

Contents lists available at [ScienceDirect](https://www.sciencedirect.com)

Current Research in Food Science

journal homepage: www.editorialmanager.com/crfs/

Research Paper

The identification and characterisation of novel bioactive peptides derived from porcine liver

Nicholas A. Pearman^{a,b}, Elena Ronander^c, Alan M. Smith^{b,**}, Gordon A. Morris^{a,*}^a Department of Chemical Sciences, School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH, UK^b Department of Pharmacy, School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH, UK^c Biofac A/S, Englandsvej 350-356, DK-2770, Kastrup, Denmark

ARTICLE INFO

Keywords:

Bioactive peptides

In silico hydrolysis

Antioxidant activity

Oxygen radical absorbance capacity assay

Cellular antioxidant activity assay

ABSTRACT

Bioactive peptides (BAPs) can be derived from a variety of sources; these could be from dietary proteins which are then broken down in the gastrointestinal tract to release BAPs, or they can be isolated from various sources *ex vivo*. Sources include plant-based proteins such as soy, and chickpeas, and animal proteins from waste from the meat industry and from fish skin. Bioinformatics is also a useful approach to assess the peptides released from digests due to the great number of possible sequences that can be isolated from proteins. Therefore, an *in silico* analysis of peptides could potentially lead to a more rapid discovery of BAPs. This article investigates a “crude” liver peptide mixture derived from papain hydrolysis of porcine liver and purified peptides derived from the hydrolysates following HPLC fractionation and *in silico* digestion of the host proteins identified using LC-MS/MS. This allowed the identification of two proteins (cytosol aminopeptidase and haemoglobin subunit alpha) present in the “crude” mixture after LC-MS/MS. *In silico* hydrolysis of these proteins identified that several peptides were predicted to be both present in the crude mixture using the BIOPEP database and to have potential bioactivity using the Peptide Ranker tool. Peptides (FWG, MFLG and SDPPLVFG) with the greatest potential bioactivity and which had not previously been reported in the literature were then synthesised. The results indicated that the predicted bioactivity of the synthetic peptides would likely include antioxidant activity. FWG and MFLG derived from the *in silico* papain hydrolysis of cytosol aminopeptidase showed activity better or comparable to Trolox in the Oxygen Radical Absorbance Capacity (ORAC) assay. The use of these *in silico* tools, alongside a robust range of biochemical assays which cover a wider range of bioactivities would be a way of improving the discovery of novel bioactive peptides.

1. Introduction

Recent trends in pharmaceutical development have seen an increased emphasis on large molecules, or biologics with regards to novel therapeutics. These biologics include proteins, monoclonal antibodies, and peptides (Prueksaritanont and Tang, 2012). Peptides are short (2–50 residues) sequences of amino acids (Banga, 2015). Generally, amino acid sequences that are under 10 residues long are termed oligopeptides; sequences over 50 residues long are regarded as proteins. Many peptide sequences that are bioactive are found in larger proteins, but in an inactive state (Capriotti et al., 2015).

Bioactive peptides (BAPs) can be derived from a variety of sources; these could be from dietary proteins which are then broken down in the gastrointestinal tract to release BAPs, or they can be isolated from various

sources *ex vivo*. Sources include plant-based proteins such as soy, and chickpeas, and animal proteins from waste from the meat industry and from fish skin (Beermann et al., 2009; Capriotti et al., 2015; Di Bernardini, Harnedy, Bolton, Kerry, O'Neill, Mullen, et al., 2011; Fu et al., 2017; Himaya et al., 2012; Howell and Kasase, 2010; Lassoued et al., 2015; Z. Xue, Gao, Zhang, Yu, Wang and Kou, 2012; Zhaohui Xue, Wen, Zhai, Yu, Li, Yu, et al., 2015; Yu et al., 2017) which enables the development of a circular economy (Bechaux et al., 2020). For example, there is only a limited market for porcine liver, however this waste material has great potential in the production of protein hydrolysates (Maluf et al., 2020). Many studies have been carried out on BAPs and their uses. Areas of greatest interest however, are where BAPs can be used in either treatment of a condition or aid of a biological process. Bioactivities that are of particular interest to this study are antioxidant activities.

* Corresponding author.

** Corresponding author.

E-mail addresses: a.m.smith@hud.ac.uk (A.M. Smith), g.morris@hud.ac.uk (G.A. Morris).

There is a significant amount of literature on the benefits of bioactive peptides when used as dietary supplements (Wolfe and Liu, 2007). Much of this focuses on BAPs derived from milk and soy proteins (Ahmed et al., 2015; Corrêa et al., 2014). These studies show that BAPs are present in proteins found in foods, and through hydrolysis in the gastrointestinal tract, they are released from this non-functional state. BAPs are released from precursor proteins by enzymes found in the gastrointestinal tract. Releasing BAPs through the hydrolysis of larger proteins using gastric proteases not only leads to peptides of various lengths, but also different structural conformations, which can also be related to activity (Himaya et al., 2012). Therefore, by using different proteases, proteins can be cleaved at different sites producing a large number of peptide fragments, which have varying levels of bioactivity (Jang and Lee, 2005).

Crude protein digests can then be purified using, for example, fast protein liquid chromatography (FPLC), and preparative reverse phase high performance liquid chromatography (RP-HPLC); the fractions collected can then be assayed for bioactive properties. Fractions that show activity can then be further purified using an analytical HPLC column, and sequenced using mass spectrometry (MS) (Ahmed et al., 2015; Corrêa et al., 2014) thus giving insight into structure/sequence and potential bioactivity. Sequencing the BAPs generated using the hydrolysis of larger peptides/proteins is a powerful tool in the analysis of the activity of BAPs. Selecting BAPs with a desired activity from a particular fraction, tandem mass spectrometry (MS/MS) can be used to sequence the peptide (Liu et al., 2015). Once the peptide sequences have been identified they can be cross-checked with databases containing known BAP sequences and/or identify known BAP sequences present in larger peptides or in proteins. Examples of types of BAP activities researched in this fashion are ACE inhibition (Lafarga et al., 2017), antioxidant activity (Bechaux et al., 2020; Borrajo et al., 2020; López-Pedrouso et al., 2020; Yu et al., 2017), immunomodulating activity, and antimicrobial activity (Borrajo et al., 2020; Capriotti et al., 2015).

Bioinformatics is also a useful approach to assess the peptides released from digests due to the great number of possible sequences that can be isolated from proteins. Therefore, an *in silico* analysis of peptides could potentially lead to a more rapid discovery of BAPs. A number of online tools are available that predict possible cleavage sites (production/bioavailability). The BIOPEP database (Minkiewicz et al., 2008) can be used to predict the peptides released from proteins after digestion with a selected enzyme. This information can be used in conjunction with experimental data to predict the type of sequences that are in a particular hydrolysate (Bechaux et al., 2020). Therefore, using an *in silico* approach to select the enzyme used to hydrolyse precursor proteins can be a useful tool (Bechaux et al., 2020; Cheung et al., 2009). The predicted peptides generated by the *in silico* digests can be further analysed using online tools (e.g. Peptide Ranker) which can predict bioactivity based on their amino acid sequence. This program assigns a rank to the likelihood of a peptide sequence being bioactive (0.0 being highly unlikely, 1.0 being highly likely) (Mooney et al., 2012).

This article investigates a “crude” liver peptide mixture derived from papain hydrolysis of porcine liver supplied by Biofac A/S (Kastrup, Denmark). The major component of this “crude” mixture was then purified using reverse-phase HPLC and amino acid sequences and hence the host proteins were intensified using LC-MS/MS. *In silico* digestion of the host proteins identified peptides which were predicted to be both to present in the crude mixture (using the BIOPEP database) (Minkiewicz et al., 2008) and to have potential bioactivity (using the Peptide Ranker tool) (Mooney et al., 2012). Three of these potentially bioactive peptides were synthesised and further assayed for anti-oxidant activity.

2. Materials and methods

2.1. Materials

Liver hydrolysates were produced and supplied by Biofac A/S (Kastrup, Denmark). Preliminary characterisation of the liver hydrolysate showed

that it contained very little carbohydrate material ($2.5 \pm 0.6\%$) and had a weight average molecular weight of 3.5 ± 1.4 kg/mol. Considering the source of the material, the presence of some carbohydrate is to be expected (Gupta et al., 2015) and the weight average molecular weight based on the manufacturing process is also in the expected range < 5 kg/mol. The amino acid composition is shown in the [Supplementary data](#). Synthetic peptides (FWG, MFLG and SDPPLVFG) were supplied (Protein Peptide Research Ltd., Fareham, UK). All other general laboratory reagents and materials used were supplied by Sigma Aldrich (Gillingham, UK), Sarsedt (Nümbrecht, Germany) and Thermo Scientific (Loughborough, UK).

2.2. Isolation of individual peaks for MS analysis

Liver peptide solution (5 mg/mL) was prepared in aqueous HPLC running buffer (ultrapure water, acetonitrile (0.2%), TFA (0.01%)) and filtered using 0.2 μ m syringe filters. These were loaded onto an analytical HPLC column (Ascentis® Express Peptide ES-C18) using a 20 μ L loading loop at a flow rate of 1 mL/min. The concentration of acetonitrile increased from 5% to 29% over the first 10 min and then from 29% to 95% over the next 5 min. The samples isolated from the largest peak (at approximately 6 min) were then selected for further analysis using LC-MS/MS.

2.3. Peptide sequencing using MS/MS

The two samples collected from section 2.2 (L6a and L6b), were shipped on dry ice to the Metabolomics & Proteomics Lab (University of York, York, UK) for peptide sequencing using LC-MS/MS using a Bruker maXisHD mass spectrometer interfaced to a 50 cm PepMap column with a Bruker CaptiveSpray ion source. Peptides were eluted from the column over a 35 min gradient at 300 nL/min. Eluting peptides were selected for MS2 fragmentation using top speed data dependent acquisition, a dynamic 7–25 Hz acquisition rate and 1 s cycle time. Product ion spectra were searched against the porcine subset of the UniProt database (The UniProt Consortium, 2017) using Mascot (Matrix Science, London, UK), with no enzyme specificity. Search results were filtered to the required expected scores of ≤ 0.05 .

2.4. *In silico* digestion, and activity prediction

In silico enzyme digestions were carried out on the protein sequences identified in section 2.3 using the analysis tool on the BIOPEP website (Minkiewicz et al., 2008). Sequences were generated using papain, bromelain, pepsin (pH 1.3), and trypsin. Generated sequences were then cross referenced against a number of bioactive peptide databases to find any active sequences which have been previously reported. Peptide sequences which had not been previously reported were then analysed using the Peptide Ranker tool by Bioware (Mooney et al., 2012). Peptide Ranker predicts the likelihood of a peptide sequence being bioactive (0.0 being highly unlikely, 1.0 being highly likely). Potentially bioactive sequences those scoring ≥ 0.75 and not having been previously reported were selected for synthesis and subsequent analyses.

2.5. Peptide synthesis and preparation

Synthetic peptides (FWG, MFLG and SDPPLVFG) from *in silico* digestion and predicted bioactivity described in section 2.4 were synthesised and supplied by Protein Peptide Research Ltd. (Fareham, UK) and used without further purification. Synthetic peptides were dissolved in deionised water to produce 10 mM stock solutions: FWG (4.08 mg/mL), MFLG (4.67 mg/mL) and SDPPLVFG (9.30 mg/mL).

2.6. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (RSA) assay

DPPH (0.01 mM) was prepared in ethanol, wrapped in foil and stored at 3–8 °C prior to use. Hydrolysates and L-Ascorbic acid (control)

stock solutions (500 µg/mL) were dissolved in 10 mM potassium phosphate buffer. Each sample was shaken vigorously before being left to incubate at room temperature for 30 min. A series of dilutions 12.5–800 µg/mL were prepared. Blank solutions were prepared for each sample by adding ethanol (37.5 µL) to the sample (112.5 µL). A negative control was prepared by combining the DPPH solution (37.5 µL) with deionised water (112.5 µL). Liver hydrolysate samples and positive control (ascorbic acid) were prepared by combining the DPPH solution (37.5 µL) with the relative sample solution (112.5 µL). The absorbance was measured using a microplate reader (Tecan Infinite F50, Männedorf, Switzerland) at 540 nm. Antioxidant activity of the samples were determined using equation (1):

$$\text{Radical Scavenging Activity} = \left[1 - \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{neg}}} \right) \right] \times 100 \quad (1)$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of the sample at 540 nm and Abs_{neg} is the absorbance of negative control at 540 nm.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The Oxygen Radical Absorbance Capacity of crude peptide mixture and purified peptides were analysed using the ORAC assay (Boxin Ou and Prior, 2001; Huang et al., 2002). Solutions of 2,2'-Azobis(2-Methylpropanamide) dihydrochloride (AAPH) solution (153 mM) and fluorescein (4×10^{-6} mM) were prepared fresh.

Black 96 well plates (Thermo Scientific™ Nunc™ FluoroNunc™/LumiNunc™ 96-Well Plate) were loaded with the sample solutions ("crude" liver hydrolysate, FWG, MFLG and SDPPLVFG) and Trolox as a standard. The plates were incubated at 37 °C for 30 min. Following incubation, fluorescence was measured using a microplate reader (Fluostar Optima, BMG Labtech Ltd., Aylesbury, UK) set to slow kinetics mode. Fluorescence readings were taken every minute for five cycles, after five cycles, the plate was removed and 153 mM AAPH solution (25 µL) was added to each experimental well. The plate was returned to the plate reader where the fluorescence was measured every minute for 115 min. AAPH was used in this particular assay as the antioxidant properties of the peptides in relation to damage to cell membranes by lipid peroxidation were of particular interest (Boxin Ou and Prior, 2001; Huang et al., 2002). The ORAC value was calculated from the areas under the curves (AUC) (equation (2)).

$$\text{Relative ORAC value (Trolox Equivalents)} = \frac{(AUC_{\text{sample}} - AUC_{\text{blank}})}{(AUC_{\text{Trolox}} - AUC_{\text{blank}})} \times \left[\frac{\text{mol}_{\text{Trolox}}}{\text{mol}_{\text{sample}}} \right] \quad (2)$$

where AUCs are the areas under the curve for the sample, Trolox and the blank, respectively and $\text{mol}_{\text{Trolox}}$ and $\text{mol}_{\text{sample}}$ are the molar concentrations of Trolox and the samples respectively.

However an important limitation of the ORAC assay is that it is not biologically relevant. It was therefore used as a screening method due to its relative simplicity. Samples of interest were further investigated using the biologically relevant Cellular Antioxidant Activity (CAA) assay which uses mammalian cells.

2.8. Cellular antioxidant activity (CAA) assay

The cellular antioxidant activity assay (CAA) was based on the method of Wolfe and Liu (2007) using 3T3 fibroblasts (Wolfe & Liu, 2007). 3T3 fibroblast cells were seeded at 3×10^4 /well on a 96 well plate (3×10^5 /mL) using DMEM supplemented with L-glutamine, HEPES buffer, and PenStrep. The cells were incubated for 24 h at 37 °C, 5 % CO₂. After 24 h the medium was aspirated and the cells washed with PBS (0.01 M). 100 µL of sample media (containing 5 µM dichlorodihydrofluorescein diacetate (DCFH-DA)) was added to the relevant wells. The

plates were returned to the incubator for a further 1 hour, after which the sample media was removed and the cells were washed with PBS (0.01 M). AAPH (120 µM) in 100 µL of Hank's Balanced Salt Solution (HBSS) was added to each experimental well and fluorescence was measured using a microplate reader (Fluostar Optima, BMG Labtech Ltd., Aylesbury, UK) set to 37 °C, with excitation at 485 nm and emission at 520 nm, readings were taken every 5 minutes for 45 minutes. All plates included control, and blank wells. Control wells contained no peptide sample and blank wells contained no AAPH and no peptide sample. The values from the blank wells were subtracted from the experimental, and control wells. The fluorescence was plotted against time, and the AUC for each sample was calculated. The AUC of the control (no peptide sample) was used to calculate the cellular CAA (equation (3)), which was expressed as a percentage. Positive values indicated that a particular sample showed antioxidant properties in relation to this assay, whereas negative values would suggest that the sample increased the production of 2',7'-dichlorofluorescein (DCF), and therefore were causing oxidative stress.

$$\text{CAA}(\%) = 100 - \left[\left(\frac{\text{AUC}_{\text{sample}}}{\text{AUC}_{\text{control}}} \right) \times 100 \right] \quad (3)$$

where $\text{AUC}_{\text{sample}}$ and $\text{AUC}_{\text{control}}$ are the integrated areas under the fluorescence versus time curves for the sample and the control respectively.

2.9. Statistical analysis

Data were analysed using one-way ANOVA with equal variances assumed. Statistical analyses were performed on triplicate data using Microsoft Excel and Minitab (version 18) with a p value < 0.05 considered significant. Standard deviation derived from the mean values were indicated with error bars.

3. Results and discussion

3.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (RSA) assay

As the concentration of the liver hydrolysate increases there is a corresponding increase in the radical scavenging activity (Fig. 1) and the activity reaches $78 \pm 5\%$ at a concentration of 800 mg/mL. At each concentration the activity of liver hydrolysate is significantly lower ($p < 0.05$) than the corresponding ascorbic acid sample. However, the highest concentration the liver hydrolysate performs as well as the lower concentrations of ascorbic acid (12.5–400 µg/mL).

The liver hydrolysates were composed of a mixture of peptides of different sizes, which are themselves composed of various amino acids with charged side chains (see Supplementary data). Amino acids with charged side chains can be either proton donors or proton acceptors. The proton donating amino acids are glutamic acid and aspartic acid and the proton accepting amino acids are arginine, lysine, and histidine. Histidine is only moderately charged at values above pH 7, therefore under the assay conditions (pH 7.2) histidine is essentially neutral (degree of ionisation < 6%), and has little or no bearing on the RSA of the hydrolysates. The pKa values for the charged amino acids are aspartic acid 3.9, glutamic acid 4.2, lysine 10.5, and arginine 12.5. This value will affect the radical scavenging activities of the peptides at pH values lower than 4.2 and at values greater than pH 10.5, only positively charged side chains will be available. This would likely lead to no radical scavenging activity for this particular assay. This limits the pH range in which this assay is useful. The proportion of charged amino acids in the hydrolysates (Supplementary data) can be used to explain the RSA performance of the liver hydrolysate, which has a net proton donor value of 13.4%. This assay is inexpensive and simple in nature therefore it is ideal to rapidly screen antioxidants. The limitations however, are that the test sample solutions must be miscible with alcohol and that the mechanism of

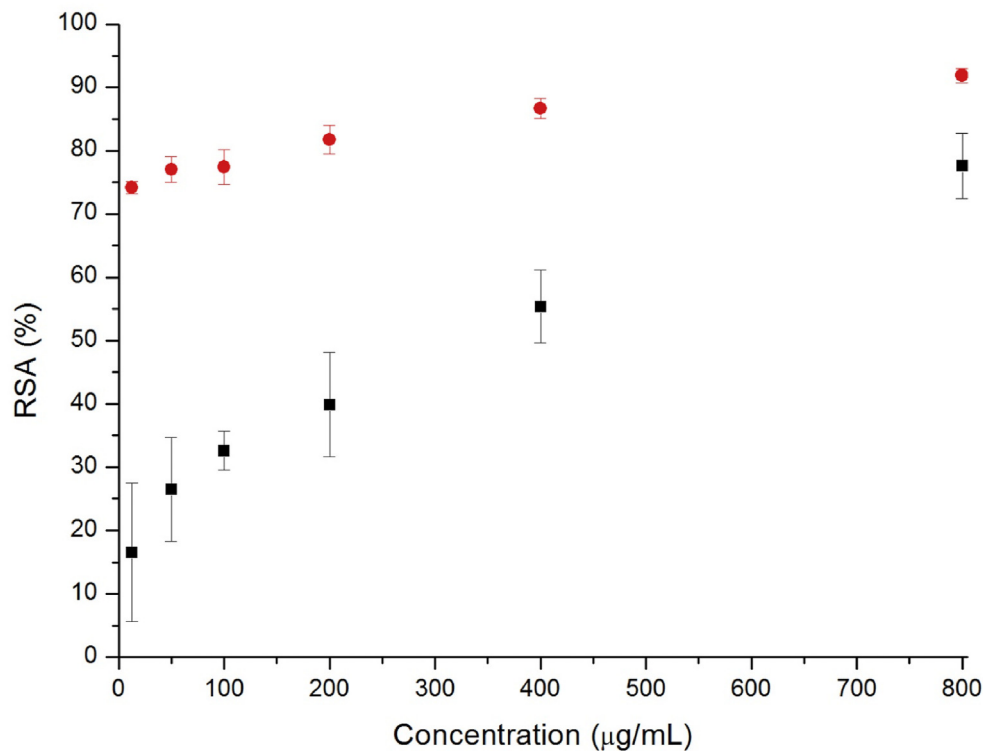


Fig. 1. RSA of the liver hydrolysate (black squares) and ascorbic acid (red circles as a positive control). Error bars indicate one standard deviation. At each concentration the activity of liver hydrolysate is significantly lower ($p < 0.05$) than the corresponding ascorbic acid sample.

reduction demonstrated is not limited to antioxidants, as other substances that exhibit no antioxidant activity can reduce the radical form of DPPH to the non-radical form (Amorati and Valgimigli, 2015). However, the concentration dependence increase in radical scavenging activity suggests that this extract contains one or more peptides which contain anti-oxidant activity. Therefore, it would be beneficial to the manufacturer to know which bioactive peptides may be present. This can be achieved through the isolation of a less “crude” peptide fraction using HPLC (section 2.2), the identification of the host protein using MS/MS.

3.2. Isolation of individual peaks for MS analysis

The results from the DPPH assay indicated that the liver hydrolysate contained one or more peptides which may have some anti-oxidant activity taking in to account the limitations of the DPPH assay. With this in mind it was decided to fractionate the liver peptide hydrolysate using reverse phase HPLC (section 2.2) and attempt to identify both potentially bioactive peptide sequences (section 2.3) and their host protein which led to the utilisation of *in silico* digestion, and activity prediction (section

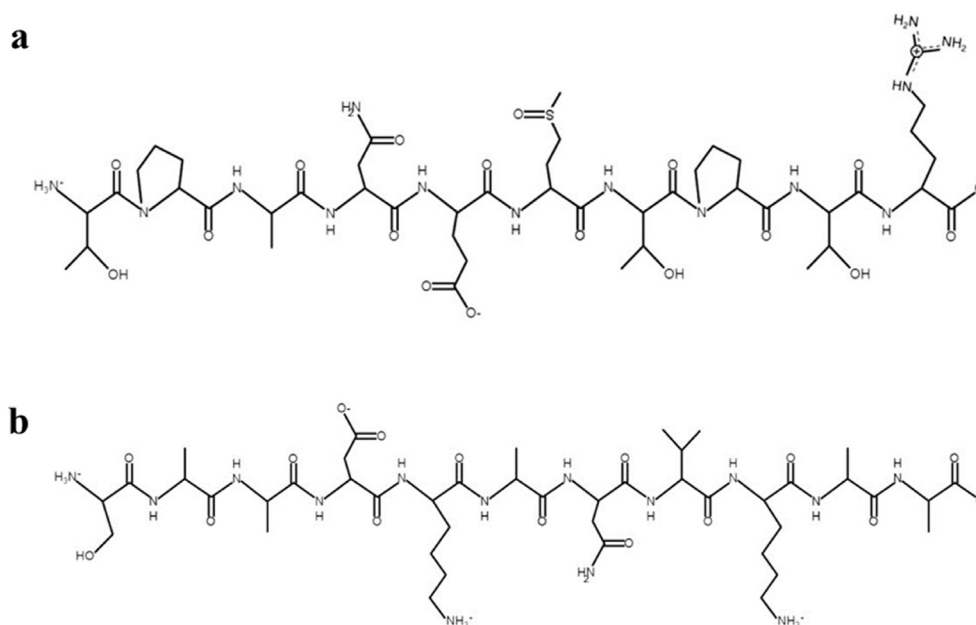


Fig. 2. a) Structures of the peptide TPANEMTPTR with methionine sulfoxide and b) of the peptide SAADKANVKAA.

2.4). Individual peptides, which were expected to show bioactivity, were then synthesised (section 2.5) and assayed in parallel with the “crude” liver hydrolysate.

3.3. Peptide sequencing using MS/MS

As the aim was to isolate as few peptides as possible. RP-HPLC was used to analyse the crude mixture (see section 2.2). A single peak was identified at ~ 6 min and collected. The following peptides were identified in the samples: TPANEMTPTR (methionine has been oxidised post-translationally) and SAADKANVKAA. The predicted peptides were cross referenced using the UniProt database (The UniProt Consortium, 2017) to find the host proteins. The structures of the peptides are shown in Fig. 2.

The host protein for TPANEMTPTR (Fig. 2a) was determined as cytosol aminopeptidase (see amino acid sequence below); an enzyme that can be found in the liver of mammals that facilitates the removal of N-terminal amino acids from peptides. It can also cleave the N-terminal of amides and arylamides. The section highlighted in yellow is the peptide identified using LC-MS/MS.

3.3.1. Cytosol aminopeptidase (*Sus scrofa*)

61 klvsqglkrei lnisgplka gktrtlyglh edfssvvvvg lgkkgagvdd qenwhgegken

I mflplpaaa rvavrqlsvr rfwgpgpdaa nmtkglvlgj yskekeddap qftsagenfd

181 gdqewarqgv lfasgqnlr hlmetpanem tptrfaevie knlksasskt dvhirpkswi

121 iraavaagr qiqdleipsv evdpcgdaqa aaegavlgly eydelkqkkk vvsaklhgs
301 lmradmgaagaa ticstivsa kldlpnlvg laplcnmps gkankpgdvv

241 eeqemgsfls vakgseppv fleihykgsp dasdpplvfv gkgitfdsgg isikasanmd

rakngkqiqv

361 dntdaegrli ladalcyahf fnpkviinaa tltgamdial gsgatgvftn sswlwnklfe

421 asietgdrvw rmpfeyhtk qivdcqladv nnigkyrsag actaaafike fvthpkwahl

481 diagvmtknd evpylrkgma grprtllief llrfsqdsa

The host protein for SAADKANVKAA (Fig. 2b) was determined as haemoglobin subunit alpha (see amino acid sequence below). The highlighted section in yellow is the peptide identified using LC-MS/MS.

3.3.2. Haemoglobin subunit alpha (*Sus scrofa*)

61 qkvadaltka vghlddplga lsalsdlhah klrvdvfnk llshcllvtl aahhpddfnp

I mvl~~saadkan vkaa~~wgkvvg qagahgaeal ermlg~~tptr~~ ktyfphfnls hgsdqvkahg

121 svhasldkfl anvstvltsk yr

3.4. *In silico* digestion, and activity prediction

3.4.1. Known bioactive peptides

Other studies have used similar techniques to analyse bioactive peptides but they generally start from peptides that have been isolated from active fractions (Cheung et al., 2009; Lafarga, O'Connor and Hayes, 2014; Lassoued et al., 2015). This method was designed as a more high-throughput approach. By sequencing straight after the initial separation, and using the BIOPEP database and Peptide Ranker utilities, it is possible to identify bioactive peptides before bioactivity assays are performed. That being said, it has been reported that there are significant differences in the predicted bioactive peptides, and actual peptides found using empirical methods (Chatterjee et al., 2015). This can be explained by the incomplete nature of these bioactive peptide databases, however, as the aim was to identify bioactive peptides that were not already in the databases, this was not a concern. There is therefore, a chance that the peptides released from experimental enzyme digestion could be different to the predicted peptides. This could be the result of miss-cleavage because of varying quality of the enzymes used (Burkhart et al., 2012). There is likely to be non-specific cleavage associated with the experimental process, therefore the complexity of the peptide profile of the hydrolysates cannot be just accreted to the specific enzyme used (in this case papain) (Picotti et al.,

2007). However, if it is likely that a predicted peptide will be present, as in the case of this investigation, the overall complex nature of the hydrolysate is not a major concern.

In silico digestion of cytosol aminopeptidase and haemoglobin subunit alpha with papain, bromelain, pepsin and trypsin resulted in a large number (34) of peptides which have previously been reported as demonstrating bioactivity, however all but one of these known bioactive peptides found using this method were dipeptides. They have been reported to exhibit the following bioactivities: dipeptidyl peptidase IV inhibitors, ACE inhibitors, renin inhibitors, Calmodulin-dependent cyclic nucleotide phosphodiesterase (CaMPDE) inhibitors, stimulating vasoactive substance release, regulating the stomach mucosal membrane activity, and antioxidant peptides (sequences not reported here). Initially FWG (1.00) and MLFG (0.96) were chosen as they had the highest scores of any peptides. It was then decided that particular attention should also be focussed on larger peptide

Table 1

Predicted peptides with a predicted rank ≥ 0.75 for all the digests (excluding dipeptides). Green highlighted peptides were selected for synthesis (and are highlighted in green in the protein sequences in section 3.3), yellow highlighted peptides are repeats which are expected to be present in more than one digest, and red highlighted peptides have been previously reported in the literature as bioactive peptides (BAPs).

Cytosol aminopeptidase derived peptides			
Papain	Bromelain	Pepsin	Trypsin
FWG (1.00)	WQRG (0.85)	WGPGDAANMTKGL (0.89)	FWGPGDAANMTK (0.86)
FLLR (0.86)	FLK (0.81)	MRADMGGAAATICSTIVSAAKL (0.83)	
WQR (0.84)	SDPPLVFG (0.75)		
FLK (0.81)			
NWH (0.78)			
SDPPLVFG (0.75)			
MPLFE (0.75)			
Haemoglobin subunit alpha derived peptides			
MFLG (0.96)	FLA (0.86)	PHF (0.94)	
FPH (0.94)	FPHFNLSHG (0.76)	ERMF (0.78)	
FLA (0.86)			

N.B.– dipeptides have been excluded.

Table 2

Amino acid sequences, host protein, Peptide Ranker score and molecular structure of selected peptides.

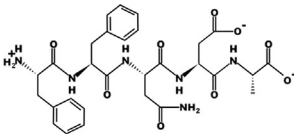
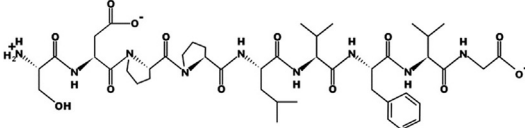
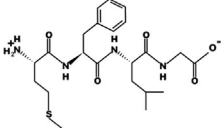
Amino acid sequence	Host protein	Peptide Ranker score	Molecular structure
FWG	cytosol aminopeptidase	1.00	
SDPPLVFG	cytosol aminopeptidase	0.75	
MFLG	haemoglobin subunit alpha	0.96	

Table 3

Trolox equivalent values for synthetic peptides and the “crude” liver hydrolysate.

Sample	Trolox equivalents (TE)
Liver hydrolysate	43.1 ± 7.6 ^a
FWG	0.3 ± 0.1 ^b
MFLG	0.7 ± 0.2 ^b
SDPPLVFG	4.6 ± 2.3 ^b

Samples with same letter are not significantly different ($p > 0.05$).

sequences not previously reported in the literature as potentially interesting bioactive target peptides, which maybe also subject to conformational changes. Taking this in to account SDPPLVFG (0.75) was chosen as like the other two smaller peptides it has a glycine C-terminus and furthermore it is predicted to be present in more than one enzymatic digest. Potentially bioactivity was estimated using Peptide Ranker as described in section 2.4. The results of which are summarised in Table 1.

3.4.2. Synthesised peptides

The following peptides were selected to be synthesised and analysed (Table 2), due to the fact they haven't previously been reported (to our

knowledge) and they all scored ≥ 0.75 using the Peptide Ranker tool.

3.5. ORAC assay

The ORAC data for the synthetic peptides indicates that FWG and MFLG have greater oxygen radical scavenging activities than Trolox (Table 3).

The data generated from the ORAC antioxidant assay indicated that the prediction of bioactivity using the peptide ranker tool could supply useful results. Peptide Ranker predicts that in small peptides (<20 amino acids), which contain phenylalanine (F) are linked with bioactivity. All the peptides selected to be synthesised contained phenylalanine. This could be related to the bioactivity demonstrated by FWG, and MFLG. The ORAC assay indicated that the two peptides that ranked the highest, FWG (1.00), and MFLG (0.96), were more potent antioxidant compounds than SDPPLVFG (0.75). The Trolox equivalent value for FWG was 0.3 ± 0.1 . This means that for the peptide to exhibit the same ORAC value as 1 g of Trolox, 0.3 g of FWG would be required, indicating that FWG is a more potent antioxidant than Trolox. MFLG demonstrated a TE of 0.7 ± 0.2 , therefore, has a similar antioxidant capacity to Trolox. The TE for SDPPLVFG was 4.6 ± 2.3 . This score was still relatively high in comparison with the other synthesised peptides but is significantly lower than the “crude” liver hydrolysate (43.1 ± 7.6). This method indicates

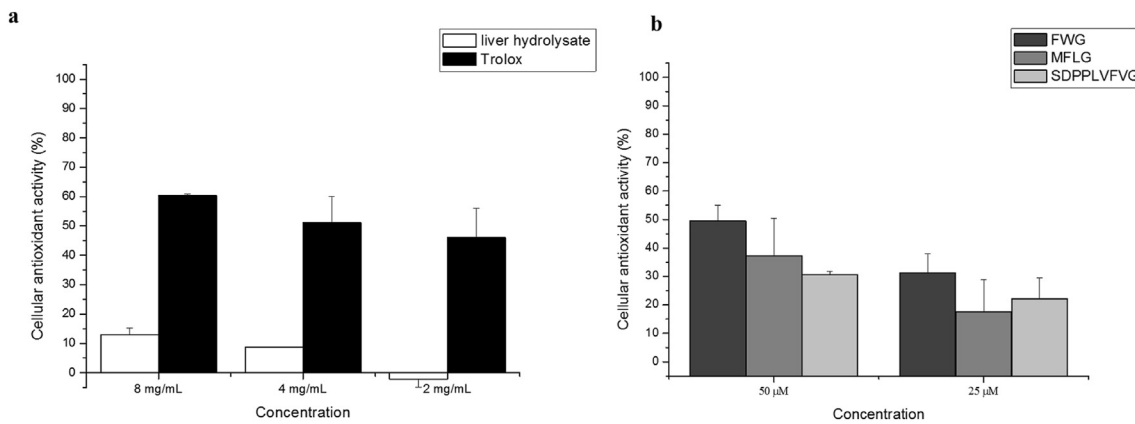


Fig. 3. a: Cellular antioxidant activity (%) of liver hydrolysate and Trolox b: Cellular antioxidant activity (%) of synthetic peptides. 50 μ M concentrations are equivalent to: FWG (0.020 mg/mL), MFLG (0.023 mg/mL) and SDPPLVFG (0.047 mg/mL) for the individual peptides, respectively. The CAA assay included a negative control (no reactive oxygen species (ROS) added). This takes into account any background ROS. The reduction in ROS is measured with this background as zero. In Fig. 3b the aim is to compare the synthetic peptides with one another rather than with Trolox and as the percentage activity is calculated from the negative control, a positive value means the peptides have demonstrated some antioxidant activity, furthermore to avoid any inter-plate variation all samples were run simultaneously.

that the bioactivity prediction for FWG and MFLG is related to antioxidant activity, but this does not mean that these peptides could not demonstrate any other bioactivity.

3.6. Cellular antioxidant activity assay (CAA)

The cellular antioxidant activity of the liver hydrolysate is significantly lower than the Trolox control at all concentrations studied (Fig. 3a). Due to the limited amount of material and potentially greater antioxidant activity the synthetic peptides were assayed at lower concentrations (50 and 25 μ M) and for comparison purposes Trolox has a CAA of 34% at 25 μ M (data not shown). The initial results for the CAA of the synthetic peptides indicate that they all demonstrated antioxidant activity, which is concentration dependant. All the test samples demonstrated some protection of the DCFH when oxidative stress was introduced (slower rate of fluorescence increase than the control). FWG demonstrated the greatest antioxidant activity at 50 μ M, which was significantly greater than SDPPLVFG; however, the difference with MFLG was not significant. At 25 μ M, there was no significant difference in antioxidant activity between the samples (Fig. 3b) and the range of activities at 25 μ M are not significantly different to Trolox (not shown).

The CAA data showed that all the peptides demonstrated antioxidant activity with only FWG at 50 μ M showing significantly more activity between the peptides. All other samples were not significantly different (Fig. 3b). The data from the antioxidant assays demonstrate that the predicted bioactivity is in some part related to antioxidant activity. The differences between the two antioxidant assays can be explained by the antioxidant mechanism of each assay. There is a great variety in mechanisms of oxidative stress, and antioxidant activity, and this is reflected in the types of *in vitro* assays used to for analysis. Two different assays may use completely different mechanisms, and compounds may generate different results. Therefore, compounds can only be determined as antioxidant in relation to a particular mechanism. For example, FWG and MFLG displayed potent antioxidant properties when measured using the ORAC assay, but all three of the synthetic peptides tested were potent antioxidant peptides when analysed using a more complex system. This would indicate that the mechanism of antioxidant activity is different for FWG and MFLG than SDPPLVFG. The CAA assay is therefore a good method to test antioxidant activity because it uses cells, and their entire metabolism to assess activity (López-Alarcón and Denicola, 2013).

4. Conclusions

The work carried out on the identification of possible bioactive peptides shows that it is possible to identify active peptides using the method developed in this study. The results indicated that the predicted bioactivity of the synthetic peptides will likely include antioxidant activity. FWG and MFLG showed activity better or comparable to Trolox in the ORAC assay. Of the other assays which may be of interest include, the anti-angiotensin converting enzyme (ACE) assay which may also be predicted using *in silico* tools, for example, AHTpin is an anti-ACE peptide predictor (Kumar et al., 2015), AntiCP is an anti-cancer peptide predictor (Tyagi et al., 2013), and AntiBP is an anti-microbial predictor (Lata et al., 2007). Certain activities are related to the secondary structures possible in larger peptides - antimicrobial peptides are an example of this. This study was primarily based on the papain digestion of proteins with this enzyme results in small peptides because of the lack of specificity. Future work with this method could investigate larger peptides generated from more specific enzymes. The use of *in silico* tools, alongside a robust range of assays which cover a wider range of bioactivities would be a way of improving the discovery, production and characterisation of novel bioactive peptides, which is an emerging challenge for the meat industry (López-Pedrouso et al., 2020; Yu et al., 2017).

Author contributions

Nicholas A. Pearman: Methodology, Investigation, Data interpretation and analysis, Writing –original draft. Elena Ronander, Alan M. Smith, Gordon A. Morris: Conceptualization, Resources, Methodology, Data interpretation and analysis, Writing – review, editing & revisions, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

NAP would like to thank Biofac A/S (Kastrup, Denmark) for their financial support during his doctoral studies. ER was an employee of Biofac A/S (Kastrup, Denmark) during this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2020.11.002>.

References

- Ahmed, A.S., El-Bassiony, T., Elmalt, L.M., Ibrahim, H.R., 2015. Identification of potent antioxidant bioactive peptides from goat milk proteins. *Food Res. Int.* 74, 80–88.
- Amorati, R., Valgimigli, L., 2015. Advantages and limitations of common testing methods for antioxidants. *Free Radic. Res.* 49 (5), 633–649.
- Banga, A.K., 2015. *Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems*. CRC press.
- Bechaux, J., Ferraro, V., Sayd, T., Chambon, C., Le Page, J.F., Drillet, Y., Gatellier, P., Santé-Lhoutellier, V., 2020. Workflow towards the generation of bioactive hydrolysates from porcine products by combining in silico and in vitro approaches. *Food Res. Int.* 132, 109123.
- Beermann, C., Euler, M., Herzberg, J., Stahl, B., 2009. Anti-oxidative capacity of enzymatically released peptides from soybean protein isolate. *Eur. Food Res. Technol.* 229 (4), 637–644.
- Borrajó, P., Pateiro, M., Gagaoua, M., Franco, D., Zhang, W., Lorenzo, J.M., 2020. Evaluation of the antioxidant and antimicrobial activities of porcine liver protein hydrolysates obtained using alcalase, bromelain, and papain. *Appl. Sci.* 10 (7).
- Boxin Ou, M.H.-W., Prior, Ronald L., 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* 49 (10), 4619–4626.
- Burkhardt, J.M., Schumbrutzki, C., Wortelkamp, S., Sickmann, A., Zahedi, R.P., 2012. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J. Proteom.* 75 (4), 1454–1462.
- Capriotti, A.L., Caruso, G., Cavaliere, C., Samperi, R., Ventura, S., Chiozzi, R.Z., Laganà, A., 2015. Identification of potential bioactive peptides generated by simulated gastrointestinal digestion of soybean seeds and soy milk proteins. *J. Food Compos. Anal.* 44, 205–213.
- Chatterjee, A., Kanawjia, S.K., Khetra, Y., Saini, P., 2015. Discordance between in silico & in vitro analyses of ACE inhibitory & antioxidative peptides from mixed milk tryptic whey protein hydrolysate. *J. Food Sci. Technol.* 52 (9), 5621–5630.
- Cheung, I.W.Y., Nakayama, S., Hsu, M.N.K., Samaranyaka, A.G.P., Li-Chan, E.C.Y., 2009. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (*Avena sativa*) proteins by in silico and in vitro analyses. *J. Agric. Food Chem.* 57 (19), 9234–9242.
- Corrêa, A.P.F., Daroit, D.J., Fontoura, R., Meira, S.M.M., Segalin, J., Brandelli, A., 2014. Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides* 61, 48–55.
- Di Bernardini, R., Hamedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A.M., Hayes, M., 2011. Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chem.* 124 (4), 1296–1307.
- Fu, Y., Young, J.F., Therkildsen, M., 2017. Bioactive peptides in beef: endogenous generation through postmortem aging. *Meat Sci.* 123, 134–142.
- Gupta, A.J., Wierenga, P.A., Gruppen, H., Boots, J.-W., 2015. Influence of protein and carbohydrate contents of soy protein hydrolysates on cell density and IgG production in animal cell cultures. *Biotechnol. Prog.* 31 (5), 1396–1405.
- Himaya, S.W.A., Ngo, D.-H., Ryu, B., Kim, S.-K., 2012. An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chem.* 132 (4), 1872–1882.
- Howell, N.K., Kasase, C., 2010. Bioactive peptides and proteins from fish muscle and collagen. In: Mine, Y., Li-Chan, E., Jiang, B. (Eds.), *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals*.
- Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A., Prior, R.L., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* 50 (16), 4437–4444.
- Jang, A., Lee, M., 2005. Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. *Meat Sci.* 69 (4), 653–661.
- Kumar, R., Chaudhary, K., Singh Chauhan, J., Nagpal, G., Kumar, R., Sharma, M., Raghava, G.P.S., 2015. An in silico platform for predicting, screening and designing of antihypertensive peptides. *Sci. Rep.* 5, 12512.
- Lafarga, T., Álvarez, C., Hayes, M., 2017. Bioactive peptides derived from bovine and porcine co-products: a review. *J. Food Biochem.* 41 (6), e12418.
- Lafarga, T., O'Connor, P., Hayes, M., 2014. Identification of novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides from meat proteins using in silico analysis. *Peptides* 59, 53–62.
- Lassoued, I., Mora, L., Barkia, A., Aristoy, M.C., Nasri, M., Toldrá, F., 2015. Bioactive peptides identified in thornback ray skin's gelatin hydrolysates by proteases from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. *J. Proteom.* 128, 8–17.
- Lata, S., Sharma, B., Raghava, G., 2007. Analysis and prediction of antibacterial peptides. *BMC Bioinf.* 8 (1), 263.
- Liu, R., Zheng, W., Li, J., Wang, L., Wu, H., Wang, X., Shi, L., 2015. Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Mactra veneriformis* by UHPLC-Q-TOF mass spectrometry. *Food Chem.* 167, 484–489.
- López-Alarcón, C., Denicola, A., 2013. Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. *Anal. Chim. Acta* 763, 1–10.
- López-Pedrouso, M., Borrajó, P., Pateiro, M., Lorenzo, J.M., Franco, D., 2020. Antioxidant activity and peptidomic analysis of porcine liver hydrolysates using alcalase, bromelain, flavourzyme and papain enzymes. *Food Res. Int.* 137, 109389.
- Maluf, J.U., Fiorese, M.L., Maestre, K.L., Dos Passos, F.R., Finkler, J.K., Fleck, J.F., Borba, C.E., 2020. Optimization of the porcine liver enzymatic hydrolysis conditions. *J. Food Process. Eng.* 43 (4), e13370.
- Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., Darewicz, M., 2008. BIOPEP database and other programs for processing bioactive peptide sequences. *J. AOAC Int.* 91, 965–980.
- Mooney, C., Haslam, N., Pollastri, G., Shields, D., 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PLoS One* 7 (10), e45012.
- Picotti, P., Aebersold, R., Domon, B., 2007. The implications of proteolytic background for shotgun proteomics. *Mol. Cell. Proteomics* 6 (9), 1589–1598.
- Pruksaritanont, T., Tang, C., 2012. ADME of biologics—what have we learned from small molecules? *AAPS J.* 14 (3), 410–419.
- The UniProt Consortium, 2017. UniProt: the universal protein knowledgebase. *Nucleic Acids Res.* 45 (D1), D158–D169.
- Tyagi, A., Kapoor, P., Kumar, R., Chaudhary, K., Gautam, A., Raghava, G.P.S., 2013. In silico models for designing and discovering novel anticancer peptides. *Sci. Rep.* 3, 2984.
- Wolfe, K.L., Liu, R.H., 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* 55 (22), 8896–8907.
- Xue, Z., Gao, J., Zhang, Z., Yu, W., Wang, H., Kou, X., 2012. Antihyperlipidemic and antitumor effects of chickpea albumin hydrolysate. *Plant Foods Hum. Nutr.* 67 (4), 393–400.
- Xue, Z., Wen, H., Zhai, L., Yu, Y., Li, Y., Yu, W., Cheng, A., Wang, C., Kou, X., 2015. Antioxidant activity and anti-proliferative effect of a bioactive peptide from chickpea (*Cicer arietinum* L.). *Food Res. Int.* 77 (Part 2), 75–81.
- Yu, H.-C., Hsu, J.-L., Chang, C.-I., Tan, F.-J., 2017. Antioxidant properties of porcine liver proteins hydrolyzed using *Monascus purpureus*. *Food Sci. Biotechnol.* 26 (5), 1217–1225.