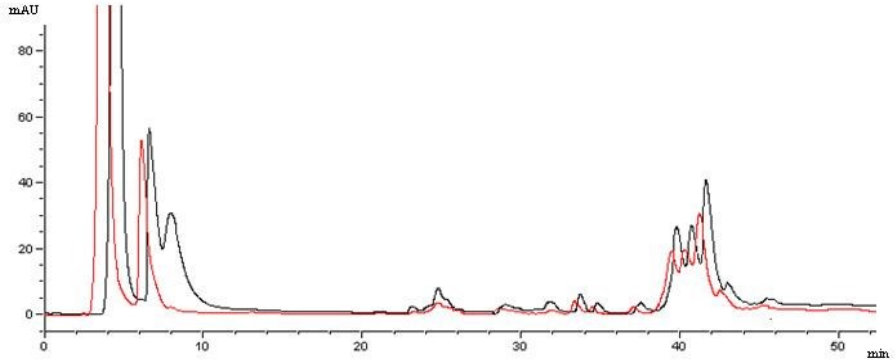

Figure 3. HPLC chromatogram of S1 (black line) and S2 (red line) (220 nm)

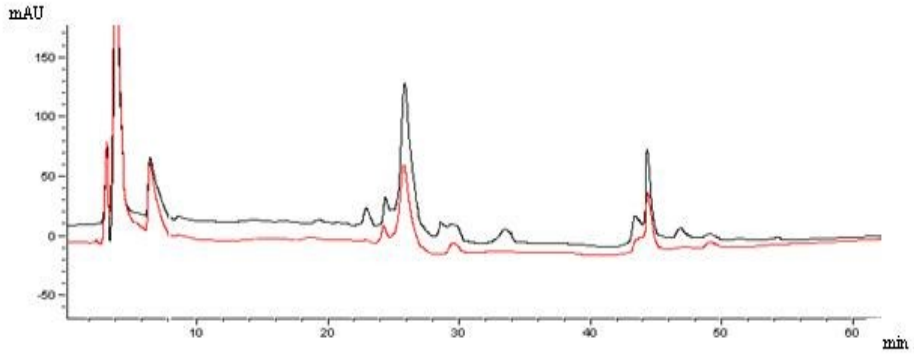

Figure 4. HPLC chromatogram of S1 (black line) and S2 (red line) albumin fraction (220 nm)

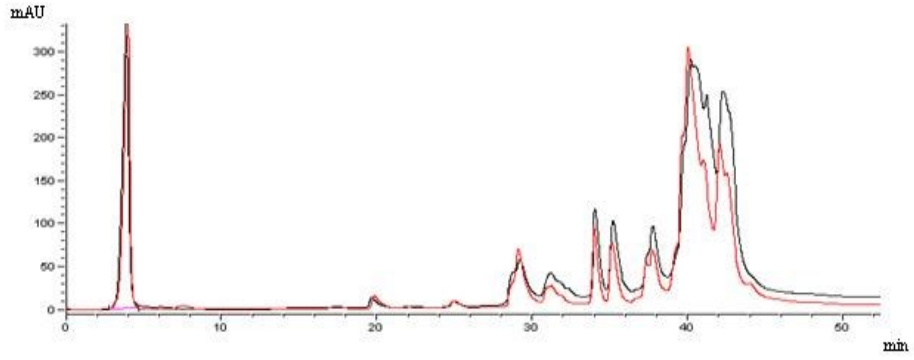

Figure 5. HPLC chromatogram of S1 (black line) and S2 (red line) vicilin fraction (220 nm)

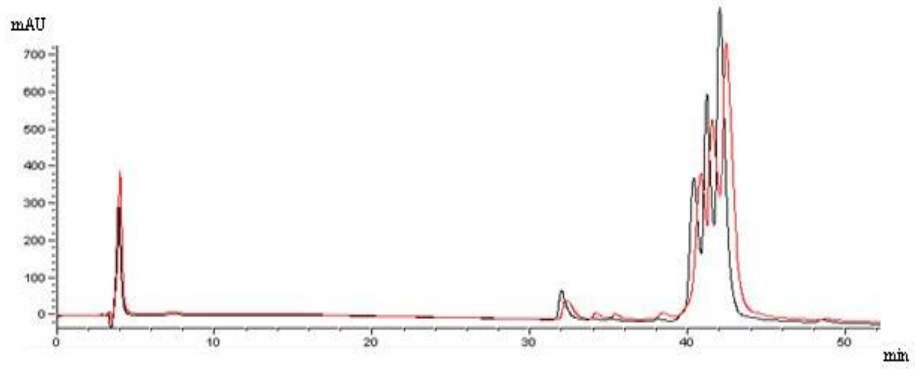

Figure 6. HPLC chromatogram of S1 (black line) and S2 (red line) legumin fraction (220 nm)

Effect of germination on chickpea flour protein digestibility and allergenicity

Although both the flour samples had a high protein digestibility, the most part of S2 proteins was completely digested after 1h of intestinal digestion (**Figure 7**). In particular, legumin fraction showed the highest protein degradation (**Figure 8**). Conversely, S1 proteins were totally digested only at the end of intestinal digestion (**Figure7**). This trend was confirmed by SDS-PAGE analysis (**Figure 2**, A and B gels) as well. The bands visible in all the samples were identified as gastrointestinal enzymes from HGJ and HDJ (**Figure 2**, C gel). A similar behaviour has also been reported for other food matrices, such as pearl millet (Khetarpaul and Chauhan, 1990).

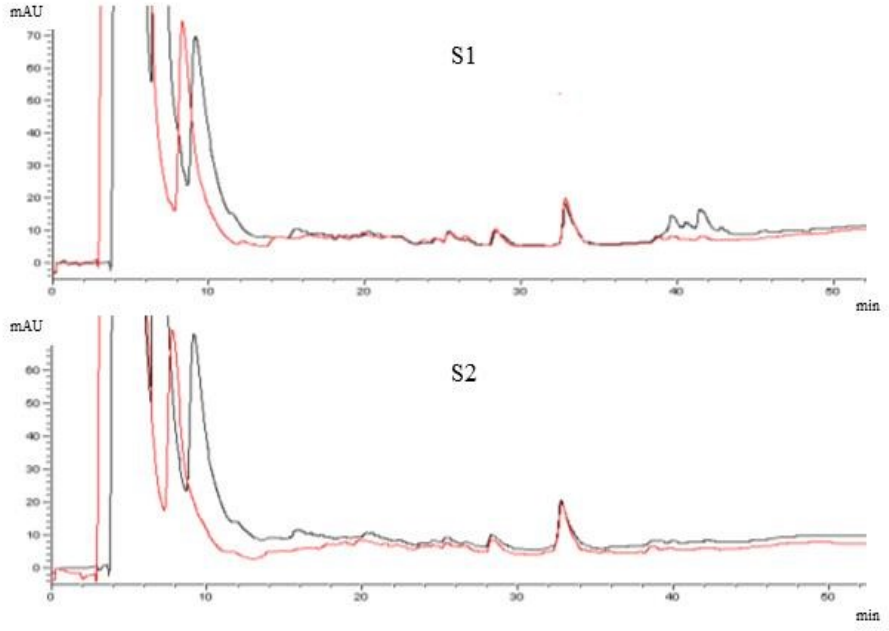

Figure 7. HPLC chromatograms of S1 and S2 after 1h (black line) and 2h (red line) of intestinal digestion (220 nm)

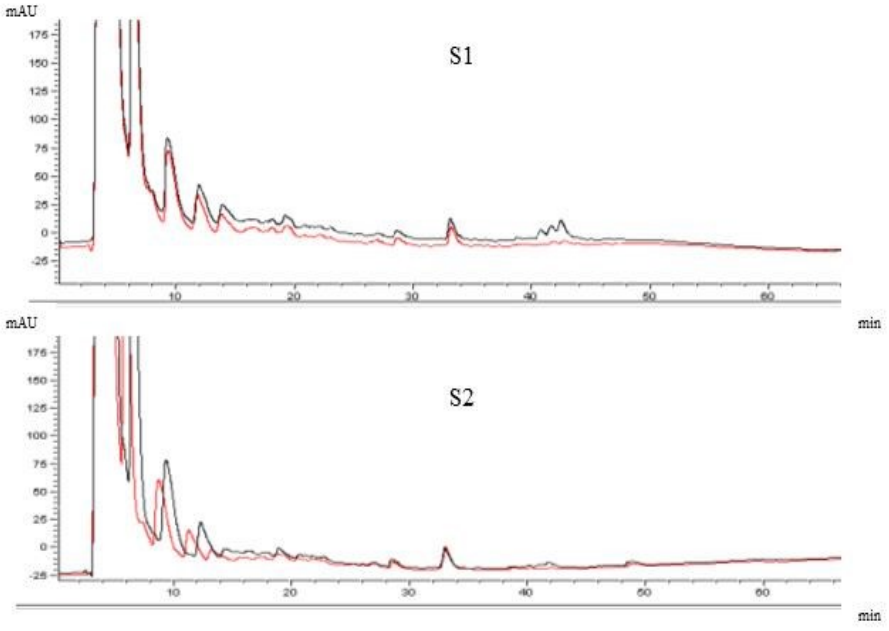

Figure 8. HPLC chromatograms of legumins 11S from S1 and S2 after 1h (black line) and 2h (red line) of intestinal digestion (220 nm)

LC-HR-MS/MS analysis allowed the identification of S1 and S2 peptides arisen from *ex vivo* gastrointestinal digestion. Interestingly albumin fraction had the highest digestibility since the identified peptides belong exclusively to legumins 11S and vicilins 7S (**Tables 1** and **2**).

Table 1. Peptide sequences detected in S1 by LC-HR-MS/MS analysis after 2h of intestinal digestion

Peptide sequence	Protein Accessions	Protein description	Theo. MH+ [Da]
ATVILVVNEGKGEVELV	828299209	vicilin-like [Cicer arietinum]	1769.00
SINASSDLFLLGFGINAQNNQR	828299209	vicilin-like [Cicer arietinum]	2379.20
KILEASFNSDYEEIER	828299209	vicilin-like [Cicer arietinum]	1942.94
DLDISLNSVEINEGSLLLPHFNSR	828299209	vicilin-like [Cicer arietinum]	2682.37
EITPEKNPQLQDLDISLNSVEINEGSLLLPHFNSR	828299209	vicilin-like [Cicer arietinum]	3960.03
SILEASFNTKYETIER	502119682	vicilin-like [Cicer arietinum]	1900.96
GGLSIITPPEKEPR	502110016	legumin-like [Cicer arietinum]	1493.83
NALEPDHRVESEAGLTETWNPNHPELQ	502114995	legumin J-like [Cicer arietinum]	3083.44
GTEHKFEDTWQEQRP	502146132	peptidyl-prolyl cis-trans isomerase FKBP42 [Cicer arietinum]	1887.86
HIVDKLQGRDEDEEKGAIVK	502110021	legumin A-like isoform X1 [Cicer arietinum]	2279.20
HIVDKLQGRDEDEEKGAIVKVK	502110021	legumin A-like isoform X1 [Cicer arietinum]	2506.36

HIVDKLQGRDEDEEKGAIV	502110021	legumin A-like isoform X1 [Cicer arietinum]	2151.10

Table 2. Peptide sequences detected in S2 by LC-HR-MS/MS analysis after 2h of intestinal digestion

Peptide sequence	Protein Accessions	Protein description	Theo. MH+ [Da]
ATVILVVNEGKGEVELV	828299209	vicilin-like [Cicer arietinum]	1769.00
FLPQHNDADFILVVLSGR AILTVLNPNDRNT	828299209	vicilin-like [Cicer arietinum]	3462.85
SILEASFNTKYETIER	502119682	vicilin-like [Cicer arietinum]	1900.96
NEDEEKGAIVKVKGGLSII TPPEKEPR	502110016	Legumin-like [Cicer arientinum]	2933.59

As shown in **Table 3**, in S1, three peptide sequences were found to be epitopes common to Q304D4 (**Figure 9**), one of the seven putative allergens from chickpea identified by Kulkarni *et al*. (2013) in their study (**Table 4**). They retrieved Q304D4 as a homologous in the BLASTp analysis of the Ara h 1, Gly m 5, Len c 1, Lup an 1 and Vig r 2 allergens from peanut, soybean, lentil, narrow-leaved blue lupin and mung bean. On the contrary, in S2, only one peptide sequence was identified as epitope from the same allergen (**Table 3**). This difference was probably due to the higher digestibility shown by S2 proteins following simulated gastrointestinal digestion.

Table 3. Putative epitopes detected in S1 and S2 after *ex vivo* gastrointestinal digestion corresponding to those of Q304D4, a chickpea allergen identified by Kulkarni *et al*. (2013)

S1 peptide sequence	S2 peptide sequence
ATVILVVNEGKGEVELV	**ATVILVVNEGKGEVELV**
SINASSDLFLLGFGINAQNNQR	
EITPEKNPQLQDLDISLNSVEINEGSLLLPHFNS	

MIVRFSLPDNENDLKLTRSINRDGEILIPKIFIIISVSQISNGASREFDGISSLKVEVFLSLGFNTV
SIALHLGLQDGSRHHCVVEERGCEVLSYFLQTVVLEVLKLLRTFVEPLEKTNVTVFVLEKSL
KVVRLKEKRILLIGISHKLRPRKQFLSSTKSGNRALIAILMIEFLLSFRIDDEIERVLLEEQEQK
PKQRRGHKDRQQSQSQSQQEADVIVKISREQIEELSKNAKSSSKKSVSSESEPFNLRSRNPIY
SNKYGNFF**EITPEKNPQLQDLDISLNSVEINEGSLLLPHFNS**R**ATVILVVNEGKGEVELV**
GLRNENEQENKKEDEEEEEDRKVQVQRFQSRLSSGDVVVIPATHPF**SINASSDLFLLGFGIN**
AQNNQRNFLAGEEDNVISQIQRPVKEVAFPGSAEEVDRLLKNQRQSHFANAQPQQKDEES
QKIRIPLSSILGGF

Figura 9. Peptide sequence of chickpea allergen Q304D4, in which are highlighted the putative epitopes detected in S1 and S2. After germination, only the epitope evidenced in blue was found

Table 4. The seven putative allergens from chickpea identified by Kulkarni *et al*. (2013)

Cicer arietinum–BLAST retrevied entry	Allergen	Molecular function	% identity
G1K3R9_CICAR	Vig r 4	Metal ion binding	57.00
G1K3S0_CICAR	Vig r 4	Metal ion binding	56.00
O23758 NLTP_CICAR	Ara h 9	Non-specific lipid-transfer protein	62.00
Q304D4_CICAR	Ara h 1	Nutrient reservoir activity	48.00
	Gly m 5		48.00
	Pis s 1		48.00
	Len c 1		48.00
	Lup an 1		48.00
	Vig r 2		48.00
Q39450_CICAR	Ara h 8	Pathogenesis-related protein	70.00
	Gly m 4		70.00
	Vig r 1		70.00
Q9SMK8_CICAR	Ara h 8	Pathogenesis-related protein	62.00
	Gly m 4		62.00
	Vig r 1		62.00
Q9SMJ4 LEG_CICAR	Ara h 3	Alpha-amylase inhibitor, seed storage protein, storage protein	50.00
	Ara h 4		49.00
	Gly m 6		49.00

A further comparison was made between the epitopes found in the chickpea flour and those reported for Ara h 1, Ara h 2 and Ara h 3 in different studies on peanut (Wesley Burks *et al*., 1997; Rabjohn *et al.,* 1999; Otsu *et al*., 2014) (**Table 5**). The scarce conformity with the examined epitopes supports the hypothesis according to which chickpea is a minor allergenic legume if compared with peanut or soybean. Interestingly, also in this case, germinated sample reported a lower number of putative epitopes as shown at the end of **Table 5**. These data, therefore, further support germination as an appropriate method for the production of hypoallergenic chickpea-based ingredients.

Table 5. Comparison between chickpea flour epitopes and those reported for Ara h 1, Ara h 2 and Ara h 3 in different studies on peanut

Allergen epitopes from peanut	S1			S2	Reference
	1	2	3	A	
ARA H1					
AKSSPYQKKT					
QEPDDLKQKA					
LEYDPRLUYD					
GERTRGRQPG					
PGDYDDDRRQ					
PRREEGGRWG					
REREEDWRQP					
EDWRRPSHQQ					
QPKKIRPEGR					
TPGQFEDFFP					Wesley
SYLQEFSRNT					Burks *et al*.,
FNAEFNEIRR					1997
EQEERGQRRW					
DITNPINLRE					
NNFGKLFEVK					
GTGNL**ELV**AV					
RRYTARLKEG					
ELHL**LGFGI**N	**LGFGI**				
HRIFLAGDKD					
IDOIEKOAKD					
KDLAFPGSGE					
KESHFVSARP					
PEKES**PEKE**D				**PEKE**	
ARA H 2					
RRCQSQQLER					
RPCEQHLMQKI					
CNELNEFE**NNQR**	**NNQR**				Otsu *et al*.,
CEALQQIIMENQSQ					2014
L**QGR**QQEQQ		**QGR**			
KRELRNLPQQ					

CGLRAPQRCDLDV			
ARA H 3			
R**PD**N**RIESE**G		**PD-R-ESE**	
GYI**ETWNPN**N		**ETWNPN**	
QEFECAGVAL			
SRLVL			
DLIAVPT			
GVAFWLYNDH			
DTDV			
AGQEEENEGG			
NIFSGFTPEF			Rabjohn *et*
LEQAFQVDDR			*al.,* 1999
QIVQNLRGE			
ES**EE**E**GAIV**T	**EE-GAIV**	**EE-GAIV**	
VRGGLRILSP			
DRKRRA**DEE**E	**DEE**	**DEE**	
EY**DEDE**YEYD	**DEDE**		
EEDRRRGRGS			
RGRGNGIE			
IETWNPNNQEFECAG			
GNIFSGFTPEFLEQA			
VTVR**GGL**RILSPDRK		**GGL**	
DEDEYEYDEEDRG	**DEDE**		

Code	S1	S2
1	SINASSDLFL**LGFGI**NAQ**NNQR**	
2	HIVDKL**QGRDEDEE**K**GAIV**KVK	
3	NALE**PD**H**RVESE**AGLT**ETWNPN**HPELQ	
A		NE**DEE**K**GAIV**KVK**GGL**SIITP**PEKE**PR

Conclusions

Protein digestibility and allergenicity are very current issues given the numerous global food safety and security emergencies, especially in developing countries. As for legumes, germination process can be responsible

for protein quali-quantitative changes due to the proteolytic activity of seed enzymes. These changes can affect protein digestibility, as much as allergenicity, of legume-based ingredients or products.

In this study, the effect of germination on chickpea protein digestibility and allergenicity was investigated. To this purpose, germinated and non-germinated chickpea flours were subjected to proteomic characterization and *ex vivo* gastrointestinal digestion. Collected data indicated that germination improved flour protein digestibility and decreased the number of potentially allergenic peptide sequences as well. Moreover, the major proteolytic activity and consequent higher protein degradation, promoted by germination, produced new peptides and amino acids that increased the flour biological value. These achievements, therefore, strongly support germination as suitable method to improve the safety and security of legume-based ingredients as in the case of chickpea flour.

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Chapter 5. Final remarks and future perspectives

The most recent FAO's definition of food security (*food security exists when all the people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life* - World Food Summit, 1996) highlights that food quality and accessibility are necessary to guarantee an active and healthy life. Evidently, this definition of food security given by FAO will soon be exceeded. In fact, the current way to produce and commerce foodstuffs, together to the consumers' food habits, will be completely changed by socio-political and ecological issues related with imminent environmental needs. Phenomena, such as the increasing of salinization and aridity or the growing of anthropogenic greenhouse gas emissions, due to the sudden climate changes, will deeply alter the agricultural system, varying the conditions of crop productions (i.e. agroecology and selective breeding) (Hocquette, 2016). The access to food will become increasingly difficult globally, and above all in developing countries. Consequently, the protein food demand of world population can no longer be supplied only by animal proteins. Moreover, intensive livestock are responsible for critical phenomena such as air pollution increase, groundwater contamination and deforestation. Thus, meat consumption negatively affects food sustainability. Hence, the need to find new alternative protein sources able to efficiently replace meat proteins. Currently, vegetal proteins represent the most effective alternative to those of animal origin. Vegetal proteins, including those from legumes, may represent a 'golden source' for the food industry; in fact, in literature, there are numerous studies that correlate them with different nutritive and biological properties. Undoubtedly, the PhD thesis achievements indicate legume proteins as the perfect matrix to improve food quality and security in full compliance with the emerging environmental and food sustainability needs. Furthermore, legume protein composition and allergenicity can be modulated by food processes like germination either to

increase their biological value or to produce hypoallergenic legume-based ingredients. These ingredients might be a low-cost, safety and ‘easy to produce’ alternative to synthetic additives, which can be harmful for consumers in certain doses. They might also be an effective means to satisfy the increasing demand for eco-sustainable and healthy protein foods, proposing themselves as suitable animal protein substitutes as well.

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You may reach any goal by yourself.
However, when you achieve it together,
you may share your satisfaction
drinking a good wine.