The Impact of Germination and *In Vitro* Digestion on the Formation of Angiotensin Converting Enzyme (ACE) Inhibitory Peptides from Lentil Proteins Compared to Whey Proteins

F. Bamdad, Sh. Dokhani, J. Keramat, and R. Zareie

Abstract—Biologically active peptides are of particular interest in food science and human nutrition because they have been shown to play several physiological roles. *In vitro* gastrointestinal digestion of lentil and whey proteins in this study produced high angiotensin-I converting enzyme inhibitory activity with 75.5±1.9 and 91.4±2.3% inhibition, respectively. High ACE inhibitory activity was observed in lentil after 5 days of germination (84.3±1.2%). Fractionation by reverse phase chromatography gave inhibitory activities as high as 86.3±2.0 for lentil, 94.8±1.8% for whey and 93.7±1.7% at 5th day of germination. Further purification by HPLC resulted in several inhibitory peptides with IC_{50} values ranging from 0.064 to 0.164 mg/ml. These results demonstrate that lentil proteins are a good source of peptides with ACE inhibitory activity that can be released by germination or gastrointestinal digestion. Despite the lower bioactivity in comparison with whey proteins, incorporation of lentil proteins in functional food formulations and natural drugs look promising.

Keywords—ACE inhibitory peptides, digestion, germination, lentil proteins, whey proteins.

I. INTRODUCTION

HIGH blood pressure is a frequently occurring condition as 15–20% of all adults are estimated to suffer from the disease [1]. Hypertension is the major controllable risk factor associated with cardiovascular disease (CVD) events such as myocardial infarction, stroke, heart failure, and end-stage diabetes. In the U.S. alone current annual antihypertensive drug costs are approximately $15 billion [2]. Hypertension treatment has been shown to lower the risk of developing heart failure among elderly patients and observational data indicate that hypertension control is associated with improved ventricular function and decreased symptoms in patients with heart failure [3], [4].

Blood pressure regulation is partially dependent on the renin–angiotensin system; renin acts on angiotensinogen, thus releasing angiotensin-I that is further converted into the active

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peptide hormone angiotensin II, by the angiotensin-I converting enzyme (ACE) [5]. Angiotensin-I converting enzyme (peptidyldipeptide hydrolase or kininase II, EC 3.4.15.1) is an unusual dipeptide-liberating exopeptidase that plays a key physiological role in the regulation of blood pressure by virtue of two different reactions that it catalyzes: conversion of the inactive decapeptide angiotensin I to a powerful vasoconstrictor and salt-retaining octapeptide angiotensin II, and inactivation of the vasodilator and natriuretic nonapeptide, bradykinin. Angiotensin II increases the production of aldosterone, which decreases the renal output while increasing water retention [6]. Therefore, this zinc ion containing metalloenzyme is an important target for the design of antihypertensive agents, as inhibition of ACE could shut down its hypertensive actions. Apart from this, angiotensin-I converting enzyme has also been related with other physiological processes, and so abnormal levels of ACE activity in serum have been related with the apparition of some diseases [7].

Synthetic inhibitors of ACE, such as captopril, enalapril, lisinopril and ramipril, are effective and widely used drugs for the therapy of hypertension, heart disease and diabetic neuropathy. As oral administration of these drugs frequently results in unwanted side effects, including hypotension, increased potassium levels, reduced renal function, cough, angioedema, skin rashes, and fetal abnormalities, a nutritional approach (consuming functional foods that naturally contain ACE inhibitory peptides) should be the preferred medium by which blood pressure is controlled [8].

It is well recognized that apart from their basic nutritional role, many food proteins contain encrypted within their primary structures, peptide sequences capable of modulating specific physiological functions. These bioactive peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing [9]. Oshima and others (1979) first reported ACE inhibitory peptides produced from food proteins by digestive proteases [10]. Afterward, many ACE inhibitory peptides have been discovered, isolated and purified from enzymatic hydrolysates of different food proteins. Some of these peptides with *in vitro* ACE inhibitory activities have been well demonstrated having *in vivo* inhibitory properties on ACE and antihypertensive effects without side effects in spontaneously hypertensive rats and hypertensive humans [11]-[14]. Despite the higher doses needed in comparison with antihypertensive drugs, the consumption of food products containing antihypertensive

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peptides has shown to significantly reduce the blood pressure of moderately hypertensive subjects [4].

Milk proteins are the most important source of bioactive peptides, though other animal as well as plant proteins are also known to contain potential bioactive sequences [9]. Casokinins and Lactokinins represent a group of bioactive peptides that have significant potential as naturally-derived agents for the prevention/control of blood pressure and related diseases. Casokinin sequences have been found in αs1-, β-, and κ-casein, and lactokinins in α-lactalbumin, β-lactoglobulin and bovine serum albumin [15]. Many milk derived peptides reveal multi-functional properties, i.e., specific peptide sequences may initiate two or more different biological activities. Due to their physiological and physicochemical versatility, milk-borne bioactive peptides are regarded as highly prominent ingredients for health-promoting functional foods or pharmaceutical preparations [9].

Peptides with biological activity can be produced from food proteins in three ways: a) enzymatic hydrolysis with digestive enzymes, b) fermentation of protein source with proteolytic starter cultures and c) through the action of enzymes derived from proteolytic microorganisms. Up to this point, enzymatic hydrolysis of proteins has been the most common way to produce bioactive peptides and pancreatic enzymes, preferably trypsin, have been used for the chemical characterization and identification of many known bioactive peptides [5].

Leguminous seeds contain large amounts of storage proteins. The storage proteins consist of a 7S component, vicilin, and an 11S component, legumin, both localized in specialized organelles called protein bodies [16], [17]. These protein bodies also contain some proteolytic enzymes, as well as other hydrolases [18], [19]. During germination the protein bodies enlarge, accompanied by an increase in the proteolytic activity in the cotyledons of legumes [20]. Germination of seeds mobilizes reserves from the seed to the growing seedling; increased metabolic activities in turn result in chemical change in the macromolecules. Increased enzymic activities in the germinating seeds are usually accompanied by interconversion and production of new compounds [21].

Although there have been a few reports on ACE inhibitory peptides in several legumes [22]-[26], to our knowledge this is the first time that lentil proteins are assessed for ACE inhibitory activity. Among leguminous species, lentil is consumed in appreciable amounts in Middle East countries. Lentil seeds are a promising source of protein and starch, although their nutritional composition varies widely depending on cultivar, growth conditions, and agricultural practice [27]. The lentil proteins, composing 22-31% of the seed dry weight, have an amino acid composition similar to those of other legumes, with methionine and cysteine being limiting [28], [29]. So, the aim of this study was to obtain ACE inhibitory active fractions from lentil protein digests by simulation of gastrointestinal digestion and compare it to whey bioactive fractions. Since extensive breakdown of the storage proteins is known to occur during germination, our second aim was to study the possibility of bioactive peptide production during germination.

II. MATERIALS AND METHODS

A. Materials

Lentils of small seeded Persian type (*Lens culinaris* Medik. cv. Gachsaran) produced in the South western area of Iran (Gachsaran), were evaluated in the present study. Whole dry Lentil seeds (harvested in 2006) were obtained from Gachsaran Agricultural Research Center, Gachsaran, Iran. They were packed in app. 500 gram tin cans or 3-layer polyethylene bags and stored at 4°C until used. The seeds were first ground by using a Moulinex coffee grinder and passed through a 40 mesh screen. The flour was stored in airtight plastic bags at refrigerator and used as starting material for analysis and production of protein isolate. Whey protein concentrate (85.02%protein DM) was a gift from Bonlac Foods, Ltd. (Melbourne, Australia). Moisture, fat, ash and nitrogen contents were determined using AOAC, (1990) approved methods [30]. Moisture content of different samples was determined by drying to constant weight in an oven at $105 \pm 1°C$.

B. Chemicals

Pepsin (EC 3.4.23.1, 10000 units/mg solid), trypsin (EC 3.4.21.4, 150 BAEE units/mg solid), and Chymotrypsin (EC 3.4.21.1, 6375 ATEE units/mg solid) were donated by National Enzyme Company (Forsyth, Missouri, USA). Hippuryl-histidyl-leucine (HHL; substrate for ACE), trifluoroacetic acid (TFA) for HPLC, Captopril, Phenyl methyl sulfonyl fluoride (PMSF) and pepstatin were from Sigma Chemical Co., Ltd. (St. Louis, MO). Hippuric acid (HA), O-phthaldialdehyde (OPA), quinoline, benzene sulfonyl chloride (BSC) and Acetonitrile ($CH3CN$) of HPLC grade were obtained from Merck (Amsterdam, The Netherlands). L-methionine was purchased from Riedel- de Haën (Seelze, Germany). Unless mentioned otherwise, all chemicals used were of analytical grade.

C. Preparation of Lentil Protein Isolate (LPI)

Lentil flour (20 g) was extracted by stirring for 1 h in 200 ml 0.2% NaOH solution at pH 12. After centrifugation at $8000 \times g$ for 15 min at 4°C, two additional extractions were carried out for another hour with half the volume of alkaline solutions. Supernatants were pooled and the pH was adjusted to 4.5 and centrifugation was performed as above. The precipitate was washed with distilled water adjusted to pH 4.5, freeze-dried and stored at refrigerator until use [31].

D. Gastrointestinal Digestion

The conditions of the physiological digestion were based on literature [32]. To simulate the digestion in the stomach, the pH of a 100 ml 4% (w/v) protein solution was brought to the desired pH for the stomach digestion (pH 2) with 1 and 10 N HCl under rigorous mixing. Pepsin was added in a ratio of enzyme and substrate of 1/250 (w/w) and the medium was incubated on a shaker for 2 h at 37°C. Samples were taken at the start, 30, 60, 90 and 120 min. After two hours, the pH was set at the desired value for small intestine digestion (pH 6.5) and trypsin and chymotrypsin were supplemented both at a ratio of enzyme and substrate of 1/250 (w/w). Then, the solution was again incubated for 2.5 h at 37 °C on a shaker. When samples were taken at the start, 30, 60, 90, 120, 150 min and the end of digestion, the pH was adjusted to 5 and then

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submerged in a boiling water bath for 10 min to terminate the digestion. As this is a pH near the isoelectric point for both proteins (lentil, pH 4.5; whey, pH 4-5), a clear separation was obtained by subsequent centrifugation ($8000 \times g$, 15 min, 4°C). During the peptic and pancreatic phases of the digestion, the pH ranged from 2.0 to 2.6 and 6.2 to 6.5, respectively.

1. Degree of Hydrolysis

Degree of hydrolysis (DH) was analyzed in triplicate using the OPA method described by Nielsen and coworkers with some modifications [33]. The OPA reagent was prepared as described by Church and coworkers [34]. The OPA solution was made by combining the following reagents and diluting to a final volume of 50 ml with water: 25 ml of 100 mM sodium tetra borate, 2.5 ml of 20% (w/v) SDS, 40 mg of OPA (dissolved in 1 ml of methanol), and 100 μl of β-mercaptoethanol. This reagent was prepared daily. The L-methionine standard was prepared as follows: 50 mg methionine was diluted in 500 mL deionized water (0.826 meqv/L).To assay the degree of hydrolysis, small aliquots (400 μl) of standard or sample was added to a test tube containing 3 mL OPA reagents and mixed for 5 s. The mixture was held for exactly 2 min before being read at 340 nm in a Unico UV-2100 spectrophotometer.

Determination of h:

methionine-NH2 = OD $_{sample}$ − OD $_{blank}$ / OD $_{standard}$ − OD $_{blank}$ × 0.826 meqv/L × 0.1 × 100 / X × P

where methionine-NH2 = meqv methionine NH2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L).

h is then: h = (methionine-NH2 - b) / a meqv/g protein

where a and b are 0.970 and 0.342 for legume proteins and 1.0 and 0.40 for Whey proteins, respectively [35] .

Calculation of DH: DH (%) = h / h_{tot} × 100

where h_{tot} for legume proteins and whey proteins are 7.8 and 8.8, respectively [35].

E. Germination

The process was carried out on a semi-pilot scale. 500 g of lentil seeds were pre-treated with 2500 ml of 0.07% sodium hypochlorite solution for 30 min at room temperature to remove surface contamination. Seeds were then drained and washed to neutral pH, and then soaked in distilled water for 5.5 h. Finally, imbibed seeds were transferred to Petri dishes lined with wet filter paper and germinated in a seed germinator (Memmert 854, Schwa Bach, Germany) in the dark at 20 °C, with 99% relative humidity. Only distilled water was sprayed daily during germination period. Seeds were harvested at different stages of germination and seedling growth (imbibed seeds, 1st, 3rd and 5th day). Germinated seeds were frozen at -18 °C for 12 hr to stop the germination process. Sprouted seeds were freeze-dried and ground to pass through a 40 mesh sieve for analysis. For extraction of peptides, flour of germinated lentils at different stages of germination, were homogenized in a ten-fold volume of 25 mM citrate-phosphate buffer, pH 5, containing 2 mM β-mercaptoethanol. The homogenates were filtered through no.1 Whatman filter paper, centrifuged at $12000 \times g$ for 20 min at 4°C and the supernatants were freeze dried.

1. Nitrogenous Compounds of Germinated Lentils

Total nitrogen was determined according to Kjeldahl's method. Percent crude protein was calculated as total nitrogen×6.25. Soluble protein, insoluble and non-protein nitrogen were measured according to the methodology described by Periago and coworkers [36].

F. Preparation of Angiotensin-I Converting Enzyme

Angiotensin-I converting enzyme was prepared following the procedure of Hayakari, Kondo, and Izumi (1978) with some modifications [37]. Rabbit lungs purchased in a local market were used as starting material. Lung tissue was diced and homogenized in 10 volumes of ice-cold Tris-HCl buffer (20 mM) pH 8.3, containing pepstatin (0.1 mM) and PMSF (0.1 mM). The homogenate was centrifuged at $6000 \times g$ for 10 min at 4 °C. ACE was solubilized from pellet using Tween 40, a non-ionic detergent. The supernatant solution brought to 50% and 70% saturation concentration of ammonium sulfate. After ammonium sulfate precipitation, the supernatant dialyzed for 24 h at 4 °C against 20 volume of the same buffer. The buffer was exchanged four times. The dialyzed $5000 \times g$ supernatant was used as the source of angiotensin-I converting enzyme. In order to verify the absence of undesirable proteases such as carboxy peptidase in ACE extract, the kinetics of HHL hydrolysis by ACE was followed in the presence and absence of captopril, a potent ACE inhibitor. Obtained data demonstrates that hydrolysis is due to ACE and not to other proteases, since hydrolysis of HHL in the presence of captopril is residual and similar to the blank (substrate alone).

1. Assay of ACE Inhibitory Activity

The lyophilized samples were dissolved in demineralized water at 10mg/ml and analyzed by an ACE inhibition assay. The ACE inhibitory activity was measured *in vitro* according to the procedure of Li and coworkers [38]. For each assay, a sample solution of ACE inhibitor (20 μl) with 50 μl of 5mM HHL in 100mM sodium borate buffer (pH 8.3) containing 300mM NaCl was pre incubated at 37 °C for 5 min. The reaction was initiated by the addition of 10 μl of ACE solution (100 mU/ml), and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 100 μl of 1M HCl. Sodium borate buffer was then added to the reaction mixture to a volume of 0.5 ml. Hippuric acid (HA) released from HHL by ACE was monitored by addition of quinoline and benzene sulfonyl chloride (BSC). Finally, the absorbance was measured in a Unico UV-2100 spectrophotometer at 492 nm and compared to the HA standard. All measurements were performed in triplicate and results are shown as averages. The 50% inhibitory concentration (IC_{50}) value is the peptide concentration that inhibits the activity of ACE by 50%.

G. Gel Electrophoresis

SDS gel electrophoresis of hydrolysate supernatants and germinated lentils was performed using 5% stacking and 13% separating gels according to the method of Laemmli (1970) with modifications [39]. The freeze-dried hydrolysates and germinated lentils were solubilized in sample buffer consisting of 50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol. The mixture was heated for 30 min at 40 °C; centrifuged ($14000 \times g$, 5min)

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and the supernatants were applied to the gel. Equal amounts of nitrogen (20 μg) were loaded in each lane. Electrophoresis was performed at a constant voltage of 150 V. gels were fixed and stained with 0.2% Coomassie Brilliant blue R-250 in methanol: acetic acid: water (5:4:1 v/v/v). Molecular weight of the protein bands was estimated from their relative mobility in gel (Ferguson Plot). The apparent relative mobility was expressed as the ratio of the distance (mm) traveled by the protein to that of dye front (120mm) [40].

H. Liquid Chromatography

Samples were chromatographed on a Hewlett Packard Model 1090 Liquid Chromatography equipped with a diode array detector and ChemStation software. HPLC profiles were obtained by analytical RP-HPLC by injecting the lyophilized sample at a concentration of 10 mg protein/ml on an analytical Nucleosil ODS column (5 μm, 4.6 mm × 250 mm; VDS Optilab chromatographic technik GmbH, Berlin, Germany) at 40°C with a flow rate of 0.7 ml/min. Solvent A was 0.09% (v/v) TFA in water and solvent B was 0.1% (v/v) TFA in 80% (v/v) acetonitrile solution. The lyophilized sample (10mg) was dissolved in 1ml of deionized water, acidified with 10% TFA solution and then centrifuged (14000 × g, 5 min). Supernatants were filtered through 0.45-μm cellulose acetate syringe filter to eliminate any turbidity and 25 μl of this solution was injected into the HPLC system. Separations were performed with a linear gradient system from 5 to 60% of solvent B for 30 min and 60 to 100% solvent B for 10 min. The column was equilibrated with 5% solvent B and tested with 10 μg of a pure protein (Ubiquitin) to verify system performance (i.e., resolution, signal intensity, bleeding, tailing, etc.). Peptide peaks were monitored at 214 nm.

1. Fractionation by RP-HPLC

Samples with highest bioactivity (L2P+2TC, W2P+2TC and G5) were fractionated using RP-HPLC by injecting a 40mg protein/ml solution. Commencing from 5 min after the start of elution, fractions were collected at every 5 min (from at least six chromatographic runs). The eight fractions obtained were concentrated in a vacuum concentrator (Eppendorf 5301, Germany) and the ACE inhibitory activity of each fraction was measured.

I. Protein Assay

Protein content of samples was assessed by the Biuret method on the Unico UV-2100 spectrophotometer. Bovine serum albumin was used as a standard. Different concentrations of albumin were mixed with the Biuret dye reagent and read, after a 30-min incubation period, at 540 nm. Samples at different stages of purification were diluted appropriately and the absorbance was read and compared to the standard.

J. Statistical Analysis

All results were expressed as the mean ± Standard error (n_{min} = 3). The statistical analysis of data was performed using SAS (1990) statistical software system (SAS institute, Cary, NC, USA). Student t-test was used to compare the ACE inhibitory activity and IC_{50} value of the samples and peptide peaks obtained by HPLC chromatography.

III. RESULT AND DISCUSSION

A. Derivation of Antihypertensive Activity from Lentil Proteins by Gastrointestinal Digestion

The lentil protein isolate used for the generation of protein hydrolysates was obtained by alkaline extraction and acid precipitation of proteins. This protein isolate with more than 85% protein content, is a good starting material for hydrolysis. In vitro digestion consisted of a simulation of the stomach digestion with pepsin and the small intestine digestion with trypsin and chymotrypsin. Before in vitro gastrointestinal digestion, the lentil protein solution showed a lower degree of proteolysis than whey, respectively, 4.2±0.02% and 12.3±0.05% (P<0.01), while the ACE inhibitory activities were similar, respectively, 11.1±0.04% and 13.4±0.06% (P<0.01) (Fig. 1).

During digestion, the degree of proteolysis augmented more for lentil than whey in both the simulated stomach and small intestine phase. But none of them reached the complete hydrolysis (DH near 100) even after small intestinal digestion. Legume proteins are only partly hydrolysed because of the inability of most proteases to cleave glycoproteins, phosphoproteins, other post-translationally modified species, or domains that contain a higher number of disulfide bridges [41].
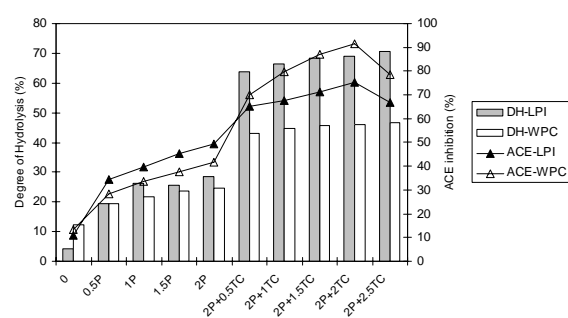

Fig. 1 Evolution in the degree of hydrolysis (%) and ACE inhibitory activity (%) during the in vitro gastrointestinal digestion of lentil and whey protein (n_{min}=3). 0.5P, 1P, 1.5P and 2P are hydrolysis at 0.5, 1, 1.5 and 2 hrs by pepsin. 2P+0.5TC, 2P+1TC, 2P+1.5TC, 2P+2TC and 2P+2.5TC are hydrolysed for 2hrs by pepsin and 0.5, 1, 1.5, 2 and 2.5 hrs by trypsin+chymotrypsin, respectively.

The major whey proteins α-lactalbumin and β-lactoglobulin partially resist gastrointestinal digestion. Pepsin degrades α-lactalbumin and only denatured β-lactoglobulin; trypsin slowly cleaves α-lactalbumin and denatured β-lactoglobulin, while chymotrypsin hydrolyzes both α-lactalbumin and β-lactoglobulin to a limited extent [42]. The higher initial degree of proteolysis of the whey protein may also have resulted in more byproduct inhibition for proteolysis.

An in vitro digestion involving only the stomach phase was not as efficient as the physiological digestion in releasing ACE inhibitory activity from lentil and whey proteins. Two hour digestion with trypsin and chymotrypsin caused a jump and further increase in the ACE inhibitory activity of the hydrolysates, particularly for whey, to maximum ACE inhibitory values of 91.4±2.3% for whey and 75.5±1.9 for

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lentil and then decreased afterwards particularly for lentil hydrolysate about 14% at more than 70%DH (Fig. 1).

The lower ACE inhibitory activity of lentil hydrolysates can be enlightened by the hypothesis that digestive enzymes split within the sequence of bioactive peptides in lentil proteins, thereby preventing their release. Therefore, there exists an optimal degree of hydrolysis; above which more ACE inhibitory peptides are degraded than new peptides are formed, decreasing the overall ACE inhibitory activity [43]. Hence, no direct relationship between the degree of proteolysis and the ACE inhibitory activity is possible, especially in the later stages of hydrolysis. Because of a reduction in ACE-inhibitory activity of lentil and whey hydrolysates at the end of simulated intestinal digestion, the hydrolysates with highest bioactivity (2hr pepsin + 2 hr intestinal digestion) selected for further purification.

B. Germination

As a result of the germination process, legume seeds undergo considerable metabolic changes in their storage proteins. Raw lentil assayed had an average total nitrogen content of 3.68 ± 0.01 g of nitrogen/100 g of DM, 13.2% of which corresponded to soluble non-protein nitrogen, 77.35% of which corresponded to soluble protein nitrogen, and the remaining 8.06% was insoluble at the basic pH conditions used for extraction. The content of soluble protein nitrogen was significantly decreased by 5 d of germination and soluble non-protein nitrogen increased progressively with increasing germination periods (1.5-fold and 2.0-fold in 3rd and 5th day of germination, respectively, versus ungerminated lentil).

Germination is a natural biological process of all superior plants by which the seed comes out of its latency stage, once the minimal environmental conditions needed for its growth and development, such as humidity, temperature, nutrients, etc., are given [44].
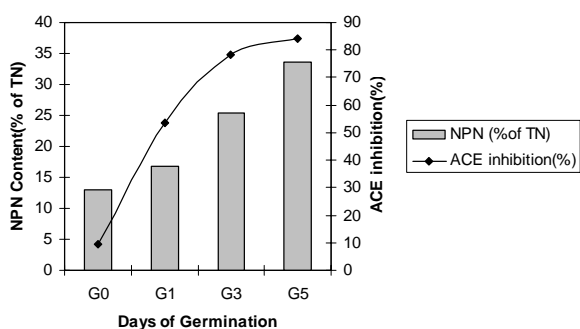

Fig. 2 Effect of germination on non-protein nitrogen content and ACE inhibitory activity of lentil. G0, ungerminated lentil; G1, germinated lentil at the first day; G3, germinated lentil at the 3rd day; G5, germinated lentil at the 5th day. Values are mean ± standard deviation ($n = 3$)

It is known that during germination a sequence of metabolic changes such as the activation of the respiratory processes and protein synthesis are triggered. The mobilization of seed storage proteins represents one of the most important post-germinative events in the growth and development of seedling [45].

Bioactivity and non-protein nitrogen content of lentil during germination are shown in Fig. 2. As observed in this Fig., there was a sharp increment, 70% in ACE inhibitory activity at the first days of germination up to 3rd day and then it increased very little gradually.

Proteolytic enzymes play central role in the biochemical mechanism of germination. The positive correlation between the developments of acid, neutral and alkaline proteases with protein depletion suggest the involvement of these proteases in the degradation of proteins in germinating legumes. These proteases increase in the early stages of germination and decrease later [46]. The presence of disulfide bonds would slow down the proteolytic process and thus prevent the indiscriminate loss of the nitrogen reserves. A limited number of specific cleavages are inflicted upon the substrate molecule at the onset of proteolysis, producing high molecular weight peptides [47].

Endopeptidases play key roles in storage protein degradation, producing oligopeptides. The latter are, in turn, hydrolysed by exopeptidases to free amino acids. Cysteine proteinases (CPs) are the major endopeptidases present in the cotyledons during early seedling growth, and are presumed to be largely responsible for the mobilization of the storage proteins [48]. They fall into two families, the papain-like CPs and the legumain-like CPs. The former exhibit low specificity for the peptide bonds cleaved, while the latter are specific for the cleavage of Asn-X peptide bonds [49]. Further proteolysis proceeds until the protein is converted into short TCA-soluble peptides. Reduction in the slope of ACE inhibitory activity may be due to breakdown of short peptides because following germination, the storage proteins are hydrolysed to free amino acids, which serve as precursors for the synthesis of new proteins and other nitrogen-containing compounds in the seedling [47].
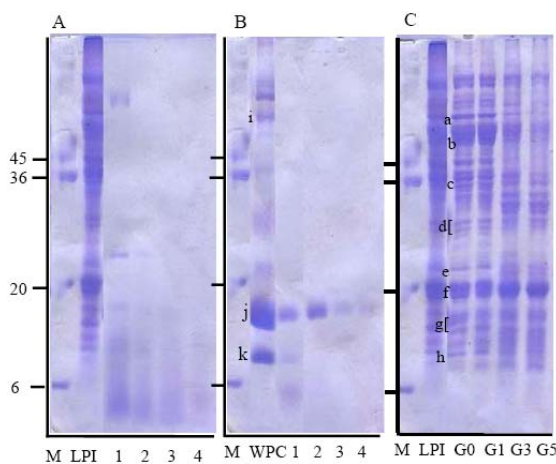

Fig. 3 SDS-PAGE pattern of lentil and whey proteins during digestion (A and B) and germination (C). M: molecular weight marker, LPI: lentil protein hydrolysate, WPC: whey protein concentrate, 1: 0.5 hr digestion with pepsin, 2: 2hr digestion with pepsin, 3: 2hr pepsin+0.5 hr trypsin & chymotrypsin, 4: 2hr pepsin+2.5 hr trypsin & chymotrypsin digestion, G0: imbibed seeds, germinated lentil at 1st day (G1), 3rd day (G3), and 5th day (G5). **a**: convicilin, **b**: vicilin, **c**: legumin α, **d**: vicilin, **e**: PA2, **f**: legumin β, **g**: vicilin, **h**: PA1, **i**: bovine serum albumin, **j**: β-lactoglobulin, **k**: α-lactalbumin

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C. SDS-PAGE Pattern of Lentil and Whey Proteins

SDS-PAGE pattern of lentil protein isolate showed a complex protein profile characterized by the presence of proteins in a wide range of molecular weights (Fig. 3).

The different SDS-PAGE bands of lentil [17], [47], [50] and whey [14], [51] protein were named according to literature. There is no research paper investigating lentil proteins and their subunits in details and the band names selected on the basis of similarity to other legume proteins.

The SDS-PAGE pattern of lentil protein isolate is similar to that of imbibed lentil seeds (G0). It indicated more than 20 bands. The largest polypeptide (about 93 KDa) may correspond to the most cathodic (largest MW) polypeptide in the legumin fraction. The band at approximately 70 KDa corresponds to a protein equal to pea convicilin. Major subunits of 50, 30-35 and 19 KDa along with a minor lower MW polypeptide (approx. 12 KDa) have been reported for vicilin fraction [52].

After the simulated stomach digestion of lentil protein, all bands lost intensity and some even disappeared. Convicilin, PA2 and to a lesser extent vicilin and legumin resisted hydrolysis by pepsin. Other proteins were degraded completely to polypeptides with molecular weights below 8 KDa and a number of faith bands with low molecular weights appeared. After the simulated small intestine phase, the proteins were almost completely broken down. Similar profiles were obtained by Deshpande and Nielsen (1987) for phaseolin subunits [53]. The time-course of hydrolysis shows that pepsin can not degrade the lentil proteins completely enough, but the central susceptible region of the protein molecules appeared to be exposed to trypsin and chymotrypsin, thus giving the characteristic breakdown products of smaller than 10 KDa. This susceptible region must contain a variety of amino acids such that the protein can be cleaved by enzymes with different specificities.

After the simulated stomach digestion of whey protein, the band of β-lactoglobulin remained present (Fig.3, B), while a fraction below 14 KDa appeared. Hence, β-lactoglobulin resisted pepsin digestion, while the other whey proteins were almost completely degraded. After the simulated small intestine phase, β-lactoglobulin was cleaved by the action of trypsin and chymotrypsin into smaller proteins. Banding patterns similar to those shown in Fig. 3 have been obtained by other researchers [54], [55].

SDS-electrophoresis patterns of extracts of lentil which had germinated for various periods of time are shown in Fig. 3, C. As germination proceeded, there was a gradual disappearance of protein subunits and a concomitant increase in components having low molecular weights. In germinated lentils there are several protein bands resistant to proteolysis. Vicilin and the basic subunit of legumin are extremely resistant to hydrolysis. As judged by the band width and intensity, the four original subunits (35, 30, 12, 11 KDa) of vicilin and the basic subunit of legumin remain intact at the 5th day of germination. On the other hand, the acidic subunit of legumin and the largest subunit of vicilin (55KDa) were degraded after 3 days of germination. Their break down was accompanied by generation of new subunits of approximately 75, 28-32, 20 and 10KDa, as well as a large amount of very low MW peptides (8-12 KDa).

This pattern of storage protein breakdown was similar to that reported for the mung bean and pea where the largest subunit of the major storage protein of these plants was the first to be broken down during germination [55].
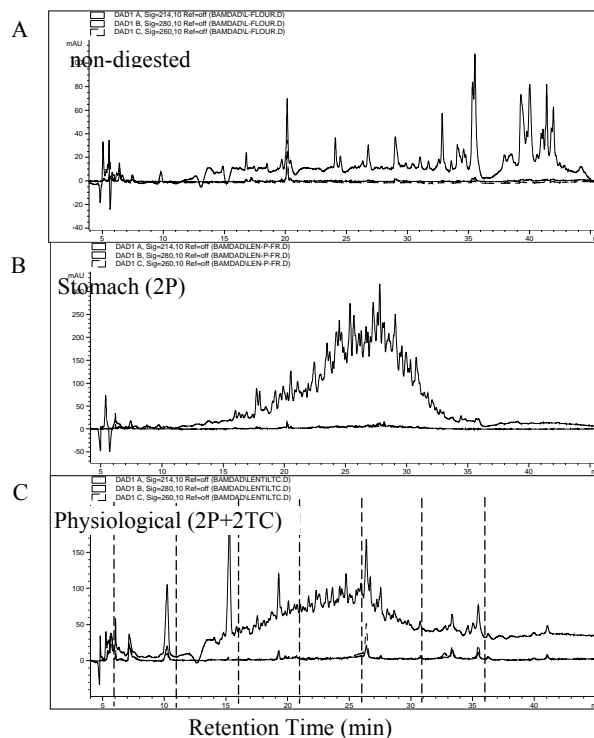
Fig. 4 HPLC chromatograms of soluble fractions of non-digested lentil protein and after simulated stomach and physiological digestion. 2P; two hour digestion by pepsin, 2P+2TC; two hour digestion by pepsin followed by 2 hour hydrolysis by trypsin+chymotrypsin, mAU; milli Absorbance Unit

Using SDS-PAGE to study changes in germinating lentil proteins, Hsu and coworkers observed a progressive decrease in large protein subunits and formation of small subunits as germination progressed [56]. They found more changes in proteins for lentils than pea and faba bean.

D. Chromatographic Analysis of Lentil and Whey Proteins

For the purification of peptide with inhibitory activity, the lyophilized germinated lentil (G5) and hydrolysates of lentil isolate and WPC (2 hr stomach + 2 hr intestinal digestion) were first solubilized in water, adjusted to pH 3, centrifuged and filtered through a 0.45 μm filter and then loaded on an HPLC C$_{18}$ reverse phase column. The HPLC chromatograms of the hydrolysates and non-digested whey and lentil proteins are shown in Figs. 4 and 5 respectively. The separation by RP-HPLC is based on molecular weight and hydrophobicity, with the higher-molecular-weight and more hydrophobic molecules eluting later in time. When the proteins are not yet digested, they appear at the end of the solvent gradient [57].

When the chromatograms of the stomach and physiological digests are compared for both proteins, a transition occurs from several peaks eluting after 17 min in the stomach digests to more peaks with lower elution times (after 10 and 15 min)

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in the physiological digests(Fig. 4). Apparently, a major breakdown of proteins and high-molecular-weight peptides still took place during the small intestine phase of digestion. As observed for the intestinal digests, the whey hydrolysate showed (Fig. 5) sharp individual peaks, while most of the peaks merged in the case of the lentil hydrolysate (Fig. 4). β-Lactoglobulin, the major whey protein, was mainly degraded by trypsin and chymotrypsin and hardly at all by pepsin. It is known that β -lactoglobulin resists pepsin
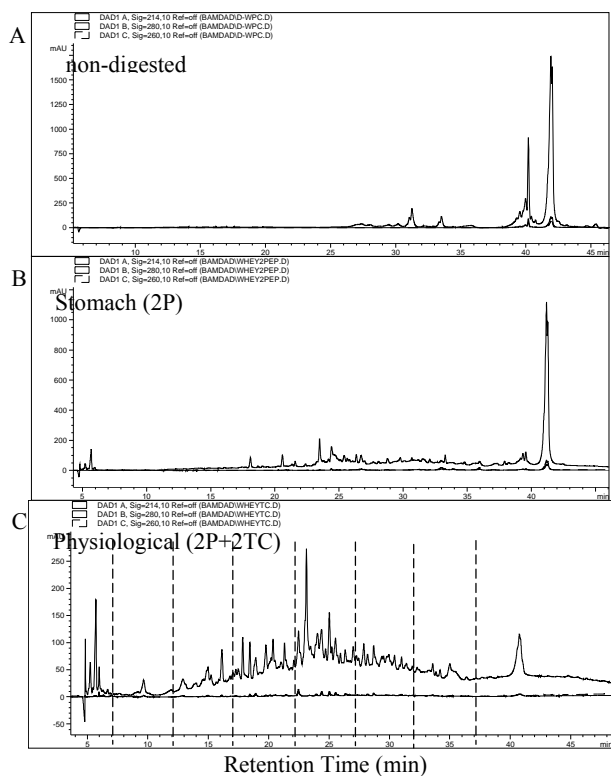

Fig. 5 HPLC chromatograms of soluble fractions of non-digested whey protein and after simulated stomach and physiological digestion. 2P; two hour digestion by pepsin, 2P+2TC; two hour digestion by pepsin followed by 2 hour hydrolysis by trypsin+chymotrypsin, mAU; milli Absorbance Unit

and largely, trypsin hydrolysis, while it is hydrolysed by chymotrypsin to a limited extent [57]. Therefore the considerable increase in ACE inhibitory activity of the whey hydrolysate during the small intestine phase of digestion is probably caused by the release of potent bioactive peptides from β-lactoglobulin. It is reported that pepsin hydrolysis for 3 h at pH 2 and 37 °C at a ratio of enzyme to substrate of 1 to 200 kg/kg is insufficient to release ACE inhibitory peptides from α–lactalbumin and β–lactoglobulin [58]. Trypsin is necessary for the formation of high ACE inhibitory activity from both whey proteins. The chromatograms of germinated lentils are shown in Fig. 6.
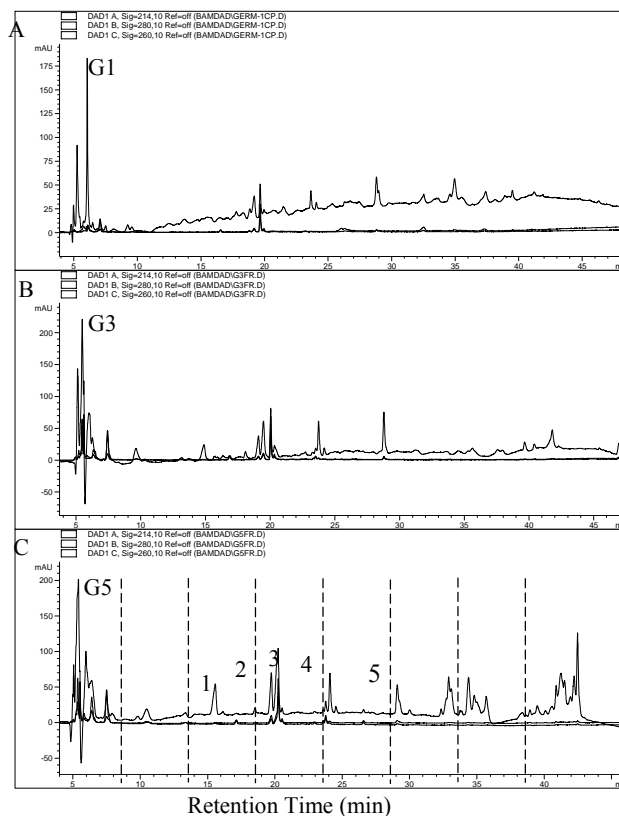

Fig. 6 HPLC chromatograms of soluble fractions of germinated lentil at different days. G1, germinated lentil at the first day; G3, germinated lentil at the 3rd day; G5, germinated lentil at the 5th day, mAU; milli Absorbance Unit

It is evident that with progression of germination during 5 days, more peptides with lower molecular weights and hydrophobicity appear (Fig. 6, G1, G3 and G5).). Some peptide peaks (2, 3, 4 and 5) are present at 1st and 3rd days of germination, although in minor quantities, but peak 1 appears at the 5th day. Quantity of these peptides, as measured by area under the peaks, increased significantly ($P<0.01$) in 5th d in comparison with the 1st day of germination. There is also an increase in the initial peak, pointing to a considerable concentration of amino acids and small hydrophilic peptides.

Bioactive peptides usually contain 2–20 amino acid residues per molecule, and the lower their molecular weight, the higher is their chance to cross the intestinal barrier and exert a biological effect. Crystallographic analysis of human ACE from testis (tACE) has revealed limited access to the active site of this enzyme, limiting its proteolytic activity to small, disordered peptides. Similarly, ACE inhibitors must be small enough to gain entry to block the active site [59].

E. Fractionation by RP-HPLC

Further purification of the lentil and whey hydrolysates (2 hr hydrolysis by pepsin + 2 hr hydrolysis by trypsin and chymotrypsin (2P+2TC)) and germinated lentil at 5th day (G5) was performed by RP-HPLC of the most bioactive fractions. Fig 3-c, 4-c and 5-c show the RP-HPLC profiles and indicate the eight fractions that were collected.

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For both lentil and whey proteins the ACE inhibitory activity in the fractions I, II and III was lower than in the physiological digests (Table I).

TABLE I
INHIBITORY CHARACTERISTICS OF FRACTIONS OBTAINED BY HPLC C$_{18}$ CHROMATOGRAPHY OF LENTIL AND WHEY HYDROLYSATES AND GERMINATED LENTIL (MEAN ± STANDARD ERROR)

	Elution times (min)	ACE inhibition (%)		Germinated Lentil(G5)
		lentil	whey	
undigested protein	---------	11.1±0.9	13.4±0.8	84.3±1.1
stomach digest	---------	49.4±1.2	41.9±1.1	----------
stomach+intestinal digest	---------	75.5±1.6	91.4±1.9	----------
fraction I	5-10	60.4±1.5	50.3±1.4	60.4±0.8
fraction II	10-15	65.7±1.6	59.4±1.4	58.5±0.8
fraction III	15-20	74.8±1.7	72.3±1.6	88.9±1.2
fraction IV	20-25	83.7±2.0	94.8±1.8	93.7±1.7
fraction V	25-30	86.3±2.0	84.2±1.6	87.2±0.9
fraction VI	30-35	79.6±1.9	79.6±1.2	78.3±0.7
fraction VII	35-40	75.2±1.5	62.3±1.5	68.4±0.6
fraction VIII	40-45	60.6±1.6	57.8±1.3	69.1±0.9

Most likely, this can be attributed to the presence of amino acids and short hydrophilic peptides with low ACE inhibitory activity. Fractions IV and V of the lentil hydrolysate (with retention times between 20 and 30 min) possessed a higher ACE inhibitory activity. In Table 1, the highest inhibitory of 86.3±1.2% was found in fraction V (eluting between 25 and 30 min), which was more than eight times higher than the inhibitory of the undigested protein. For whey the fractionation resulted in a slight enrichment of ACE inhibitory activity. Fraction IV had higher ACE inhibitory activities compared with the physiological digest. This was expected, since ACE inhibitory peptides are usually constituted by hydrophobic amino acids [60], which will increase the interaction of the peptides with the column and delay elution.

In germinated lentil at 5th day, fractions III, IV and V possessed higher bioactivity than ungerminated lentils. ACE inhibition activity is noticeably higher in fraction IV, probably because it contains three major peaks. Since in the chromatogram of G5 the peptides were eluted individually, the separated peaks were collected from several runs and assayed for their bioactivity (Fig. 6-c).

The fractions with the highest ACE inhibitory activity corresponded with a gradient of about 23–27% acetonitrile. Hence these fractions contained more hydrophobic peptides, which are more likely to exert an ACE inhibitory effect according to the structure–activity relationship of ACE inhibitory peptides [15]. Cheung and coworkers have reported binding of ACE and synthesized peptides and showed the importance of hydrophobic (aromatic or branched-chain aliphatic) amino acid residues at each of the three C-terminal positions [7]. Pepsin (in the stomach) as well as chymotrypsin (in the pancreatic secretions) cleave at the carboxyl end of hydrophobic and aromatic amino acids (Phe, Tyr, Trp, Leu), while pancreatic trypsin preferentially cleaves at the carboxyl end of arginyl and lysyl residues [61]. Peptides with hydrophobic and aromatic residues as well as the imino acid proline at the C-terminal position appear to be the most favorable substrates or competitive inhibitors of ACE [59]. Quantitative structure-activity relationship (QSAR) modeling by Pripp and coworkers [62] indicated that ACE-inhibitory potential of milk-derived peptides increased with greater side chain hydrophobicity and the absence of positive charge at the C-terminal position, while an increased side chain size of the amino acid next to the C terminal position decreased ACE-inhibitory potential. Fractionation was more effective for lentil than for whey. This suggests that potent ACE inhibitory peptides were present alongside low active peptides in lentil hydrolysate. As shown, several peaks were not resolved in the first chromatographic run. The most bioactive fractions (IV, V for lentil and IV for whey) were collected, concentrated and re-chromatographed with a slower gradient on the same column. In this stage, two shallower gradients were designed (15-20% solvent B for 17min (slope: 0.3) and 20-50% for 37 min (slope: 0.8)) and several unresolved peptides became fully resolved by this procedure. The elution profiles are shown in Fig. 7.
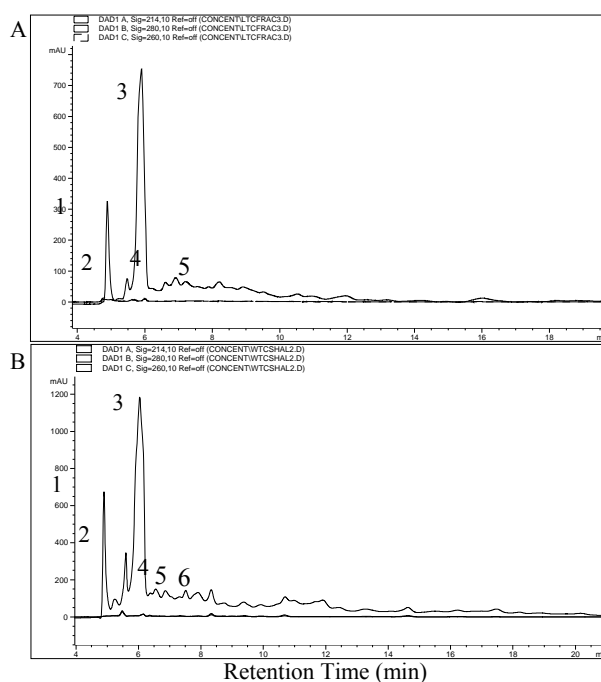

Fig. 7 Elution profiles of active fractions in C$_{18}$ chromatography with shallow gradient (15-20% solvent B for 17min (slope: 0.3) and 20-50% for 37 min (slope: 0.8)). A; lentil hydrolysate fractions (IV and V), B; Whey hydrolysate fraction (V), mAU; milli Absorbance Unit

Depicted peptide peaks were collected, concentrated and were also found to be biologically active. Table II shows the inhibitory characteristics of these peptides.

As shown in Table II, bioactive peptide peaks obtained from lentil proteins showed similar IC$_{50}$ values, between 0.08

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and 0.12, while peptides released from whey proteins had lower IC_{50} values.

TABLE II
IC_{50} ± STANDARD ERROR OF MEAN (MG PROTEIN/ML) OF PEPTIDE PEAKS OBTAINED BY SHALLOW GRADIENT HPLC CHROMATOGRAPHY OF LENTIL AND WHEY HYDROLYSATES AND GERMINATED LENTIL (n_{min}=3)

Peptide peaks	Germinated lentil (G5)	Lentil hydrolysate (2P+2TC)	Whey hydrolysate (2P+2TC)
1	0.107±0.04	0.098±0.04	0.075±0.05
2	0.088±0.03	0.12±0.03	0.097±0.06
3	0.078±0.03	0.087±0.03	0.064±0.04
4	0.126±0.04	0.157±0.04	0.113±±0.07
5	0.134±0.03	0.164±0.05	0.143±0.05
6	---------	---------	0.127±0.04

It is known that whey proteins are a rich source of ACE inhibitory peptides. Vermeirssen and coworkers [63] showed that whey proteins possess an almost threefold higher potential for the presence of ACE inhibitory peptides than pea protein. Maeno and coworkers [64] also found a potent antihypertensive effect in the whey fraction of the milk that was fermented by *Lactobacillus helveticus* CP790 in spontaneously hypertensive rats. Abubakar and coworkers [65] reported that thermolysin digest of whey protein showed strong ACE inhibitory activity (98.6% *in vitro*).

IV. CONCLUSION

In vitro simulated digestion of lentil and whey by digestive proteases produced ACE-inhibitory activity that was dependent on the time of digestion and the source of protein. Pepsin digestion in the stomach phase produced increasing ACE-inhibitory activity with increased digestion time. The subsequent action of trypsin and chymotrypsin in the intestinal phase initially produced peptides with higher ACE-inhibitory activity than the products of pepsin digestion, but decreasing inhibitory activity was observed with longer digestion time. Whey proteins possess more bioactive fractions than lentil proteins. It may be due to the presence of much more bioactive sequences within different whey proteins and also the specifity of digestive enzymes for releasing these sequences.

Germination of legume seeds has been investigated as a means of reducing the antinutritional factors and improving the nutritional quality by increasing the level of some amino acids, vitamins and minerals [66]. Our results suggest the potential production of bioactive peptides during first stages of germination.

Overall, the results from this study suggest the potential production of peptides with ACE-inhibitory activity upon germination of lentil seeds and physiological digestion of lentil proteins. To exert an antihypertensive effect, the peptides also have to pass the intestinal barrier to arrive in the blood in an active form [25]. Clinical trials would be required to provide final evidence of the efficacy of peptides released by germination and digestion.

The inhibitory activities of the peptide fractions mentioned in this paper can be improved. Their sequences provide a starting point for molecular modeling and thermodynamic studies. The residues critical for binding can then be derived

and homologues can be synthesized to produce compounds of more potent inhibitory activity [21]. It is also important to study the technological properties of the active peptide fractions and to develop model foods which contain these peptides and retain their activity for a certain period.

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