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Generation of bioactive hydrolysates and peptides from bovine hemoglobin with in vitro renin, angiotensin-I-converting enzyme, and dipeptidyl peptidase-IV inhibitory activities

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Abbreviations: RAAS: renin-angiotensin-aldosterone system; ACE-I: angiotensin-I-converting enzyme; DPP-IV: dipeptidyl peptidase-IV; GLP-1: glucagon-like peptide-1; GIP: gastric-inhibitory peptide; MWCO: molecular weight cut-off; HPLC: high performance liquid chromatography; FA: formic acid; DMSO: dimethyl sulfoxide; ACN: acetonitrile; MS: mass spectrometry; HBA_BOVIN: α-chain of bovine hemoglobin; HBB_BOVIN: β-chain of bovine hemoglobin; HB-NUFH: non-ultrafiltrated hydrolysate; TOF: time of flight; DDA: data-dependent acquisition; MW-SPPS: microwave-assisted solid phase peptide synthesis; MALDI: matrix assisted laser desorption/ionization; S.D.: standard deviation; CMC: carboxymethyl cellulose; QSAR: quantitative structure-activity relationship.

ABSTRACT

Bovine hemoglobin was selected for use in the generation of bioactive hydrolysates with potential for use as functional food ingredients for prevention of disorders such as hypertension, obesity and diabetes. Bovine hemoglobin was isolated and hydrolysed with papain, which was selected using *in silico* analysis. The generated hydrolysate was enriched by ultrafiltration and further purified by high performance liquid chromatography. A number of peptides were identified using *de novo* peptide sequencing and these peptides were chemically synthesised to confirm their bioactivity *in vitro*. Three multifunctional peptides with both, angiotensin-I-converting enzyme- (ACE-I) and renin-inhibitory properties and one peptide with ACE-I-inhibiting properties were identified. These included the di-peptide HR with ACE-I and renin IC_{50} values of 0.19 and 7.09 mM, respectively. The generated papain hydrolysate of bovine hemoglobin not only inhibited the enzymes ACE-I and renin but also the enzyme dipeptidyl peptidase-IV (DPP-IV), which has been linked to type-2 diabetes.

PRACTICAL APPLICATIONS

Slaughterhouse blood represents a problematic co-product to meat processors due to the large volumes generated and its high pollutant load, and it is usually discarded as waste or used for low value purposes such as blood meal. However, bovine blood represents a valuable source of protein which is underutilized in the food industry. In order to find potential applications for this largely underutilized co-product, the ACE-I, renin, and DPP-IV inhibitory properties of a papain hydrolysate of bovine hemoglobin were studied and a number of novel multifunctional bioactive hydrolysates and peptides were identified. Results obtained may not only reduce blood disposal but also have a role in improving public health. In addition, this study demonstrates the potential of bovine hemoglobin as a resource for generation of

bioactive peptides and opens new commercial opportunities for its use beyond current applications in the food industry.

Keywords: hemoglobin, bioactive peptides, renin, ACE-I, hypertension, DPP-IV.

INTRODUCTION

The inhibition of enzymes involved in the renin-angiotensin-aldosterone system (RAAS) such as angiotensin-I-converting enzyme (ACE-I; EC 3.4.15.1) and renin (EC 3.4.23.15) plays a key role in the treatment of hypertension. In addition, a new approach in the management of type-2 diabetes is inhibition of the enzyme dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5). DPP-IV degrades and inactivates glucagon-like peptide-1 (GLP-1) and gastric-inhibitory peptide (GIP), two incretin hormones which contribute to the enhancement of glucose-induced insulin secretion (Drucker 2006). Various chemically synthesised DPP-IV inhibitors are at different stages of development and registration worldwide (Mkele 2013), and common antihypertensive drugs such as Captopril®, Enalapril®, Tekturna®, and Rasilez® are used as pharmaceutical agents in the control of ACE-I and renin. However, DPP-IV, ACE-I, and renin can be inhibited using naturally sourced compounds including bioactive peptides derived from food sources, with milder side-effects compared to their chemically synthesised counterparts (Danquah and Agyei 2012).

Bioactive peptides are short sequences of amino acids that are inactive within the sequence of the parent protein but have positive impacts on systems of the body including the circulatory, gastrointestinal, and nervous system once released (Korhonen and Pihlanto 2006). Such peptides can be released by chemical or enzymatic hydrolysis and have been generated from a wide variety of natural compounds such as milk (Hernández-Ledesma *et al.* 2014), fish (Mora and Hayes 2015), and co-products of the meat industry including meat, bone, and blood (Bah *et al.* 2013, Lafarga and Hayes 2014). The use of bioactive peptides as ingredients in the functional foods industry, as well as nutraceuticals or pharmaceutical agents has gained much interest in recent years, and may provide a commercial opportunity for many companies (Lalor and Wall 2011).

Blood proteins are not only used in the food industry as ingredients in numerous foods, but also as binders, natural colour enhancers, emulsifiers, and fat replacers (Bah *et al.* 2013). Blood proteins are also used in the pharmaceutical industry (Xie *et al.* 2012). For example, hemoglobin is the main constituent of Proferrin® (Colorado Biolabs, Inc., CO, USA), a heme iron supplement used to address iron deficiency (Hudson and Schonder 2002). However, blood is largely underutilized as a protein source and only 30% of the blood generated in slaughterhouses is used in the food industry (Bah *et al.* 2013). As well as being an excellent source of iron, hemoglobin is a major constituent protein found in blood and therefore a potential resource for the generation of bioactive hydrolysates and peptides for use as functional ingredients (Bah *et al.* 2013, Lafarga and Hayes 2014). Indeed, previous studies have demonstrated the potential of blood-derived peptides and have reported physiological bioactivities including antioxidant, antihypertensive, and antimicrobial properties (Bah *et al.* 2013, Lafarga and Hayes 2014).

The aim of this work was to generate and characterize a papain (EC 3.4.22.2) hydrolysate of bovine hemoglobin and to assess its DPP-IV, ACE-I, and renin inhibitory activities *in vitro*. The generated hydrolysate was enriched by molecular weight cut-off (MWCO) filtration and further purified by high performance liquid chromatography (HPLC). A number of peptides were identified by *de novo* peptide sequencing and were chemically synthesised for confirmation of bioactivity *in vitro*. Moreover, the concentration of peptide required to inhibit the activity of ACE-I and renin by half (IC_{50}) was calculated for active peptides and their bitterness and resistance to gastrointestinal digestion were predicted using Ney's '*Q* rule' (Ney 1971) and ExPASy PeptideCutter (Gasteiger *et al.* 2003), respectively.

MATERIALS AND METHODS

Materials and reagents

Formic acid (FA), dimethyl sulfoxide (DMSO), acetonitrile (ACN), sodium citrate, chloroform, papain from *Carica papaya*, the specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, and the ACE-I inhibitor Captopril® were supplied by Sigma Aldrich (Dublin, Ireland). The DPP-IV inhibitor screening assay kit, containing the DPP-IV inhibitor Sitagliptin, and the renin inhibitor screening assay kit were supplied by Cambridge BioSciences (Cambridge, England, UK). The ACE-I inhibition assay kit was supplied by NBS Biologicals Ltd. (Cambridgeshire, England, UK). All other chemicals used were of analytical grade.

Protein isolation

Whole bovine blood was collected at time of slaughter under hygienic conditions at the abattoir at the Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland. All animals slaughtered were female, Charolais cross heifer breed, and were aged between 23 and 24 months at the time of slaughter. Sodium citrate solution was used as an anticoagulant and was added immediately to blood following collection at a final concentration of 1.5% (w/v). Blood was chilled to 4 ºC and handled carefully to minimize hemolysis.

Whole blood cells were separated from plasma by centrifugation at 4ºC and 10,000 × g for 10 minutes using a Sigma 6K10 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The cellular fraction was frozen and freeze-dried. Hemoglobin was obtained from this dehydrated cellular fraction by dilution in distilled water (1:3, w/v) to induce hemolysis, following the procedure described in Figure 1. Hemoglobin was extracted using chloroform in a chloroform to cellular solution ratio of 1:4 (v/v). The sample was agitated for 10 minutes

and subsequently centrifuged at 4 ºC and 5,000 × g for 10 minutes using a Sigma 6K10 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) (Álvarez *et al.* 2009). The supernatant containing the hemoglobin was frozen, freeze-dried, and stored at -20 ºC until further use.

The total protein content was determined in duplicate using a LECO FP628 Protein analyser (LECO Corp., MI, USA) based on the Dumas method and according to AOAC method 992.15, 1990. The conversion factor of 6.25 was used to convert total nitrogen to protein. The total protein content and yields were calculated per litre of blood and are shown in Figure 1. Moisture and ash content were determined gravimetrically in accordance with previously described methods (Kolar 1992).

In silico analysis

The amino acid sequence of the α-chain (UniProt ID: HBA_BOVIN | UniProt AC: P01966) and the β-chain of bovine hemoglobin (UniProt ID: HBB_BOVIN | UniProt AC: P02070) were accessed from the UniProt database, available at http://www.uniprot.org/. An *in silico* analysis was carried out on these sequences following a previously described method (Lafarga *et al.* 2014). Computer simulations of proteolysis were used to study the bioavailability of the studied peptides after simulated gastrointestinal digestion with ExPASy PeptideCutter, available at http://web.expasy.org/peptide_cutter/ using enzymes found in the gastrointestinal tract including pepsin (pH 1.3 and pH>2; EC 3.4.23.1), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1) (Gasteiger *et al.* 2003). Moreover, the '*Q* rule' formulated by Ney (1971) was used to predict the bitterness of the studied peptides based on its amino acid composition, whereby a *Q-value* was calculated from the solubility data of the individual amino acids.

Enzymatic hydrolysis

Papain hydrolysates of bovine hemoglobin were prepared in triplicate using a BioFlo 110 Modular Benchtop fermenter (New Brunswick Scientific Co., Cambridge, England, UK) with agitation, temperature, and pH control. A substrate solution was prepared by resuspending the dried hemoglobin in MilliQ purified water at a concentration of 30 g/L at a total volume of 500 mL. Agitation, temperature, and pH conditions were adjusted to 350 rpm, 65 ºC, and 6.5, respectively. The pH was kept constant using 0.1 M NaOH. Once the optimum pH and temperature conditions were achieved, the enzyme papain (activity ≥3 U/mg) was added in a substrate to enzyme ratio of 100:1 (w/w). After 24 h, papain was heat-deactivated at 95 ºC for 10 min in a Grant JB Aqua 12 water bath (Grant instruments, England, UK).

Four protein fractions were generated from the whole papain hydrolysate of bovine hemoglobin. Fraction one was termed non-ultrafiltered hydrolysate (HB-NUFH). Fractions two, three, and four were obtained by MWCO filtration of the whole hydrolysate using 1, 3, and 10 kDa MWCO membranes separately (Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland). These fractions were labelled as HB-1UFH, HB-3UFH, and HB-10UFH, respectively. All fractions were frozen, freeze-dried, and stored at -20 ºC until further use.

Renin inhibition assay

This assay was carried out using a renin inhibitor screening assay kit (Cayman Chemical Company, MI, USA) in accordance with the manufacturers' instructions. All fractions were assayed at a concentration of 1 mg/mL DMSO in triplicate and standard deviations (S.D.) calculated. The known renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe was used as a positive control at a concentration of 1 mg/mL. Fluorescence intensity was recorded with a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg,

Germany) using an excitation wavelength of 340 nm and an emission wavelength of 500 nm. Renin IC_{50} values were determined for active peptides and hydrolysates by plotting the percentage of renin inhibition as a function of the concentration of test compound.

ACE-I inhibition assay

This assay was carried out using an ACE-I inhibitor assay kit (Dojindo Molecular Technologies, Inc., Munich, Germany) in accordance with the manufacturers' instructions. All fractions were assayed at a concentration of 1 mg/mL HPLC grade water in triplicate and means and S.D. were calculated. Absorbance was measured with a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany) at 450 nm. ACE-I IC_{50} values were determined for active hydrolysates and peptides by plotting the percentage of inhibition as a function of the concentration of test compound.

DPP-IV inhibition assay

This assay was carried out using a DPP-IV inhibitor screening assay kit in accordance with the manufacturers' instructions (Cayman Chemical Company, MI, USA). All hydrolysates were assayed in triplicate and means and S.D. were calculated. The known DPP-IV inhibitor sitagliptin was used as a positive control at a final concentration of 0.4 mg/mL, as recommended by the manufacturer. Fluorescence intensity was recorded with a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Protein and peptide identification using mass spectrometry

A direct infusion of the isolated hemoglobin, dissolved in HPLC grade water at a final concentration of 50 μg/mL, was carried out in 1:1 ACN:HPLC grade water, containing 0.1%

FA, into the electrospray source of the Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA). The mass spectra data were acquired in positive ion mode from m/z 600 to 1400.

The HB-1UFH hydrolysed fraction was re-suspended in HPLC grade water at a concentration of 1 mg/mL and filtered through a 0.45 μm CHROMAFIL® Xtra PVDF-45/25 syringe filter (MACHEREY-NAGEL GmbH & Co., Düren, Germany). The filtered hydrolysate was analysed on a Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA), coupled to an Alliance 2695 HPLC system (Waters Corp., Milford, MA, USA). The chromatographic separation was carried out at a flow rate of 0.2 mL/min with an injection volume of 10 μL on an Atlantis dC18 column – 100mm × 2.1 mm, 3μm particle size (Waters Corp., Milford, MA, USA). Peptides were separated using 0.1% FA in HPLC grade water (solvent A) and 0.1% FA in ACN (solvent B). Column temperature was maintained at 40 ºC. The gradient program was as follows: (i) 0 min, 98% A; (ii) 0-0.1 min, 98% A; (iii) 0.1-18 min, 90% A; (iv) 18-20 min, 85% A; (v) 20-21 min, 40% A; (vi) 21-22 min, 20% A; (vii) 22-25 min, 98% A, and (viii) 25-30 min, 98% A. The HPLC-MS/MS was performed using a data-dependent acquisition (DDA) on positive ion mode at 1 s scan. Argon was used as collision gas with the collision energy ramp from 15 eV for low molecular weight (MW) peptides to 60 eV for high MW peptides. The tandem mass spectrometry (MS/MS) spectral data were deconvoluted using the MaxEnt 3 algorithm and their amino acid sequences were determined using the peptide sequencing software available in the Waters Biolynx^{TM} software package.

Microwave-assisted solid phase peptide synthesis

Peptides were synthesised by microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty CEM microwave peptide synthesizer (Mathews, North Carolina,

USA) following a previously described method (Lafarga *et al.* 2014). Fractions containing the desired MW were identified using MALDI-TOF-MS and were pooled and lyophilized on a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswich, UK).

Statistical analysis

All tests were replicated three times and mean values and S.D. were calculated. ANOVA one-way analysis was carried out using Minitab® v17 (Minitab Ltd., England, UK). Where significant differences were present, a Tukey pairwise comparison of the means was conducted to identify where the sample differences occurred.

RESULTS AND DISCUSSION

Hemoglobin is the main protein in blood and represents approximately 10.3% of the total content of whole bovine blood (Bah *et al.* 2013). The yield of hemoglobin was calculated per litre of blood and was found to be 16.5 ± 0.2 g/L. The total protein content of the hemoglobin fraction, generated following the procedure described in Figure 1, was 95.5 ± 0.2%. In addition, the total moisture and ash content of the isolated protein were measured as 0.95 ± 0.05 and 0.23 ± 0.02%, respectively. The isolated hemoglobin was analysed by MS by direct injection of the sample into a Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source. The ESI-MS analysis of the isolated protein revealed a series of multiple charged species of bovine α- and β-globin chains ($[M+11H]^{11+}$ to $[M+22H]^{22+}$). The deconvoluted mass spectrum of the sample, shown in Figure 2, showed the true MW of bovine α- and β-chains, which were 15054.5 and 15956.0 Da, respectively. The differences between the expected and the deconvoluted masses for both α- and β-chains of bovine hemoglobin were less than 0.01%. Both, the raw and deconvoluted mass spectral data suggested that the isolated hemoglobin was a pure protein.

In silico analysis

Before *in vitro* hydrolysis, an *in silico* study was carried out in order to select a suitable enzyme to generate a hydrolysate rich in bioactive peptides with potential to inhibit ACE-I, renin, and DPP-IV. The use of *in silico* methods was shown previously as effective for predicting the release of bioactive peptides from known protein sequences and in the selection of enzymes and proteins for generation of bioactive peptides (Lacroix and Li-Chan 2012, Lafarga *et al.* 2014, Lafarga *et al.* 2015). The hemoglobin protein fraction isolated in this study was found to be a good reservoir for generation of renin, ACE-I, and DPP-IV inhibitory peptides and hydrolysates for potential use in the food industry. Papain was the

most effective enzymes for the generation of known ACE-I and DPP-IV inhibitors from bovine hemoglobin *in silico* when compared to the enzymes pepsin, thermolysin, trypsin, and bromelain. No renin inhibitory peptides were generated from bovine hemoglobin by *in silico* cleavage using selected enzymes. This may be due to the limited reports of renin inhibitory peptides found in the literature to date. Indeed, only five renin-inhibitory peptides derived from food sources are currently available in BIOPEP compared to over 500 ACE-I-inhibitory peptides (Data accessed on April 2015) (Minkiewicz *et al.* 2008). Results are comparable to previous studies where no renin inhibitory peptides were predicted to be generated *in silico* from blood proteins (Lafarga *et al.* 2016). Bioactive peptides identified using *in silico* analysis included the di-peptide VE, generated using papain and corresponding to sequence *f*(71-72) of the α-chain of bovine hemoglobin. The peptide VE, was previously identified by Van Platerink *et al.* (2008) by LC-MS in a commercial milk hydrolysate and was reported to inhibit ACE-I. Moreover, papain was used previously for the generation of numerous ACE-I and renin inhibitors as well as antioxidant peptides from animal sources including bovine brisket sarcoplasmic proteins (Di Bernardini *et al.* 2012), plant proteins (He *et al.* 2013), blood (Lafarga *et al.* 2015), and seaweed (Fitzgerald *et al.* 2012).

ACE-I- and renin-inhibitory activities in vitro

The *in vitro* ACE-I and renin inhibitory activity of the generated hydrolysates was measured and is shown in Figure 3. At a concentration of 1 mg/mL, HB-1UFH inhibited ACE-I by over 40% compared to the control which was assayed at a concentration of 1 mg/ml. The ACE-I inhibitory activity of the papain hydrolysate of bovine hemoglobin generated in this study was low compared to a previously generated hydrolysate obtained during hydrolysis of porcine hemoglobin with pepsin which inhibited ACE-I by over 60% (Deng *et al.* 2014). Purification by ultrafiltration using 10, 3 and 1 kDa membranes led to increased ACE-I-

inhibitory activity compared to the HB-NUFH ($p<0.05$). Inhibition was also significantly higher in the HB-1UFH fraction compared to the HB-3UFH and HB-10UFH fractions ($p<0.05$). These results were consistent with previous studies where similar increases in bioactivities resulted from enrichment (Hyun and Shin 2000). It is well documented that peptidic ACE-I inhibitors usually consist of short amino acid sequences (Wu *et al.* 2006). The presence of high MW peptides in the HB-NUFH fraction may have contributed to the decrease in ACE-I-inhibitory activity not only by decreasing the concentration of small MW peptides in the sample but also by blocking the activity of smaller peptides.

Furthermore, the renin-inhibitory activity of the generated papain hydrolysates of hemoglobin was measured at a concentration of 1 mg/mL compared to a positive control. It is well known that the inhibition of ACE-I is easier to achieve when compared to that of renin (Udenigwe *et al.* 2009). However, in this study, the renin-inhibitory activity was similar compared to that of ACE-I. At a concentration of 1 mg/mL, the fraction HB-1UFH was the most active with a percentage of renin inhibition of 40.25 ± 8.37%. Although it was previously suggested that the nature and position of the amino acid residues rather than the size of the peptide play a major role in the enhancement of renin inhibition (Mundi and Aluko 2014), purification by ultrafiltration led to a significant increase in the renin inhibitory activity of the HB-1UFH fraction compared to the HB-NUFH, HB-3UFH, and HB-10UFH fractions ($p<0.05$). These results are consistent with the fact that di-peptides were previously suggested as the most effective peptidic renin inhibitors (Li and Aluko 2010). The papain hydrolysate generated herein was more active inhibiting renin than previous blood protein hydrolysates reported in the literature. For example, Lafarga *et al.* (2015) generated a papain hydrolysate of a bovine fibrinogen-rich protein fraction which inhibited renin by 18.8% when tested at a concentration of 1 mg/mL. This is, to the best of our knowledge, the first study that used hemoglobin as a resource for generation of renin inhibiting hydrolysates and peptides.

However, the renin inhibitory values achieved in this study are similar to those obtained from a variety of natural sources in previous studies including an Alcalase® hydrolysates of kidney bean protein, which was found to inhibit renin by 20-40% at a concentration of 1 mg/mL (Mundi and Aluko 2014), and hydrolysates of flaxseed protein with renin IC_{50} values ranging between 1.22-2.81 mg/mL (Udenigwe *et al.* 2009).

DPP-IV-inhibitory activities in vitro

The *in vitro* DPP-IV-inhibitory activity of the studied fractions was calculated and results are shown in Figure 3. The most active fractions, HB-1UFH and HB-10UFH inhibited DPP-IV by 45.26 ± 0.35 and 49.93 ± 1.28%, respectively when tested at a concentration of 1 mg/mL. DPP-IV IC_{50} values of HB-NUFH, HB-1UFH, HB-3UFH, and HB-10UFH were 1.10 ± 0.09, 1.04 ± 0.22, 1.09 ± 0.09, and 0.99 ± 0.12 mg/mL, respectively. As well as for ACE-I and renin inhibitory properties, a slight increase in the DPP-IV inhibitory activity of HB-1UFH compared to the HB-NUFH was observed ($p<0.005$). Results obtained in this study are consistent with the fact that most of the peptides with DPP-IV-inhibitory activity described in the literature to date contain between two and eight amino acid residues in length (Lacroix and Li-Chan 2012). Indeed, the peptides IPI (diprotin A) and VPL (diprotin B), isolated for the first time from culture filtrates of *Bacillus cereus* BMF673-RF1, are known peptidic inhibitors of DPP-IV (Umezawa *et al.* 1984), and are used as positive controls in numerous screening studies. Even smaller MW components such as the amino acid residues tryptophan, methionine, and leucine were shown to present DPP-IV inhibitory activities (Nongonierma *et al.* 2013). The papain hydrolysates generated in this study presented DPP-IV IC_{50} values between 0.99 and 1.10 mg/mL. These values compared favourably with those obtained by trypsin hydrolysates of *Amaranthus hypochondriacus* L. proteins with IC_{50} values ranging from 1.2 to 2.0 mg/mL, depending on the enzyme to substrate ratio (Velarde-Salcedo *et al.*

2013), and a Flavourzyme® hydrolysate of Atlantic salmon gelatine with a DPP-IV IC_{50} value of 1.35 mg/mL (Li-Chan *et al.* 2012). The IC_{50} values obtained herein, were also lower than those obtained from a trypsin hydrolysate of whey protein with an IC_{50} value of 1.51 mg/mL, where the penta-peptide IPAVF, corresponding to β-lactoglobulin *f*(78-82) and with an IC_{50} value of 44.7 µM was responsible for the observed activity (Silveira *et al.* 2013).

Peptide identification by de novo peptide sequencing

The LC-MS/MS chromatogram of the papain hydrolysed peptides in the HB-1UFH fraction is shown in Figure 4. Figure 4 also shows the MS spectrum for the elucidated peptide HLP, where Y and B ions and possible internal ions were labelled. The HPLC profile from this enriched protein hydrolysate showed great peptide generation throughout the separation as papain cleaves at several amino acid sites. The identification of peptides was carried out using the Biolynx^{TM} peptide sequencing software for each MS/MS spectral data generated via DDA. Thirty short peptides of between two and six amino acids in length were identified by *de novo* peptide sequencing. Table 1 lists the identified amino acid sequences, their corresponding position in the parent protein, their observed masses, and their calculated masses. According to the manufacturer, the preferential cleavage sites of papain are the C-terminal sides of arginine and lysine. Consequently, the majority of the peptides identified herein showed a prevalence of arginine and lysine residues in the C-terminal position. Identified peptides were compared to previously reported ACE-I, renin, and DPP-IV inhibitors available in the database BIOPEP (Minkiewicz *et al.* 2008). There were sequence similarities between the peptides identified in this study and previously reported ACE-I and DPP-IV inhibitors available in BIOPEP. Other amino acids sequences that are similar in peptides derived from other sources are also listed in Table 1. For example, the peptide LKG, corresponding to *f*(90-92) of the β-chain of bovine hemoglobin, was found to share the same

termination with the previously reported chemically synthesized ACE-I inhibitor KG (Cheung *et al.* 1980) and the DPP-IV inhibitor VFRELKDLKG, derived from milk (Lacroix and Li-Chan 2014).

A number of the identified peptides were selected for chemical synthesis and bioassay assessment. Selected peptides were the di-peptides HR, YR, HF, and the tri-peptide HLP. These peptides were chosen for chemical synthesis as it is well known that di- and tri-peptides are more likely to be resistant to degradation by digestive enzymes and are expected to be absorbed directly from the gastrointestinal tract into the blood circulatory system (Vermeirssen *et al.* 2004). Indeed, Iwai *et al.* (2005) identified several food-derived collagen short peptides rich in proline and hydroxyproline in human blood after oral ingestion of gelatine hydrolysates. Moreover, peptides chosen for chemical synthesis were selected based on the current knowledge of bioactive peptides and the known attributes of ACE-I and renin inhibitors (Lacroix and Li-Chan 2012, Li and Aluko 2010, Mundi and Aluko 2014, Wu *et al.* 2006). The amino acid residue composition of a peptide and the position of the amino acid in the sequence are determinant in the ACE-I, renin, and DPP-IV inhibitory bioactivity of a peptide (Korhonen and Pihlanto 2006). The scores assigned by PeptideRanker to the identified peptides, shown in Table 1, were also taken into consideration when selecting the peptides for synthesis. PeptideRanker, available at http://bioware.ucd.ie, is a useful *in silico* tool that may be used to identify among a set of peptides those that may be more likely to be bioactive (Mooney *et al.* 2012). The peptide HF, for example, had a PeptideRanker score of 0.95 and presented the hydrophobic residue phenylalanine in its C-terminus position, as previously suggested by Wu *et al.* (2006). Moreover, the peptide HLP had a proline in its C-terminus residue which was suggested to aid resistance to degradation (Vermeirssen *et al.* 2004) and to encourage ACE-I inhibition in tri-peptides (Wu *et al.* 2006).

Chemical synthesis and confirmation of bioactivity

The di-peptides HR, YR, HF, and the tri-peptide HLP were chemically synthesised by MW-SPPS and tested for ACE-I and renin inhibition *in vitro*. ACE-I and renin IC_{50} values were determined for active peptides by plotting the percentage of inhibition as a function of the concentration of test compound as shown in Figure 5. At a concentration of 1 mg/mL, the peptide HF did not inhibit renin. In turn, the peptides HR, YR and HLP showed renin IC_{50} values of 7.09, 8.78, and 6.43 mM, respectively. These results were comparable to previously reported renin inhibitory peptides from natural sources such as the peptide IR, generated by enzymatic hydrolysis of pea protein with Alcalase® (Li and Aluko 2010), and the peptide IRLIIVLMPILMA, generated by enzymatic hydrolysis of seaweed protein with papain (Fitzgerald *et al.* 2012). The peptides IR and IRLIIVLMPILMA had renin IC_{50} values of 9.2 and 3.3 mM, respectively. The peptides HR, YR, and HLP described in this study are the first renin-inhibitory peptides generated and characterized from bovine hemoglobin to date. These peptides were found not only to inhibit renin but also inhibit the enzyme ACE-I. Hydrolysates and peptides presenting both, renin and ACE-I inhibitory properties were recently suggested to provide better antihypertensive properties compared to hydrolysates or peptides that inhibit ACE-I alone (Udenigwe *et al.* 2009).

ACE-I IC_{50} values found following bioassay were calculated as 191.66, 124.12, and 715.48 μM for the peptides HR, YR and HLP, respectively. The peptide HF which did not inhibit renin, showed an ACE-I IC_{50} value of 221.28 μM. The ACE-I-inhibiting activity of the identified peptides was comparable to previously reported ACE-I-inhibiting peptides generated from bovine hemoglobin including TKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHSLL, LDDLPGALSELSDLHAHKLRVDPVNFKLLSHSL, and LLSHSL which inhibited the

activity of ACE-I by half at a concentration of 366, 518, and 1095 μM, respectively (Adje *et al.* 2011). It was lower, however, than the activity of the peptides VVYPWT and LGFPTTKTYFPHF, which were generated from porcine hemoglobin and showed IC_{50} values of 6.02 and 4.92 μM (Yu *et al.* 2006).

Prediction of resistance to gastrointestinal degradation and bitterness

Many biologically active peptides never become clinically useful because of a number of factors including poor stability, low bioavailability and permeability through biological barriers, and low water solubility (Wang *et al.* 1999). The resistance of peptides to gastrointestinal enzyme degradation determines to a large extent their bioavailability (Vermeirssen *et al.* 2004). Computer simulations of proteolysis were carried out on bioactive peptides identified in this study in order to predict their stability to gastrointestinal digestion. Results suggest that the peptides HR, YR, HF, and HLP would be further broken down by the action of the enzymes pepsin, trypsin, and chymotrypsin, found in the gastrointestinal tract. Using *in silico* analysis, the peptides YR and HF were predicted to be cleaved by both, pepsin and chymotrypsin, into the amino acids tyrosine, arginine, histidine, and phenylalanine, respectively. The peptide HR was predicted to be cleaved by the action of chymotrypsin into the amino acid residues histidine and arginine. In addition, the tri-peptide HLP was cleaved by both, chymotrypsin and pepsin, to generate the di-peptides LP and HL. The peptide LP is a known bioactive peptide previously generated from rice bran protein and reported to inhibit DPP-IV *in vitro* (Hatanaka *et al.* 2012). Although *in silico* analysis is a useful tool to predict resistance of a protein or a peptide to enzymatic cleavage, *in vivo* studies in animal models are required in order to assess the bioavailability of a biologically active hydrolysate or peptide.

Moreover, although humans accept bitterness in certain products including coffee, we have a natural preference for sweet and fat tastes (Temussi 2012). Bioactive hydrolysates and peptides usually have a bitter taste and Western consumers do not seem to be willing to compromise taste for health benefits (Grasso *et al.* 2014). A number of methods have been used to predict the bitterness of a peptide. For example, the '*Q* rule' formulated by Ney (1971), quantifies the bitterness of a peptide based on its amino acid composition, whereby a *Q-value* is calculated from the solubility data for each individual amino acids. According to this method, when the *Q*-value of a peptide, with a MW under 6 kDa, exceeds 1400 cal/mol, this peptide will be almost certainly bitter (Ney 1971). The *Q*-values for the peptides HR, YR, HF, and HLP were 625.0, 1525.0, 1500.0, and 1633.3 cal/mol, respectively. Obtained *Q*-values suggest that the peptides YR, HF, and HLP may present bitter taste. However, although Ney's rule can be applied to the majority of known peptides, there are exceptions. For example, Cho *et al.* (2004) characterized for flavor, chemical properties, and hydrophobicity a number of peptides derived from two commercial enzymatic hydrolysates of soy protein. In this study, the hydrophobicity data based on *Q*-values did not support Ney's rule as a predictor of bitterness. Although it's an expensive and time-consuming activity, the ideal route for quantifying bitterness is to use sensory evaluation panels (Spellman *et al.* 2005). Despite the fact that bioactive hydrolysates and peptides usually have a bitter taste, industrial production of food-derived bioactive peptides does exist and include the tri-peptides IPP and VPP which are found in the food product Calpis® sour milk (Calpis Food Industry Co., Ltd., Tokyo, Japan).

CONCLUSION

This study demonstrates, for the first time, the potential of bovine hemoglobin as source of hydrolysates with renin as well as DPP-IV inhibitory properties. In addition, the renin inhibitory peptides identified herein are the first renin inhibitors identified from bovine hemoglobin. One novel ACE-I inhibitor, the bioactive peptide HF, as well as three novel multifunctional peptides which inhibited ACE-I and renin were identified. These multifunctional peptides were identified as HR, YR, and HLP and presented IC_{50} values comparable to previously reported biologically active peptides from animal and plant sources. The *in vitro* ACE-I and renin inhibitory activity of a peptide is not enough to evaluate their activity in the human body after ingestion, and *in vivo* studies should be carried out in order to assess the bioavailability of the generated hydrolysates and peptides after ingestion. Potential toxicity, allergenicity should as well be considered before inclusion of the generated hydrolysates and peptides into a food product and before human consumption.

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TABLE 1.

PEPTIDES IDENTIFIED FROM THE GENERATED PAPAIN HYDROLYSATE OF BOVINE HEMOGLOBIN BY HPLC-MS/MS.

Amino acid sequence	Parent protein	Obtained mass charge (Da)	Calculated mass (Da)	PeptideRanker score*	Similar ACE-I-inhibiting peptides**	Similar DPP-IV-inhibiting peptides**
AADK	*f*(6-8) HBA	403.28	403.20	0.11	N/A	AA, AAATP, AAAAG
NFK	*f*(98-100) HBA; *f*(101-103) HBB	407.28	407.21	0.58	PGTAVFK	IPAVFK
HGAK	*f*(59-62) HBA	411.28	411.22	0.20	FQKVVAK, IAK	N/A
KLR	*f*(91-93) HBA	415.28	415.29	0.29	LDAQSAPLR	N/A
ALTK	*f*(66-69) HBA	431.28	431.27	0.10	RMLGNTPTK, RMLGQTPTK	AL, ALGGA, ALAV, MWPTSSSTK
HAAE	*f*(21-24) HBA	426.28	426.18	0.08	IAE	N/A
ERM	*f*(31-33) HBA	434.28	434.19	0.34	WRM	WRM
PVLQ	*f*(123-126) HBB	455.28	455.27	0.18	GKKVLQ, LQ	N/A
NNPK	*f*(55-58) HBB	471.28	471.24	0.22	MKPWIQPK, EMPFPK, VPK, AMKPWIQPK, EMPFPK, MNPPK, PPK, VPAAPPK	N/A
GHAAE	f*(20-24) HBA*	483.28	483.20	0.12	IAE	N/A
HF	*f*(46-47) HBA	302.17	302.13	0.95	GHF	N/A
HR	*f*(142-143) HBB	311.17	311.17	0.33	N/A	N/A
LKG	*f*(90-92) HBB	316.27	316.21	0.30	KG, MKG	VFRELKDLKG, LKPTPEGD, LKPTPEGDLEIL, VFRELKDLKG
AVH	*f*(121-123) HBA	325.17	325.17	0.09	N/A	N/A
YR	*f*(141-142) HBA	337.17	337.17	0.53	N/A	N/A
HGK	*f*(62-64) HBB	340.17	340.18	0.23	FGK, FFVAPFPFEVFGK, FPFEVFGK, GK, FPEVFGK, FFVAPFPEVFGK	N/A
AHK	*f*(89-91)HBA	354.17	354.20	0.13	HLAHK, VGINYWLAHK, HK	LAHKALCSEK

LSH	*f*(49-51; 102-104) HBA	355.17	355.18	0.18	YIPIQYVLSR	N/A
LGR	*f*(27-29) HBB	344.27	344.21	0.62	GR	N/A
LAR	*f*(113-115) HBB	358.27	358.23	0.33	ALKAWSVAR, YLYEIAR, AR	LAPSLPGKPKPD
LTK	*f*(67-69) HBA	360.27	360.23	0.07	RMLGNTPTK, RMLGQTPTK	EQLTKCEVFR, MWPTSSSTK
HLP	*f*(113-115) HBA	365.17	365.20	0.58	PAVVLP, NILP, PQNILP, ILP, VLP, PLP, LPLP, LLP, GLP	HL
VGGH	f(18-21) HBA	368.14	368.18	0.31	GH	VGL
HLDDLK	*f*(86-91) HBB	739.32	739.38	0.28	FALPQYLK	HL, VGGSDLQALK
EFT	*f*(120-122)	395.28	395.16	0.24	QAFT, SSIQSQPQAFT, VHSSIQSQPQAFT	N/A
SELSDLHAHK	*f*(82-91) HBA	1048.48	1048.53	0.18	HLAHK, VGINYWLAHK, HK	N/A
LSDLHAHKLR	*f*(84-93)HBA	1188.61	1188.67	0.40	LDAQSAPLR	N/A
ELSDLHAHKLR	*f*(83-93) HBA	1317.67	1317.71	0.37	LDAQSAPLR	N/A
SELSDLHAHKLR	*f*(82-93) HBA	1404.70	1404.74	0.41	LDAQSAPLR	N/A
HGSA	*f*(51-54) HBA	370.18	370.16	0.22	CMENSA	N/A

* Data accessed from PeptideRanker, available at http://bioware.ucd.ie/ on April 2015

** Data accessed from BIOPEP, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep on April 2015

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