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In silico and *in vitro* analyses of the angiotensin-I converting enzyme inhibitory activity of hydrolysates generated from crude barley (*Hordeum vulgare*) protein concentrates

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ABSTRACT

Angiotensin-I-converting enzyme (ACE-I) plays a key role in control of hypertension, and type-2 diabetes mellitus, which frequently co-exist. Our current work utilised *in silico* methodologies and peptide databases as tools for predicting release of ACE-I inhibitory peptides from barley proteins. Papain was the enzyme of choice, based on *in silico* analysis, for experimental hydrolysis of barley protein concentrate, which was performed at the enzyme's optimum conditions (60 °C, pH 6.0) for 24 h. The generated hydrolysate was subjected to molecular weight cut-off (MWCO) filtration, following which the non-ultrafiltered hydrolysate (NUFH), and the generated 3 kDa and 10 kDa MWCO filtrates were assessed for their *in vitro* ACE-I inhibitory activities. The 3 kDa filtrate (1 mg/ml), that demonstrated highest ACE-I inhibitory activity of 70.37%, was characterised in terms of its peptidic composition using mass spectrometry and 1882 peptides derived from 61 barley proteins were identified, amongst which 15 peptides were selected for chemical synthesis based on their predicted ACE-I inhibitory properties. Of the synthesized peptides, FQLPKF and GFPTLKIF were most potent, demonstrating ACE-I IC_{50} values of 28.2 μM and 41.2 μM respectively.

1. Introduction

Barley (*Hordeum vulgare*) is a cereal crop widely used as animal feed and for the purpose of malting and brewing (Sullivan, Arendt, & Gallagher, 2013). Approximately 2% of barley produced worldwide is used for food directly. Apart from being a source of the cholesterol reducing dietary fibre β-glucan, barley is also a source of relatively nutritious proteins (Cavazos & Gonzalez de Mejia, 2013). In fact protein is the second most abundant component in barley grain after starch (Sullivan et al., 2013). Barley grain typically consists of 10–17% protein, though this range decreases when the grain is de-hulled (Quinde-Axtell, Ullrich, & Baik, 2005). Despite the high content of protein in barley, there is limited recognition of the nutritional and bio-functional properties of barley proteins.

In the last five decades, there has been growing interest in the use of food-derived bioactive peptides as agents in the control of chronic diseases (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011; Udenigwe & Aluko, 2012), to reduce the risk of side effects arising from synthetic drugs usage. Bioactive peptides are latent or encrypted in the primary structure of the protein, which become active after their release by enzyme catalysed hydrolysis, fermentation, food processing or by proteolytic cleavage by proteolytic enzymes during gastrointestinal transit and digestion (Gobbetti, Minervini, & Rizzello, 2004). Hypertension, the main risk factor for cardiovascular diseases accounts for 45% and 51% of heart disease and stroke related deaths worldwide respectively, causes more than 9 million deaths annually (World Health Organization, 2013). Inhibition of enzymes involved in various stages of the renin angiotensin-aldosterone system (RAAS) can play a key role in combating hypertension and other related diseases such as type-2 diabetes mellitus. Two enzymes found within the RAAS that have been well-studied to date include angiotensin-I converting enzyme (ACE-I, EC 3.4.23.15) and renin. A number of

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synthetic drugs that inhibit ACE-I and renin such as Captopril® and Aliskiren® are currently used to lower blood pressure. Natural ACE-I inhibitory peptides from various sources including fish, meat, soy, and milk have been studied and their antihypertensive effect has been reported in hypertensive animal models and human subjects (Muir, 2005). However, few studies to date have focussed on the potential of barley as a source of ACE-I inhibitory peptides.

Enzymatic hydrolysis using one or more proteases is an effective way of releasing bioactive peptides from their protein sources (Korhonen & Pihlanto, 2006). Screening for ACE-I inhibitors from novel substrates using conventional methods is an expensive and time consuming process. It involves using previous reported studies to select proteases that demonstrate the highest potential to liberate ACE-I inhibitory peptides, and further experimentally testing each one of them for their *in vitro* activity. The process can be simplified by using *in silico* analysis and tools such as BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) and Expasy Peptide Cutter (http://web.expasy.org/peptide_cutter/). These tools allow for the theoretical prediction of potential of various substrates with known protein sequences to generate bioactive peptides, using enzymes with known cleavage specificities. Recently, *in silico* analysis was used to predict the presence of potential ACE-I inhibitory peptides in the sequences of barley proteins (Cavazos & Gonzalez de Mejia, 2013), however, no ACE-I inhibitory hydrolysates and/or fractions were generated in this study, that demonstrate potential heart health benefits *in vitro*.

Thus the objectives of this study were (a) To use *in silico* methods to select suitable enzymes for use in the generation of ACE-I inhibitory peptides from barley proteins; (b) To use the chosen enzyme to generate hydrolysates from crude barley protein concentrates; (c) To enrich the generated barley protein hydrolysates using molecular weight cut-off filtration (MWCO) and verify the *in vitro* ACE-I inhibitory activity of the MWCO fractions; (d) To identify the peptides and their parent proteins in the most active MWCO fractions by liquid chromatography tandem mass spectrometry (LC-MS/MS) and (e) To chemically synthesize and determine the IC_{50} values of the most active peptides.

2. Materials and methods

2.1. Materials and reagents

Hulled Irish spring barley cultivar 'Propino' from the 2013 harvest was provided by Seedtech (Waterford, Ireland). Whole barley grains were milled using a Perten Lab mill 3100 (Perten Instruments, AB, Kungens Kurva, Sweden). The enzyme papain from Papaya latex, ammonium sulphate, and sodium hydroxide (NaOH) were obtained from Sigma Aldrich (Wicklow, Ireland). The 3.5 kDa dialysis tubing was obtained from the Medical Supply Company, Dublin, Ireland. The 10 kDa and 3 kDa MWCO membranes were purchased from Millipore (Cork, Ireland). The ACE-I inhibition assay kit was supplied by NBS Biologicals Ltd. (Cambridgeshire, England, UK).

2.2. In silico analysis

In silico analysis was used to select a suitable enzyme for hydrolysis of barley protein. Four enzymes (thermolysin, papain, pepsin and trypsin) were chosen for use in this work based on previous reports and evidence from databases including BIOPEP. The databases BIOPEP and ExPASy peptide cutter were then used to predict the ability of the above mentioned enzymes to theoretically release peptides from the major barley storage proteins. These proteins included the β3-hordein (UnitProt ID: P06471), C-hordein (Uniprot ID: Q41210), D-hordein (Uniprot ID: Q84LE9) and Globulin (Uniprot ID: Q84NG7) accessed from the UniProt database, available at http://www.uniprot.org/. The hypothetically released peptides were examined for their theoretical ACE-I inhibitory properties, based on their hydrophobic amino acids content and relative abundance of proline residues in their sequences (Wilson, Hayes, & Carney, 2011).

2.3. Preparation of barley protein concentrate

Extraction of proteins from barley was carried out in water following a method used by Fitzgerald et al. (2012). Briefly, milled barley flour was mixed in Milli-Q water at flour to water ratio of 1:50 w/v. To enhance release of proteins into the solution, the milled barley solution was ultrasonicated for 1 h, following which it was left to stir overnight at 4 °C on a magnetic stirrer plate (C-MAGHS 7KAMAG, IKA-Werke GmbH & Co. KG, Staufen, Germany). The supernatant, obtained from centrifugation of the solution at 10,000 g for 1 h, was decanted and stored at 4 °C. The pellet obtained from the centrifugation was used for re-extraction to achieve an exhaustive extraction of proteins from the sample matrix. The pellet was re-suspended in Milli-Q water at water to flour ratio of 25:1, and subjected to a second extraction procedure with the exact same steps described above. Supernatants from the sequential extractions were pooled together and the proteins were precipitated using ammonium sulphate. The solution was brought to 80% saturation with ammonium sulphate, stirred for 1 h at 4 °C and centrifuged at 17,000 g for 1 h to obtain a protein precipitate. The precipitate was subsequently suspended in a small amount of water and dialyzed overnight at 4 °C using a 3.5 kDa MWCO dialysis tube against Milli-Q water. The dialysed retentate (fraction greater than 3.5 kDa) was then freeze dried and stored at −20 °C until further use. The process followed for bioactivity guided fractionation of the crude barley protein hydrolysates is shown in Fig. 1.

2.4. Hydrolysis of barley proteins

Based on *in silico* analysis (Section 2.2), the enzyme papain (EC number: 3.4.22.2) was chosen for experimental hydrolysis of barley proteins, due to its ability to produce the highest number of theoretical ACE-I inhibitory peptides. The hydrolysates of the barley protein concentrate were generated using a New Brunswick (Cambridge, UK) 1.5 l bioreactor with temperature and pH control. 5 g of freeze-dried barley protein concentrate was dissolved in Milli-Q water in a 1:10 w/v ratio and added to the bioreactor. Prior to initiating hydrolysis, the temperature of the mixture was adjusted to 60 °C and pH to 6.0 using 0.1 M NaOH. The enzyme papain was added to the bioreactor in a substrate to enzyme ratio of 0.033:1 w/w, and barley protein hydrolysis was carried out for 24 h with agitation at 300 rpm. Temperature and pH were maintained during the course of hydrolysis. Following hydrolysis, the enzyme was deactivated thermally at 95 °C for 10 min in a water bath. A supernatant was obtained after centrifugation at 10,000 g for 10 min and subdivided into three fractions. The three fractions were obtained and named following similar nomenclature to that of Di Bernardini et al. (2012). Fraction 1 was referred as the non-ultrafiltered hydrolysate (NUFH). Fractions 2 and 3 were obtained by ultrafiltration of the hydrolysate supernatants using 10 kDa and 3 kDa MWCO membranes and hence were termed the 10 kDa ultrafiltered hydrolysate (10 kDa-UFH) and 3 kDa ultrafiltered hydrolysate (3 kDa-UFH), respectively. All the hydrolysates were freeze dried, vacuum-packed and stored at −20 °C until further use.

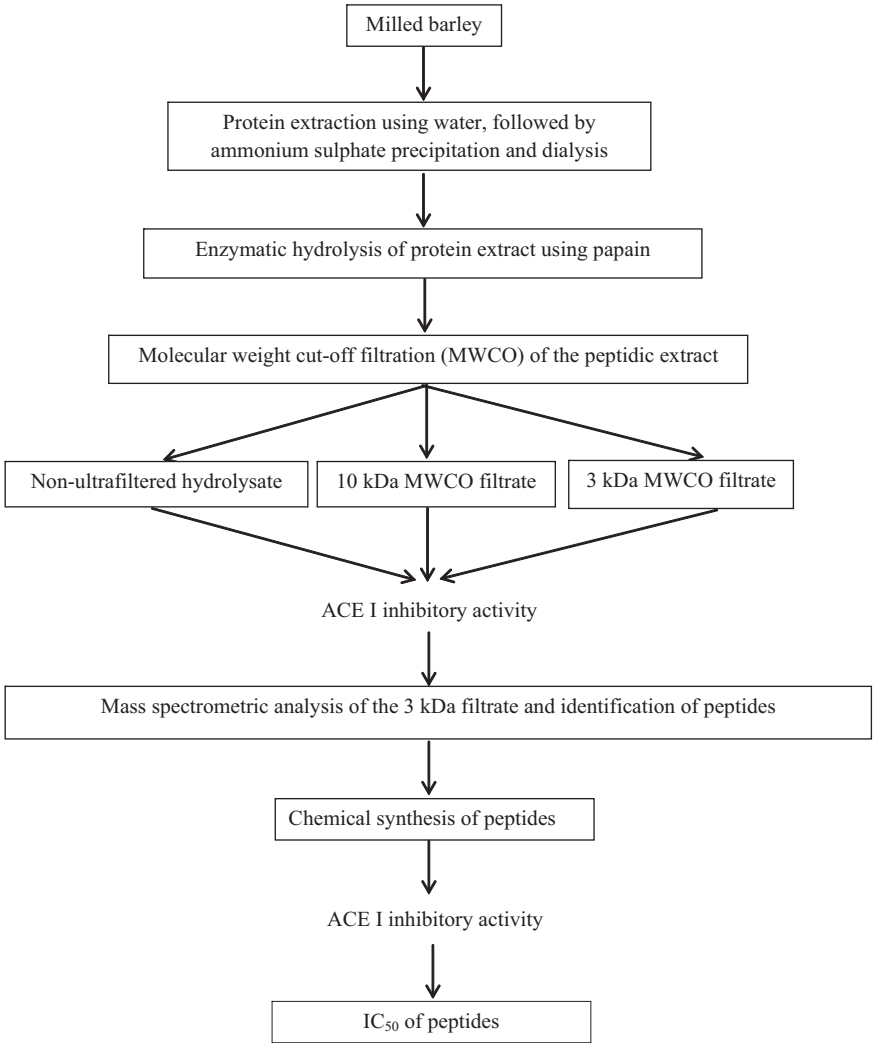

Fig. 1. Schematic representation of the main steps used in this study to generate and characterise ACE-I inhibitory peptide fractions from barley (*Hordeum vulgare*).

2.5. Determination of protein content and extraction yield

The barley protein extraction method was assessed by estimating the protein content and resultant extraction yield. The protein content is defined as the percentage of protein in the isolated dry powders. The total nitrogen content of raw barley, protein concentrate and the barley protein hydrolysate and filtrates (3 kDa and 10 kDa) was determined by combustion in a LECO FP-428 Nitrogen Analyzer (Leco Corporation, MI, USA), which was calibrated with the analytical agent EDTA. A factor of 5.70 was used for converting the nitrogen content of the samples into their protein content (Tkachuk, 1969).

Extraction efficiency (EE) describes the recovery as the percentage of protein recovered in the isolated fraction relative to the total quantity of starting material. The EE of the above mentioned samples were determined by using the following Eq. (1):

$$\text{Extraction efficiency}(\%) = \frac{\text{Total amount of protein recovered after extraction}}{\text{Total amount of sample used for extraction}} \times 100$$

2.6. Measurement of in vitro ACE-I inhibitory activity

This assay was carried out using an ACE-I inhibitor assay kit (NBS Biologicals, Cambridgeshire, England) in accordance with the manufacturers' instructions. The protocol used by Lafarga, O'Connor, and Hayes (2014) was used for conducting the assay. All barley protein hydrolysate and filtrate fractions were assayed at a concentration of 1 mg/ml in triplicate, and means and standard deviations (SD) for each were calculated. The known ACE-I inhibitor Captopril® was used as a positive control at a concentration of 0.05 mg/ml. The percentage of ACE-I inhibition was calculated for each barley fraction/filtrate and the positive control using the following Eq. (2):

$$\text{ACE-Iinhibitory activity}(\text{inhibition rate }\%) = \frac{\text{Blank1} - \text{Inhibitor}}{\text{Blank1} - \text{Blank2}} \times 100 \quad (2)$$

where, Blank 1 is the positive control (with no ACE-I inhibition) which contained both the substrate and the enzyme, Blank 2 is the negative control (reagent blank) which only contained the substrate, and Inhibitor is the ACE-I inhibitory test reaction which contained the sample (Captopril or barley protein hydrolysate/filtrates), the substrate and the enzyme.

2.7. Identification of peptides and sequence analysis

The peptidic content of the 3 kDa-UFH was analysed using a Thermo Scientific Q-exactive mass spectrometer connected to a Dionex Ultimate 3000 nano-liquid chromatography system. The

samples were suspended in 0.1% formic acid and desalted using Millipore C18 ziptips prior to the LC-MS/MS analysis. The sample was loaded onto Biobasic Picotip emitter (120 mm length, 75 μm ID) packed with Reprocil Pur C18 (1.9 μm) reversed phase media and the separation of the peptides was achieved by increasing acetonitrile gradient over 60 min at a flow rate of 250 nl/min. The mass spectrometer was operated at 2.3 kV capillary voltage in positive ion mode with a source temperature of 220 °C. The mass spectrometer was operated in an automatic data dependant switching mode for acquiring all data. A high resolution (70,000 FWHM) MS scan (m/z 300 to m/z 1600) was performed using the Q-Exactive to select the 12 most intense ions prior to MS/MS analysis using higher-energy collisional dissociation (HCD).

The raw data was *de novo* sequenced and searched against *H. vulgare* subset of the Uniprot Swissprot database using the search engine PEAKS Studio 7, for peptides cleaved with no specific enzyme. At least one unique peptide was required to identify a protein. Each peptide used for protein identification met specific PEAKS parameters, i.e. only peptide scores that corresponded to a false discovery rate (FDR) of $\leqslant$1% were accepted from the PEAKS database search. The amino acid sequences, the positions inside the parent proteins, the observed masses expressed in Dalton (Da) and the retention times at which the peptides were obtained, were compiled by the PEAKS software program.

2.8. Selection of peptides for synthesis

The potential bioactivities of all the peptides identified within the 3 kDa-UFH were predicted, and their peptide scores calculated using an *in silico* tool PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/) (Mooney, Haslam, Pollastri, & Shields, 2012). Peptides labelled over the score threshold of 0.8 were selected and subjected to the rules of ACE-I inhibition according to Wilson et al., 2011. These rules define the correlation between structure of amino acids present in peptide sequences, and their effective ACE-I inhibitory activity. Sequences containing aromatic, hydrophobic or branched side chains (such as proline, phenylalanine and tyrosine) as their C-terminal amino acid residues, contribute to ACE-I inhibitory potency of the peptides. Arginine and lysine are other amino acids at the C-terminus that favour the ACE-I inhibitory activity of the peptide sequence.

A total of 15 peptides were selected after the above screening for chemical synthesis.

2.9. Microwave-assisted solid phase peptide synthesis

The procedure used for chemical synthesis of the peptides was similar to methods described by Fitzgerald et al. (2012). The selected bioactive peptides from the above analysis were synthesized using microwave-assisted solid phase peptide synthesis performed on a Liberty CEM microwave peptide synthesizer (Mathews, NC, USA). Peptides were synthesized on a H-Ala-HMPB-ChemMatrix and H-Ile HMPB-ChemMatrix resins (PCAS Biomatrix Inc., Quebec, Canada). Reversed-phase-high-performance liquid-chromatography on a semi-preparative Jupiter Proteo (4 μm, 90 A) column (Phenomenex, Cheshire, UK), was used for purification of the synthetic peptides. Fractions containing the desired molecular mass were identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and were pooled and lyophilized on a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswitch, UK).

IC_{50} values of those chemically synthesized peptides that showed the highest ACE-I inhibition was determined. The IC_{50} is a measure of the effectiveness of a substance in inhibiting a specific substance and indicates the amount of inhibitor needed to inhibit the enzyme by half. The IC_{50} values were determined for the bioactive peptides by plotting the percentage of inhibition as a function of the concentration of test compound.

3. Results

3.1. Selection of enzyme for hydrolysis using in silico analysis

Preliminary analysis using *in silico* methodologies (Section 2.2) indicated that papain generated the highest number of theoretical ACE-I inhibitory peptides from the barley proteins, in comparison to the enzymes thermolysin, trypsin and pepsin. For example, papain generated 23 hypothetical ACE-I inhibitory peptides from the protein sequence of β-hordein, whereas thermolysin generated 19, and trypsin and pepsin generated 4 and 3 ACE-I inhibitory peptides respectively (Supplementary Table S1). Therefore, papain was chosen for further experimental work.

3.2. Nitrogen content and extraction efficiency

The nitrogen content of the dried and milled hulled barley flour, the isolated barley protein concentrate, the NUFH, the 10 kDa-UFH and the 3 kDa-UFH were determined and converted to protein content using the conversion factor of 5.7 as described in Section 2.5. The raw milled barley flour had a protein content of 8.8%, whereas upon extraction the protein content of the freeze dried protein concentrate was 94.3%. The protein contents of the NUFH, 3 kDa-UFH and 10 kDa-UFH were 91.9%, 99.5% and 98.3% respectively.

EE of protein from the raw barley was 8.5%. This indicates that almost all of the protein present in raw barley (about 8.8%), was recovered during the protein extraction process. The EEs of the NUFH, 3 kDa-UFH and the 10 kDa-UFH were 79.3%, 56.3% and 65% respectively.

3.3. ACE-I inhibitory activity for the barley protein concentrates, the NUFH, the 10 kDa-UFH and the 3 kDa-UFH

Fig. 2 shows the ACE-I inhibitory activities of the whole protein extract of barley, and the barley hydrolysate fractions NUFH, 10 kDa-UFH and 3 kDa-UFH. All the samples were tested for ACE-I inhibitory activity at 1 mg/ml. The whole protein extract demonstrated ACE-I inhibitory activity of 37.90 ± 2.24%. For the various hydrolysate fractions, ACE-I inhibitory activity was highest for the 3 kDa UFH (70.37 ± 0.67%), followed by 10 kDa UFH (57.42 ± 4.68%) and the NUFH fraction (47.23 ± 2.62%). These results indicated that ultrafiltration helps in enriching the barley protein derived ACE-I inhibitory peptides. Captopril©, which was used as a positive control, had an ACE-I inhibitory activity of 87.46 ± 1.71% at a concentration of 0.05 mg/ml.

3.4. Peptides characterised in the 3 kDa-UFH

Since the 3 kDa-UFH showed the highest ACE-I inhibitory activity (70.4%), compared to the 10 kDa-UFH (57.4%) and NUFH (47.2%), the peptides present in the 3 kDa-UFH were further analysed using the liquid chromatography-tandem mass spectrometry. 1882 peptide sequences originating from 61 barley proteins were identified in the 3 kDa-UFH. The amino acid sequences of the characterised peptides were between 4 and 18 amino acids in length possessing molecular weights ranging from 702.3 Da to 2477.4 Da. Some of the major barley proteins from which the peptides in the 3 kDa-UFH originated were beta amylase (Accession number P16098), B3-hordein (Accession number P06471), Serpin Z4 (Accession number P06293), Subtilisin chymotrypsin inhibitor (Accession number P16063).

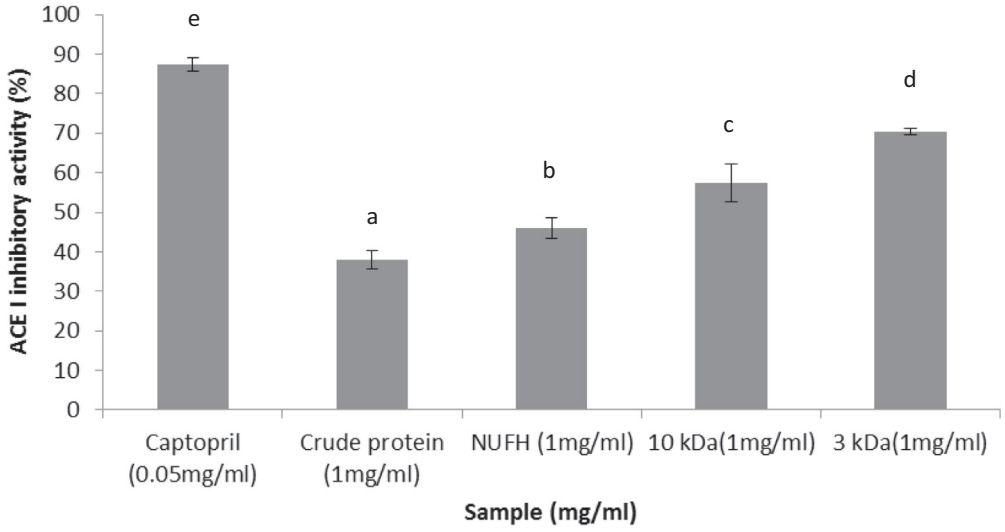

Fig. 2. *In vitro* ACE-I inhibitory activity of the positive control (Captopril), crude protein concentrate, papain hydrolysed non ultra-filtered hydrolysate (NUFH), 10 kDa-UFH and 3 kDa-UFH fractions, expressed as % inhibition. Each sample was tested three times and the ACE-I percentage of inhibition values represent the means of three replicates ± SD. Bars bearing different letters (a, b, c, d, e) are significantly different ($p < 0.05$).

3.5. Peptide synthesis and IC_{50} determination

Following *in silico* analysis of the peptides identified in the 3 kDa-UFH, a total of 15 peptides were selected for chemical synthesis, and tested for their *in vitro* ACE-I inhibitory activities. Table 1 lists the peptides chosen for chemical synthesis, along with their source proteins, position within the proteins, retention time and observed molecular weights. ACE-I inhibitory enzyme activities of the 15 chemically synthesized peptides have been illustrated in Fig. 3. The ACE-I inhibitory activities of the synthesized peptides ranged from 25.80 ± 4.96% for the peptide DSWRPW to 91.83 ± 0.33% for the peptide FQLPKF. Amongst the 15 synthesized peptides, four peptides FQLPKF, ALRYFM, NFLARF, and GFPTLKIF showed the highest ACE-I inhibitory activities of 91.8%, 90.3%, 89.0%, and 88.3% respectively, and were chosen for IC_{50} determination. The IC_{50} values of the above mentioned four peptides were 28.2 μM, 200 μM, 100 μM, and 41.2 μM respectively (Fig. 4). FQLPKF harboured the lowest IC_{50} value, and hence was the most effective peptide in inhibiting the ACE-I enzyme, amongst the synthesized peptides. The MS/MS spectrum of this peptide is shown in Fig. 5.

Table 1
Peptides from 3-kDa-UFH selected for chemical synthesis, their source proteins, position within the proteins, retention time on the mass chromatograph and observed molecular weights in Daltons (Da).

Amino acid sequence	Protein (accession number)	Position	Retention time (min)	Molecular weight (Da)
PMAPLPR	Beta-amylase (P16098)	451–457	14.52	780.43
SGPPEHKLF		404–412	14.60	1010.51
GIHWWYK		296–302	22.43	988.49
GVDNQPLF		136–143	25.18	888.43
KLQPFPF		474–480	27.34	875.49
VPLDFNPNRIFIL	Subtilisin-chymotrypsin inhibitor (P16063)	61–73	39.63	1556.87
QFMLPKFK	Serpin-Z7 (Q43492)	284–291	29.79	1020.54
GFPTLKIF	Protein disulfide-isomerase (P80284)	114–121	31.87	921.53
WPFRL	Aldose reductase (P23901)	122–126	28.21	717.39
DHPFLFL	Serpin-ZX (Q40066)	370–376	37.27	887.45
FQLPKF	Serpin-Z4 (P06293)	284–289	27.65	778.44
FIDNIFR	ATP synthase subunit (P00828)	271–277	26.40	923.49
NFLARF	UTP–glucose-1-phosphate uridylyltransferase (Q43772)	409–414	24.29	766.41
DSWRPW	Serine carboxypeptidase (P07519)	444–449	25.69	845.38
ALRYFM	Alpha-amylase/ trypsin inhibitor (P11643)	102–106	22.04	728.37

4. Discussion

Treating hypertension and blood pressure control by inhibition of enzymes present within the RAAS is a well-established approach (Ibrahim, 2006). Peptides released from proteins from a number of food sources like milk, fish and soy (Balti et al., 2015; Jäkälä & Vapaatalo, 2010; Singh, Vij, & Hati, 2014) have been assessed previously for their ACE-I inhibitory and antihypertensive properties *in vitro* and *in vivo*. While the potential of barley proteins to produce ACE-I inhibitory peptides was analysed before by *in silico* analysis (Cavazos & Gonzalez de Mejia, 2013), this study, to the best of authors' knowledge, is the first to generate peptides from barley proteins and to determine their ACE-I inhibitory activities *in vitro*.

In silico analysis carried out in this study helped in predicting the hypothetical potential of barley as a substrate, to release ACE-I inhibitory peptides, using the enzymes thermolysin, papain, pepsin, and trypsin. Based on the *in silico* analysis, enzyme papain was chosen for performing hydrolysis to release bioactive peptides from barley proteins. Use of proteolytic enzymes such as papain for releasing bioactive peptides from plant and other proteins is well documented (Udenigwe & Aluko, 2012). Furthermore, papain has the status of a generally recognised as safe enzyme.

Papain hydrolysed barley protein (NUFH) was found to inhibit ACE-I by 47.23 ± 2.62% as compared to the whole barley protein concentrate, which inhibited ACE-I by 37.90 ± 2.24%. This may be related to the fact that enzyme-catalysed hydrolysis of the protein concentrates is necessary to release the latent bioactive peptides that are encrypted in the primary structure of the protein

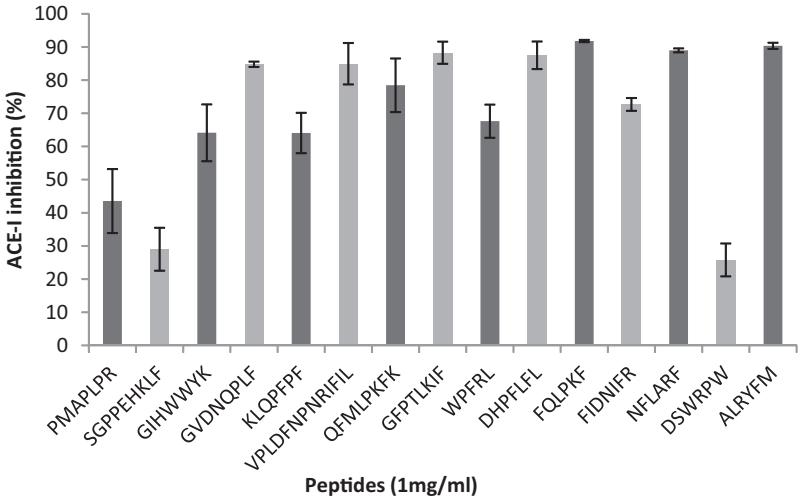

Fig. 3. *In vitro* ACE-I inhibitory activities (expressed as % inhibition) of the chemically synthesized peptides, tested at a concentration of 1 mg/ml.

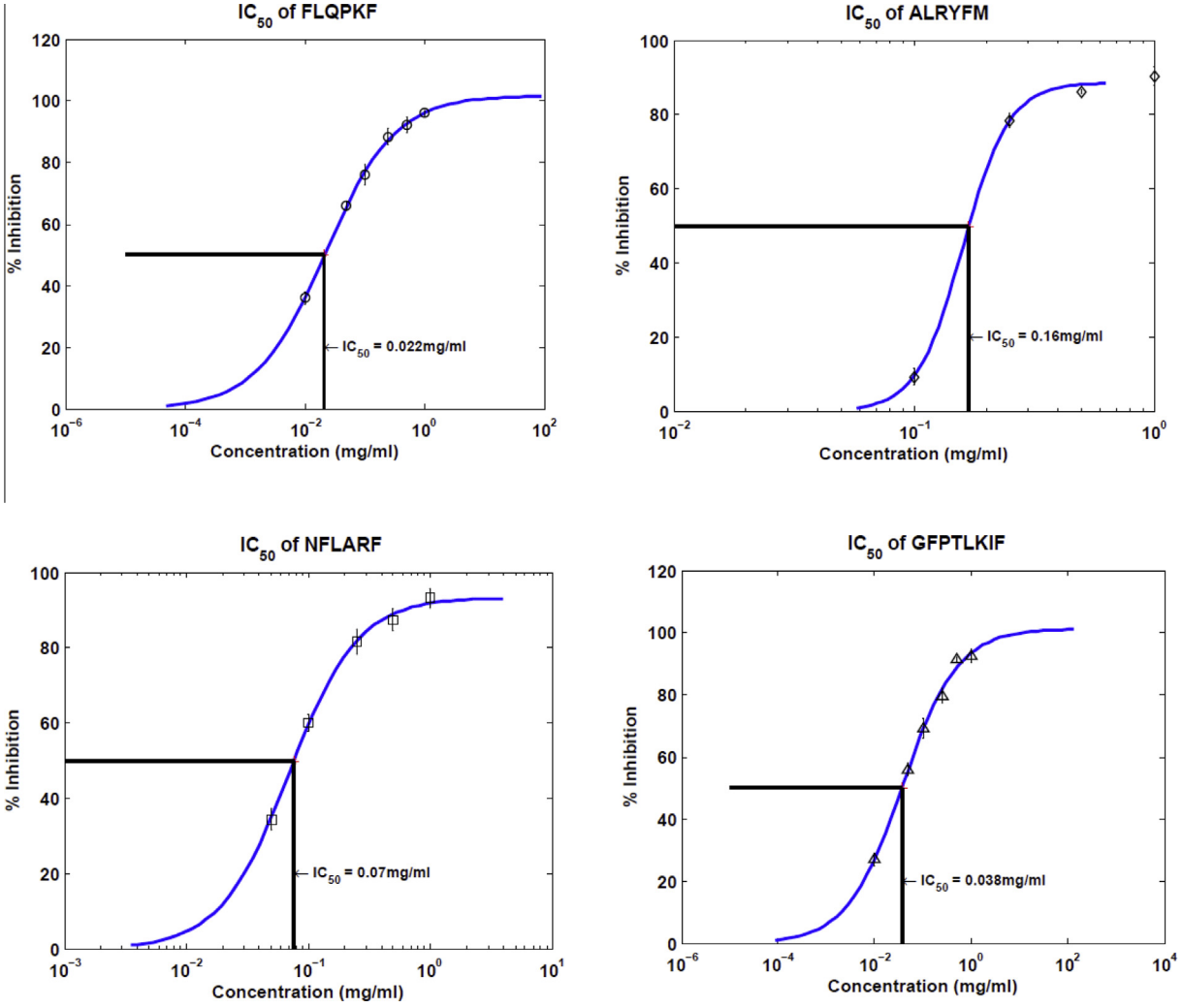

Fig. 4. ACE-I IC_{50} values of chemically synthesized peptides. IC_{50} values of active peptides were calculated by plotting the percentage (%) of enzyme inhibition as a function of concentration (mg/ml) on a log scale. Data were fitted using a four parameter logistic model. ACE-I percentage of inhibition values represent the means of three replicates ± S.D.

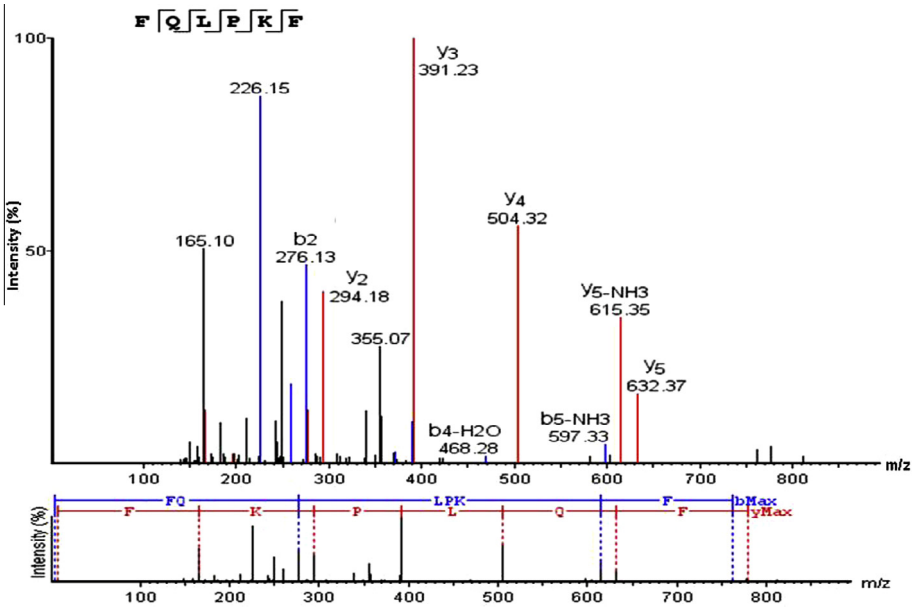

Fig. 5. MS/MS spectrum with amino acid sequence assignment of the peptide FQLPKF.

(Quinde-Axtell et al., 2005). Hydrolysis of the barley protein followed by MWCO ultrafiltration further enhanced the ACE-I inhibitory activity of the resulting fractions. The 3 kDa-UFH showed the highest ACE-I inhibition of 70.37 ± 0.67% compared to the 10 kDa-UFH and the NUFH at a concentration of 1 mg/ml. This indicated that the ACE-I inhibitory peptides are mostly enriched in the 3 kDa-UFH fraction. This also reflected the fact that these bioactive peptides have molecular weights of less than 3 kDa. This is in accordance with previously reported studies stating that ACE-I inhibitory peptides are composed of a small number of amino acids and are typically less than 3 kDa (Li & Aluko, 2010). This result also justifies the use of the process of membrane filtration which enriches the bioactive peptides in the ultra-filtered fraction, while removing the deactivated enzyme molecules, large protein molecules and aggregated peptides that may be present in the NUFH.

Although the peptides identified in this study have not been previously reported in the database BIOPEP, or in the literature, some of the peptide sequences share homologies with previously reported ACE-I inhibitory peptides. For example, the segment **GIH** contained in the peptide sequence **GIH**WWYK, identified in the 3 kDa-UFH, was previously identified as a part of the ACE-I inhibitory peptide **GIH**ETTY generated from the hydrolysis of cuttlefish (*S. officinalis*) muscle protein (Balti et al., 2015). Another peptide fragment **PFP,** present in peptide KLQ**PFP**F of the 3 kDa-UFH, was previously identified as a part of the ACE-I inhibitory peptides GPVRG**PFP**II and EM**PFP**K generated from hydrolysis of fermented milk and casein fraction of milk respectively (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Pihlanto-Leppälä, Rokka, & Korhonen, 1998). Moreover the fragment **PFL** in the peptide sequence DH**PFL**FL of barley 3 kDa-UFH, is present in the peptide sequence of the antihypertensive peptide RADH**PFL** generated from the hydrolysis of egg white with pepsin (Miguel, Alonso, Salaices, Aleixandre, & López-Fandiño, 2007). The presence of amino acid fragments in the peptides identified in this study, that were previously reported in literature as having ACE-I inhibitory activities, may have contributed to the activities of the hydrolysates in this study.

Chemical synthesis of individual peptides is carried out to confirm that the bioactivity demonstrated by a particular MWCO fraction is truly due to the peptides that have been identified in the fraction. Since all the 1882 peptides characterised in the 3 kDa-UFH could not be chemically synthesized, an *in silico* tool called PeptideRanker was used for scoring the identified peptides based on the prediction of their bioactivity. For every peptide, PeptideRanker predicts the probability (between 0 and 1) of that peptide being bioactive. The closer the predicted probability is to 1, higher are the chances of the peptide being bioactive (Mooney et al., 2012). About 349 peptides having a score of 0.8 and more were chosen for a further *in silico* screening for their ACE-I inhibitory potentials. Based on the criteria mentioned in Section 2.8, a total of 15 peptides were finally chosen for chemical synthesis, with preference being given to sequences containing bulky side chains and hydrophobic amino acids at the C-terminus. For example, the peptide PMAPLPR was chosen based on presence of amino acid arginine (R) and the hydrophobic proline (P) at the ultimate and penultimate positions of the C-terminal end respectively. Similarly, the peptide SGPPEHKLF was chosen because of the hydrophobic phenylalnine (F) at the C-terminal end, and presence of two proline (PP) residues in the sequence. Most of the chemically synthesized peptides inhibited the enzyme ACE-I by more than 50%. Thus, these peptides among others, possibly contribute towards the ACE-I inhibitory activity of the entire 3 kDa-UFH.

Amongst those evaluated for their IC_{50}, FQLPKF and GFPTLKIF were the most potent in inhibiting ACE-I enzyme with an IC_{50} of 28.2 μM. and 41.2 μM respectively, while ALRYFM (100 μM) and NFLARF (200 μM) exhibited moderate to high ACE-I IC_{50} values. These IC_{50} values compare favourably with the values from other food-derived ACE-I inhibitory peptides. Recently peptides from brewers' spent grain exhibited IC_{50} values ranging from 80.4 μM to 226 μM (Connolly, O'Keeffe, Piggott, Nongonierma, & FitzGerald, 2015), whereas the peptide in the most bioactive fraction of rice protein isolate showed ACE-I IC_{50} of 18.4 μM (Li, Qu, Wan, & You, 2007). It has been observed from previous studies that the IC_{50} of food derived peptides are generally 10–1000 times higher (thus indicating lower ACE-I inhibitory activity) as compared to the commercial available drugs such as Captopril, which has an IC_{50} of 0.3 μM (Li, Le, Shi, & Shrestha, 2004). In the present study, the ACE-I inhibitory activity of the most active peptide (FQLPKF) was 94 times lower than Captopril. In general, food derived ACE-I inhibitory peptides with IC_{50} values in the range of

100–500 μM can be of nutritive/physiological importance in that they could be active following oral administration (FitzGerald & Meisel, 2000).

5. Conclusions

The increasing consumer concern on the health and safety linked with synthetic drugs, has spurred a constant search for natural compounds that could replace synthetic drugs for the treatment of hypertension. Some blood pressure targeting products containing peptide mixtures from natural sources such as milk and fish already exist in the market. Barley is a relatively rich source of proteins, and this study appears to be the first to investigate the potential of peptide enriched ultra-filtered hydrolysate from barley, to inhibit the enzyme ACE-I, while also identifying the peptide sequences with high ACE-I inhibitory activities. Moreover, *in silico* analysis used in this study was efficient in assessing the potential of producing ACE-I inhibitors from barley proteins. The enhanced *in vitro* ACE-I inhibitory activity of the 3-kDa peptidic fraction generated as a result of ultrafiltration, indicates the possibility of incorporating this bioactive fraction in functional food preparations targeted at managing hypertension. *In vivo* studies are warranted to further validate these findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.097.

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