Research Article • DOI: 10.1515/ijafr-2017-0002 IJAFR • 56(1) • 2017 • 12-24

Irish Journal of Agricultural and Food Research

Effect of pre-treatment on the generation of dipeptidyl peptidase-IV- and prolyl endopeptidase-inhibitory hydrolysates from bovine lung

T. Lafarga, M. Hayes†

Teagasc, The Irish Agricultural and Food Development Authority, Food BioSciences Department, Ashtown, Dublin 15, Dublin, Ireland

Abstract

The aim of this work was to study the effect of two different pre-treatments, high temperature (100 °C, 5 min) and high pressure (600 MPa, 3 min), on the potential of the enzymes papain, collagenase and Alcalase® to generate bioactive hydrolysates containing dipeptidyl peptidase-IV- (DPP-IV; EC 3.4.14.5) and prolyl endopeptidase- (PEP; EC 3.4.21.26) inhibitory peptides from bovine lung. Both pre-treatments resulted in an increase in the degree of hydrolysis over a 24 h period ($P < 0.001$) and significantly increased the DPP-IV- and PEP-inhibitory activities of the generated hydrolysates ($P < 0.001$). Generated hydrolysates included an Alcalase hydrolysate of pressure-treated bovine lung, which was the most active, and showed DPP-IV and PEP half-maximal inhibitory concentration (IC_{50}) values of 1.43 ± 0.06 and 3.62 ± 0.07 mg/mL, respectively. The major peptides contained in this hydrolysate were determined by liquid chromatography–tandem mass spectrometry, and results demonstrated that bovine lung is a good substrate for the release of bioactive peptides when proper pre-treatment and enzymatic treatment are applied.

Keywords

bioactive peptides • bovine lung • DPP-IV • high pressure • PEP • pre-treatment

Introduction

Bioactive peptides consist of short sequences of amino acids encrypted within the sequence of a parent protein, which may be released by fermentation or hydrolysis using microorganisms, enzymes or acids (Korhonen and Pihlanto, 2006). Several studies in the past decade have demonstrated that peptides can be biologically active and may therefore serve as potential therapeutic agents in the body (Agyei and Danquah, 2011). The majority of the biologically active peptides identified to date can be found in the BIOPEP database (Minkiewicz *et al*., 2008) and include antimicrobial, dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5), angiotensin-I-converting enzyme (ACE-I; EC 3.4.15.1) and prolyl endopeptidase (PEP; EC 3.4.21.26)-inhibitory peptides, as well as antioxidant peptides (Nedjar-Arroume *et al*., 2008; Di Bernardini *et al*., 2012; Bah *et al*., 2013; Lafarga and Hayes, 2014).

Human PEP is a serine peptidase, which digests a variety of proline-containing small peptides, including hormones, neuroactive peptides and various cellular factors (García-Horsman *et al*., 2007). Although PEP is found in the central nervous system (CNS), the testis, skeletal muscle, kidney, liver and lung (Yaron *et al*., 1993), the one found in the CNS is the main pharmacological target. Most of the published PEP inhibitors are chemically synthesised compounds based on the *N*-acyl-l-prolyl-pyrrolidine structure (Wilson *et al*., 2011). However, a number of naturally occurring peptidic PEP inhibitors have been reported (Wilson *et al*., 2011). Although no physiological effect has been reported after administration of PEP-inhibitory hydrolysates or peptides to date, several studies carried out using animal models demonstrated the anti-amnestic and the memory- and cognition-enhancing properties of PEP inhibitors (Morain *et al*., 2002; Cheng *et al*., 2005; Lawandi *et al*., 2010). Furthermore, transport of a PEP-inhibitory peptide across the blood–brain barrier was recently demonstrated using a transcytosis model (Hayes *et al*., 2015). In addition, both PEP and DPP-IV have been recently associated with externalising and aggressive behaviours in normal and autistic adolescents as a result of these peptidases cleaving behavioural neuropeptides (Frenssen *et al*., 2015). Human DPP-IV also degrades the incretin hormones such as glucagon-like peptide 1 and gastric inhibitory peptide, which aid the physiological control of postprandial blood glucose concentration in the body (Drucker, 2006). DPP-IV is expressed in a variety of tissues, primarily on endothelial

†Corresponding author: Maria Hayes
E-mail: maria.hayes@teagasc.ie

and epithelial cells (Lambeir *et al*., 2003). However, DPP-IV inhibitors have been suggested to act locally in the gastrointestinal tract by inhibiting intestinal DPP-IV (Waget *et al*., 2011). The use of bioactive hydrolysates containing PEP and DPP-IV inhibitors, as well as their inclusion into food products, shows potential in the realm of functional food research as a strategy for potential prevention of mental health disorders and type-2 diabetes through tailored diet.

Co-products from the meat processing industry constitute an excellent source of high-quality proteins including collagen. Recently, Darine *et al*. (2010) determined the collagen content of bovine lung, which was approximately 4.1% (w/w). Collagen is used extensively in the pharmaceutical, cosmetic, biomedical and food industries. A number of bovine collagen–derived hydrolysates with health-promoting properties, including Peptan® B 5000 HD (Rousselot SAS, Angoulême, France), are currently available. In addition, bovine lung has been used as a substrate for the release of bioactive peptides with antioxidant activities (Damgaard *et al*., 2015) and has been also suggested to be a potential source of novel PEP- and DPP-IV-inhibitory peptides, mainly because of its high collagen concentration (Minkiewicz *et al*., 2011; Lafarga *et al*., 2014, 2015). However, due to its structure, collagen is not easily accessible for enzymatic hydrolysis. High-pressure and heat treatments have been previously used before enzymatic hydrolysis of collagen to facilitate the destabilisation of its structure and the exposure of the inner sites of the protein to the proteolytic enzyme (Zhang *et al*., 2013). Pressure and temperature pre-treatments have also been used successfully in the release of peptides with other bioactivities, including antioxidant peptides and inhibitors of ACE-I from different animal and plant sources (Quirós *et al*., 2007; Singh and Ramaswamy, 2014; Garcia-Mora *et al*., 2015; Girgih *et al*., 2015).

The aim of this study was to generate hydrolysates with PEP- and DPP-IV-inhibitory bioactivities from bovine lung using the enzymes Alcalase, collagenase and papain, in addition to studying the effect of heat and pressure pre-treatments on the DPP-IV- and PEP-inhibitory activities of bovine lung hydrolysates. Hydrolysates generated using Alcalase, collagenase and papain after pre-treatment with temperature or pressure were screened for their ability to inhibit the enzymes PEP and DPP-IV *in vitro*. The concentration of hydrolysate needed to inhibit the activity of PEP and DPP-IV by 50% (half-maximal inhibitory concentration [IC_{50}] value) was determined for the generated protein hydrolysates, and a number of peptides were identified using liquid chromatography (LC) and mass spectrometry (MS) in an Alcalase hydrolysate obtained from high pressure–treated bovine lung.

Materials and methods

Materials and reagents

Trichloroacetic acid (TCA), formic acid (FA), acetonitrile (ACN), collagenase from *Clostridium histolyticum*, papain from *Carica papaya* and valproic acid were supplied by Sigma Aldrich (Dublin, Ireland). Alcalase® 2.4 L FG was kindly supplied by Novozymes (Bagsvaerd, Denmark). The DPP-IV inhibitor screening assay kit, containing the DPP-IV inhibitor and positive control sitagliptin, was supplied by Cambridge BioSciences (Cambridge, England, UK). The PEP assay kit was supplied by Tebu-Bio Ltd. (Peterborough, England, UK). All other chemicals used were of analytical grade.

Sample preparation and pre-treatment

The three animals slaughtered were of Charolais cross heifer breed and were aged between 23 and 24 mo at the time of slaughter. One whole lung from each animal was collected at the time of slaughter under hygienic conditions at the abattoir at the Teagasc Food Research Centre, Ashtown, Dublin, Ireland. Collected lungs were frozen, freeze-dried using an industrial-scale FD 80 model freeze-drier (Cuddon Engineering, Marlborough, New Zealand), milled and stored at -20 °C until further use, as shown in Figure 1.

Three bovine lung samples were used. One lung sample was left untreated and was labelled as UT. The UT substrate solution was prepared by suspending the dried, untreated lung co-product in MilliQ purified water at a concentration of 10 g/L at a total volume of 500 mL. Heat-treated samples were prepared by suspending the dried, untreated lung co-product in MilliQ purified water at a concentration of 10 g/L at a total volume of 500 mL and immediately immersing in a boiling water bath for 5 min. The samples were then chilled with tap water for 10 min and labelled as HT. Dried lung samples that were treated with pressure were transferred into polyethylene bags and vacuum-sealed. High-pressure pre-treatment was performed in the pressurising chamber of a Hiperbaric 135 equipment (Hiperbaric, Burgos, Spain) at 600 MPa for 3 min, and samples were labelled as PT. After pressure treatment, PT samples were immediately stored at -20 °C for a maximum of 24 h. The high-pressure substrate solutions were prepared by suspending the pressure-treated dried samples in MilliQ purified water at a concentration of 10 g/L at a total volume of 500 mL.

The total protein content of the UT, HT and PT lung samples was determined using a LECO FP628 Protein analyser (LECO Corp., St. Joseph, MI, USA) based on the Dumas method and according to Association of Official Analytical Chemists (AOAC) method 992.15 (AOAC, 1990). The protein content of enzymatic hydrolysates was assessed using the same method. The conversion factor of 6.25 was used to

convert total nitrogen to protein. Moisture and ash content were determined gravimetrically in accordance with previously described methods (Kolar, 1992).

Amino acid composition analysis

For total and free amino acid composition analysis, the dried bovine lung (UT sample) was hydrolysed in 6 M HCl at 110 °C for 23 h following the method of Hill (1965). Briefly, the sample was de-proteinised by mixing equal volumes of 24% (w/v) TCA and sample. It was then allowed to stand for 10 min before centrifuging at 14,400 × *g* for 10 min in an MSE Micro Centaur Plus centrifuge (MSE, London, UK). The supernatant was removed and diluted with 0.2 M sodium citrate buffer (pH 2.2) to give approximately 250 nmol of each amino acid residue. The sample was then diluted 1:1 with the internal standard, norleucine, to give a final concentration of 125 nmol/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high-performance cation exchange column.

Enzymatic hydrolysis

The UT, PT and HT bovine lung samples were hydrolysed in triplicate separately with papain, Alcalase or collagenase. The sample solutions were pre-incubated at the optimal temperature and pH of each enzyme for 10 min before addition of the enzyme: 65 °C and pH 6.5 for papain, 37 °C and pH 7.5 for collagenase and 60 °C and pH 9.5 for Alcalase. Agitation was kept constant at 350 rpm. Once the optimum conditions were achieved, the enzymes were added in a substrate-to-enzyme ratio of 100:1 (w/w). After 24 h, the enzymes were heat-deactivated at 95 °C for 10 min in a water bath. The pH was kept constant at the optimum for each enzyme using 0.1 M NaOH.

The degree of hydrolysis (DH) was calculated using the pH-stat technique after 1, 30, 60, 120, 240, and 1,440 min (Adler-Nissen, 1986). The DH was calculated using the equation:

$$\mathrm{DH} = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{\mathrm{tot}} \times 100\ \%,$$

where B (mL) is the volume of NaOH consumed, N_B is the normality of the NaOH used, $1/\alpha$ is the average degree of dissociation of the a-amino groups related with the pK of the amino groups at particular pH and temperatures, M_P (g) is the amount of protein in the reaction mixture, and h_{tot} (meq/g) is the sum of the millimoles of individual amino acids per gram of protein associated with the source of protein used in the experiment. Values for h_{tot} and $1/\alpha$ were obtained from the study conducted by Adler-Nissen (1986).

DPP-IV inhibition assay

This assay was carried out using a DPP-IV inhibitor screening assay kit in accordance with the manufacturer's instructions. Fluorescence intensity was recorded with a FLUOstar Omega microplate reader (BMG Labtech, Ortenburg, Germany) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Sitagliptin was used as a positive control and it has a known IC_{50} value of 19 nM (Thomas *et al*., 2008). The IC_{50} values of the enzymatic hydrolysates generated in this study were determined by plotting the percentage of inhibition as a function of the concentration of test hydrolysate (0.5, 1.0, 2.0, 4.0 and 5.0 mg/mL).

PEP-inhibitory assay

The PEP-inhibitory assay was carried out using a fluorogenic PEP assay kit in accordance with manufacturer's instructions. Fluorescence intensity was recorded with a FLUOstar Omega microplate reader (BMG Labtech) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Valproic acid was used as a positive control and it has an IC_{50} value of 1–2 mM (Cheng *et al*., 2005). PEP IC_{50} values were determined for UT, PT and HT samples hydrolysed with Alcalase, collagenase and papain by plotting the percentage of PEP inhibition as a function of the concentration of test hydrolysate (0.5, 1.0, 2.0, 4.0 and 5.0 mg/mL).

Peptide identification by LC-MS/MS

The peptidic content of the Alcalase hydrolysate of PT lung proteins, which showed the highest bioactivity values, was analysed by LC-MS/MS using a Thermo Scientific Q-Exactive™ mass spectrometer connected to a Dionex UltiMate® 3000 RSLCnano LC System. The freeze-dried sample was suspended in 0.1% FA in high-performance LC-grade water at a concentration of 1 mg/L and cleaned using Millipore® C18 ZipTips before characterisation. The sample was then loaded onto a Biobasic Picotip Emitter (120 mm length, 75 mm internal diameter) packed with Reprocil-Pur C18 (1.9 mm) reversed phase medium and was separated using an increasing ACN gradient over 60 min at a flow rate of 250 nL/min. The mass spectrometer was operated in a positive ion mode with a capillary temperature of 220 °C and a potential of 2.3 kV applied to the emitter. All data were acquired by operating in an automatic data-dependent switching mode. A high-resolution – 70,000 full width at half maximum – MS scan (*m*/*z* 300 to *m*/*z* 1,600) was performed using Q-Exactive mass spectrometer to select the 12 most intense ions before MS/MS analysis using higher-energy collisional dissociation.

The raw data were *de novo* sequenced and searched against the bovine subset of the UniProtKB/Swiss-Prot database using the search engine of PEAKS Studio 7 for peptides cleaved with no specific enzyme. At least one unique peptide was required to identify a protein. Each peptide used for protein identification

met specific PEAKS parameters (only peptide scores that corresponded to a false discovery rate of ≤1% were accepted from the PEAKS database search). The sample was run three times, and the results shown are the combined technical replicates. Peptide sequences, their positions inside the parent proteins, their observed masses and their retention times were provided by the PEAKS Studio software.

Statistical analysis

The statistical analyses were performed using SPSS for Windows (v. 18.0) (IBM Corp., Armonk, NY, USA). Normality of the data was tested using the Kolmogorov–Smirnov test, and the data were logarithmically transformed when needed. A univariate general linear model was used to test the different bioactivity responses observed for DPP-IV and PEP, with the effects of pre-treatment, enzyme and the interaction of both factors included in the model and Tukey's honest significant difference (HSD) *post hoc* test to check for statistical differences. Repeated-measures general linear model was used to test the response of the DH with time, time × pre-treatment, time × enzyme and time × pre-treatment × enzyme interactions included in the model. *Post hoc* Scheffe test was used to test for statistically significant differences when needed. The criterion for statistical significance was $P < 0.05$.

Table 1. Total and free amino acid contents of bovine lung proteins

Amino acid	Total amino acids (mg/100 g) ± s.d.	Free amino acids (mg/100 g) ± s.d.
Met	1.29 ± 0.03	0.77 ± 0.02
Asx	4.49 ± 0.09	8.31 ± 0.17
Thr	2.11 ± 0.04	3.30 ± 0.07
Ser	2.46 ± 0.05	4.34 ± 0.09
Glx	6.81 ± 0.14	28.88 ± 0.58
Gly	5.86 ± 0.12	26.45 ± 0.53
Ala	4.04 ± 0.08	6.25 ± 0.13
Cys	1.28 ± 0.03	1.79 ± 0.09
Val	3.44 ± 0.07	4.41 ± 0.09
Ile	1.81 ± 0.04	2.26 ± 0.05
Leu	4.65 ± 0.09	4.46 ± 0.09
Tyr	1.43 ± 0.03	2.31 ± 0.05
Phe	2.54 ± 0.05	2.96 ± 0.06
His	1.88 ± 0.04	1.40 ± 0.03
Lys	3.74 ± 0.07	4.49 ± 0.09
Arg	3.69 ± 0.07	4.07 ± 0.08
Pro	3.51 ± 0.07	3.15 ± 0.06

Results

Protein content and amino acid composition

The protein content of whole bovine lung was 19.2 ± 0.4%. The amino acid composition of bovine lung proteins was calculated, and the results are shown in Table 1. Whole bovine lung was cut into smaller pieces of approximately 3 × 3 × 3 cm and freeze-dried as shown in Figure 1. The total protein, ash and moisture contents of the dried lung were 71.6 ± 0.3%, 4.2 ± 0.1% and 13.2 ± 0.6%, respectively. In addition, bovine lung samples were hydrolysed individually with the enzymes papain, collagenase and Alcalase. These hydrolysates were freeze-dried and were found to contain 70.3 ± 0.3, 60.0 ± 0.1 and 68.0 ± 0.2% protein; and 7.31 ± 0.02, 13.69 ± 0.12 and 9.87 ± 0.11% moisture; and 7.27 ± 0.05, 6.21 ± 0.07 and 14.38 ± 0.26% ash, respectively.

In vitro *assessment of bioactivity: effect of pre-treatment on the release of DPP-IV- and PEP-inhibitory peptides*

The papain, collagenase and Alcalase hydrolysates of UT bovine lung were assessed for their *in vitro* DPP-IV- and PEP-inhibitory activities, and the results are shown in Figure 2. Results demonstrated that Alcalase was the most effective enzyme in releasing DPP-IV-inhibitory peptides from the

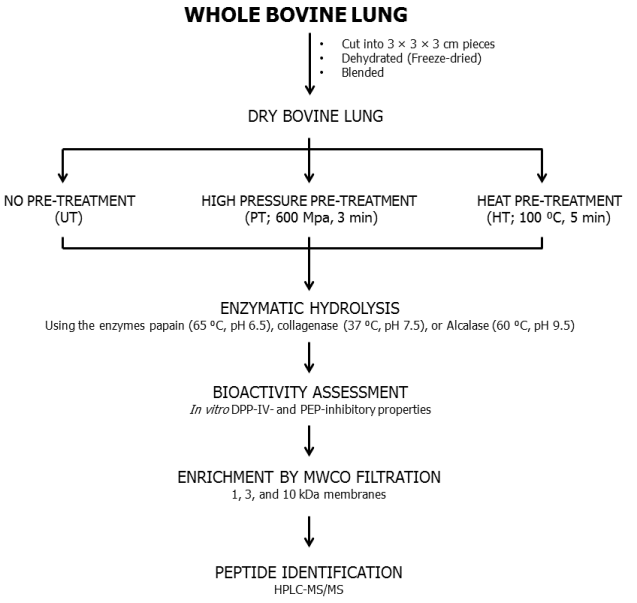

Figure 1. Schematic representation of the procedure followed for the generation of bioactive peptides from bovine lung. UT, HT and PT refer to untreated, heat-treated and high pressure-treated samples, respectively. MWCO = molecular weight cut-off; HPLC-MS/MS = high-pressure liquid chromatography-tandem mass spectrometry; DPP-IV = dipeptidyl peptidase-IV; PEP = prolyl endopeptidase.

bovine lung. Hydrolysates generated using non–pre-treated lung were also assessed for their PEP-inhibitory activities, and the results are shown in Figure 2. No inhibitory activity was observed in the unhydrolysed bovine lung proteins. At a concentration of 1 mg/mL, the studied hydrolysates generated using papain, collagenase and Alcalase, as well as the UT bovine lung, showed PEP-inhibitory activity in the range of 5–10%, compared to the positive control, which inhibited PEP by more than 60%. PEP IC_{50} values were 6.11 ± 0.70, 4.70 ± 0.03 and 7.10 ± 0.30 mg/mL for the hydrolysate generated with papain, collagenase and Alcalase, respectively. The percentage of DPP-IV inhibition was calculated for hydrolysates generated using papain, collagenase and Alcalase after heat and high-pressure pre-treatments. Results demonstrated that the DPP-IV-inhibitory activity of the hydrolysates was significantly influenced by pre-treatment ($P < 0.001$), enzyme ($P < 0.001$) and the interaction of both factors ($P < 0.05$). As mentioned earlier, Alcalase generated the most active hydrolysate from the UT samples. This trend was also observed after hydrolysis of both HT and PT samples. Alcalase hydrolysates of PT and HT bovine lung were found to inhibit the enzyme DPP-IV by 43.70 ± 0.63% and 28.28 ± 1.18%, respectively, when tested at a sample concentration of 1 mg/mL. The IC_{50} values of these two hydrolysates were 1.43 ± 0.06 and 2.18 ± 0.03 mg/mL, respectively. Both pre-treatments, heat and high pressure, also increased the activity of hydrolysates generated using papain and collagenase. The IC_{50} values of papain hydrolysates of PT and HT samples were lower when compared to that of the papain hydrolysate of UT samples ($P < 0.05$). In addition, collagenase hydrolysates of PT and HT samples were also more active when compared to the UT sample, and the IC_{50} values were 2.60 ± 0.18 and 2.70 ± 0.01 mg/mL, respectively. PEP-inhibitory activity was also significantly influenced by pre-treatment ($P < 0.001$), enzyme ($P < 0.05$) and the interaction of both factors ($P < 0.05$). As mentioned earlier, collagenase generated the most active PEP-inhibitory hydrolysate after hydrolysis of the UT lung samples (Figure 2). Again, this trend was maintained after hydrolysis of both HT and PT lung samples. The use of heat and high pressure as pre-treatments significantly increased the PEP-inhibitory activity of the Alcalase hydrolysates of lung proteins ($P < 0.05$). No significant differences were observed between the IC_{50} values obtained after hydrolysis of pre-treated samples with Alcalase or collagenase. Alcalase hydrolysates of PT and HT bovine lung had PEP IC_{50} values of 3.62 ± 0.07 and 3.87 ± 0.10 mg/mL, respectively. These values were lower than the one obtained after hydrolysis of the UT sample ($P < 0.05$). The PEP IC_{50} values of collagenase hydrolysates of PT and HT bovine lung were 3.70 ± 0.02 and 4.18 ± 0.11 mg/mL, respectively (Figure 2). In addition, heat and high-pressure pre-treatments also resulted in hydrolysates with lower IC_{50} values (increased the PEP-inhibitory activity of hydrolysates) when using papain. Papain hydrolysates of PT and HT samples showed lower PEP IC_{50} values (4.96 ± 0.41 and 5.67 ± 0.47 mg/mL, respectively) compared to the IC_{50} value of the papain hydrolysate of UT samples, which was 6.11 mg/mL ($P < 0.05$).

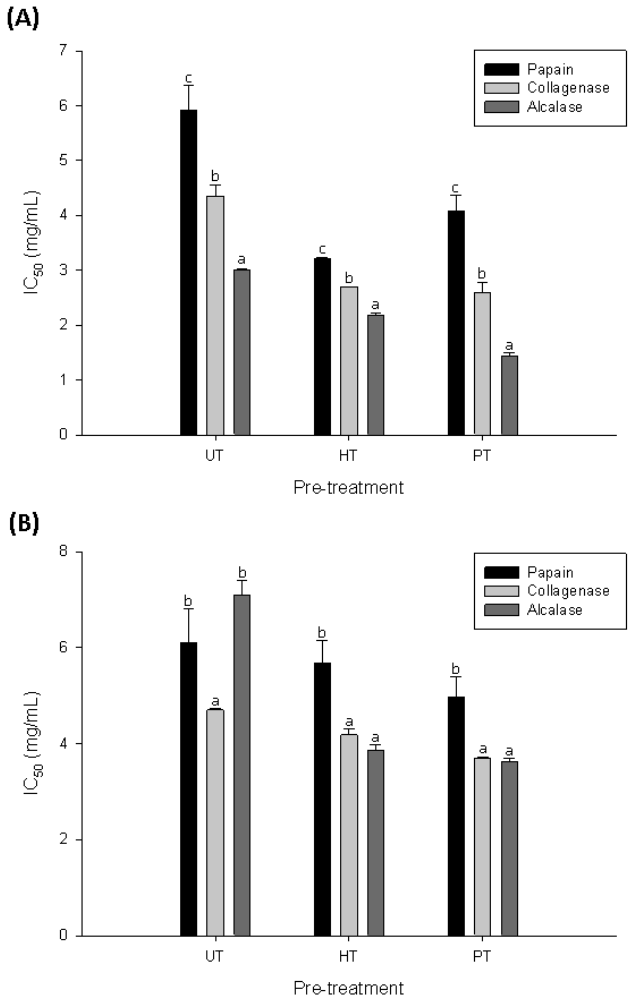

Figure 2. *In vitro* DPP-IV (A) and PEP (B) IC_{50} values of the studied enzymatic hydrolysates. IC_{50} values represent the means of three independent experiments ± standard deviation. For each bioactivity, bars with different letters have mean values that are significantly different ($P < 0.05$). UT, HT and PT refer to untreated, heat-treated and high pressure-treated samples, respectively. DPP-IV = dipeptidyl peptidase-IV; PEP = prolyl endopeptidase; IC_{50} = half-maximal inhibitory concentration.

Effect of pre-treatment and hydrolysis time on the DH

The DH was calculated using the pH-stat technique after 1, 30, 60, 120, 240 and 1,440 min in each enzymatic treatment, and the results are shown in Figure 3. The DH was significantly influenced by time ($P < 0.001$), time × pre-treatment ($P<0.001$), time × enzyme ($P<0.001$) and the

interaction between all factors ($P < 0.05$). The differences in the DH were analysed at each time point for the different pre-treatments and enzymes used. As expected, when all the enzymes were considered together, there was a significant effect of hydrolysis time on the DH. The DH increased for all enzymatic treatments with time for both the UT and the pre-treated (HT and PT) samples ($P < 0.001$). Pre-treatment did not affect all the enzymes in the same way. The DH after 24 h was measured as 6.1 ± 0.3%, 10.8 ± 0.4% and 13.7 ± 0.3% for the papain hydrolysate of the UT, HT and PT lung proteins, respectively. High-pressure pre-treatment also increased the DH for hydrolysates generated with collagenase. However, the observed increase was lower, and after 24 h, the DH was 6.4 ± 0.3%, 7.6 ± 0.1% and 6.9 ± 0.1% for the hydrolysates of UT, HT and PT samples, respectively. A similar trend was observed after 24 h for the Alcalase hydrolysates of UT, HT and PT samples, which showed a DH of 28.2 ± 1.8%, 38.8 ± 2.0% and 39.6 ± 1.2%, respectively. Although a slight increase in the DH of the Alcalase hydrolysate of the PT sample, compared to the HT samples, was observed, this was not significantly different.

Identification of proteins and peptides in pre-treated bovine lung Alcalase hydrolysate

A total of 306 proteins were identified in the bovine lung sample (supplementary data). Four types of collagen were identified: (i) alpha-1 (P02453|CO1A1_BOVIN) and alpha-2 (P02465|CO1A2_BOVIN) type I collagen, (ii) alpha-1 (P02459|CO2A1_BOVIN) type II collagen, (iii) alpha-1 (P04258|CO3A1_BOVIN) type III collagen and (iv) alpha-I (Q7SIB2|CO4A1_BOVIN) and alpha-2 (Q7SIB3|CO4A2_BOVIN) type IV collagen. Other proteins commonly found in blood, such as haemoglobin (P01966|HBA_BOVIN and P02070|HBB_BOVIN), fibrinogen (P02672|FIBA_BOVIN, P02676|FIBB_BOVIN and P12799|FIBG_BOVIN) and bovine serum albumin (P02769|ALBU_BOVIN), as well as actin (P63258|ACTG_BOVIN, P60712|ACTB_BOVIN and P62739|ACTA_BOVIN) and myosin (Q27991|MYH10_BOVIN), were also identified.

Discussion

The use of co-products, such as lung or blood, in the food industry is not new, and these are staple foods found in the diets of persons from several countries (Toldrá *et al*., 2012). However, traditional markets for edible co-products are disappearing, and the meat industry is making efforts to add value to protein sources beyond their current use as techno-functional ingredients (Toldrá *et al*., 2012). As mentioned earlier, a potential application of meat co-products is as a resource for the generation of bioactive hydrolysates. Most

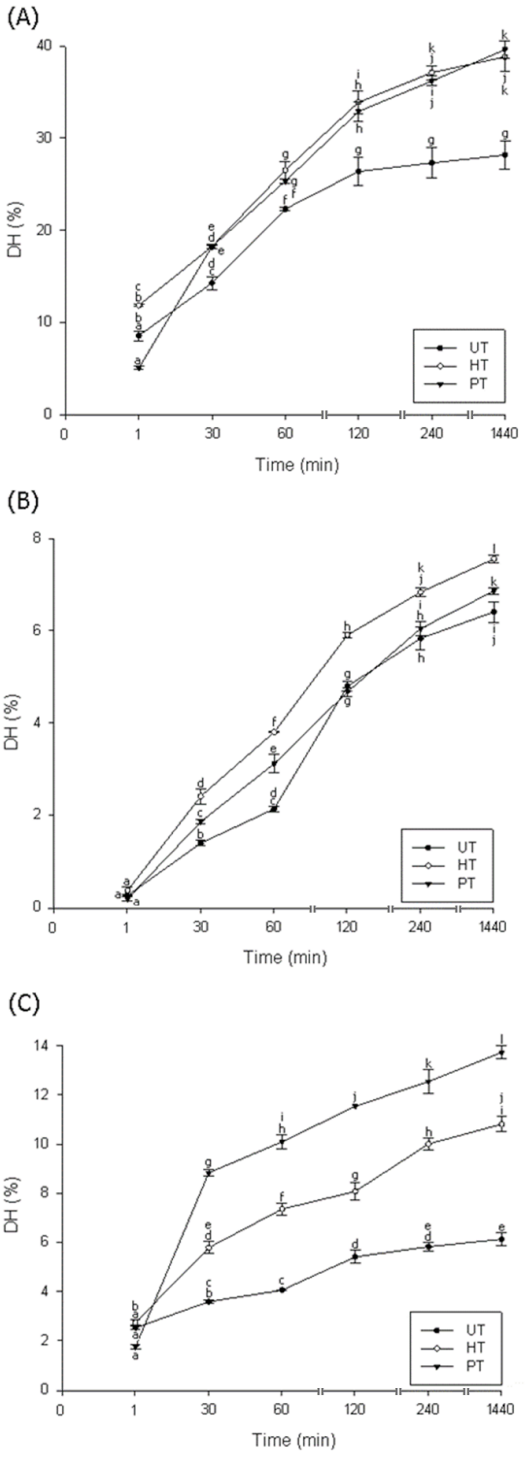

Figure 3. Degree of hydrolysis of bovine lung treated with Alcalase (A), collagenase (B) and papain (C) over a 24 h period. Results represent the means of three independent experiments ± standard deviation. Different letters indicate mean values that are significantly different for treatments at different times ($P < 0.05$). UT, HT and PT refer to untreated, heat-treated and high pressure-treated samples, respectively.

of the biologically active hydrolysates and peptides generated from meat co-products to date show antihypertensive, antimicrobial and antioxidant activities (Bah *et al*., 2013; Lafarga and Hayes, 2014). A number of DPP-IV- (Gallego *et al*., 2014) and PEP- (Ohmori *et al*., 1994) inhibitory peptides have been isolated from animal sources. In addition, bovine lung has been used as a source of antioxidant hydrolysates previously (Damgaard *et al*., 2015). However, to the best of our knowledge, this is the first study that has examined the potential of bovine lung as a source of DPP-IV and PEP inhibitors.

Protein content and amino acid composition

The total protein content of bovine lung and the generated hydrolysates was calculated, and the results were comparable to those obtained in previous hydrolysis studies where high protein yields were obtained from peanut or blood proteins (Lafarga *et al*., 2016). The amino acid composition of bovine lung (Table 1) showed the presence of a high concentration of glutamic acid, glycine, leucine, lysine, arginine and proline. Proline is a key amino acid in DPP-IV- and PEP-inhibitory peptides (Lawandi *et al*., 2010). The relatively high concentration of collagen and the amino acid residue proline, together with the high protein content, of bovine lung suggests its potential for use as a resource for the generation of PEP- and DPP-IV-inhibitory peptides. No significant differences were observed in the protein or moisture content of the generated hydrolysates. However, the ash content of the hydrolysates generated using Alcalase was higher ($P < 0.05$). This could be caused by the higher amount of NaOH added during hydrolysis to keep the pH constant at the optimum of the enzyme.

In vitro *assessment of bioactivity: effect of pre-treatment on the release of DPP-IV- and PEP-inhibitory peptides*

The DPP-IV IC_{50} value of the hydrolysates generated using Alcalase and that of the UT samples was similar to those obtained in the study by Sila *et al*. (2015), who recently reported the generation of peptidic hydrolysates from barbel skin gelatine with DPP-IV IC_{50} values ranging between 2.2 and 3.7 mg/mL depending on the enzymatic treatment used. As mentioned earlier, bovine collagen and elastin have been suggested as a potential source of DPP-IV inhibitors (Minkiewicz *et al*., 2011). However, although bovine lung is a resource rich in collagen and elastin, the *in vitro* DPP-IV-inhibitory properties obtained herein were lower than those obtained previously from enzymatic hydrolysates of bovine proteins, including serum albumin (Lafarga *et al*., 2016). In addition, the PEP-inhibitory activity of the generated hydrolysates was also higher than that reported in previous studies including enzymatic hydrolysates of barbel skin gelatine, which inhibited the activity of PEP by half at concentrations ranging from 0.91 to 3.79 mg/mL depending on the enzymatic treatment (Sila *et al*., 2015). This could be caused because the study by Sila *et al*. (2015) used fish-derived gelatine, which is different from bovine collagen and was already partially hydrolysed. In addition, use of different enzymes under different conditions results in different peptide profiles with different bioactivities.

PEP- and DPP-IV-inhibitory peptides are characterised by short lengths (Lawandi *et al*., 2010; Lacroix and Li-Chan, 2012). Collagen molecules are composed of a triple helix formed by the interaction of three a-chains, which are mainly stabilised by hydrogen bonds produced by a repetition of the GXY sequence, where X is generally proline and Y is generally hydroxyproline (Ichikawa *et al*., 2010). The number of short peptides generated from collagen by enzymatic hydrolysis was expected to be limited, and this may be the cause of the observed low inhibitory values. Hence, bovine lung was pre-treated with high temperature and high pressure to aid the enzymatic cleavage of proteins and the release of short peptides with potential bioactivities. Treatments with high pressure and heat were used previously to aid hydrolysis and to increase the biological activity of proteins. For example, high-pressure treatment was used previously to generate ACE-I-inhibitory peptides from ovalbumin (Quirós *et al*., 2007) and lentil proteins (Garcia-Mora *et al*., 2015), as well as for the release of antioxidant peptides from egg (Singh and Ramaswamy, 2014), lentils (Garcia-Mora *et al*., 2015) and pea (Girgih *et al*., 2015).

In this study, high pressure was the most efficient pre-treatment used to release DPP-IV-inhibitory peptides from bovine lung before hydrolysis with the enzyme Alcalase. Previous studies suggested that high pressure is more efficient when compared to heat (100 °C) in inducing conformational changes, enhancing enzymatic hydrolysis and releasing bioactive peptides (Girgih *et al*., 2015). Results obtained herein are similar to those obtained previously, where high pressure and Alcalase were found to be the most efficient treatment modes for generating ACE-I-inhibitory peptides from bovine collagen (Zhang *et al*., 2013). However, this trend was not observed after hydrolysis with papain, in which case heat pre-treatment was found to be more efficient in aiding the release of DPP-IV inhibitors. The IC_{50} value obtained for the papain generated after pressure treatment was significantly higher than that for the hydrolysate obtained after heat treatment ($P < 0.001$). In addition, both heat and high-pressure pre-treatment resulted in a comparable increase in the DPP-IV-inhibitory activity of the hydrolysate generated by hydrolysis using collagenase. Previous studies also suggested that the effect of pre-treatments such as high pressure and heat was dependant on the enzymatic treatment (Zhang *et al*., 2013). In this study, the most active DPP-IV-inhibitory hydrolysates were generated by hydrolysing pre-treated bovine lung with

Alcalase. The IC_{50} values of the Alcalase hydrolysates of PT and HT bovine lung were comparable to those obtained previously by proteolytic or microbial hydrolysis of East Asian azuki bean, where a 10-kDa fraction showed 52% inhibition of DPP-IV at a concentration of 1 mg/mL (Patil *et al*., 2015). The hydrolysates generated using Alcalase in this study were more active than DPP-IV-inhibitory hydrolysates reported previously in the literature, including a tuna cooking juice hydrolysate generated using fungal proteases, which inhibited DPP-IV by 45.2% at a concentration of 2 mg/mL (Huang *et al*., 2012).

Although numerous bioactive peptides with PEP-inhibitory activities have been isolated from natural sources, including wine (Yanai *et al*., 2003), bovine brain (Ohmori *et al*., 1994) and cheese (Sorensen *et al*., 2004), the number of active hydrolysates reported in the literature is still limited. Combinations or mixtures of peptides were suggested to ensure a better nutraceutical effect than single peptides (Sorensen *et al*., 2004). The PEP-inhibitory value of the Alcalase hydrolysate of PT bovine lung obtained in this study was comparable to that of chemically synthesised peptides, including MPPPLPARVDALD with an IC_{50} value of 38.4 mM (Ohmori *et al*., 1994) and to a peptidase hydrolysate of barbel skin, which had an IC_{50} value of 3.79 mg/mL (Sila *et al*., 2015). However, a number of hydrolysates generated by Sila *et al*. (2015), including the Alcalase, trypsin and neutrase hydrolysates of fish gelatine, showed lower IC_{50} values.

Overall, the generated enzymatic hydrolysates of UT bovine lung showed low DPP-IV- and PEP-inhibitory activities. However, heat and pressure treatment increased the DPP-IV- and PEP-inhibitory activities of bovine lung after hydrolysis with selected enzymes, and their *in vitro* inhibitory values were comparable to previously reported hydrolysates with PEP- and DPP-IV-inhibitory properties.

Effect of pre-treatment and hydrolysis time on the DH

A secondary aim of this study was to evaluate the effect of heat and high-pressure pre-treatments on the DH. In this study, high pressure was the most efficient pre-treatment among those studied in aiding the release of peptides using papain. The optimum temperature used for hydrolysis with papain was already high, probably denaturing part of the collagen triple helical structure and other proteins. High-pressure pre-treatment also increased the DH for hydrolysates generated with collagenase. However, the observed increase was lower, and after 24 h, the DH was 6.4 ± 0.3%, 7.6 ± 0.1% and 6.9 ± 0.1% for hydrolysates of UT, HT and PT samples. It is probable that pre-treatment of bovine lung facilitated the action of collagenase on peptide bonds of bovine collagen but did not cleave other proteins such as actin or haemoglobin present in the sample. In addition, Alcalase is a non-specific enzyme, and it is probable that the observed increase in the DH using Alcalase was caused by an increase in the activity of this enzyme on all the proteins contained in the bovine lung and not only on collagen.

Previous studies reported an increase in the DH after pre-treatment. For example, high pressure (300 MPa) was previously reported to result in the enhancement of the proteolytic efficacy of a number of enzymes, including Alcalase, Protamex, Savinase and Corolase 7089, on lentil proteins (Garcia-Mora *et al*., 2015). Moreover, high-pressure treatments also increased proteolysis of ovalbumin with pepsin, trypsin and chymotrypsin (Quirós *et al*., 2007) and proteolysis of bovine collagen with Alcalase, collagenase, thermolysin, pepsin and trypsin (Zhang *et al*., 2013). Although only one high-pressure pre-treatment was tested in this study (600 MPa, 3 min), a previous study carried out by Singh and Ramaswamy (2014) suggested that the DH was increased when both pressure level and pressure holding time were increased. In the study carried out by Singh and Ramaswamy (2014), the maximum DH was achieved when samples were treated at 500 MPa for 15 min (maximum pressure and highest treatment time tested). Therefore, we would expect an increase in DH with an increase in the pressure treatment duration, although this would need to be assessed *in vitro*. For hydrolysates generated with Alcalase, both heat and high-pressure pre-treatments increased the DH as shown in Figure 3. Treatment at 100 °C for 30 min was used previously (Girgih *et al*., 2015) for inducing structural changes in pea proteins, and although the DH was not measured in that study, the authors suggested that the observed increase in
the antioxidant activity was caused by a favoured release of peptides after hydrolysis of the pre-treated sample compared to the control (Girgih *et al*., 2015). In this study, heat was more effective in increasing the DH than high pressure after hydrolysis with collagenase. This aligns with a previous study in which both heat and high pressure were applied to bovine collagen, and thereafter boiling of collagen was found to give rise to the highest DH as well as ACE-I-inhibitory activity (Zhang *et al*., 2013). However, this trend was not observed in this study during hydrolysis using papain, whereby high-pressure pre-treatment resulted in the highest increase in the DH after 24 h.

Results obtained in this study suggested that heat and high-pressure pre-treatments can increase the DH of the papain, collagenase and Alcalase hydrolysates of bovine lung over a 24 h period, and this increase was found to be enzyme dependent. In addition, the observed increase in the DH was not directly related to an increase in bioactivity. For example, in the case of HT samples, the DH obtained after hydrolysis with Alcalase was five fold bigger than that obtained after hydrolysis with collagenase. However, the DPP-IV- and PEP-inhibitory bioactivities of the enzymatic hydrolysate of Alcalase were only slightly higher (P<0.05). Similar results were

Table 2. Collagen-derived peptides released from pressure-treated bovine lung by the action of Alcalase

Amino acid sequence	Protein Accession	Position	MW (Da)	PeptideRanker score
GFPGADGVAGPK	P02453|CO1A1_BOVIN	493–505	1,072.19	0.70
GADGAPGKDGVRGL	P02453|CO1A1_BOVIN	751–764	1,269.38	0.76
GADGAPGKDGVR	P02453|CO1A1_BOVIN	751–762	1,099.17	0.53
GDDGEAGKPGRPGERGPP	P02453|CO1A1_BOVIN	229–246	1,748.83	0.60
GAVGPAGKDGEAGAQ	P02453|CO1A1_BOVIN	604–618	1,284.35	0.26
GERGFPGERGVQ	P02453|CO1A1_BOVIN	676–687	1,288.39	0.32
GDRGDAGPK	P02453|CO1A1_BOVIN	742–750	871.91	0.41
DGAPGKDGVR	P02453|CO1A1_BOVIN	753–762	971.04	0.45
DRGDAGPK	P02453|CO1A1_BOVIN	743–750	814.85	0.38
GDRGIKGH	P02453|CO1A1_BOVIN	1102–1109	838.92	0.40
AGEEGKRGARGEPGPA	P02453|CO1A1_BOVIN	461–476	1,538.64	0.36
TGSPGSPGPDGK	P02453|CO1A1_BOVIN	539–550	1,056.10	0.51
GAPGDKGEAGPS	P02453|CO1A1_BOVIN	775–786	1,042.04	0.40
SGEPGAPGSK	P02453|CO1A1_BOVIN	432–441	885.93	0.41
DGAPGKDGVRGL	P02453|CO1A1_BOVIN	752–763	1,141.25	0.73
KDGVRGLTGPI	P02453|CO1A1_BOVIN	757–767	1,112.29	0.28
GFPGER	P02453|CO1A1_BOVIN	679–684	661.72	0.76
FPGER	P02453|CO1A1_BOVIN	680–684	604.66	0.73
SGERGPP	P02453|CO1A1_BOVIN	990–996	698.73	0.52
GEKGAPGADGPA	P02453|CO1A1_BOVIN	931–942	1,026.07	0.37
KGDRGDAGPK	P02453|CO1A1_BOVIN	741–750	1,000.08	0.33
GPRGETGPAGRPG	P02453|CO1A1_BOVIN	907–919	1,208.30	0.47
FLPQPPQEK	P02453|CO1A1_BOVIN	1199–1207	1,083.25	0.41
GERGFPGLP	P02453|CO1A1_BOVIN	967–975	929.04	0.78
FGFDGDFYRA	P02465|CO1A2_BOVIN	1108–1117	1,194.27	0.83
GDQGPVGR	P02465|CO1A2_BOVIN	821–828	784.83	0.53
GFDGDFYRA	P02465|CO1A2_BOVIN	1109–1117	1,047.09	0.82
GEKGETGLR	P02465|CO1A2_BOVIN	653–661	946.03	0.20
GEDGHPGKPGRPGER	P02465|CO1A2_BOVIN	140–154	1,545.63	0.62
GIDGRPGPIGPA	P02465|CO1A2_BOVIN	470–481	1,106.25	0.60
GRPGPIGPA	P02465|CO1A2_BOVIN	473–481	820.95	0.69
DGDFYR	P02465|CO1A2_BOVIN	1111–1116	771.78	0.78
GKEGPVGLPGIDGRP	P02465|CO1A2_BOVIN	461–475	1,448.64	0.60
GLPGADGR	P02465|CO1A2_BOVIN	404–411	741.80	0.49
GFDGDFYR	P02465|CO1A2_BOVIN	1109–1116	976.01	0.87
GKEGPVGLPGID	P02465|CO1A2_BOVIN	461–472	1,138.29	0.39
AGARGSDGSVGPVGPA	P02465|CO1A2_BOVIN	229–244	1,354.44	0.35
RGEVGLPGLSGPV	P02465|CO1A2_BOVIN	280–292	1,237.42	0.39
GEEGKRGSTGEIGPA	P02465|CO1A2_BOVIN	374–388	1,444.52	0.23
GSVGEPGPL	P02465|CO1A2_BOVIN	887–895	811.89	0.61
GERGPPGESG	P02465|CO1A2_BOVIN	587–596	941.95	0.28
GDKGEPGDKGPR	P02465|CO1A2_BOVIN	1004–1015	1,212.28	0.52
SGLPGER	P02465|CO1A2_BOVIN	637–643	714.78	0.49
EGPVGLPGIDGRP	P02465|CO1A2_BOVIN	463–475	1,263.42	0.53
GERGLPGVA	P02465|CO1A2_BOVIN	878–886	854.96	0.31

Amino acid sequence	Protein Accession	Position	MW (Da)	PeptideRanker score
FDGDFYRA	P02465|CO1A2_BOVIN	1110–1117	990.04	0.83
DGNKGEPGVV	P02465|CO1A2_BOVIN	616–625	971.03	0.22
FDGDFYR	P02465|CO1A2_BOVIN	1110–1116	918.96	0.89
KEGPVGLPGID	P02465|CO1A2_BOVIN	462–472	1,081.23	0.31
GEVGLPGL	P02465|CO1A2_BOVIN	281–288	740.85	0.52
AGPAGERGEQGAP	P02459|CO2A1_BOVIN	647–659	1,196.24	0.32
GERGEQGAP	P02459|CO2A1_BOVIN	651–659	899.92	0.13
GERGAPGEKGEG	P04258|CO3A1_BOVIN	669–680	1,143.18	0.27
GERGVPGF	P04258|CO3A1_BOVIN	327–334	817.90	0.58
GVDGAPGKDGPR	P04258|CO3A1_BOVIN	594–605	1,125.21	0.53
GLPGGPGLR	P04258|CO3A1_BOVIN	372–380	822.96	0.84
GERGGPGGPGPQ	P04258|CO3A1_BOVIN	438–449	1,065.11	0.43
VDGAPGKDGPR	P04258|CO3A1_BOVIN	595–605	1,068.15	0.48
GSSGVDGAPGKDGPR	P04258|CO3A1_BOVIN	591–605	1,356.41	0.56
RGEPGPQGHAGAP	P04258|CO3A1_BOVIN	212–224	1,230.31	0.44
GNRGERGSEGSP	P04258|CO3A1_BOVIN	1011–1022	1,202.21	0.13
ERGVPGF	P04258|CO3A1_BOVIN	328–334	760.85	0.55
GVPGIAGPR	P04258|CO3A1_BOVIN	630–638	822.96	0.62
KGEPGSSGVDGAPGKDGP	P04258|CO3A1_BOVIN	587–604	1,611.69	0.44
GIPGAPGLI	P04258|CO3A1_BOVIN	246–254	793.96	0.73
GVPGFRGPA	P04258|CO3A1_BOVIN	330–338	856.98	0.72
TTAPLPMMPVAEEDIRPY	Q7SIB3|CO4A2_BOVIN	84–101	2,031.37	0.16
EDIRPY	Q7SIB3|CO4A2_BOVIN	96–101	791.86	0.27

PeptideRanker is available at http://bioware.ucd.ie/~compass/biowareweb/. MW = molecular weight.

obtained for PT samples, whereby no significant differences were observed between the IC_{50} values of the hydrolysates generated using Alcalase and collagenase, and the DH of the former was five times bigger than the latter. This was caused by a higher content of peptides derived from collagen in the hydrolysate generated using collagenase compared to the one generated using Alcalase, which contains a larger number of peptides but which were generated from proteins such as haemoglobin or serum albumin.

Identification of proteins and peptides in pre-treated bovine lung Alcalase hydrolysate

Identification of small peptides in biological or food samples is mainly done by MS coupled with LC. In this study, a total of 2,350 peptides were identified by LC-MS/MS in the Alcalase hydrolysate of PT lung proteins (supplementary data). The majority of these (73.5%) were cleaved from type I collagen, which is the most common type of collagen in the body (approximately 90%) and is found in the skin, tendon, bones and internal organs including the lungs (Di Lullo *et al*., 2002). Numerous peptides were also generated from bovine haemoglobin (323) and actin (373). The molecular weight (MW) of most of the peptides identified in this study ranged between 600 and 1,200 Da. As mentioned earlier, PEP- and DPP-IV-inhibitory peptides are short in length (Lawandi *et al*., 2010; Lacroix and Li-Chan, 2012). Because of the sequence diversity of short peptides, *de novo* sequencing or the use of standards for matching the accurate masses and retention times is difficult and time consuming. The use of database searches for peptide identification is commonly used. However, current search algorithms are often limited to peptides containing more than four amino acids in length (Tang *et al*., 2014). Shorter peptides do not produce a sufficient number of unique fragment ions and are not retained well on LC columns, making their identification very complicated (Tang *et al*., 2014). Many efforts are being put into the identification and quantification of di-, tri-, and other small peptides. For example, Tang *et al*. (2014) recently published a novel database search methodology for the identification of di- and tripeptides from complex biological samples based on the use of MS/MS spectra of both unlabelled and dimethyl-labelled peptides.

Table 2 lists the identified unique collagen-derived peptides generated from PT bovine lung by the action of Alcalase,

together with their positions in the parent protein and their calculated MWs. Table 2 also lists the PeptideRanker scores of the identified peptides. PeptideRanker, available at http://bioware.ucd.ie, is a useful *in silico* tool that may be used to identify, among a set of peptides, those that are more likely to be bioactive (Mooney *et al*., 2012). A number of peptides, including GFDGDFYRA, FDGDFYRA and FDGFY, which corresponded to *f*(1109-1117), *f*(1110-111b7) and *f*(1110-1115) of CO1A2_BOVIN, respectively, showed high (more than 0.80) scores assigned by PeptideRanker. In addition, it has been shown that DPP-IV-inhibitory peptides consist of short sequences of between two and nine amino acids in length (Hatanaka *et al*., 2012). Potent DPP-IV inhibitors contain proline or alanine residues as the penultimate position at the N-terminal end of the peptide (Power *et al*., 2014). These peptides usually possess specific amino acid sequences composed of hydrophobic residues, together with proline, arginine and lysine (Hatanaka *et al*., 2012). Numerous dipeptides with a proline residue at the C-terminus have been identified as DPP-IV inhibitors (Hatanaka *et al*., 2012). However, dipeptides that do not contain proline have also been shown to inhibit DPP-IV (Lacroix and Li-Chan, 2012). Structural studies of PEP have shown that the access of the substrate or inhibitor to the active site of the enzyme is difficult: the active site is hidden by a seven-bladed b-propeller that works as a filter, excluding large peptides and proteins (Fülöp *et al*., 1998). Effective PEP-inhibitory peptides are substrate-like compounds, which contain a proline or a proline analogue (Lawandi *et al*., 2010). A quantitative structure–activity relationship study carried out by Pripp (2006) suggested that increased hydrophobicity and molecular bulkiness of amino acids increased the bioactivity of PEP-inhibitory peptides. Peptides with high PeptideRanker scores and those rich in proline and hydrophobic residues were expected to be bioactive.

Acknowledgements

Tomas Lafarga is in receipt of a Teagasc Walsh Fellowship. This work formed part of the ReValueProtein Research Project (Grant Award No. 11/F/043), which was supported by the Irish Department of Agriculture, Food and the Marine and the Food Institutional Research Measure, both funded by the Irish Government under the National Development Plan 2007–2013. The authors would like to acknowledge Mr Kieran Wynne from the University College Dublin and Ms Anne Marie McAuliffe from Teagasc Food Research Centre, Moorepark, Cork, Ireland, for their collaboration in the identification of the peptides and in the amino acid composition analysis, respectively.

References

Adler-Nissen, J. 1986. “Enzymatic Hydrolysis of Food Protein”. Elsevier Applied Science Publishers LTD, London, UK, 451 pages.

Agyei, D. and Danquah, M.K. 2011. Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnology Advances* **29**: 272–277.

AOAC. 1990. “Official Methods of Analysis of the Association of Official Analytical Chemists”, 15th Edition. Association of Official Analytical Chemists Inc., Virginia, USA, 771 pages.

Bah, C.S.F., Bekhit, A.E.D.A., Carne, A. and McConnell, M.A. 2013. Slaughterhouse blood: an emerging source of bioactive compounds. *Comprehensive Reviews in Food Science and Food Safety* **12**: 314–331.

Cheng, L., Lumb, M., Polgar, L. and Mudge, A.W. 2005. How can the mood stabilizer VPA limit both mania and depression? *Molecular and Cellular Neuroscience* **29**: 155–161.

Damgaard, T., Lametsch, R. and Otte, J. 2015. Antioxidant capacity of hydrolyzed animal by-products and relation to amino acid composition and peptide size distribution. *Journal of Food Science and Technology* **52**: 6511–6519.

Darine, S., Christophe, V. and Gholamreza, D. 2010. Production and functional properties of beef lung protein concentrates. *Meat Science* **84**: 315–322.

Di Bernardini, R., Mullen, A.M., Bolton, D., Kerry, J., O’Neill, E. and Hayes, M. 2012. Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. *Meat Science* **90**: 226–235.

Di Lullo, G.A., Sweeney, S.M., Korkko, J., Ala-Kokko, L. and San Antonio, J.D. 2002. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *Journal of Biological Chemistry* **277**: 4223–4231.

Drucker, D.J. 2006. The biology of incretin hormones. *Cell Metabolism* **3**: 153–165.

Frenssen, F., Croonberghs, J., Van den Sttene, H. and Maes, M. 2015. Prolyl endopeptidase and dipeptidyl peptidase IV are associated with externalizing and aggressive behaviors in normal and autistic adolescents. *Life Sciences* **136**: 157–162.

Fülöp, V., Böcskei, Z. and Polgár, L. 1998. Prolyl oligopeptidase: an unusual β-propeller domain regulates proteolysis. *Cell* **94**: 161–170.

Gallego, M., Aristoy, M.C. and Toldra, F. 2014. Dipeptidyl peptidase IV inhibitory peptides generated in Spanish dry-cured ham. *Meat Science* **96**: 757–761.

García-Horsman, J.A., Mannisto, P.T. and Venalainen, J.I. 2007. On the role of prolyl oligopeptidase in health and disease. *Neuropeptides* **41**: 1–24.

Garcia-Mora, P., Peñas, E., Frias, J., Gomez, R. and Martinez-Villaluenga, C. 2015. High-pressure improves enzymatic proteolysis and

the release of peptides with angiotensin I converting enzyme inhibitory and antioxidant activities from lentil proteins. *Food Chemistry* **171**: 224–232.

Girgih, A.T., Chao, D., Lin, L., He, R., Jung, S. and Aluko, R.E. 2015. Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment. *Food Chemistry* **188**: 510–516.

Hatanaka, T., Inoue, Y., Arima, J., Kumagai, Y., Usuki, H., Kawakami, K., Kimura, M., and Mukaihara, T. 2012. Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran. *Food Chemistry* **134**: 797–802.

Hayes, M., Moen, L.F., Auty, M.A.E. and Lea, T.E. 2015. Transport of a proly endopeptidase inhibitory peptide across the blood brain barrier demonstrated using the hCMEC/D3 cell line transcytosis assay. *Journal of Agricultural and Food Chemistry* **64**: 146–150.

Hill, R.L. 1965. Hydrolysis of proteins. *Advances in Protein Chemistry* **20**: 37–107.

Huang, S.L., Jao, C.L., Ho, K.P. and Hsu, K.C. 2012. Dipeptidyl-peptidase IV inhibitory activity of peptides derived from tuna cooking juice hydrolysates. *Peptides* **35**: 114–121.

Ichikawa, S., Morifuji, M., Ohara, H., Matsumoto, H., Takeuchi, Y. and Sato, K. 2010. Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of gelatin hydrolysate. *International Journal of Food Sciences and Nutrition* **61**: 52–60.

Kolar, K. 1992. Gravimetric determination of moisture and ash in meat and meat products: NMKL interlaboratory study. *Journal of AOAC International* **75**: 1016–1022.

Korhonen, H. and Pihlanto, A. 2006. Bioactive peptides: production and functionality. *International Dairy Journal* **16**: 945–960.

Lacroix, I.M.E. and Li-Chan, E.C.Y. 2012. Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. *Journal of Functional Foods* **4**: 403–422.

Lafarga, T., Aluko, R.E., Rai, D.K., O'Connor, P. and Hayes, M. 2016. Identification of bioactive peptides from a papain hydrolysate of bovine serum albumin and assessment of an antihypertensive effect in spontaneously hypertensive rats. *Food Research International* **81**: 91–99.

Lafarga, T. and Hayes, M. 2014. Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients. *Meat Science* **98**: 227–239.

Lafarga, T., O'Connor, P. and 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.

Lafarga, T., O'Connor, P. and Hayes, M. 2015. *In silico* methods to identify meat-derived prolyl endopeptidase inhibitors. *Food Chemistry* **175**: 337–343.

Lambeir, A.M., Durinx, C., Scharpé, S. and De Meester, I. 2003. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical Reviews in Clinical Laboratory Sciences* **40**: 209–294.

Lawandi, J., Gerber-Lemaire, S., Juillerat-Jeanneret, L. and Moitessir, N. 2010. Inhibitors of prolyl oligopeptidases for the therapy of human diseases: defining diseases and inhibitors. *Journal of Medicinal Chemistry* **53**: 3423–3438.

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dzuiba, M. and Darewicz, M. 2008. BIOPEP database and other programs for processing bioactive peptide sequences. *Journal of AOAC International* **91**: 965–980.

Minkiewicz, P., Dziuba, J. and Michalska, J. 2011. Bovine meat proteins as potential precursors of biologically active peptides – a computational study based on the BIOPEP database. *Food Science and Technology International* **17**: 39–45.

Mooney, C., Haslam, N.J., Pollastri, G. and Shields, D.C. 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PLoS ONE* **7**: e45012.

Morain, P., Lestage, P., De Nanteuil, G., Jochemsen, R., Robin, J.L., Guez, D. and Boyer, P.A. 2002. S 17092: a prolyl endopeptidase inhibitor as a potential therapeutic drug for memory impairment. Preclinical and clinical studies. *CNS Drug Reviews* **8**: 31–52.

Nedjar-Arroume, N., Dubios-Del Val, V., Adje, E.Y., Traisnel, J., Krier, F., Mary, P., Kouach, M., Briand, G. and Guillochon, D. 2008. Bovine hemoglobin: an attractive source of antibacterial peptides. *Peptides* **29**: 969–977.

Ohmori, T., Nakagami, T., Tanaka, H. and Maruyama, S. 1994. Isolation of prolylendopeptidase-inhibiting peptides from bovine brain. *Biochemical and Biophysics Research Communications* **202**: 809–815.

Patil, P., Mandal, S., Tomar, S.K. and Anand, S. 2015. Food protein-derived bioactive peptides in management of type 2 diabetes. *European Journal of Nutrition* **54**: 863–880.

Power, O., Nongonierma, A., Jakeman, P. and Fitzgerald, R. 2014. Food protein hydrolysates as a source of dipeptidyl peptidase IV inhibitory peptides for the management of type 2 diabetes. *Proceedings of the Nutrition Society* **73**: 34–46.

Pripp, A.H. 2006. Quantitative structure-activity relationship of prolyl oligopeptidase substrate/inhibitory peptides derived from β-casein using simple amino acid descriptors. *Journal of Agricultural and Food Chemistry* **54**: 224–228.

Quirós, A., Chichon, R., Recio, I. and Lopez-Fandiño, R. 2007. The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry* **104**: 1734–1739.

Sila, A., Martinez-Alvarez, O., Haddar, A., Gomez Guillen, M.C., Nasril, M., Montero, M.P. and Bougatef, A. 2015. Recovery, viscoelastic and functional properties of Barbel skin gelatine: investigation of anti-DPP-IV and anti-prolyl endopeptidase activities of generated gelatine polypeptides. *Food Chemistry* **168**: 478–486.

Singh, A. and Ramaswamy, H.S. 2014. Effect of high-pressure treatment on trypsin hydrolysis and antioxidant activity of egg white

proteins. *International Journal of Food Science and Technology* **49**: 269–279.

Sorensen, R., Kildal, E., Stepaniak, L., Pripp, A.H. and Sorhaug, T. 2004. Screening for peptides from fish and cheese inhibitory to prolyl endopeptidase. *Nahrung/Food* **48**: 53–56.

Tang, Y., Li, R., Lin, G. and Li, L. 2014. PEP Search in MyCompoundID: detection and ddentification of dipeptides and tripeptides using dimethyl labeling and hydrophilic interaction liquid chromatography tandem mass spectrometry. *Analytical Chemistry* **86**: 3568–3574.

Thomas, L., Eckhardt, M., Langkopf, E., Tadayyon, M., Himmelsbach, F. and Mark, M. 2008. (R)-8-(3-Amino-piperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinazolin-2-ylmethyl)-3,7-dihydropurine-2,6-dione (BI 1356), a novel xanthine-based dipeptidyl peptidase 4 inhibitor, has a superior potency and longer duration of action compared with other dipeptidyl peptidase-4 inhibitors. *Journal of Pharmacology and Experimental Therapeutics* **325**: 175–182.

Toldrá, F., Aristoy, M.C., Mora, L. and Reig, M. 2012. Innovations in value-addition of edible meat by-products. *Meat Science* **92**: 290–296.

Waget, A., Cabou, C., Masseboeuf, M., Cattan, P., Armanet, M., Karaca, M., Castel, J., Garret, C., Payros, G., Maida, A., Sulpice, T., Holst, J.J., Drucker, D.J., Magnan, C., and Burcelin, R. 2011. Physiological and pharmacological mechanisms through which the DPP-IV inhibitor sitagliptin regulates glycemia in mice. *Endocrinology* **152**: 3018–3029.

Wilson, J., Hayes, M. and Carney, B. 2011. Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from natural sources with a focus on marine processing by-products. *Food Chemistry* **129**: 235–244.

Yanai, T., Suzuki, Y. and Ssato, M. 2003. Prolyl endopeptidase inhibitory peptides in wine. *Bioscience, Biotechnology, and Biochemistry* **67**: 380–382.

Yaron, A., Naider, F. and Scharpe, S. 1993. Proline-dependent structural and biological properties of peptides and proteins. *Critical Reviews in Biochemistry and Molecular Biology* **28**: 31–81.

Zhang, Y., Olsen, K., Grossi, A. and Otte, J. 2013. Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides. *Food Chemistry* **141**: 2343–2354.