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Identification of novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides from meat proteins using *in silico* analysis

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ABSTRACT

Angiotensin-I-converting enzyme (ACE-I, EC 3.4.15.1), renin (EC 3.4.23.15), and dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) play key roles in the control of hypertension and the development of type-2 diabetes and other diseases associated with metabolic syndrome. The aim of this work was to utilize known *in silico* methodologies, peptide databases and software including ProtParam (http://web.expasy.org/protparam/), Basic Local Alignment Tool (BLAST), ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter/) and BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) to assess the release of potentially bioactive DPP-IV, renin and ACE-I inhibitory peptides from bovine and porcine meat proteins including hemoglobin, collagen and serum albumin. These proteins were chosen as they are found commonly in meat by-products such as bone, blood and low-value meat cuts. In addition, the bioactivities of identified peptides were confirmed using chemical synthesis and *in vitro* bioassays. The concentration of peptide required to inhibit the activity of ACE-I and DPP-IV by 50% was determined for selected, active peptides. Novel ACE-I and DPP-IV inhibitory peptides were identified in this study using both *in silico* analysis and a literature search to streamline enzyme selection for peptide production. These novel peptides included the ACE-I inhibitory tri-peptide Ile-Ile-Tyr and the DPP-IV inhibitory tri-peptide Pro-Pro-Leu corresponding to sequences f (182–184) and f (326–328) of both porcine and bovine serum albumin which can be released following hydrolysis with the enzymes papain and pepsin, respectively. This work demonstrates that meat proteins are a suitable resource for the generation of bioactive peptides and further demonstrates the usefulness of *in silico* methodologies to streamline identification and generation of bioactive peptides.

Introduction

The term metabolic syndrome (MetS) consists of a combination of metabolic disorders which increase the risk of developing cardiovascular diseases (CVDs) and type-2 diabetes (T2D) [12]. CVDs and T2D are a global public health issue. Diabetes mellitus currently affects 170 million people and is expected to affect over 366 million people worldwide by 2030 [46]. Early detection and treatment of high blood pressure (HBP) and T2D can contribute to a reduction in mortality related to the development of MetS [44].

Inhibition of enzymes such as renin (EC 3.4.23.15) and angiotensin-I-converting enzyme (ACE-I, EC 3.4.15.1) within the renin–angiotensin–aldosterone system (RAAS) plays a key role in the treatment of HBP, and this strategy has potential for use in the treatment of T2D, hypercholesterolemia, insulin resistance and other diseases related to MetS [12,15,43]. A new therapeutic strategy for the treatment of T2D is the inhibition of DPP-IV. DPP-IV

Abbreviations: MetS, metabolic syndrome; CVD, cardiovascular disease; T2D, type-2 diabetes; HBP, high blood pressure; 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; BLAST, basic local alignment search tool; GI, gastrointestinal; DMSO, dimethylsulfoxide; UK, United Kingdom; MW, molecular weight; IEP, isoelectric point; SVM, support vector machine; MW-SPPS, microwave-assisted solid phase peptide synthesis; RP-HPLC, reversed-phase-high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; AMC, aminomethylcoumarin; ALBU, serum albumin; HBA, hemoglobin subunit alpha; HBB, hemoglobin subunit beta; MYG, myoglobin; MYH2, myosin-2; ACTS, actin alpha skeletal muscle; CO1A1, collagen alpha-1 (I) chain; CO1A2, collagen alpha-2 (I) chain; QSAR, quantitative structure–activity relationships study.

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degrades and inactivates a number of incretin hormones including glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) which contribute to the enhancement of glucose-induced insulin secretion [11]. Meat and meat products are not ordinarily associated with health benefits and indeed, consumption of red meat products is usually associated with high cholesterol and subsequently, HBP [10]. However, health-promoting bioactive peptides including ACE-I, renin and DPP-IV inhibitors can be naturally generated or generated from meat protein substrates using chemical/enzymatic hydrolysis or microbial fermentation [2,33].

In silico analysis is a useful technique for predicting the release of bioactive peptides from known protein sequences and in the selection of precursor proteins not previously studied as sources of bioactive peptides [8,39]. The aim of this work was to predict the release of ACE-I, renin and DPP-IV inhibitory peptides from proteins commonly found in bovine and porcine by-products using commercially available enzymes and secondly to identify novel bioactive peptide sequences. Five enzymes were selected for use in this work based on a literature search and evidence from databases, including BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), which document the release of bioactive peptides from other resource materials. These enzymes included bromelain, thermolysin, pepsin, ficain and papain [30]. Use of *in silico* analysis was used recently to identify potential sources of antithrombotic, antioxidant, ACE-I and DPP-IV inhibiting peptides from a variety of sources including egg, milk, pea, oat, barley and fish [7,16,18,19,24]. However, these studies, which used *in silico* analysis to predict bioactive peptide production, have not, to date, confirmed the bioactivities of predicted bioactive peptides from meat proteins and have largely, identified known bioactive peptides [7,16,18,19,24,31].

The concentration of peptide required to inhibit the activity of ACE-I and DPP-IV by 50% (IC_{50}) for the chemically synthesized novel ACE-I and DPP-IV inhibitory peptides was calculated. Synthesized peptides were then assessed using *in silico* analysis in order to predict their potential stability during gastrointestinal (GI) digestion and *in silico* analysis was also used to predict the toxicity of active peptides. Future work will include assessment of the absorption and bioavailability of identified peptides using tissue culture and *in vivo* studies. The results obtained in this study demonstrate that *in silico* analysis is a suitable method to assess the potential of meat proteins and selected enzymes to generate bioactive peptides from meat protein sources.

Materials and methods

Materials and reagents

Dimethylsulfoxide (DMSO), the specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, the DPP-IV inhibitor Ile-Pro-Ile 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.

Proteins and enzymes selected for the prediction of bioactive peptides

The bovine and porcine proteins from meat by-product sources used in this work are listed in Table 1. Proteins were selected based on a number of criteria including their documented abundance in common meat processing by-products, the availability of sequence information for the proteins and the amino acid composition. The methodology used in this study to identify bioactive peptides from bovine and porcine protein sources is shown in Fig. 1. The sequence and molecular weight (MW) for each protein were accessed from the UniProt database at http://www.uniprot.org/ [38].

Enzymes used for *in silico* hydrolysis in this study were pepsin (EC 3.4.26.1), papain (EC 3.4.22.2), bromelain (EC 3.4.22.4), ficain (EC 3.4.22.3) and thermolysin (EC 3.4.24.27). The selection of such enzymes was based on the availability of cleavage information in peptide/protein databases such as BIOPEP and their documented ease of use in industry and their use in previous meat hydrolysis studies [1,4,25].

In silico analysis

The amino acid concentration of the selected porcine and bovine proteins was determined using ProtParam (http://web.expasy.org/protparam/), an *in silico* analysis program which computes the physicochemical properties of a peptide or protein from its amino acid sequence [13]. BLAST was used to compare protein sequences and to calculate significant areas of commonality of amino acid sequences of porcine and bovine proteins [3].

The cleavage sites of the enzymes pepsin (pH 1.3) and thermolysin within the protein sequences listed in Table 1 were predicted using PeptideCutter (http://web.expasy.org/peptide_cutter/) [13]. The profiles of potential ACE-I, renin and DPP-IV inhibitory peptides generated using ficain, bromelain and papain, were predicted using BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). Following *in silico* digestion, BIOPEP was also used to compare the generated peptides with previously described bioactive peptides in their database. The theoretical MW and isoelectric point (IEP) of the studied peptides were calculated using the ExPASy Compute PL/MW Tool at http://web.expasy.org/compute_pi/ [13]. A number of potentially novel bioactive peptides were predicted and are shown in Table 2. The potential bioactivities of these peptides were predicted using PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/), and their peptide scores calculated [32].

The toxicity of the peptides generated was predicted using ToxinPred, available at http://www.imtech.res.in/raghava/toxinpred/ [17]. The support vector machine (SVM) based prediction method and the SVM threshold value of 0.0 were chosen for toxicity prediction.

Simulated GI digestion of identified ACE-I and DPP-IV peptides

In silico analysis to determine potential survival of synthesized ACE-I and DPP-IV peptides *in vivo*, was carried out. The most active peptide sequences were assessed for potential cleavage by GI tract enzymes using the program Expasy PeptideCutter (http://ca.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl). The peptides were evaluated against a number of enzymes that are found in the GI tract using *in silico* analysis including pepsin (pH > 1.3 and pH 2.0), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1).

Microwave-assisted solid phase peptide synthesis

Peptides were synthesized using microwave-assisted solid phase peptide synthesis (MW-SPPS) 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) and purified using reversed-phase-high-pressure liquid chromatography (RP-HPLC) on a Semi Preparative Jupiter Proteo (4u, 90A) column (Phenomenex, Cheshire, UK). Fractions containing the