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Identification of bioactive peptides from a papain hydrolysate of bovine serum albumin and assessment of an antihypertensive effect in spontaneously hypertensive rats

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ABSTRACT

Inhibition of angiotensin-I-converting enzyme (ACE-I), renin, and dipeptidyl peptidase-IV (DPP-IV) plays a key role in the treatment of hypertension and type-2 diabetes. The aim of this study was to isolate and characterize novel ACE-I, renin, and DPP-IV inhibitory peptides from a papain hydrolysate of bovine serum albumin (BSA). BSA was obtained from whole bovine blood and hydrolyzed with the food-grade enzyme papain. The generated hydrolysate was further purified using ultrafiltration and high performance liquid chromatography (HPLC), and a number of novel bioactive peptides were identified using de novo peptide sequencing. These included SLR, YY, ER, and FR which inhibited the activity of the enzyme ACE-I by half at a concentration of 0.17 ± 0.02, 0.18 ± 0.04, 0.27 ± 0.01, and 0.42 ± 0.02 mM, respectively. In addition, the 1 kDa fraction of the papain hydrolysate was assessed for antihypertensive activity in vivo using spontaneously hypertensive rats (SHRs) and reduced systolic blood pressure over a 24 h period when compared with the control ($p < 0.001$). Results demonstrated the potential of bovine serum albumin as a source of bioactive peptides with health-promoting properties and potential for use as functional food ingredients.

1. Introduction

The term metabolic syndrome describes a combination of medical disorders which increase the risk of developing cardiovascular disease, namely diabetes, obesity, hypertension, lipid disorders, and alterations in the thrombotic potential related to insulin resistance (Fulop, Tessier, & Carpentier, 2006). Inhibition of dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) is one of the new approaches in the management of type-2 diabetes. 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, 2003). In addition, a recent study carried out by Koibuchi et al. (2014) suggested that administration of linagliptin, a known DPP-IV inhibitor, after onset of hypertension and cardiac hyperthrophy limited cardiovascular injury, fibrosis, vascular dysfunction, and inflammation in salt-sensitive hypertensive rats. These beneficial effects were associated by the authors with the attenuation of oxidative stress and cardiac angiotensin-I-converting enzyme (ACE-I; EC 3.4.15.1). Meanwhile, high blood pressure results from a combination of genetic and environmental factors but contributing factors include: (i) increased sympathetic nervous system activity, (ii) increased levels of sodium intake, (iii) altered renin (EC 3.4.23.15) secretion, (iv) increased ACE-I activity and several others (Majumder & Wu, 2014). Inhibition of renin and ACE-I plays a key role in the treatment of high blood pressure.

Bioactive peptides are short sequences of amino acids that are inactive within the sequence of the parent protein but have a positive impact on systems of the body once released (Korhonen & Pihlanto, 2006). Functional foods may be used by consumers for preventative healthcare and bioactive peptides from food sources can prevent the development of high blood pressure and diabetes by inhibiting the enzymes ACE-I, renin, and DPP-IV. Indeed, there are a number of

Abbreviations: DPP-IV, dipeptidyl peptidase-IV; GLP-1, glucagon-like peptide-1; GIP, gastric-inhibitory peptide; ACE-I, angiotensin-I-converting enzyme; RAAS, renin-angiotensin-aldosterone system; EFSA, European Food Safety Authority; BSA, bovine serum albumin; MWCO, molecular weight cut-off; HPLC, high performance liquid chromatography; SHRs, spontaneously hypertensive rats; FA, formic acid; DMSO, dimethyl sulfoxide; ACN, acetonitrile; NUFH, non-ultrafiltrated hydrolysate; 1UFH, hydrolysate ultrafiltrated with a 1 kDa MWCO membrane; 3UFH, hydrolysate ultrafiltrated with a 3 kDa MWCO membrane; 10UFH, hydrolysate ultrafiltrated with a 10 kDa MWCO membrane; DDA, data-dependent acquisition; MW, molecular weight; MW-SPPS, microwave-assisted solid phase peptide synthesis; MALDI, matrix assisted laser desorption/ionization; TOF, time of flight; MS, mass spectrometry; S.D., standard deviation; PBS, phosphate buffer saline; SBP, systolic blood pressure; QSAR, quantitative structure–activity relationship; SEM, standard error of the mean.

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functional foods containing bioactive hydrolysates and peptides including Calpis® sour milk, containing the peptides IPP and VPP, and Valtyron® which contains the di-peptide VY. However, although these products were repeatedly found to have blood pressure lowering effects when orally administered to hypertensive patients, the European Food Safety Authority (EFSA) concluded that a cause and effect relationship between the consumption of IPP and VPP and maintenance of normal blood pressure has not yet been established (EFSA, 2011). Bioactive peptides with ACE-I-, renin-, and DPP-IV-inhibiting properties have been generated from a wide variety of natural sources previously including animal and plant sources (Jamdar et al., 2010; Mora & Hayes, 2015). Although blood is known as an excellent source of bioactive peptides, few biologically active peptides to date have been generated from bovine serum albumin (BSA) (Bah, Bekhit, Carne, & McConnell, 2013; Lafarga & Hayes, 2014). BSA is a single polypeptide chain with a long history of use in pharmaceutical applications (Xie et al., 2012), research (Tanaka et al., 2001), and in the food industry (Ofori & Hsieh, 2013). BSA is commercially available in numerous food additives including Plasma Powder FG (Sonac BV, Netherlands) and Prolican 70 (Lican Functional Protein Source, Chile). The use of bioactive peptides as functional ingredients and pharmaceutical agents has gained much interest in recent years, and may not only have a role in improving public health but may also provide a commercial opportunity for many companies.

The aim of this work was to generate a bioactive peptide containing hydrolysate using the enzyme papain (EC 3.4.22.2) from BSA, to identify bioactive peptides and to assess the potential of this hydrolysate to inhibit DPP-IV, ACE-I, and renin. The generated hydrolysate was enriched using 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 synthesized to confirm their DPP-IV, renin, and ACE-I inhibitory properties in vitro. The concentration of peptide required to inhibit the activity of ACE-I, DPP-IV, and renin by half (IC_{50}) was calculated for active peptides. Moreover, the 1 kDa fraction of the generated papain hydrolysate of BSA was assessed for antihypertensive effects in vivo in spontaneously hypertensive rats (SHRs).

2. Materials and methods

2.1. Materials and reagents

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

2.2. Blood collection and fractionation procedure

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 females, 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 °C and handled carefully to minimize hemolysis.

Whole blood cells were separated from plasma by centrifugation at 4 °C and 10,000 ×*g* for 10 min using a Sigma 6 K10 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Plasma was kept at 4 °C, filtered through glass wool and freeze-dried using an industrial scale freeze-drier, FD 80 model (Cuddon Engineering, Marlborough, New Zealand). The temperature was maintained at less than 35 °C during the freeze-drying process. BSA was obtained from the freeze dried extract by precipitation following a previously described method (Fig. 1) (Álvarez, Bances, Rendueles, & Díaz, 2009). Briefly, dehydrated plasma proteins were resuspended in MilliQ water to a final concentration of 35 g/L. The pH was adjusted using 0.1 M HCl and ethanol was added as the fractionation agent. Bovine serum was obtained by precipitation of fibrinogen by addition of ethanol to plasma at a final concentration of 8% (v/v) and adjustment of the pH to 7.2. Ethanol was added drop by drop and the process was carried out in an ice bath to minimize protein denaturation. A protein fraction rich in BSA was separated by centrifugation at 4 °C and 10,000 ×*g* for 5 min, re-suspended in MilliQ water, frozen, and freeze-dried.

The total protein content was determined in duplicate using a LECO FP628 Protein analyzer (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.

2.3. In silico analysis

The amino acid sequence of BSA (UniProt ID: ALBU_BOVIN|UniProt AC: P02769) was accessed from the UniProt database at http://www.uniprot.org/. The predicted cleavage sites of BSA using the enzymes papain, pepsin (EC 3.4.23.1), bromelain (EC 3.4.22.4), ficain (EC 3.4.22.3) and thermolysin (EC 3.4.24.27) were calculated using the "Enzyme(s) action" option in BIOPEP, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). These enzymes were selected based on the availability of cleavage information in BIOPEP and their documented use in previous hydrolysis studies. BIOPEP was also used to compare the peptides generated in silico and the peptides identified by de novo peptide sequencing with previously described ACE-I-, DPP-IV- and renin-inhibiting bioactive peptides in its database. PeptideRanker, available at http://bioware.ucd.ie, was used to predict the bioactivity of the identified peptides (Mooney, Haslam, Pollastri, & Shields, 2012).

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), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1) (Gasteiger et al., 2003).

2.4. Enzymatic hydrolysis

Papain hydrolysates of BSA were prepared in triplicate using a BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Cambridge, England, UK) with agitation, temperature, and pH control. A substrate solution was prepared by resuspending the dried BSA in MilliQ purified water at a concentration of 10 g/L at a total volume of 500 mL. Temperature and pH conditions were adjusted to 65 °C and pH 6.5 respectively. Agitation was maintained at a constant of 350 rpm. 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 °C for 10 min in a water bath.

Four protein fractions were generated from the whole hydrolysate of BSA. Fraction one was termed as the non-ultrafiltered hydrolysate (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). The fractions obtained using 1, 3, and 10 kDa MWCO membranes were labelled as 1UFH, 3UFH, and 10UFH, respectively. All fractions were frozen, freeze-dried and stored at −20 °C until further use.

2.5. De novo sequencing

The 1UFH fraction was resuspended 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 analyzed 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 — 100 mm × 2.1 mm, 3 μm 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 °C. The gradient programme 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 LC–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 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™ software package.

2.6. Chemical synthesis

Peptides were synthesized using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty CEM microwave peptide synthesizer (Mathews, North Carolina, USA). Peptides were synthesized on H-Ala-HMPB-ChemMatrix and H-Ile-HMPB-ChemMatrix resins (PCAS Biomatrix Inc., Quebec, Canada) and purified using RP-HPLC on a Semi Preparative Jupiter Proteo (4u, 90 A) column (Phenomenex, Cheshire, UK). Fractions containing the desired molecular mass were identified using matrix assisted laser deionization-time of flight-mass spectrometry (MALDI-TOF-MS) and were pooled and lyophilized on a Genevac HT 4× lyophilizer (Genevac Ltd., Ipswich, UK).

2.7. Renin inhibition assay

This assay was carried out using a renin inhibitor screening assay kit 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. 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. The known renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe was used as a positive control and renin IC_{50} values were determined in triplicate for active peptides and hydrolysates by plotting the percentage of renin inhibition as a function of the concentration of test compound.

2.8. ACE-I inhibition assay

This assay was carried out using an ACE-I inhibitor assay kit 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. The known ACE-I inhibitor captopril was used as a positive control at a concentration of 1 mg/mL. 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.

2.9. DPP-IV inhibition assay

This assay was carried out using a DPP-IV inhibitor screening assay kit in accordance with the manufacturers' instructions. All hydrolysates were assayed in triplicate and means and S.D. were calculated. The known DPP-IV inhibitor IPI was used as a positive control. 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. DPP-IV IC_{50} values were determined for active hydrolysates by plotting the percentage of inhibition as a function of the concentration of test compound.

2.10. Assessment of the antihypertensive effect in vivo

All in vivo experiments were performed according to protocols approved by the University of Manitoba Animal Care Protocol and Management Review Committee in accordance with the Canadian Council on Animal Care Regulations. Adult SHRs weighing between 275 and 300 g were kept under a 12 h day/night cycle at 21 °C and fed with a standard chow diet and water ad libutum. The SHRs were divided into three groups of four rats each and administered the following treatment: (a) 1UFH fraction of the generated papain hydrolysate of BSA dissolved in phosphate buffer saline (PBS; pH 7.2) at 200 mg protein/kg body weight, (b) the positive control (captopril) dissolved in PBS at 10 mg/kg body weight, and (c) PBS only (negative control). Each group received 1 mL dose of each treatment via oral gavage. Before blood pressure was determined, rats were first anesthetized in a chamber at 40 °C with 4% isofluorane for 4 min to avoid stress-related blood pressure effects. The systolic blood pressure (SBP) of the SHRs was measured at 0, 2, 4, 6, 8, and 24 h by the tail cuff method while the rats were in an unconscious state using the Mouse Rat Tail Cuff Blood Pressure System (IITC Life Sciences, Woodland Hills, CA, USA). The change in SBP was determined by subtracting the SBP at time *n* (where *n* was equal to 2, 4, 6, 8, and 24 h) from the SBP at time 0.

2.11. Statistical analysis

All tests were replicated three times and mean values and S.D. were calculated. All statistical analyses were performed using SPSS for Windows v. 18.0. Normality of the data and equality of variances were tested using the Kolmogorov–Smirnov and Levene tests, respectively. Repeated measures general linear model was used to test differences in SBP between treatments with the effects of time, treatment, and time * treatment interaction included in the model, and post-hoc HSD Tukey tests were used to check the differences. The criterion for statistical significance was $p < 0.05$.

3. Results and discussion

BSA is the main constituent of blood plasma and represents approximately 3.61% of the total content of whole blood (Bah et al., 2013). The total protein content of the generated fraction was 89.0 ± 0.4%, which is comparable with the protein content of other resources for peptide generation including peanut (Jamdar et al., 2010) and bovine fibrinogen (Lafarga, Rai, O'Connor, & Hayes, 2015). In addition, moisture and ash contents of the isolated BSA were calculated as 1.08 ± 0.01 and 0.60 ± 0.03%, respectively.

3.1. In silico analysis

The use of in silico methods was previously shown to be efficient for predicting the release of bioactive peptides from known protein sequences and in the selection of enzymes and proteins for the generation of bioactive peptides (Lacroix & Li-Chan, 2012; Lafarga, O'Connor, & Hayes, 2014). A number of enzymes were trialled using in silico analysis

in order to generate peptides from BSA and to select a suitable enzyme to generate a hydrolysate rich in bioactive peptides with potential to inhibit ACE-I, renin, and DPP-IV. Enzyme selection was based on the availability of cleavage information in BIOPEP. A total of 24, 2, 18, 8, and 24 previously reported ACE-I-inhibiting bioactive peptides were generated in silico using the enzymes papain, pepsin, bromelain, thermolysin, and ficain, respectively (data accessed from BIOPEP in January 2015). These included the ACE-I inhibitors LY and FY corresponding to *f*(162–163) and *f*(172–173) of BSA, respectively. These peptides were previously reported to inhibit ACE-I and were generated by hydrolysis of sardine muscle (Matsufuji et al., 1994) and maize protein (Yano, Suzuki, & Funatsu, 1996), respectively. In this study, no renin inhibitors were generated in silico using the selected enzymes. However, there are limited reports of renin inhibitors in the literature to date. Furthermore, a number of previously described DPP-IV-inhibiting peptides, available in BIOPEP, were generated using papain (25) and bromelain (7). Moreover, papain is a food-grade enzyme which was also used previously for the generation of a number of ACE-I and renin inhibitors as well as antioxidant peptides (Di Bernardini et al., 2012; He et al., 2013; Lafarga et al., 2015).

3.2. ACE-I- and renin-inhibitory activities in vitro

Fig. 2 shows the in vitro ACE-I- and renin-inhibitory activity of the generated hydrolysates. When tested at a concentration of 1 mg/mL, fractions 1UFH and 3UFH inhibited ACE-I by over 70%. The ACE-I IC_{50} values were calculated for fractions 1UFH, 3UFH, and 10UFH, and were 0.104 ± 0.001, 0.054 ± 0.007, and 0.150 ± 0.013 mg/mL, respectively. Enrichment using MWCO led to an increase in the ACE-I-inhibitory activity compared with the NUFH ($p < 0.05$). A significant difference was also observed between the ACE-I inhibitory activity of fractions 1UFH and 10UFH ($p < 0.05$). It is well documented that peptidic ACE-I inhibitors usually consist of short amino acid sequences (Wu, Aluko, & Nakai, 2006). High MW peptides in the NUFH and in the 10UFH fractions may be responsible for the observed decrease in ACE-I-inhibitory activity. However, although a similar trend was observed, no significant differences were detected between the 1UFH and the 3UFH fractions and observed inhibition may be due to the collective effect of these peptides. Sequential MWCO filtration of blood protein hydrolysates led to increased bioactivity previously (Hyun & Shin, 2000; Lafarga et al., 2015). Results obtained herein are similar to those

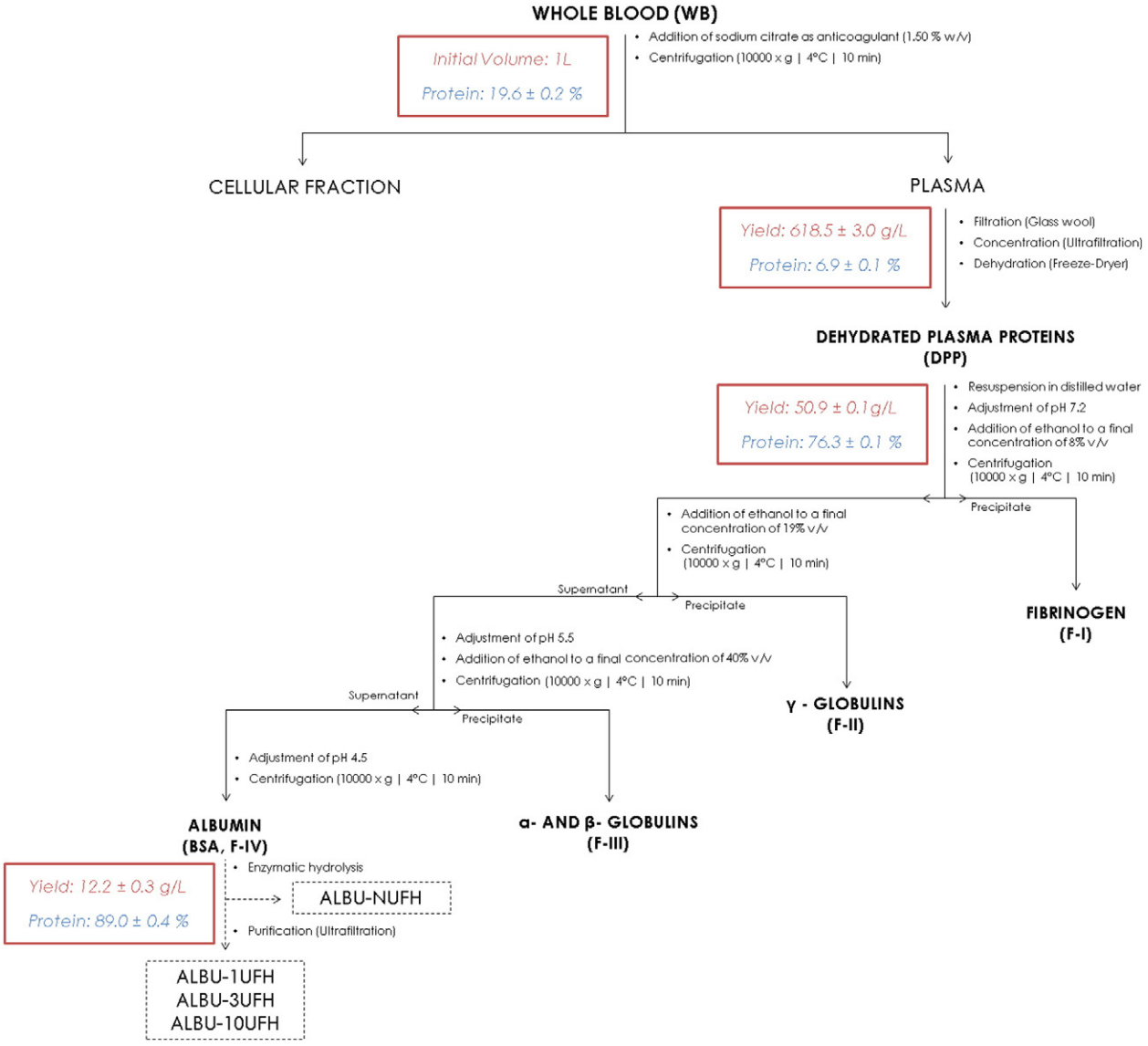

Fig. 1. Schematic representation of the procedure followed for the generation of bioactive peptides from BSA. BSA was obtained from bovine serum by cold ethanol precipitation, frozen, and freeze-dried. The isolated protein fraction was hydrolysed using papain and four sub-fractions were generated by MWCO filtration.

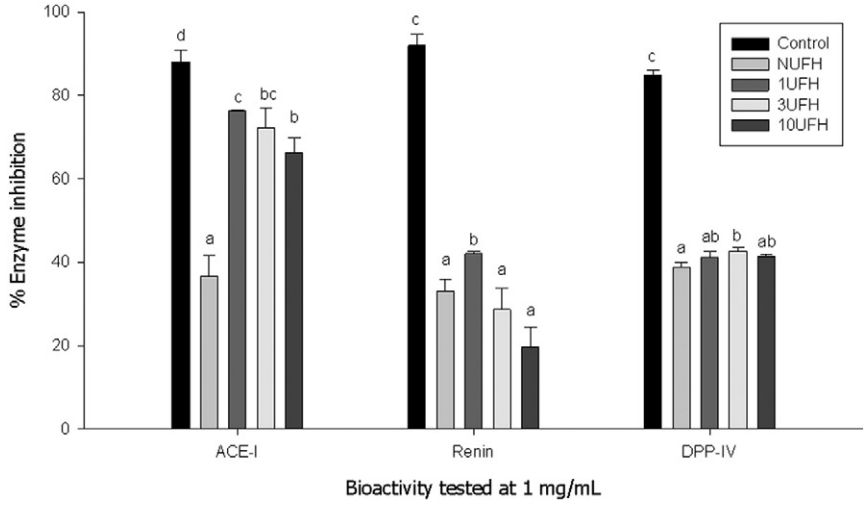

Fig. 2. ACE-I-, renin- and DPP-IV-inhibitory properties of the papain hydrolysates in vitro. ACE-I-, renin-, and DPP-IV-inhibitory activity of the papain hydrolysates of BSA when tested in vitro at a sample concentration of 1 mg/mL. Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, IPI, and captopril were used as positive controls at a concentration of 1 mg/mL for renin, DPP-IV, and ACE-I inhibition, respectively. Enzyme inhibition is expressed as per cent inhibition and the values represent the means of three independent experiments ± standard error of the mean (SEM). For each bioactivity, bars with different letters have mean values that are significantly different ($p < 0.05$).

obtained previously from hydrolysates of rapeseed (He et al., 2013), peanut (Jamdar et al., 2010), and kidney bean (Mundi & Aluko, 2014). Results obtained in this study also compared favourably with previous studies where hydrolysates from blood proteins were generated. For example, Hyun and Shin (2000) generated hydrolysates of whole bovine plasma and plasma proteins with different commercially available enzymes. In this study, the most active fraction, the 1 kDa fraction generated from an Alcalase® hydrolysate of BSA, presented an IC_{50} value of 0.12 mg/mL.

The renin-inhibitory activity of the enriched papain hydrolysates was measured at a concentration of 1 mg/mL. 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 renin inhibition (Mundi & Aluko, 2014). However, purification by ultrafiltration led to an increase in the renin-inhibiting activity of 1UFH compared with NUFH ($p < 0.05$). These results are consistent with the fact that di-peptides were previously suggested as the most effective for renin inhibition (Li & Aluko, 2010). The renin-inhibitory activity was lower compared with that of ACE-I. At a concentration of 1 mg/mL, the fraction 1UFH was the most active and was found to inhibit renin by 42.10 ± 0.26 with an IC_{50} value of 1.182 ± 0.028 mg/mL. Few renin-inhibitory hydrolysates have been generated to date. These include a papain hydrolysate of bovine fibrinogen which inhibited renin by 32.09 ± 1.93% when tested at a concentration of 1 mg/mL (Lafarga et al., 2015). Similar results were obtained from Alcalase® hydrolysates of kidney bean protein, which inhibited renin by 20–40% at a concentration of 1 mg/mL (Mundi & Aluko, 2014), to enzymatic hydrolysates of flaxseed protein with renin IC_{50} values ranging 1.22–2.81 mg/mL (Udenigwe, Lin, Hou, & Aluko, 2009), and to a papain hydrolysate of seaweed protein which inhibited renin by 41.89% when tested at a concentration of 1 mg/mL (Fitzgerald et al., 2012).

3.3. In vitro DPP-IV-inhibitory activity

The in vitro DPP-IV-inhibitory activity of the papain hydrolysate of BSA, the 1UFH, 3UFH and 10UFH fractions are shown in Fig. 2. DPP-IV IC_{50} values were calculated as 1.124 ± 0.015, 1.060 ± 0.011, and 1.047 ± 0.022 mg/mL for 1UFH, 3UFH, and 10UFH, respectively. Interestingly, although a slight increase in the DPP-IV-inhibiting activity of the 3UFH compared with the NUFH was observed ($p < 0.005$), no significant variations were observed between NUFH, 1UFH and 10UFH. This contrasts with previous studies where the removal of higher MW fractions led to an increase in the DPP-IV-inhibitory activity in vitro (Velarde-Salcedo et al., 2013). It is possible that some of the peptides with higher MW generated during enzymatic hydrolysis of BSA have structural features recognized by DPP-IV and act as substrates for the enzyme (Lacroix & Li-Chan, 2012). The hydrolysates generated herein presented lower DPP-IV IC_{50} values than those obtained previously from 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 Flavourzyme® hydrolysates of Atlantic salmon gelatine which displayed an IC_{50} value of 1.35 mg/mL (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012). The DPP-IV-inhibiting potential of the papain hydrolysates generated in this study also compared favourably with that of a trypsin hydrolysate of whey proteins which previously reported 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, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013).

3.4. Peptide identification by LC–MS/MS

The resulting peptide patterns contained in the 1UFH fraction are shown in Fig. 3. The identification of peptides was carried out using the Biolynx™ peptide sequencing software for each MS/MS spectral data generated via DDA. A total of five di-peptides, eight tri-peptides, and five tetra-peptides were identified. The MS/MS ESI^+ spectra for de novo sequence determination of the di-peptide ER, corresponding to *f*(121–122), *f*(231–232) and *f*(467–468) of BSA, and the tri-peptide GPK, corresponding to *f*(595–597) of BSA is shown in Fig. 3. Table 1 lists the amino acid sequences of identified peptides, their position within the parent protein, and the observed peptide masses as well as their calculated masses. Identified peptides were compared with previously reported DPP-IV, ACE-I, and renin inhibitory peptide sequences available in the database BIOPEP (Minkiewicz et al., 2008). Sequence similarities were observed between the peptides identified in this study and previously reported ACE-I and DPP-IV inhibitors available in BIOPEP, shown in Table 1 from other protein sources including animal, dairy, and vegetable proteins. Peptides identified in this study that had similarities to those found in BIOPEP included the ACE-I-inhibiting peptides LVL, which was previously generated from porcine plasma and showed IC_{50} values of 4.2, 0.6 and 0.9 μg/mL for ACE-I isolated from guinea pig serum, rabbit lung and monkey brain, respectively (Hazato & Kase, 1986).

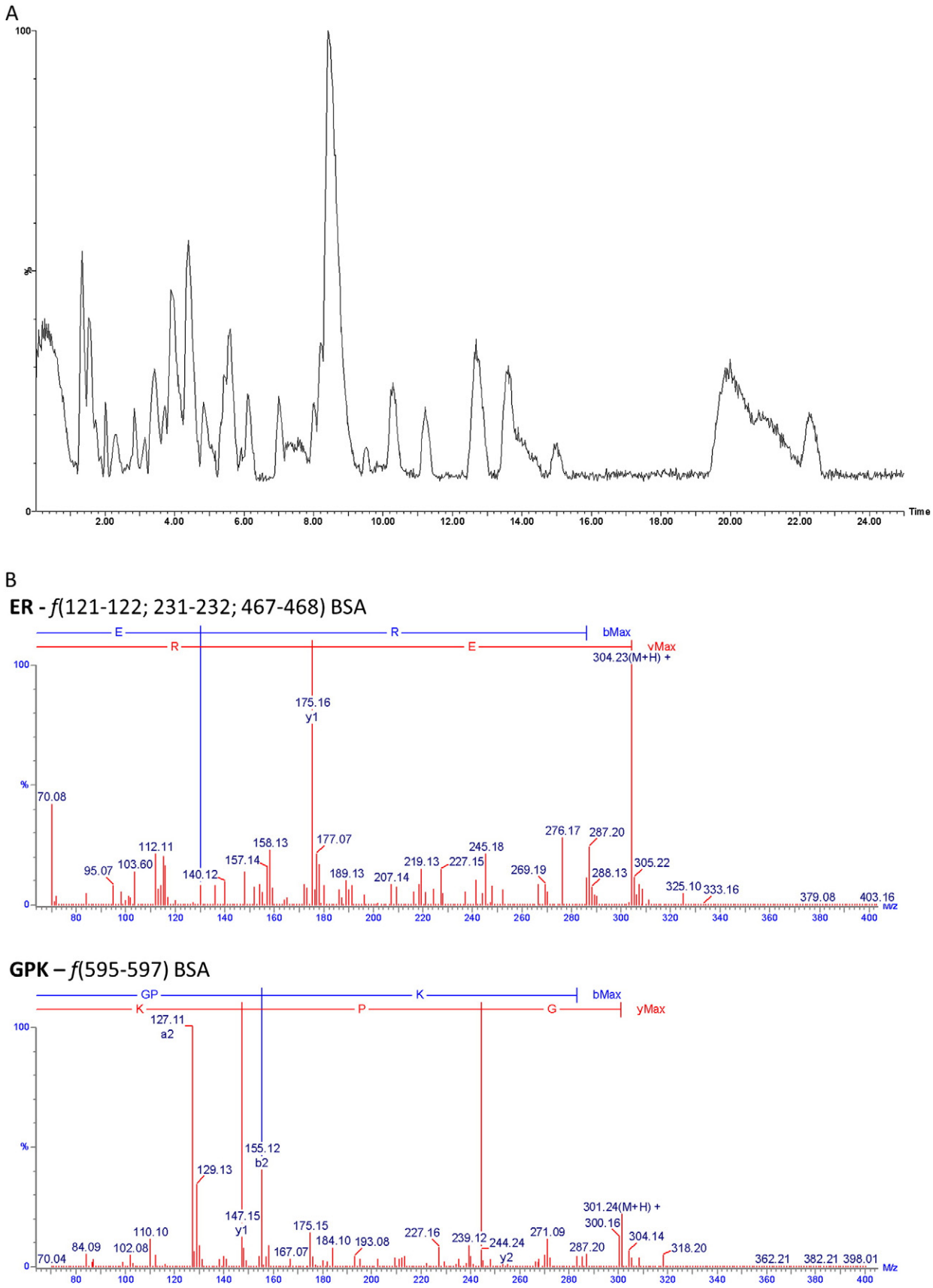

Fig. 3. A. Base peak chromatogram of the 1 kDa fraction of the generated papain hydrolysate. The 1UFH enriched fraction was analyzed on a Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA), coupled to an Alliance 2695 HPLC system (Waters Corp., Milford, MA, USA). The chromatographic separation was carried out using an Atlantis dC18 column – 100 mm × 2.1 mm, 3 μm particle size (Waters Corp., Milford, MA, USA) as previously described. B. ESI-MS/MS spectra for de novo sequence determination of peptides ER and GPK. Peptides KR and GPK were identified by de novo peptide sequencing. Mass spectral data were acquired in the DDA mode. Automated spectra processing and peak list generation was performed using the software ProteinLynx Global Server v2.4 (Waters Corporation, Milford, MA, USA).

Table 1
Peptides identified using de novo sequencing from a 1kDa fraction of bovine serum albumin hydrolysed with papain.

Amino acid sequence	Parent protein	Obtained mass (Da)	Calculated mass (Da)	Retention time (min)	PeptideRanker score[a]	Similar ACE-I inhibitors[b]	Similar DPP-IV inhibitors[b]
GPK	*f*(595–597) BSA	300.17	300.17	1.69	0.57	MKPWIQPK, EMPFPK, VPK, AMKPWIQPK, EMPFPK, MNPPK, PPK, VPAAPPK	GPA, GPGA, GPGE
FH	*f*(532–533) BSA	302.17	302.13	3.58	0.95	RFH	LPEWVCTTFH, FHTSGYDTQA
ER	*f*(121–122; 231–232; 467–468) BSA	303.17	303.15	3.60	0.07	GKKIATYQER, KKIATYQER	N/A
MR	*f*(208–209) BSA	305.17	305.15	3.45	0.85	N/A	MRPVDPNIE
FR	*f*(22–23) BSA	321.17	321.18	4.76	0.99	FR, WTFR, AFKDEDTEEVPFR	EQLTKCEVFR, VFRELKDLKG
VPK	*f*(521–523) BSA	342.27	342.22	3.14	0.17	MKPWIQPK, EMPFPK, VPK, AMKPWIQPK, EMPFPK, MNPPK, PPK, VPAAPPK	VPITPT, VPPFIQPE
YY	*f*(179–180) BSA	343.87	344.13	0.68	0.48	MYY, QVSLNSGYY, KYY, YKYY, PSGQYY, FFYY	YPYY
LVL	*f*(46–48) BSA	343.17	343.24	7.19	0.17	LVL, YQQPVL, YQEPVL	VLVLDTDYK, LVSGM
VTK	*f*(254–256; 496–498) BSA	346.27	346.22	4.44	0.03	RMLGNTPTK, RMLGQTPTK	MWPTSSSTK
LTK	*f*(261–263) BSA	360.27	360.23	4.85	0.07	RMLGNTPTK, RMLGQTPTK	MWPTSSSTK, EQLTKCEVFR
SLR	*f*(103–105) BSA	374.28	374.22	3.63	0.40	LDAQSAPLR	SL
MEN	*f*(571–573) BSA	392.18	392.13	1.80	0.13	N/A	MEPVDPNIE
SLGK	*f*(452–455) BSA	403.28	403.24	10.96	0.28	FGK, FFVAPFPFEVFGK, FPFEVFGK, GK, FPEVFGK, FFVAPFPEVFGK	SL
TMR	*f*(207–209) BSA	406.28	406.19	3.39	0.41	N/A	N/A
SVAR	*f*(238–241) BSA	431.28	431.24	11.98	0.13	ALKAWSVAR, YLYEIAR	N/A
FVAF	*f*(574–577) BSA	482.18	482.25	1.66	0.89	AF	N/A
VLLR	*f*(368–371) BSA	499.28	499.34	2.81	0.21	LDAQSAPLR	VLGP, VLVLDTDYK

[a] Data accessed from PeptideRanker, available at http://bioware.ucd.ie/ on April 2015.
[b] Data accessed from BIOPEP, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep on April 2015.

3.5. Chemical synthesis and confirmation of bioactivity in vitro

Four peptides were selected for chemical synthesis and for use in in vitro bioassays. All selected peptides were di- and tri-peptides. Di- and tri-peptides, with a proline residue and/or hydroxyproline residues at their C-terminus, 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, Camp, & Verstraete, 2004). Peptides chosen for chemical synthesis were selected based on current knowledge concerning the known attributes of ACE-I, renin, and DPP-IV inhibitors, and the score assigned by PeptideRanker to each peptide as shown in Table 1. PeptideRanker, available at http://bioware.ucd.ie, is a useful in silico tool that may be used to identify from a set of peptides those that are likely to be bioactive (Mooney et al., 2012). In addition, a quantitative structure–activity relationships study (QSAR) carried out previously, suggested that amino acid residues with large bulky-chains as well as hydrophobic side chains such as phenylalanine, tryptophan, and tyrosine as the most effective residues in a di-peptide (Wu et al., 2006). Amino acid residues with small as well as hydrophobic side chains, which include valine, leucine, and isoleucine were suggested for the N-terminal side and hydrophobic residues with high electronic properties such as proline, phenylalanine, and tryptophan were recommended as the amino acids present at the C-terminal end of active tri-peptides (Wu et al., 2006). In addition, di-peptides with hydrophobic residues at the N-terminus and a bulky or aromatic group at the C-terminus were previously observed to be the most effective for renin inhibition (Li & Aluko, 2010). It has been suggested that di- and tri-peptides can inhibit DPP-IV (Lacroix & Li-Chan, 2012), and it is known that DPP-IV acts as a cleaving enzyme with specificity for cleaving Xaa-Pro and Xaa-Ala di-peptides from the N-terminus of polypeptides, where Xaa represents an amino acid residue (De Meester, Lambeir, Proost, & Scharpé, 2003). Several DPP-IV peptide inhibitors described to date contain proline and/or hydrophobic amino acids within their sequence, and substrates bearing residues with bulky side chains such as tryptophan in the P_2 position appear to show enhanced binding (Lacroix & Li-Chan, 2012).

Selected peptides were chemically synthesized by MW-SPPS and tested for bioactivity in vitro at a concentration of 1 mg/mL. The IC_{50} values of active peptides were calculated by plotting the percentage of inhibition as a function of the concentration of test peptide. The peptides SLR, YY, ER, and FR inhibited the activity of the enzyme ACE-I by half at a concentration of 0.17 ± 0.02, 0.18 ± 0.04, 0.270 ± 0.01, and 0.42 ± 0.02 mM, respectively. These values are comparable with previous ACE-I inhibitory di-peptides containing the residue arginine at the C-terminus position including RR, generated by the action of dipeptidyl peptidases purified from porcine skeletal muscle with an IC_{50} value of 0.26 mM (Sentandreu & Toldrá, 2006). The peptide SLR did not only inhibit ACE-I but also the enzyme renin with an IC_{50} value of 7.29 ± 0.16 mM. This value is comparable with previous renin-inhibitory peptides generated from blood proteins such as the di-peptide YR which presented an IC_{50} value of 8.78 mM (Lafarga et al., 2015). In addition, the peptide ER inhibited DPP-IV with an IC_{50} value of 4.48 ± 0.38 mM. However, this value was lower than previously described bioactive di-peptides including WR, WV, and IP with IC_{50} values of 0.02, 0.03, and 0.06 mM (Lan et al., 2015). Although bioavailability of a peptide should be assessed in vivo, the resistance of a peptide to enzymes found in the gastrointestinal tract can be predicted in silico. In this study, the peptide ER was predicted to be resistant to gastrointestinal degradation. To the best of our knowledge, the tri-peptide SLR and the di-peptide ER are the first reported peptide renin and DPP-IV inhibitors generated from BSA.

3.6. In vivo determination of the antihypertensive activity in SHR

The results shown in Fig. 4 represent the short-term changes in SBP of SHRs observed over a 24 h period. SHRs were fed a diet of the generated papain hydrolysate of BSA, a positive control (capropril), and a negative control (saline solution). Differences in SBP were analyzed at each time for the different treatments with respect to the control as shown in Fig. 4. SBP was significantly affected by time ($p < 0.001$), diet ($p < 0.001$), and by the interaction between both factors ($p < 0.001$). Although the protein hydrolysate and the control were fed at different concentrations, both showed a similar trend in lowering the SBP of the SHRs and this was found to be statistically significantly different to the negative control at times 2, 4, 6, and 8 h. After 2 h, a drop of SBP of 14.33 ± 2.19 and 19.50 ± 4.01 mm Hg was observed in the group fed the test hydrolysate and the group fed the positive control, respectively. The maximum decrease in SBP in rats fed with the protein hydrolysate was 32.25 ± 3.62 mm Hg and was observed 8 h after oral administration. After 24 h, the SBP decreased 6.25 ± 3.20 and 18.17 ±

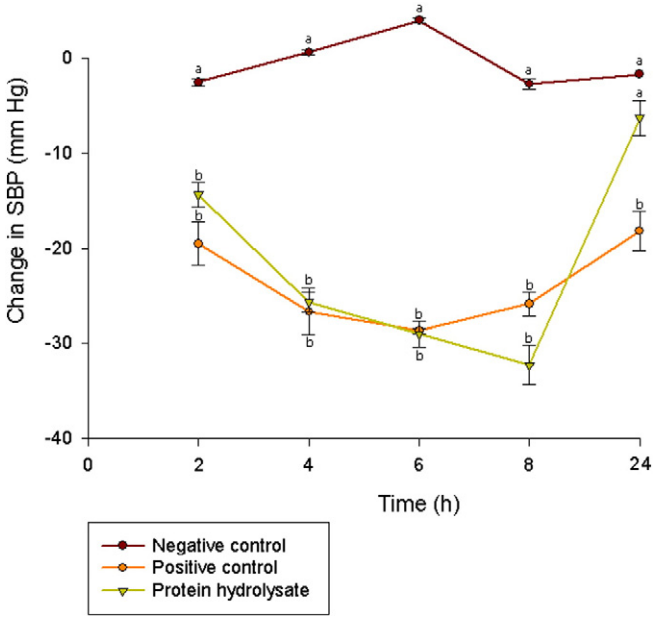

Fig. 4. Short-term antihypertensive effect on SHRs. The change in SBP of SHRs was measured after oral administration of the enriched papain hydrolysate of BSA (200 mg/kg body weight) and was compared with captopril (10 mg/kg body weight), and a negative control (saline solution). Results are expressed as mean ± SEM. Different letters indicate statistically significant differences ($p < 0.05$) in the change in SBP between the different animal groups at different times.

3.54 mm Hg in rats fed with the protein hydrolysate and captopril, respectively. However, no significant differences were observed between the groups fed with the negative control and the protein hydrolysate after 24 h. Results obtained are comparable with those obtained in a previous study where the in vivo antihypertensive effect of an enzymatic hydrolysate of hemp seed protein was studied (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011). In this study, a drop in SBP of 20 and 30 mm Hg was observed 2 and 8 h after oral administration at a dosage of 200 mg/kg body weight. Results obtained in this study were lower than those obtained by Fitzgerald, Aluko, Hossain, Rai, and Hayes (2014) who observed a drop of 34 mm Hg in SBP 24 h after oral administration at a dose of 50 mg/kg body weight. However, the hydrolysate generated herein showed a higher hypotensive effect than an enzymatic hydrolysate of pea protein with drops of 19 and 13 mm Hg after 4 and 8 h of oral administration at a dosage of 200 mg/kg body weight (Li et al., 2011).

4. Conclusions

Although BSA has been in the generation of ACE-I-inhibiting peptides previously, our study reports for the first time the renin- and DPP-IV-inhibiting properties of hydrolysates and peptides derived from BSA. Few renin and DPP-IV inhibitory peptides generated from natural sources such as blood have been reported to date. The generation and identification of novel renin and DPP-IV-inhibiting peptides, such as those identified in this study, would help the development and validation of computer-based models which would, in turn, aid the identification of novel inhibitors. Bioactive hydrolysates and peptides show potential for use as ingredients in the functional foods market. This study demonstrated that bioactive peptides and hydrolysates with antihypertensive effects and in vitro ACE-I-, renin-, and DPP-IV-inhibiting properties can be generated by enzymatic hydrolysis of BSA. Biologically active hydrolysates and peptides generated from natural sources, including the papain hydrolysate of BSA generated in this study, show potential for use in the functional foods market. Future studies include toxicity assessment as well as study of the interactions of the generated hydrolysates with other compounds in a food matrix.

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