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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.