SPECIAL GUEST EDITOR SECTION

Determination of Nutritional and Bioactive Properties of Peptides in Enzymatic Pea, Chickpea, and Mung Bean Protein Hydrolysates

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Within the primary structure of many pea and mung bean proteins are peptide sequences that can potentially be used in the formulation of therapeutic products for the treatment and prevention of human diseases. However, these peptide sequences need protease treatments before they can be released free of the parent proteins. Unlike chemical hydrolysis, enzymatic treatment enables more efficient tailoring of peptide products without formation of toxic by-products or destruction of amino acids. This review provides information on current methods that have been used to convert inactive pea and mung bean proteins into bioactive peptides. It focuses on 3 main bioactive properties, such as inhibitions of (*1*) angiotensin converting enzyme (ACE) activity; (*2*) calmodulin (CaM)-dependent enzymes; and (*3*) copper-chelating activity. ACE is an established marker for hypertension, high levels of some CaM-dependent enzymes are risk factors for various human diseases including cancer and Alzheimer's disease, and high vascular copper concentrations may potentiate atherosclerosis. Also reviewed are the production and evaluation of activity of hypoallergenic peptides that may offer protection against anaphylactic reactions. The 3 main proteins discussed are chickpea, mung bean, and field pea.

Recently, there has been a tremendous increase in research activities that investigate the production, characterization, and efficacy of bioactive ingredients present in foods. Although these ingredients have less physiological effectiveness, they do not have the toxic or severe adverse side effects that are associated with therapeutic drugs. Bioactive peptides represent one of these food-derived therapeutic ingredients. These peptides are inactive as long as they remain trapped within the primary structure of their parent proteins, until they are liberated and can become active. Production and evaluation of bioactive peptides have been reported for several food proteins, including milk (1), soybean (2–4), wheat (5), and corn (6).

Field peas, chickpeas, and mung bean are rich sources of proteins (19–35% as-is basis) with a diverse content of amino acid sequences (7) that can be released through enzymatic hydrolysis as bioactive peptides. Several reports in the literature detail the production, characterization, and in vitro and in vivo evaluations of bioactive protein hydrolysates and peptides produced from the enzymatic hydrolysis of field peas, chickpeas, and mung bean. Production of hypoallergenic protein hydrolysates has also been reported as a means of reducing or eliminating the allergenic proteins in these pea and bean products. The objective of this review is to provide detailed information on the various methods that have been used to convert the inactive native proteins in field peas, chickpeas, and mung bean into bioactive peptides and nutritionally desirable products.

Preparation of Protein Substrates

Various methods for preparing protein isolates from peas have been reported. Figure 1 shows a general outline that is common to most methods used to prepare protein isolates from seeds. In general, extraction is performed at alkaline pH values because seed proteins have highest solubility at pH values >7.0. For example, chickpea seeds were cleaned of foreign matter, ground, and defatted for 9 h using hexane in a Soxhlet apparatus (8, 9) and then ground into flour. Lipids were removed to prevent formation of an emulsified phase that could limit protease activity and reduce recovery of hydrolyzed peptides. Initially, the flour was treated with a pH 10.5 aqueous solution that contained 0.25% sodium sulfite followed by shaking for 1 h at room temperature. Sodium sulfite was added to prevent dark color formation in the final protein isolate preparation. The aqueous extract was centrifuged and the supernatant saved while the residue was re-extracted twice with the aqueous solution (half the volume of the first extraction). The 3 supernatants were pooled and adjusted to pH 4.3 to precipitate most of the proteins at their

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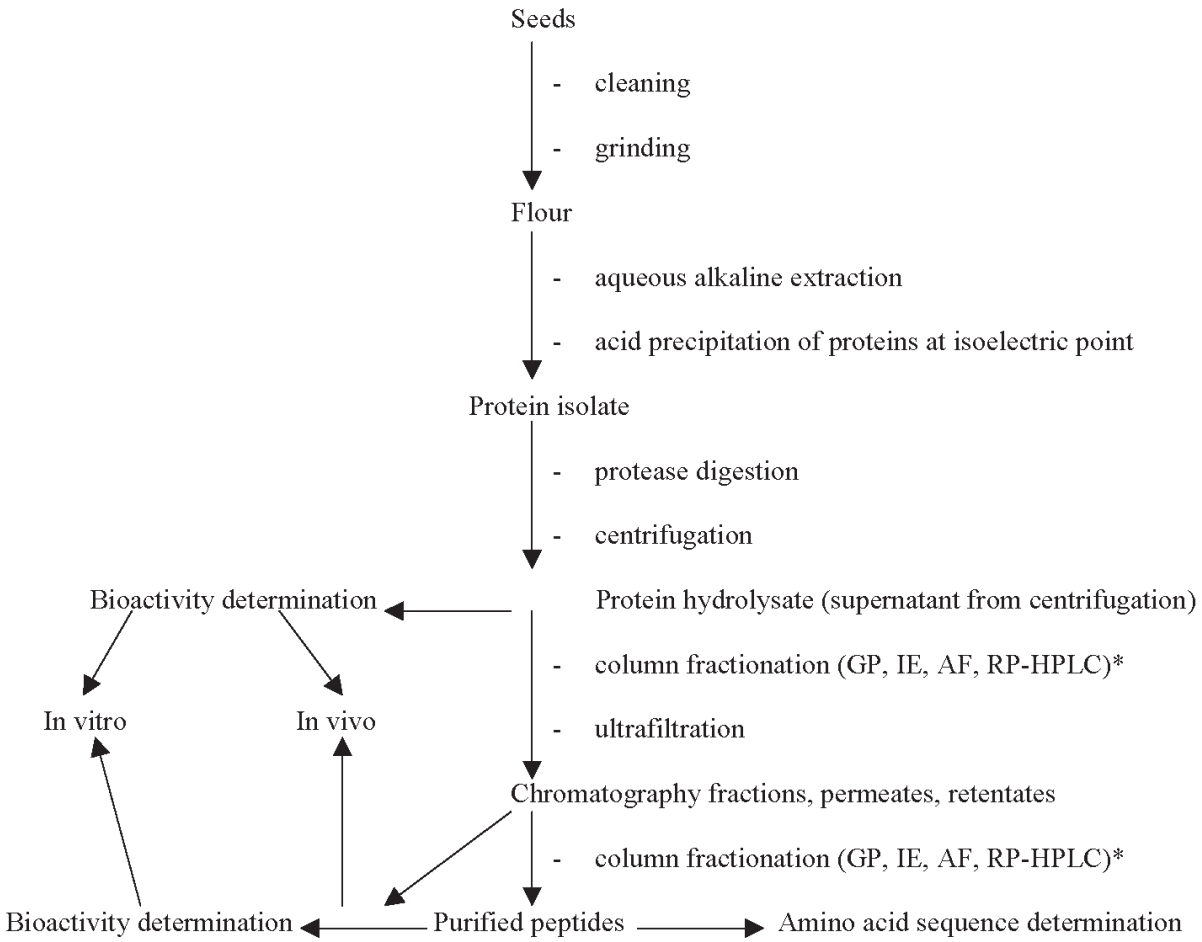

Figure 1. Typical flow chart for the production and evaluation of bioactive peptides from plant seeds. GP = Gel permeation; IE = ion-exchange; AF = affinity; RP-HPLC = reversed-phase high-performance liquid chromatography.

isoelectric point (pI). In order to remove some of the water-soluble nonprotein contaminants, the isoelectric precipitate was washed with pH 4.3 water. Aqueous washing was followed sequentially with ethanol and acetone washing, and the residue was dried at room temperature. In other similar methods, the defatted chickpea flour was extracted with 0.2% NaOH solution at pH 12.0 (10), or an aqueous slurry was adjusted to pH 9 using 0.1 M NaOH solution (9) prior to centrifugation and acid-induced protein precipitation at pH 4.3 or 4.5, respectively.

Mung bean protein isolate was produced by using a procedure similar to that for chickpea as described above, except that the hexane-defatted meal was extracted with 10 volumes of alkaline water (pH 9.0) but without addition of sodium sulfite. The supernatant was adjusted to pH 4.6 with dilute HCl solution, and the precipitate washed with distilled water and then freeze-dried (11). By omitting sodium sulfite, there is an increased probability of polymerization of the seed coat polyphenols, especially if the seed coat is highly colored. Increased protein–polyphenol interactions reduce protein solubility, which could lead to decreased protein extraction when the alkaline reagent is added. However, there is no information on whether the presence of polyphenols influences the yield of peptides during protein hydrolysis.

Bioactive Protein-Derived Peptides

Production and Evaluation of Protein Hydrolysates and Peptides

Generally, protein isolates are treated with appropriate proteases to release short-chain peptide sequences that are usually more soluble than the native proteins. Protease reaction is stopped by adjusting the pH to the pI of the native proteins and where the enzyme is inactive, or the reaction mixture is heated to temperatures >70°C, which inactivates the enzyme and coagulates undigested proteins. Usually, the

use of pI is preferable because it helps to increase clarity of the supernatant (contains the desirable peptides) after centrifugation, but there is the potential for further proteolysis if the protease possesses some residual activity at the pI of the protein substrate. Therefore, the desired degree of hydrolysis (DH) may not be maintained. However, high temperature inactivation ensures that protease activity is permanently stopped; this maintains the desired DH even if the pH of the hydrolysate during post-processing operations is in the optimum range for protease activity. As shown in Figure 1, the protein hydrolysate obtained can then be tested for various bioactive properties or subjected to further separation, fractionation, and peptide purification. The following sections describe specific targets for some pulse seed-derived bioactive peptides and provide detailed descriptions of the production and evaluation protocols.

Angiotensin-Converting Enzyme Inhibition

Angiotensin-converting enzyme (ACE) inhibition assay methods.—ACE (EC 3.4.15.1) is an enzyme that modulates the renin-angiotensin system (RAS) and therefore regulates blood pressure. Excessive levels of ACE have been implicated in the development and progression of hypertension. Clinical management of hypertension utilizes ACE-inhibitory drugs, but the use of natural food protein-derived peptides is seen as a natural and effective alternative to drugs. A few methods have been used to determine ACE-inhibitory activities of protein hydrolysates and purified peptides. The most widely used in vitro method involves measuring the ACE-mediated liberation of hippuric acid (HA) from hippuryl-histidinyl-leucine (HHL) as shown in Equation 1, which was originally developed by Cushman and Cheung (12):

$$\text{HHL} \xrightarrow{\text{ACE}} \text{HA} + \text{HL} \qquad (1)$$

In the presence of inhibitors such as protein hydrolysates, ACE activity is reduced and the amount of HA liberated will be less than in the absence of inhibitors. HA can be quantified in several ways: First, by extraction into ethyl acetate followed by evaporation of the solvent; the residue (HA) is dissolved in distilled water and absorbance intensity at 228 nm is measured (13). The spectrophotometric method has been used to determine the ACE-inhibitory properties of a pea protein hydrolysate (14) and a mung bean protein hydrolysate (11). Second, the HA can be separated by high-performance liquid chromatographic (HPLC) methods from the other reaction mixture ingredients and concentration determined from absorbance at 228 nm after calibration of the column with pure HA (13). Third, HA has been quantified by measuring the colorimetric adduct formed in the presence of quinoline at 492 nm, which was used to determine ACE-inhibitory activities of mung bean protein hydrolysates and purified peptides (15, 16). Fourth, in a separate report, the ACE-inhibitory properties of chickpea protein hydrolysates were determined by measuring absorbance at 382 nm of the complex formed between 2,4,6-trichloro-s-triazine and HA (10, 17). Apart from HHL, furanacryloyl-Phe-Gly-Gly (FAPGG) is another substrate that has been used to determine ACE activity in the presence of pea bioactive peptides (18, 19). ACE cleaves FAPGG to yield FAP and GG, and is then accompanied by a blue shift in the absorption spectrum; when the assay is performed at 340 nm, ACE activity is monitored as a decrease in absorbance intensity.

A typical drawback of the spectrophotometric measurement of HA concentration through ethyl acetate extraction is that both HA and HHL have intense absorption at 228 nm. Therefore, during extraction of the reaction mixture with ethyl acetate, some unreacted HHL may be solubilized along with HA, which can then lead to overestimation of the reaction rate. In our previous work, HPLC analysis of the ethyl acetate extract contained a residual peak identified as HHL (13). Therefore, direct quantification of HA through HPLC analysis seems to be faster, more accurate, and highly sensitive than the traditional ethyl acetate extraction and spectrophotometric measurement method. HPLC analysis is also more accurate for performing enzyme inhibition kinetics than the spectrophotometric estimation of HA. The uses of quinoline and FAPGG have not been compared to the HPLC method but it is reasonable to assume that the latter will provide a more sensitive, accurate, and reproducible estimation of ACE inhibition than either of the spectrophotometric methods. However, although it is faster and more accurate, the HPLC method requires expensive hardware that may not be readily available in some laboratories. On the other hand, virtually every laboratory has a spectrophotometer, and non-HPLC methods may be the only methods that can still be used to obtain a meaningful and publishable set of data.

Mung bean protein hydrolysates.—Preparation of a mung bean protein hydrolysate involved treatment of a 4% (w/v) suspension of the protein isolate with alcalase (EC 3.4.21.62) and neutrase (EC 3.4.24.28) at pH 8.0 (55°C) and 7.0 (45°C), respectively, for 10 h (14). Samples were withdrawn at certain time intervals, heated in a water bath (to inactivate protease), and centrifuged, and the supernatant was lyophilized for testing. Results from these time interval samples will provide an idea of the effect of rate of protein degradation by the proteases on the bioactive properties of hydrolysates. In order to prepare protein hydrolysate samples for in vitro and in vivo testing, the mung bean protein isolate was hydrolyzed for 2 h with alcalase or neutrase. Mung bean protein hydrolysate was centrifuged, the supernatant was then passed through a 6 kilodalton (kDa) molecular weight cut-off (MWCO) ultrafiltration membrane, and the permeate was collected and freeze-dried (20). The use of an ultrafiltration membrane determines the molecular size of the peptides that will be tested for bioactivity. In the case of the mung bean hydrolysate, most of the peptides tested will be <6 kDa in size. A major rationale for the fractionation of protein hydrolysates by size is that smaller-sized peptides are believed to be more bioactive than the large-sized peptides. Small-sized peptides have a better chance than large-sized peptides of escaping digestion in the gastrointestinal tract (GIT) and can be readily

absorbed intact into the circulatory system where they are transported to target organs (21). In order to estimate the potential of the protein hydrolysate peptides to escape structural degradation and loss of ACE-inhibitory activity in the GIT, a simulated GIT digestion was performed. The mung bean protein hydrolysate permeate was treated at 37°C consecutively with pepsin (EC 3.4.23.1) at pH 2.0 for 3 h and pancreatin at pH 8.0 for 4 h to simulate digestion in the stomach and small intestine, respectively. The reaction was terminated by placing the mixture in boiling water for 5 min; the mixture was then centrifuged, and ACE-inhibitory activity of the supernatant was compared to original values obtained without simulated GIT digestion. This type of test is useful to determine the potential loss of activity in the digestive tract if protein hydrolysate is ingested. A substantial loss in ACE-inhibitory activity after simulated GIT digestion will most likely stop further in vivo evaluation of the sample, which prevents unnecessary use of animals.

Mung bean protein hydrolysate permeate obtained from a 6 kDa MWCO membrane was fractionated on a Sephadex G-15 column that had been equilibrated with acetate buffer, pH 4.0 (16). Peptides in the pooled active fractions that inhibited ACE activity were purified by 2 consecutive rounds of reversed-phase HPLC using a Sephasil peptide C_{18} column (12 μm) in the first round and a similar column (but 5 μm particle size) for the second round. Elution of peptides from the HPLC column used 2 solvent systems with detection at 220 nm: (A) 0.1% trifluoroacetic acid (TFA) in water, and (B) 60% aqueous acetonitrile that contained 0.1% TFA. To determine amino acid composition, the purified mung bean peptides were hydrolyzed for 24 h using 6 M HCl at 110°C under vacuum. Amino acid composition of the hydrolyzed peptides was determined by precolumn derivatization with *O*-phthalaldehyde using an automatic amino acid analyzer. Amino acid sequence of the purified peptides was determined by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry after the peptides were dissolved in water that contained 0.1% TFA. Results identified the 3 most active peptides present in the mung bean protein hydrolysate as KDYRL, VTPALR, and KLPAGTLF with ACE-inhibitory concentration (IC_{50}) values of 26.5, 82.4, and 13.4 μM, respectively (15).

For the in vivo test, male spontaneously hypertensive rats (SHR, 12 weeks old) with a systolic blood pressure (SBP) >180 mm Hg were orally fed a solution of the 6 kDa ultrafiltration permeate of mung bean protein hydrolysate at a dose of 600 mg/kg of body weight (20). A control equivalent was similarly fed distilled water only. SBPs of the rats were then measured using a tail pulse pickup method after warming the rats to 37–39°C for 10 min to improve blood circulation to the tail blood vessels. SHRs have been traditionally bred to develop hypertension starting from the age of 10 weeks because of high level of activity of the RAS, which leads to high levels of ACE activity and hence excessive amounts of angiotensin II (a potent vasoconstrictor) in the blood vessels. Males exhibit average SBP >200 mm Hg by 3–4 months of age, which mimics the level of blood pressure in hypertensive humans. Peptides present in the protein hydrolysate are negative modulators of RAS, and the rats provide a perfect model to demonstrate blood pressure-lowering effects. The SHR feeding experiment showed that the 2 h alcalase mung bean protein hydrolysate caused a gradual reduction in SBP with a maximum value of 30 mm Hg after 6 h. The SBP returned to the initial value 12 h after oral administration of mung bean protein hydrolysate. Such a long-lasting effect is suggestive of high resistance of the peptides to structural degradation by ACE and other proteolytic factors in tissues and blood. Therefore, the mung bean protein hydrolysate could be an effective blood pressure reduction agent in human beings, depending on results from clinical trials. Even though there was a reduction in SBP, there was no change in heart rate, which suggests that the effects were primarily due to reduction in the contractile properties (reduced ACE activity) of blood vessels. However, the protein hydrolysate could have contained small quantities of antihypertensive low MW polyphenols or other phytochemicals. Therefore, purification of the active peptides is desirable but the in vivo activities of the purified mung bean peptides have not been reported.

Chickpea protein hydrolysates.—Enzymatic hydrolysis of a chickpea pI isolate was performed by batchwise method using 2 proteases, alcalase and flavourzyme, either individually (for 3 h) or by sequential addition (alcalase for 3 h followed by flavourzyme for 5 h) at optimum pH, enzyme/substrate ratio, and temperature (8; Figure 2). At the end of protein hydrolysis, enzyme reaction was stopped by heating the reaction mixture at 85°C for 10 min. The DH was determined using the trinitrobenzene sulfonic acid method as described by Adler-Nissen (22). Peptides in an ACE-inhibitory chickpea protein hydrolysate were fractionated into purified peptides by consecutive gel filtration and HPLC protocols (10). The first fractionation used a Superose 12 HR 10/30 column; the second used a Biogel P2 column. An active fraction was identified, isolated, and further separated on a C_{18} HPLC column from which 4 ACE-inhibitory peptides were recovered. Two of the peptides inhibited ACE in a competitive manner with IC_{50} values of 0.108 and 0.117 mg/mL. The other 2 peptides behaved as uncompetitive inhibitors of ACE with IC_{50} values of 0.103 and 0.105 mg/mL. There was no indication of significant differences between these IC_{50} values (10). Purification of peptides eliminates potential contaminating compounds such as polyphenols and enables determination of amino acid sequence. Knowledge of the amino acid sequences facilitates studies involving determination of structure–function relationships, which could lead to the identification of peptides with greater inhibitory capacity. Synthesis of active peptides and development of peptidomimetics are also facilitated by knowledge of amino acid sequences of known ACE-inhibitory peptides.

Results of the chickpea and mung bean protein hydrolysates showed that alcalase was more effective than flavourzyme or neutrase in generating ACE-inhibitory peptides. For the chickpea protein hydrolysate, the highest ACE inhibition (about 38%) was achieved after 30 min

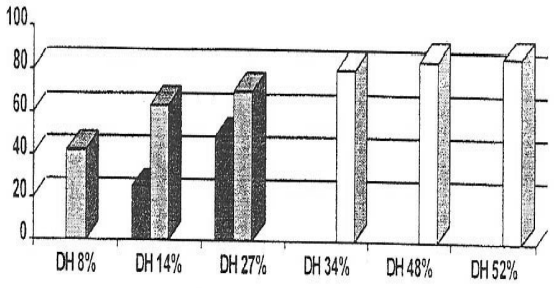

Figure 2. Hypoantigenic activity of chickpea protein hydrolysates with respect to protein isolates. Hydrolysates: solid box, flavourzyme; shaded box, alcalase; open box, alcalase + flavourzyme sequentially. From Clemente et al. (8). Reproduced with copyright permission of the American Chemical Society.

hydrolysis with alcalase (about 30% DH). In contrast, about 80% inhibition of ACE activity was achieved after 2 h hydrolysis of mung bean protein isolate with alcalase, whereas the value for neutrase hydrolysate was only 15%. The DH of the alcalase reaction was more than twice that of neutrase, which confirms a well-known fact that smaller-sized peptides in comparison to bigger peptides are better inhibitors of ACE; peptide chain length is inversely proportional to DH. However, there seem to be an optimum peptide size, and further reduction could lead to reduced ACE inhibition. For example, the maximum ACE-inhibitory activity of the mung bean protein hydrolysate was achieved after 2 h of alcalase hydrolysis (about 16% DH), whereas that of chickpea was achieved after 30 min. Beyond 2 h (>16% DH), ACE inhibition by the mung bean protein hydrolysate gradually decreased until it reached 50% at 21% DH. ACE inhibition by the chickpea protein hydrolysate also decreased after 30 min even though the DH increased. From these results, we can deduce that when hydrolysis of the mung bean and chickpea proteins was extended beyond 2 h and 30 min, respectively, the structural degradation of the ACE-inhibitory peptides occurred with concomitant reduction in ability to block ACE activity.

Field pea protein hydrolysates.—A pea protein isolate was subjected to in vitro GIT digestion first by treatment with pepsin at pH 2.0 and 37°C (23). After 2 h of digestion with pepsin, the reaction mixture was adjusted to pH 6.5, followed by addition of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), and incubated at 37°C for 2.5 h. Enzyme reaction was stopped by adjusting the reaction mixture to pH 5.0 during which undigested proteins were precipitated and soluble peptide products remained in solution. After centrifugation, the supernatant containing the peptides was saved and freeze-dried. Freeze-dried pea protein hydrolysate obtained from in vitro GIT digestion of pea protein isolate was redissolved in water, passed through a 3 kDa MWCO membrane, and the permeate and retentate were freeze-dried (23). The 3 kDa ultrafiltration membrane permeate was fractionated by a preparative reversed-phase HPLC using a C_{18} column with a linear gradient of water containing 0.1% TFA (solvent A) and acetonitrile containing 0.085% TFA (solvent B; 23). The hydrolysate permeate and HPLC fractions were further subjected to another in vitro GIT digestion and then tested again for ACE-inhibitory activity.

Results showed that the in vitro GIT digestion produced pea protein hydrolysate with an IC_{50} value of 0.07 mg/mL against ACE (23). After ultrafiltration, the 3 kDa had a slightly higher ACE-inhibitory activity with a reduced IC_{50} value of 0.055 mg/mL (Table 1). Fractionation of the 3 kDa permeate by reversed-phase HPLC further reduced the IC_{50} value to a maximum of 0.016 mg/mL, indicating an enrichment in ACE-inhibitory peptides as a result of removal of inactive peptides by the column (Table 1). The combination of ultrafiltration and HPLC produced ACE-inhibitory low MW peptide fractions that also had more hydrophobic characters than the inactive fractions. It was concluded that the hydrolysate permeate and HPLC fractions will be resistant to digestion in the GIT and the products may be used to formulate functional foods that play significant roles in the prevention and treatment of hypertension (23).

In a recent report, commercial pea protein isolate was individually hydrolyzed for 4 h with 5 different enzymes that included alcalase, flavourzyme, papain (EC 3.4.22.2), trypsin, and chymotrypsin at the optimum pH and temperature of each enzyme (14). Results showed that papain produced high MW peptides (10–178 kDa), whereas the other 4 enzymes produced peptides of <25 kDa. ACE-inhibitory activity of the papain hydrolysate was the highest and that of the flavourzyme hydrolysate was the lowest. The authors suggested that the low ACE-inhibitory activity of the flavourzyme hydrolysate may be due to high levels of free amino acids produced by the exo-peptidase component of the enzyme; free amino acids are less effective inhibitors of ACE than peptides. However, the flavourzyme hydrolysate was the most effective free radical scavenger as determined from the quenching of absorbance of 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Calmodulin-Binding Peptides

Calmodulin (CaM) is an important calcium-binding protein that serves as an activator of several metabolic enzymes involved in cellular hemostasis and the well being of humans (24). Typical enzymes that require CaM for activity include phosphodiesterase I (EC 3.1.4.17), protein kinase II (EC 2.7.11.17), and the nitric oxide synthases (EC 1.14.13.39). Typical processes that involve CaM-dependent metabolic enzymes include cell division, neurotransmission, and cell proliferation (25). Therefore, excessive activities of CaM-dependent metabolic enzymes are believed to be involved in the pathogenesis of various diseases. Because these enzymes require CaM for activation, a therapeutic approach is to use compounds that bind to CaM and thereby prevent CaM from binding effectively to the target enzymes. CaM exists as a negatively charged protein at physiological pH, which enhances its ability to bind calcium and become

Table 1. IC_{50}[a] values of pea protein hydrolysates against angiotensin converting enzyme activity[b]

Protein sample	IC_{50}, mg/mL
Native protein	14.130
Pepsin digest	0.080
Pepsin + trypsin + α-chymotrypsin	0.069
3 kDa MWCO[c] permeate	0.055
3 kDa MWCO retentate	0.087
HPLC fraction I	0.120
HPLC fraction II	0.040
HPLC fraction III	0.023
HPLC fraction IV	0.016
HPLC fraction V	0.058

[a] IC_{50} = Inhibitory concentration that reduces enzyme activity by 50%.
[b] Adapted from Vermeirssen et al. (ref. 23).
[c] MWCO = Molecular weight cut-off ultrafiltration membrane.

active towards target enzymes. Thus, for peptides to act as effective CaM inhibitors, they should have several basic groups and a net positive charge at physiological pH to allow for maximum protein-peptide interactions (26).

A commercial field pea protein isolate (5%, w/v, in water) was hydrolyzed with alcalase for 6 h at 50°C and pH 9.0 (24). Reaction was terminated by adjusting the slurry to pH 4.0, followed by centrifugation to obtain a peptide-rich supernatant, which was then passed through a 1 kDa MWCO membrane at room temperature using an Amicon ultrafiltration setup. The permeate, which contains low MW peptides was freeze-dried and fractionated on a cation-exchange column (SP-Sepharose) using a Fast Protein Liquid Chromatography System (FPLC). The freeze-dried sample was dissolved in 0.1 M ammonium acetate buffer (pH 7.5), filtered through a 0.2 μm membrane, and loaded onto the column. After washing off unbound peptides with 3 column volumes of buffer, the bound peptides were eluted using a linear gradient between 0 and 0.5 M ammonium carbonate in 0.1 M ammonium acetate buffer; peaks were detected at 280 nm.

Freeze-dried FPLC pea peptide fractions were evaluated as CaM inhibitors against protein kinase using a reaction system that measures the rate of phosphorylation of hydrolyzed and dephosphorylated casein. Results showed that the FPLC fractions (I and II) were able to inhibit CaM-dependent protein kinase II (CaMKII) in a dose-dependent manner (24). Fraction II, which had greater contents of lysine and arginine (15 and 23%, respectively), inhibited CaM-dependent protein kinase II to a greater extent than fraction I, which had lower concentrations (3 and 4%, respectively). At lower CaM concentrations (5–25 μg/mL), the IC_{50} values of fraction II were 10 times less than those obtained for fraction I. Even more significant is that at a high CaM concentration of 35 μg/mL, the IC_{50} values of fraction II were 50 times less than those obtained for fraction I. Similarly, the Km values showed that the CaMKII reaction was several times slower in the presence of fraction II (Km = 25 mM at 1 mg/mL peptide concentration) than in the presence of fraction I (Km = 0.4 mM at 1.5 mg/mL peptide concentration). The results provide further evidence that reductions in CaM-dependent enzyme reactions are more effective when the peptide inhibitor has more positively charged amino acid residues. Furthermore, the inhibition constant (Ki) was calculated to provide estimates of binding affinity between the peptides and CaM. The Ki of fraction II was 20 times less than that of fraction I, which indicates greater binding affinity between fraction II and CaM when compared to binding between fraction I and CaM. Results confirm that positively charged peptides that modulate the function of CaM and the dependent enzymes can be produced through enzymatic proteolysis of pea seed proteins.

In order to better understand some of the mechanisms involved in the inhibitory activities of cationic pea peptides, the structure of CaM and CaMKII were studied using fluorescence and circular dichroism methods (27). Using intrinsic fluorescence, it was shown that addition of pea protein-derived peptides led to dose-dependent increases in protein (CaM and CaMKII) maximum emission (Fmax) but without any substantial change in the wavelength (λmax; Table 2). The results indicate that binding of the peptides led to partial unfolding of the CaM molecule to expose hydrophobic groups. Fraction II with almost 4 times the basic amino acid content was more effective than fraction I in unfolding CaM structure, which correlates with results obtained from the enzyme inhibition reaction assay. Similarly, Fmax of the protein kinase II was increased in the presence of peptides in a dose-dependent manner, and fraction II was more effective than fraction I (Table 2). The authors concluded that the mode action of the inhibitory pea protein-derived peptides involves partial unfolding of CaM molecule, which then leads to intense interactions between CaM and the enzyme (27). Ultimately, increased CaM-enzyme interactions led to unfolding of the enzyme from its optimum structural conformation and hence loss of some catalytic activity. The greater the affinity of the peptides for CaM, the more damaging to catalytic efficiency of CaMKII as a result of unfavorable consequences to the enzyme's structural conformation. Overall, this preliminary work showed the potential for the production of bioactive pea protein-derived peptides that may be used to formulate functional food and nutraceuticals against diseases caused by excessive activities of CaM-dependent metabolic enzymes. Although the pea protein-derived peptides were partially purified, there is the likelihood of contributions from low MW phytochemicals, especially polyphenols, to the observed inhibition of CaM-dependent enzyme reactions.

Copper Chelating and Antioxidant Peptides

As a trace element in human nutrition, copper is required for various vital functions including activation of metabolic enzymes and amino acid transport (28). However, copper is

Table 2. Structural changes in calmodulin (CaM) and CaM-dependent protein kinase II (CaMKII) as a consequence of binding with inhibitory pea protein-derived cationic protein hydrolysate fractions[a]

	Fmax/Fo[b]		λmax, nm[c]	
Treatment	CaM	CaMKII	CaM	CaMKII
Calcium	2.23	1.89	301	325
Fraction I[d] plus				
Calcium + 10 μg	2.33	2.52	301	325
Calcium + 20 μg	3.00	2.68	301	325
Calcium + 40 μg	3.72	3.22	301	325
Calcium + 80 μg	4.71	5.71	301	330
Fraction II[e] plus				
Calcium + 5 μg	2.76	2.56	300	325
Calcium + 10 μg	2.90	2.71	301	325
Calcium + 20 μg	3.56	2.79	301	325
Calcium + 40 μg	5.38	7.14	301	330

[a] Adapted from Li and Aluko (ref. 27). Fractions were obtained after eluting bound pea protein hydrolysate peptides from an SP-Sepharose column.

[b] Fmax = Maximum fluorescence intensity (arbitrary units); Fo = fluorescence intensity of CaM or enzyme alone.

[c] λmax = Wavelength of maximum fluorescence intensity.

[d] Less cationic fraction with approximately 10% content of basic amino acids.

[e] More cationic fraction with approximately 43% content of basic amino acids.

also a powerful pro-oxidant that can cause oxidation of low-density lipoproteins (LDL) to release peroxides and damaging free radicals. The products of LDL oxidation are believed to promote cardiovascular diseases by enhancing formation of atherogenic plaques and transformation of macrophages into foam cells as well as development of vasoconstrictor and prothrombotic properties (29). A potential therapeutic approach to minimizing copper-induced cardiovascular damage is the use of copper binding agents such as peptides that are derived from the enzymatic hydrolysis of food proteins.

Chickpea protein isolate was hydrolyzed consecutively with alcalase and flavourzyme followed by enzyme inactivation by heating at 80°C for 20 min. After clarification using a 0.45 μm filter, the hydrolysate was freeze-dried and its antioxidant activity determined by measuring the inhibition of β-carotene oxidation (29). Hydrolysates with the highest antioxidant activities were dissolved in 0.1 M phosphate buffer, pH 7.0, and loaded onto a Metal Chelate Maxi (MCM) spin column that had been charged with copper. The column was washed with several volumes of phosphate buffer to remove unbound peptides. Bound peptides were then eluted from the column by increasing their protonation, and hence dissociation from copper, using phosphate buffer, pH 3.5. Eluted peptides from the MCM column were further purified by HPLC using a reversed-phase C_{18} column. HPLC elution was performed with linear gradient of acetonitrile in water containing 0.1% TFA at 30°C and a flow rate of 4 mL/min.

Results showed that hydrolysis of chickpea proteins for 10 and 100 min produced hydrolysates with the highest antioxidant ability to decrease oxidation of β-carotene (29). The 10 min hydrolysate had 1.2 times the antioxidant activity of the 100 min hydrolysate. Affinity chromatography of the 10 and 100 min hydrolysates on the MCM column led to isolation of peptides that have significantly higher antioxidant properties than the parent hydrolysates. For example, the MCM column eluates of the 10 min hydrolysate had 8.3 times the antioxidant activity of the parent protein hydrolysate. Amino acid composition analysis showed that histidine and arginine were the 2 amino acids that increased substantially in the 10 and 100 min protein hydrolysates. Correlation plot showed a positive relationship between histidine content and antioxidant activity in the β-carotene model, which confirms this amino acid as the major determinant of peptide activity. The purified peptides were more active as antioxidants than free histidine, which indicates that other structural components (apart from the most active amino acid residue) of peptides contribute to activity and provides additional evidence of the superior bioactive properties of peptides when compared to free amino acids. Therefore, knowledge of the amino acid sequence of the copper-chelating antioxidant peptides should provide further information on the structure-function properties of these bioactive peptides. An interesting finding was that the protein hydrolysate was more effective than the purified peptides as an antioxidant when copper was not included in the reaction mixture, which confirms that the observed property of the peptides is due to their copper-chelating ability (29).

Since the recognition of the potential damaging effects of oxidative stress to human health, scientists have investigated the production and properties of antioxidant peptides from food proteins. Lipid peroxidation and production of free radicals are believed to contribute to the development of some chronic human diseases; therefore, food protein-derived antioxidant and free radical scavenging peptides may serve protective functions in the body (30). An aqueous suspension of chickpea protein isolate was hydrolyzed with alcalase at pH 8.0 and 50°C for 40 min (31). After enzyme inactivation at 95°C for 10 min, the reaction mixture was cooled, centrifuged, freeze-dried, and fractionated on a Sephadex G-25 gel filtration column using a flow rate of 20 mL/h; eluted fractions were detected at 280 nm. Reducing power of the fractionated peptides (fractions I–IV) was determined by mixing the peptides solutions with 1% potassium ferricyanide followed by incubation at 50°C for 20 min. After addition of 10% (w/v) trichloroacetic acid, the mixture was centrifuged and the upper layer was mixed with 0.1% (w/v) ferric chloride; absorbance was read at 700 nm, and the intensity is proportional to the reducing power of the peptides (31). This method is based on the principle that peptides with reducing power (antioxidants) will cause reduction of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) measured as a Perl's

Prussian blue at 700 nm (32). Fraction IV was the most hydrophobic and contained the smallest peptides, which may have been responsible for its better reducing power when compared to fractions I–III.

Inhibition of lipid peroxidation was measured as the ability of the chickpea peptide fractions (I–IV) to inhibit linoleic acid autoxidation. The phosphate-buffered peptide solutions were mixed with an ethanolic solution of linoleic acid, sealed tightly, and kept at 60°C in the dark for 8 days (32). An aliquot of the incubated reaction mixture was mixed with 75% ethanol, 30% (w/v) ammonium thiocyanate, and a solution of 0.02 M acidified (with 1 M HCl) ferrous chloride. The color formed was measured at 500 nm and is directly proportional to degree of linoleic acid oxidation. Fraction IV produced the lowest absorbance and was the most active in preventing oxidation of linoleic acid.

Free radical scavenging ability of the chickpea protein hydrolysate fractions was determined using 3 types of radicals: DPPH, hydroxyl, and superoxide. For DPPH, the peptide solutions were mixed with an ethanolic solution of DPPH and left to stand at room temperature for 30 min, followed by absorbance measurement at 517 nm (32). Peptides with high DPPH scavenging activity will produce lower absorbance values because the DPPH radical has an intense absorption at 517 nm. The inhibition of pyrogallol autoxidation was used to measure the superoxide radical scavenging activity of the peptide fractions. A solution of peptide fractions in Tris-HCl-EDTA buffer, pH 8.0, was mixed with pyrogallol solution, and absorbance at 320 nm was measured. Hydroxyl radical scavenging activity (HRSA) was determined by mixing a solution containing hydrogen peroxide (H_2O_2) and peptide fraction with a solution containing 1,10-phenanthroline and $FeSO_4$; results were calculated using Equation 2.

$$\text{HRSA, \%} = [(\text{As} - \text{A1})/(\text{Ao} - \text{A1})] \times 100 \quad (2)$$

where As is sample absorbance, A1 is control (solution of 1,10-phenanthroline, H_2O_2, and $FeSO_4$) absorbance, and Ao is blank solution (1,10-phenanthroline and $FeSO_4$) absorbance (32).

Similar to the results of the reducing power experiment, fraction IV was the most active as a free radical scavenging agent in the DPPH, superoxide radical, and hydroxyl radical experiments (32). The fact that the 5 analytical methods yielded similar results in terms of reducing and free radical scavenging power indicates that any of the methods can be used to accurately determine the antioxidant properties of bioactive peptides. All of the 5 methods are straightforward, although the DPPH seems to involve the least amount of time and uses a minimal number of reagents. However, the linoleic acid autoxidation better reflects the potential situation in the human body and may be a more meaningful type of measurement for estimating the physiological activity of antioxidant peptides. Phytochemical contaminants in the protein hydrolysates may have contributed to the observed antioxidant properties because some polyphenols are also powerful proton donors.

Hypoimmunoreactive Peptides

Antigenic activities of a chickpea protein hydrolysate were determined as a means of verifying its hypoimmunoreactive properties. This type of protein hydrolysate could be used in the formulation of nutritional foods for people that have allergic reactions to chickpea proteins, because allergic reactions are believed to be caused by the presence of epitopes (a linear sequence of certain amino acids) in proteins that bind to antibodies. During random in vitro protein hydrolysis, it is possible that the epitopes will be broken into several shorter sequences that have weak or no reactivity against antibodies. Therefore, protein hydrolysates are believed to contain peptides with fewer antigenic properties than intact native proteins.

Enzymatic chickpea protein hydrolysate was produced with alcalase and flavourzyme as described above (8) and their antigenic activities were determined by comparing the sera from 6 allergic patients with that from healthy nonallergenic people. First, electrophoresis was used to separate proteins in the native intact chickpea proteins followed by immunoblotting onto 45 mm nitrocellulose membranes (8). Second, following several steps of blocking and washing, the blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 1 M borate buffer at room temperature for 10 min. A final wash of the nitrocellulose membranes with distilled water for 15 min ended the procedure (8). The developed blot showed that the basic polypeptide chains were the most strongly bound to immunoglobulin E (IgE), thus identifying the proteins of about 25 kDa as mostly responsible for the antigenic properties of chickpea (8). However, the acidic polypeptides (39–46 kDa) were also recognized by IgE but not as strong as the reaction between IgE and the basic polypeptides. The 2 types of chickpea proteins that reacted in some way with IgE belonged to glycinin (11S globulins). There was no detectable reaction between the β-conglycinin (7S) chickpea globulins (60–65 kDa) and IgE. This type of information suggests that the 11S chickpea globulin should be a target during enzymatic hydrolysis to produce hypoimmunoreactive protein hydrolysates.

In order to quantify the hypoimmunoreactive properties of the chickpea protein hydrolysate in comparison to the protein isolate, an antibody-capture assay format was used (8). A serial dilution (10–1000 mg/mL) of the protein isolate was prepared in phosphate-buffered saline (PBS), pH 7.2, and the protein hydrolysate was prepared as a 200 mg/mL solution; both samples were then incubated at 4°C for 12 h. After incubation, samples were washed 3 times (15 min each) with PBS buffer that contained 0.05% Tween 20 (TPBS). This was followed by blocking with 0.7% bovine serum albumin in PBS, and washing twice with TPBS. A solution of patient sera sensitized against chickpea proteins was then added. After a series of washings and treatment with alkaline phosphatase-conjugate mouse antihuman IgE, a solution of

p-nitrophenyl phosphate in 10.5% diethanolamine and 0.02% $MgCl_2$, pH 9.8, was added to the samples as well as to the control (nonallergenic patient serum) and positive control (allergic patient serum). The intensity of the color was developed at room temperature and measured at 405 nm using a microplate spectrophotometer. Hypoimmunoreactivity was estimated as a percentage loss of immunoreactive activity, which was calculated using equation 3 (8):

$$(1 - A_{CPH}/A_{CPI}) \times 100 \quad (3)$$

where A_{CPH} and A_{CPI} represent absorbance of the chickpea protein hydrolysate and chickpea protein isolate, respectively. The results showed that hypoimmunoreactivity was directly proportional to the DH of the hydrolysates. For example, hypoimmunoreactivity of the chickpea protein hydrolysate with 8% DH was 40% when compared to 60% for the 14% DH, and almost 80–90% for DH values from 34 to 52%. Alcalase was more effective in producing hypoimmunoreactive chickpea peptides when compared to flavourzyme (8). This is probably due to the fact that alcalase was effective in breaking down the chickpea proteins, especially the reactive 11S polypeptides, as evident from the lack of visible polypeptide bands after electrophoresis of the alcalase hydrolysate. In contrast, the 11S proteins were resistant to flavourzyme digestion and were still detectable after gel electrophoresis of the flavourzyme hydrolysates (8). It is not surprising, therefore, that sequential hydrolysis of chickpea protein isolate with alcalase and flavourzyme produced hydrolysates with the most hypoimmunoreactive properties, which was attributed to the more effective destruction of flavourzyme-resistant epitopes by alcalase. A different report (33) showed that bromelain (EC 3.4.22.32 or 3.4.22.33) was more effective than chymotrypsin in hydrolyzing chickpea proteins but the hypo immunoreactivity of chymotrypsin hydrolysate was higher than that of bromelain hydrolysate. The result could be attributed to greater epitope hydrolysis by chymotrypsin even though its overall protein hydrolysis was less than that of bromelain.

Conclusions

Different pulse proteins contain several bioactive peptide sequences that may be used as therapeutic tools in the prevention and treatment of human diseases. Pulse seed proteins are less expensive and therefore offer a valuable alternative to the more expensive animal proteins for the production of food protein-derived bioactive peptides. Unlike chemical hydrolysis, enzymatic hydrolysis offers a unique tool in liberating these peptide sequences hidden within the primary structures of pulse seed proteins. Recent developments in enzyme technology have greatly improved our ability to select specific enzymes that will yield desired types of peptides; alcalase seems to be the most effective protease in converting the pulse seed proteins into potentially bioactive peptides. Peptides that possess multifunctional properties need to be further explored for therapeutic uses. For example, protein hydrolysate fractions that are rich in arginine or histidine have been shown to have in vitro activities as inhibitors of CaM-dependent enzymes (24) and to function as copper-chelating agents (29). Further work is required to confirm the in vivo activities of such multifunctional peptides. However, one of the limiting factors in the utilization of food proteins as sources of therapeutic peptides is the low potency of the initial hydrolysate fractions when compared to currently available drugs. Even though processing methods such as ultrafiltration and column chromatography can be used to enrich the protein hydrolysates into very potent fractions, the economic viability of such processes is doubtful. Therefore, efforts must continue in developing more efficient hydrolytic and cheaper separation or purification methods that are compatible with industrial production practices and have commercial viability. However, it is important that even though food protein hydrolysates are less potent, they can be consumed in much larger quantities that will provide physiological benefits without the toxic side effects of drugs. It is also important that the presence of low MW phytochemicals such as polyphenols that are present in pulse seed coats may contribute to the observed properties of unpurified protein hydrolysates and their fractions.

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