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Atlantic salmon (*Salmo salar*) co-product-derived protein hydrolysates: A source of antidiabetic peptides

Short title: Antidiabetic Atlantic salmon (*Salmo salar*) co-product hydrolysates


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Abstract

Large quantities of low-value protein rich co-products, such as salmon skin and trimmings, are generated annually. These co-products can be upgraded to high-value functional ingredients. The aim of this study was to assess the antidiabetic potential of salmon skin gelatin and trimming-derived protein hydrolysates in vitro. The gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L exhibited significantly higher ($p<0.001$) insulin and GLP-1 secretory activity from pancreatic BRIN-BD11 and enteroendocrine GLUTag cells, respectively, when tested at 2.5 mg/mL compared to hydrolysates generated with Alcalase 2.4L or Promod 144MG. The gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L showed significantly more potent ($p<0.01$) DPP-IV inhibitory activity than those generated with Alcalase 2.4L or Promod 144MG. No significant difference was observed in the insulinotropic activity mediated by any of the trimming-derived hydrolysates when tested at 2.5 mg/mL. However, the trimmings hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L exhibited significantly higher DPP-IV inhibitory ($p<0.05$: Alcalase 2.4L and $p<0.01$: Promod 144MG) and GLP-1 ($p<0.001$, 2.5 mg/mL) secretory activity than those generated with Alcalase 2.4L or Promod 144MG. The salmon trimmings hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L when subjected to simulated gastrointestinal digestion (SGID) was shown to retain its GLP-1 secretory and DPP-IV inhibitory activities, in addition to improving its insulin secretory activity. However, the gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L was shown to lose GLP-1 secretory activity following SGID. A significant increase in membrane potential ($p<0.001$) and intracellular calcium ($p<0.001$) by both co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L suggest that both hydrolysates mediate their insulinotropic activity through the $K_{\text{ATP}}$ channel-dependent pathway. Additionally, by stimulating a significant increase in intracellular cAMP release ($p<0.05$) it is likely that the trimming-derived hydrolysate may also
mediate insulin secretion through the protein kinase A pathway. The results presented herein demonstrate that salmon co-product hydrolysates exhibit promising \textit{in vitro} antidiabetic activity.

\textbf{Keywords:} salmon skin, co-products, gelatin, muscle, protein hydrolysate, peptide, antidiabetic, trimmings

1. \textbf{Introduction:}

In response to the increased global demand for seafood, food production from fisheries and aquaculture exceeded 160 million tons in 2014 (FAO, 2016). With the world population due to reach 9.8 billion by 2050, the demand for such food products is set to increase further (UN, 2017). At present only 50–60\% of total marine landings are utilized for direct human consumption with the remainder used as fertiliser or for animal and farmed fish/shellfish feed (Norris, Harnedy, & FitzGerald, 2013). With the projected escalation in seafood production and the fact that a significant quantity of the co-product material generated during fish/shellfish processing, if handled and stored correctly, is suitable for human consumption, co-products from marine food processing are potential added-value components capable of ensuring a sustainable food supply. Low-value co-products such as salmon trimmings and skin, which are generated during the salmon filleting process, contain significant quantities of high quality protein and gelatin.

Gelatin, a partially hydrolysed form of the triple helix protein collagen, is widely used in the food, pharmaceutical and cosmetics industries because of its unique functional and technological properties (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). The most abundant current sources of commercial gelatin arise from terrestrial animal by-products such as porcine and bovine skin/hide and bones (Gómez-Guillén et al., 2011). However, the food, pharmaceutical and cosmetic industries are now searching for alternative sources of gelatin and gelatin derivatives (e.g., hydrolysates) to meet the global consumer demand for certifiably safe food products that can
be utilised by certain religious groups (e.g., Halal and Kosher certified) and customers who have made specific lifestyle choices in relation to diet (e.g., vegetarians and vegans).

In addition to acting as potential sustainable food sources to meet future demand, marine proteins contain a large range of bioactive components in the form of peptides encrypted within their primary sequences. Evidence indicates that novel proteins and peptides from the marine environment can have a favourable impact on health enhancement and may be utilised to combat the ever-increasing incidences of non-communicable diseases such as cardiovascular disease, cancer, chronic respiratory disease and diabetes (Harnedy & FitzGerald, 2012).

T2DM is a chronic progressive metabolic disorder that is characterised by hyperglycaemia due to impaired insulin secretion and tissue insulin resistance. In the early stages of T2DM elevated pancreatic insulin secretion (hyperinsulinaemia) can compensate for tissue insulin resistance thus avoiding hyperglycaemia. Insulin resistance is characterised by impaired responsiveness of cells in the muscles, liver and fat tissue towards insulin, which leads to a reduction in glucose uptake and hyperglycaemia, when pancreatic insulin production can no longer compensate (Goldstein, 2002). Approximately 415 million individuals worldwide are currently living with the disorder with approximately 12% ($673 billion) of global healthcare expenditure spent on diabetes management/treatment (IDF Atlas, 2015). This is expected to increase to 642 million people by 2040 if immediate interventions are not made (IDF Atlas, 2015). Drug therapies such as biguanides, meglitinides, sulphonylureas, thiazolidinediones, insulin, insulin analogues, sodium glucose co-transporter-2 (SGLT-2) inhibitors, incretin-based therapies, DPP-IV and α-glucosidase inhibitors are currently used for treatment of T2DM (Olokoba, Obateru, & Olokoba, 2012). These therapies are expensive and often display adverse side effects. Therefore, there is an ever-increasing need to identify natural functional components for the management of this metabolic disorder. Evidence-based research has shown that blood glucose levels in humans can be beneficially regulated by dietary protein, protein hydrolysates, peptides and amino acids through
direct and/or indirect mechanisms (Oseguera-Toledo, de Mejía, Reynoso-Camacho, Cardador-Martínez, & Amaya-Llano, 2014; Promintzer & Krebs, 2006). These mechanisms include direct stimulation of insulin secretion from pancreatic β-cells or indirectly through the release of the gut incretin hormone GLP-1 (Oseguera-Toledo et al., 2014; Promintzer & Krebs, 2006). The insulinotropic potency of protein and protein hydrolysates can differ significantly depending on the primary sequence of the peptides and amino acids generated during digestion (McGregor & Poppitt, 2013; Ranawana & Kaur, 2013).

Salmon protein hydrolysates/peptides exhibit antidiabetic activity in vitro and in vivo. In previous studies we have shown that muscle protein and gelatin hydrolysates derived from Atlantic salmon trimmings inhibit dipeptidyl peptidase-IV (DPP-IV) in vitro (Neves, Harnedy, O’Keeffe, Alashi, et al., 2017; Neves, Harnedy, O’Keeffe, & FitzGerald, 2017). This was also seen with hydrolysates generated from salmon muscle protein and salmon skin derived gelatin (Falkenberg, Stagsted, & Nielsen, 2014; Li-Chan, Hug, Jao, Ho, & Hsu, 2012). Salmon protein hydrolysates were shown to stimulate insulin-induced glucose uptake in L6 myocytes (Jin, 2012). Salmon skin gelatin derived peptides were reported to reduce blood glucose concentrations in diabetic rat models through enhancement of circulating GLP-1 concentration and reduction in T2DM-related islet cell apoptosis (Hsieh, Wang, Hung, M.C., & Hsu, 2012; Zhu et al., 2017; Zhu, Peng, Liu, Zhang, & Li, 2010). Salmon protein incorporation into a high-fat, high-sucrose diet was shown to improve whole-body insulin sensitivity and glucose tolerance in male Wistar rats (Pilon et al., 2011). In addition, low molecular weight peptides from a salmon protein hydrolysate were reported to prevent obesity-linked glucose intolerance in LDLR$^{+/+}$/ApoB$^{100/100}$ mice (Chevrier et al., 2015). Furthermore, marine collagen peptides have been shown to beneficially modulate glucose metabolism in T2DM human subjects (Zhu, Li, et al., 2010a; Zhu, Li, et al., 2010b). These individuals showed lower fasting blood glucose and glycated haemoglobin (HbA$_{1c}$) concentrations, as well as increased insulin secretion and tissue insulin sensitivity, following
ingestion of marine collagen peptides. Furthermore, we have recently shown that a blue whiting (Micromesistius poutassou) muscle protein hydrolysate exhibited antidiabetic activity in vitro and in normal healthy mice (Harney et al., 2018).

The objective of this study was to employ in vitro analysis to investigate the antidiabetic potential of salmon co-product hydrolysates and to determine the mechanism by which the hydrolysates may mediate their antidiabetic activity.

2. Materials and Methods

2.1. Materials and chemicals

Abz-Gly-p-nitro-Phe-Pro-OH, Abz-Gly-OH-HCl, H-Gly-Pro-AMC (7-amino-4-methyl coumarin) and Diprotin A were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Promod 144MG was kindly provided by Biocatalysts Ltd, (Cardiff, Wales, UK). HPLC grade water and acetonitrile were from VWR International (Dublin, Ireland) and trinitrobenzenesulphonic acid (TNBS) reagent was from Medical Supply Co Ltd. (Dublin, Ireland). Calcium chloride dihydrate (CaCl₂×2H₂O), D-glucose, HEPES, hydrochloric acid (HCl), magnesium sulphate (MgSO₄×7H₂O), potassium dihydrogen orthophosphate (KH₂PO₄), potassium chloride (KCl), sodium bicarbonate (NaHCO₃) and sodium chloride (NaCl) were purchased from BDH Chemicals Ltd (Poole, Dorset, UK). Fetal bovine serum (FBS), Hank’s buffered saline solution (HBSS 10× stock), penicillin-streptomycin (0.1 g/L), RPMI-1640 culture media, Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose and trypsin/EDTA (10×) were obtained from Gibco Life Technologies Ltd (Paisley, Strathclyde, UK). Radio-labelled sodium iodide (Na¹²⁵I, IMS 100 mCi/mL stock) was purchased from Perkin Elmer (Buckinghamshire, UK). Rat insulin standard was purchased from Novo Industria, Copenhagen, Denmark. All other reagents including DPP-IV, from porcine kidney (≥10 units/mg protein), Alcalase® 2.4L and Flavourzyme® 500L were supplied by Sigma Chemical Company Ltd. (Wicklow, Ireland). The Good Fish Processing
Company Ltd., Carrigaline, Co. Cork, Ireland, kindly provided samples of Atlantic salmon (*Salmo salar*) trimmings and skin.

2.2 Extraction and quantification of gelatin from salmon skin

Salmon skin which were stored at -20°C were thawed at room temperature cut into pieces (about 5×5 cm) and were then washed by stirring in distilled water (1:5 (w/v)) for 15 min. The skins were soaked in 0.2 M NaOH (1:5 (w/v)) and stirred at room temperature for 15 min to remove non-collagenous proteins. This procedure was repeated three times. The skins were separated by filtration using a double layer of cheesecloth between each step of the extraction procedure. The NaOH was then removed by sequentially soaking (×3) the skins for 15 min in distilled water at a mass:volume ratio of 1:5 (w/v) until the pH was neutral. The skins were suspended in distilled water (1:6 (w/v)); the pH was adjusted to pH 3.0 with 1N HCl, and stirred for 1 h at room temperature. The HCl was removed by sequentially soaking (×3) the skins for 15 min in distilled water (1:5 (w/v)) until the pH was neutral. The gelatin in the swollen skins was then extracted in distilled water 1:5 (w/v) during heating at 50°C for 16 h. The gelatin-containing supernatant was freeze-dried and stored at -20°C until required. The protein content of the gelatine isolate and salmon trimmings protein hydrolysate was determined by the macro-Kjeldahl procedure as described previously by Connolly, Piggott, and FitzGerald (2013) using a nitrogen to protein conversion factor of 5.55 and 6.25, respectively (FAO/INFOODS, 2012; Kristinsson & Rasco, 2000).

2.3 Enzymatic hydrolysis of salmon skin gelatin and trimmings

A 7% (w/v) gelatin solution (on a protein basis) was preheated to 50°C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L, Alcalase 2.4L and Flavourzyme 500L, and Promod 144MG at an enzyme:substrate (E:S) ratio of 0.74% (w/w or v/w) at 50°C. The hydrolysis reaction was
maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland) and enzyme activity was terminated after 4 h incubation by heating at 90°C for 20 min. Salmon trimming proteins were hydrolysed by direct hydrolysis. The minced salmon trimmings were suspended at 1:1.75 (w/v) in distilled water and homogenised at room temperature at 24,000 rpm over 4 × 15 sec treatments (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany). Additional water was added following homogenisation to obtain a 6.83% (w/v) protein suspension. Hydrolysis was performed as described above and the peptides generated were separated by 2× filtration (Whatman grade 1: 11 μm). The filtrate were freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

2.4 In vitro insulin secretion in BRIN-BD11 cells

The effect of the hydrolysates on in vitro insulin secretion was measured using clonal pancreatic BRIN-BD11 cells as previously described (McClenaghan et al., 1996). Briefly, cells were seeded (150,000 cells per well) into 24-well plates and allowed to attach overnight at 37°C. After a 40 min pre-incubation (1.1 mM glucose; 37°C) cells were incubated (20 min; 37°C) in the presence of 5.6 mM glucose with a range of hydrolysate concentrations (0.039–2.500 mg/mL). Following 20 min incubation, sample were removed from each well and aliquots (200 μL) stored at -20°C prior to measurement of insulin by radioimmunoassay (RIA) as described previously (Flatt & Bailey, 1981).

In order to determine if the hydrolysate samples were cytotoxic, the lactate dehydrogenase (LDH) concentrations in cellular supernatants were measured using a CytoTox96 non-radioactive cytotoxicity assay kit according to the manufacturers’ protocol (Promega, Madison, WI, USA). The release of cytoplasmic LDH from cells treated with the hydrolysates indicates that the integrity of the plasma membrane has been compromised (Owolabi et al., 2016).

2.5 In vitro determination of dipeptidyl peptidase-IV (DPP-IV) inhibitory activity
In vitro DPP-IV inhibitory activity of the hydrolysates was determined as described by Harnedy, O’Keeffe, and FitzGerald (2015). The results were expressed as IC\textsubscript{50} values (concentration that inhibits DPP-IV activity by 50%, n=3). Diprotin A (Pro-Ile-Pro) was used as a positive control.

2.6 Determination of GLP-1 secretion from GLUTag cells

The GLP-1 secretory effects of hydrolysates were measured in vitro using a murine GLUTag cell line as described previously (Ojo, Conlon, Flatt, & Abdel-Wahab, 2013). In brief, cells were seeded on matrigel coated plates (150,000 cells/well) and incubated overnight at 37°C. Following a 40 min pre-incubation step (37°C), cells were treated with the hydrolysates (2.5 mg/mL) prepared in 2 mM glucose and incubated for 2 h at 37°C. Following incubation, the supernatant buffer was removed and stored at -20°C prior to measurement of total GLP-1 concentration using a sandwich ELISA kit according to the manufacturer’s protocol (GLP-1 Total ELISA, EZGLP-1T-36K, Millipore, MA, USA).

2.7 Membrane potential, intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) and cAMP production

Changes in membrane potential and [Ca\textsuperscript{2+}]\textsubscript{i} were assessed fluorimetrically utilizing monolayers of BRIN-BD11 cells as described previously (Srinivasan, Mechkarska, Abdel-Wahab, Flatt, & Conlon, 2013) using a membrane potential assay kit or a Ca\textsuperscript{2+} assay kit (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer’s protocols. In brief, cells were incubated at 37°C for 10 min with the hydrolysates generated using Alcalase 2.4L and Flavourzyme 500L (2.5 mg/mL). Control incubations with 10 mM KCl and 10 mM alanine in the presence of 5.6 mM glucose were also performed. Data were acquired using a FlexStation scanning fluorimeter with an integrated fluid transfer workstation (Molecular Devices, Rockville, MD, USA). The effect of the hydrolysate on the production of cAMP was also assessed in BRIN-BD11 cells. Cells were seeded (150,000 cells per well) into 24-well plates. After overnight incubation, cells were washed with
HBSS buffer before incubation with the sample (2.5 mg/mL) in the presence of 200 μM of isobutyl-l-methylxanthine (IBMX) for 20 min at 37°C. After incubation, media was removed and cells lysed before measurement using a cAMP detection kit (R&D Systems Parameter, Abingdon, UK; Hogan et al., 2011).

2.8 Physicochemical characterisation

The degree of hydrolysis (DH) was estimated using a modification of the TNBS method described by Spellman, McEvoy, O'Cuinn, and FitzGerald (2003) as reported in Harnedy and FitzGerald (2013). All samples were analysed in triplicate (n=3). DH was calculated as follows: DH = 100 × ((AN2 - AN1)/Npb). Where AN1 is the amino nitrogen content of the unhydrolysed protein (mg/g protein). An AN1 value of 5.6 mg amino nitrogen/g protein was used for determination of the % DH of salmon trimming proteins. This value was as recommended by Adler-Nissen (1979) when the source protein value is not known. AN2 is the amino nitrogen content of the hydrolysate (mg/g protein) and Npb is the nitrogen content of the peptide bonds in the protein substrate which was 102.3 and 155.5 for fish muscle and gelatin, respectively (Adler-Nissen, 1979). Molecular mass distribution profiles of hydrolysates were determined using gel permeation-high performance liquid chromatography (GP-HPLC) by the method described by Spellman, Kenny, O'Cuinn, and FitzGerald (2005). Reverse-phase ultra-performance liquid chromatography (RP-UPLC) was performed as described previously by Nongonierma and FitzGerald (2012) using MS grade water and ACN and a flow rate of 0.2 mL/min.

2.9 Total and free amino acid content

Total and free amino acid analysis of salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L was performed at the Technical Services Laboratory, Teagasc Food
Research Centre, Moorepark, Fermoy, Co. Cork, Ireland using methods previously described (Hill, 1965; McDermott et al., 2016).

2.10 Peptide identification

The peptides in the salmon co-product hydrolysate generated using Alcalase 2.4L and Flavourzyme 500L were separated and identified by UPLC-ESI-MS/MS with the use of an ACQUITY UPLC (Waters, Milford, MA, USA) connected to an Impact HD Ultra high resolution (UHR) Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). An aliquot (7 µl) of the hydrolysate (0.1 mg/mL in mobile phase A (0.1% (v/v) formic acid (FA) in MS-grade H₂O) was injected onto an Acquity BEH 300 C18 column (2.1×50 mm, 1.7 µm; Waters, Dublin, Ireland) and separated as follows where mobile phase B was 0.1% (v/v) formic acid in 80% ACN (v/v): 0–0.28 min: 0% B; 0.28-45 min: 0-80% B; 45-46 min: 80-100% B; 46-48 min 100% B; 48-49 min: 100-50% B; 49-50 min 50% B; 50-51 min 50-0% B; 51-53 min 0% B at a flow rate of 0.2 mL/min. Peptide identification was performed using the MS/MS methods in positive ion mode as previously described (O’Keeffe & FitzGerald, 2015; O’Keeffe, Norris, Alashi, Aluko, & FitzGerald, 2017). Mobile phase A was 0.1 % (v/v) FA in MS grade H₂O. Peptides were identified by searching the MS/MS spectra against a database (Chordata limited SwissProt) and by de novo sequencing. Peptides ≥ 6 amino acids in length identified by de novo sequencing were searched for homology within fish proteins using the FASTAM tool (available at http://www.ebi.ac.uk/Tools/sss/fastm/). Short peptide sequences (≤5 amino acids) in the salmon skin gelatin hydrolysate were searched for homology with collagen proteins using an ‘in-house’ database consisting of the reviewed protein sequences of 288 collagen proteins from the phylum Chordata. Short peptide sequences (≤5 amino acids) in the salmon trimmings-derived hydrolysate were searched for homology with Salmo salar (Atlantic salmon) proteins using an ‘in-house’
database consisting of the reviewed protein sequences of 158 proteins from the phylum *Salmo salar*.

### 2.11 Simulated gastrointestinal digestion (SGID)

SGID was performed by the method as described by Walsh et al. (2004) with modifications. In brief, a 2.0% (w/v) hydrolysate on a protein basis was incubated with pepsin at an E:S of 2.5% (w/w) at 37 °C and pH 2.0 for 90 min. Following adjustment to pH 7.0 and heat treatment at 90°C for 20 min the sample was incubated at 37 °C with Corolase PP (E:S of 1% (w/w)). After 150 min the sample was heat treated as described above. All samples were subsequently freeze-dried and stored at -20 °C.

### 2.12 Statistical analysis

SPSS (version 20, IBM Inc., Chicago, IL, USA) was utilized to perform statistical analysis on the degree of hydrolysis and DPP-IV inhibition data. Statistical significance at a level of p<0.05 was determined using one-way analysis of variance (ANOVA) followed by Tukey’s and Games–Howell post-hoc tests, where applicable. All other results were analysed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were performed using the Students unpaired t-test. Where appropriate, data were compared using one-way and two-way analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test. Incremental area under the curve (AUC) for plasma glucose and insulin were calculated using GraphPad Prism. Groups of data were considered to be significantly different if p<0.05. All data is presented as mean ± S.E.M.

### 3. Results & Discussion

#### 3.1. Effect of salmon-derived hydrolysates on insulin release from BRIN-BD11 cells *in vitro*
The insulin secretory activity mediated by salmon co-product hydrolysates was determined between 0.039 and 2.5 mg/mL. Cell cytotoxicity analysis (LDH assay) performed at each of the sample concentrations (0.039-2.5 mg/mL) tested indicate that the insulinotropic activity observed was due to the action of the hydrolysate and not due to cell death (data not shown). The salmon skin gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L induced a significantly higher insulinotropic response from BRIN-BD11 cells at concentrations ranging from 0.625 to 2.500 mg/mL compared to the glucose control (p<0.001-0.05, Fig. 1). A 3.2-fold increase in insulin secretion above the basal rate (5.6 mM glucose alone) was observed following incubation with 2.5 mg/mL of the gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L. Salmon skin gelatin hydrolysates generated with Alcalase 2.4L or Promod 144MG were shown to mediate an insulin stimulatory response similar to the glucose control at each of the concentrations tested (Fig. 1). In contrast, all salmon trimming-derived hydrolysates were shown to induce a significantly higher (p<0.001-0.05) insulinotropic response when tested at 2.5 mg/mL compared to the glucose control. No significant difference was observed in the concentration of insulin released from pancreatic cells when all salmon trimming hydrolysates were tested at 2.5 mg/mL (Fig. 1). To the best of our knowledge, this is the first report showing the direct action of salmon-derived hydrolysates on insulin secretion from BRIN-BD11 cells. The insulin secretory activity observed with the salmon gelatin and trimming hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L, which showed a 3.2- and 3.0-fold increase, respectively, relative to the glucose control when tested at 2.5 mg/mL. This was in the same order (3.8-fold) as the increase seen with a blue whiting hydrolysate generated with the same enzymes (Harnedy et al., 2018).

Differences in the RP-UPLC (Supplementary Fig. S1) and the molecular mass distribution profiles (Table 1) for each of the gelatin hydrolysates demonstrate that different peptides were generated during hydrolysis of the salmon skin-derived gelatin. It is possible that these differences in the
size and sequence of the peptides had a significant effect on the insulinotropic potency of the hydrolysate. However, this was not observed with the salmon trimming derived hydrolysates where the differences in the DH, abundance of peptides < 1 kDa and RP-UPLC profiles had no effect on insulin secretory activity (Table 1 and Supplementary Fig. S1).

3.2. Effect of salmon-derived hydrolysates on GLP-1 release from GLUTag cells and DPP-IV inhibition in-vitro

The incretin hormone, GLP-1 released from the enteroendocrine L-cells of the intestine, stimulates the release of insulin from β-cells through distinct G-protein-coupled receptors highly expressed on the cells (Mignone, Wu, Horowitz, & Rayner, 2015). GLP-1 is rapidly degraded by DPP-IV, an ubiquitous enzyme expressed on the surface of most cell types (Green, Gault, O’Harte, & Flatt, 2004). Different strategies have been explored to aid glycaemic management in T2DM including the development of long acting GLP-1 analogues (incretin mimetics) and/or increasing the half-life of GLP-1 through the development of specific DPP-IV inhibitors. However, new treatments that could beneficially modulate endogenous enteroendocrine cell secretory mechanisms and increase GLP-1 release are currently being researched (Wang, Liu, Chen, Li, & Qu, 2015).

As shown in Fig. 2 the hydrolysates generated from both salmon co-products with Alcalase 2.4L and Flavourzyme 500L showed significantly higher GLP-1 secretory potential (p<0.001), than those generated with Alcalase 2.4L or Promod 144MG. The hydrolysate generated from skin-derived gelatin and trimming muscle proteins demonstrated a 3.8- and 2.4-fold (p<0.001) increase in GLP-1 secretion above the basal level, respectively (Fig. 2). This was higher than that observed with a blue whiting protein hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L that showed a 1.4- fold increase in GLP-1 secretion above the basal level when tested at the same concentration (2.5mg/mL; Harnedy et al., 2018). For both co-products the hydrolysates generated with Alcalase 2.4L mediated a response significantly higher (gelatin: 1.4-fold increase, p<0.05) or
similar (trimming protein: 1.1-fold increase) to the glucose control whereas the hydrolysates generated with Promod 144MG elicited a response lower than the glucose control. Whilst a salmon skin gelatin hydrolysate has previously been reported to enhance circulating GLP-1 concentrations in vivo through increased DPP-IV inhibition (Hsieh et al., 2012), to our knowledge this is the first report of the direct action of salmon gelatin and muscle protein hydrolysates on GLP-1 release from enteroendocrine GLUTag cells.

In comparison, the gelatin hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L, when tested at 2.5 mg/ml, mediated both a significantly higher insulin and GLP-1 secretory response than those mediated by gelatin hydrolysates generated with Alcalase 2.4L and Promod 144MG (Fig. 1 & 2). However, while the salmon trimmings hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and tested at 2.5 mg/ml, was more potent at mediating the release of GLP-1 compared to the other salmon trimmings hydrolysates, no significant difference was observed in the amount of insulin released from pancreatic cells when all salmon trimming hydrolysates were tested at 2.5 mg/mL (Fig. 1 & 2).

The salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L herein were also shown to be more potent ($p<0.05$) inhibitors of DPP-IV activity in vitro than those hydrolysates generated with Alcalase 2.4L and Promod 144MG (Table 1). In our previous study, a similar trend was observed where gelatin and protein hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L from salmon trimmings had more potent DPP-IV inhibitory activity than those generated with Alcalase 2.4L and Promod 144MG (Neves, Harnedy, O’Keeffe, Alashi, et al., 2017; Neves, Harnedy, O’Keeffe, & FitzGerald, 2017). However, the IC$_{50}$ values obtained herein for hydrolysates generated from salmon skin gelatin (0.90 ± 0.05 mg/mL) and trimmings derived proteins (0.84 ± 0.04 mg/mL), with Alcalase 2.4L and Flavourzyme 500L, were significantly higher than values obtained previously for salmon trimmings derived gelatin (0.55 mg/mL) and protein (0.10 mg/mL) hydrolysates generated with the same enzymes (Neves,
This difference in DPP-IV inhibitory response may be due to the source of the gelatin isolate and the method used to generate the muscle protein hydrolysates. In the previous study, the gelatin was isolated from salmon trimmings that contained both muscle and skin derived gelatin, whereas in the present study the gelatin was derived solely from the skin. It may be possible that salmon muscle derived gelatin contains peptide sequences with more potent DPP-IV inhibitory activity than that derived from skin. In the present study, salmon protein hydrolysates were generated by direct hydrolysis of homogenised salmon trimmings, whereas in the previous study salmon muscle proteins were extracted prior to hydrolysis. Direct hydrolysis was utilised in this study as it is believed to be a more industrially efficient method for generation of muscle protein hydrolysates and to be a process which maybe more easily transferred to semi-pilot or pilot scale. While the DPP-IV inhibition results presented herein were less potent than the results reported previously they were more potent than those values reported elsewhere for salmon skin gelatin hydrolysates generated with Alcalase 2.4 L, Flavourzyme 1000 L and Bromelain (Li-Chan et al. 2012).

In general, DPP-IV inhibitory activity has been associated with peptides having low molecular masses. The fact that the degree to which the gelatin and trimming muscle proteins were hydrolysed was higher with Alcalase 2.4L and Flavourzyme 500L and that the proportion of peptides <1 kDa in hydrolysates generated with these enzymes was higher than those generated with the other enzymes may explain the higher DPP-IV inhibitory potency of these hydrolysates (Table 1). Interestingly, the salmon co-product hydrolysates generated herein exhibit both DPP-IV inhibitory and GLP-1 secretory activity and as such may indirectly aid in improving glucose homeostasis in TD2M patients by increasing the concentrations of biological active GLP-1 (GLP-1(7-36) amide) circulating in the blood.
Overall the salmon trimming and gelatin hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L showed the most promising *in vitro* antidiabetic activity and warrants further investigation.

### 3.3. Effect of salmon co-product hydrolysates on intracellular Ca\(^{2+}\) and cAMP concentration in BRIN-BD11 cells

The two main signalling pathways that regulate insulin secretion from β-cells are the K\(_{\text{ATP}}\) channel-dependent and K\(_{\text{ATP}}\) channel-independent pathways (Henquin, 2000). In the K\(_{\text{ATP}}\) channel-dependent pathway, secretion of insulin is mediated by an increased [ATP]/[ADP] ratio, closure of ATP-sensitive potassium channels and opening of voltage-dependent calcium channels. This leads to calcium influx and elevated cytoplasmic intracellular calcium, inducing rapid insulin exocytosis. The effects of salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L on membrane potential, intracellular [Ca\(^{2+}\)] and cAMP concentration in BRIN-BD11 cells were investigated to determine the potential mechanism by which these hydrolysates enhanced insulin secretion.

A significant (2.9-fold) increase in membrane potential was observed with BRIN-BD11 cells exposed to the salmon skin gelatin hydrolysates compared to glucose alone (*p*<0.001, Fig. 3a). A similar response was observed with the trimmings protein hydrolysates, where a 2.0-fold increase in membrane potential was observed (*p*<0.001, Fig. 3a). Both hydrolysates were shown to mediate a significant increase in intracellular calcium concentration compared to the glucose basal response, with a 14- and 17-fold increase being observed for the gelatin and trimmings hydrolysates, respectively (*p*<0.001, Fig. 3b). These increases in membrane depolarisation and intracellular calcium concentration would suggest that both hydrolysates mediate their insulinotropic activity through the K\(_{\text{ATP}}\) channel-dependent pathway. The trimmings-derived hydrolysate was also shown to significant increase intracellular cAMP concentration in BRIN-
BD11 cells ($p<0.05$, data not shown). This indicates that the insulin secretory response observed with the trimmings-derived hydrolysate may also be via the protein kinase A (PKA) pathway (Doyle & Egan, 2007). In contrast, the gelatin hydrolysate did not mediate a significant increase in cAMP concentration in BRIN-BD11 cells (data not shown).

### 3.4 Free amino acid composition

Although the mechanism by which proteinaceous components exert their antidiabetic effect has not yet been fully elucidated, it is believed that the post prandial change in key amino acids in vivo is a contributing factor (Power-Grant et al., 2015). Amino acids such as Gln, Ala, Arg, Leu, Phe, Val, Ile, and Lys have strong insulin secretagogue activity (Dixon, Nolan, McClenaghan, Flatt, & Newsholme, 2003; Morifuji et al., 2010; Newsholme, Brennan, & Bender, 2006; Power-Grant et al., 2015). Furthermore, amino acids such as Glu, Ala, Ser and Gln, Gly, Asp, Leu and Met can stimulate the release of GLP-1 from intestinal cells and Phe, Arg and Tyr residues exhibit DPP-IV inhibitory activity (Gameiro et al., 2005; Neves, Harnedy, O’Keeffe, Alashi, et al., 2017; Neves, Harnedy, O’Keeffe, & FitzGerald, 2017; Reimann, Williams, da Silva Xavier, Rutter, & Gribble, 2004).

As shown in Table 2 both salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L contain significant quantities of key insulinotropic amino acids. Two of the most abundant free amino acids present in the hydrolysate generated from salmon skin gelatin include Arg and Phe at 1.15 and 1.07 g/100g, respectively, while Phe, Leu and Arg were present at 2.13, 1.17 and 1.01 g/100 g in the hydrolysate generated from salmon trimmings. The presence of these amino acids with known insulinotropic activity, in addition to other insulin secretory residues present at low to moderate levels (Ala, Lys, Val and Ile) may contribute to the potent insulinotropic activity observed with these hydrolysates. The positively charged amino acid Arg accounted for 12.57% and 7.06% (w/w) of the total free amino acid content in the salmon gelatin.
and trimmings hydrolysate, respectively. This residue is believed to enhance insulin secretion through direct depolarization of the plasma membrane, which in turn activates voltage-dependent Ca\(^{2+}\) channels leading to an increase in intracellular Ca\(^{2+}\) and a subsequent stimulation of insulin secretion (Newsholme et al., 2006).

The majority of the amino acids with known GLP-1 secretory activity, with the exception of Leu, which was present at 1.17 g/100 g in the free amino acid complement of the hydrolysate generated from salmon trimmings, were only found at moderate levels. However, the levels of these known GLP-1 secretory amino acids or the combination of such amino acids present in the hydrolysate may have been high enough to mediate the GLP-1 secretory response observed. Furthermore, residues such as Phe, Arg and Tyr, which are known to exhibit DPP-IV inhibitory activity, were found in high abundance in the free amino acid complement of both hydrolysates. These amino acids with concentrations ranging 1.07-2.13 g/100 g may contribute to the DPP-IV inhibitory activity observed with both hydrolysates.

3.5 Total amino acids and peptide profiles

The total amino acid composition of the salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L is shown in Table 2. Both co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L contain all the essential amino acids with higher quantities generally being found in the salmon trimming-derived hydrolysate. Trp, was not determined during total amino acid analysis.

Table 2 indicates that the salmon skin gelatin hydrolysate contains a higher quantity of free Tyr than that determined in the hydrolysate as a whole. The reason for this is unknown; however, Tyr has been reported to be sensitive to destruction/modification under certain acid hydrolysis conditions (Fountoulakis & Lahm, 1998).
The most abundant amino acids found in the gelatin hydrolysate include Gly and Pro, which account for 27.12 and 12.76% (w/w) of the total amino acid content. This is not surprising as the sequence of gelatin (collagen) is composed of the repeat sequence Gly-X-Y, where X is mostly Pro and Y is mostly hydroxyproline. The most abundant amino acids in the trimmings-derived hydrolysate were Glx and Asx (Table 2).

Although a large number of food-protein derived peptides have been shown to exhibit DPP-IV inhibitory activity \textit{in vitro}, limited research has been performed on the identification of peptides with GLP-1 and insulin secretory activity. To the best of our knowledge, only three peptides (Leu-Gly-Gly, Gly-Leu and Gly-Pro) have been reported to date which stimulate the release of GLP-1 (Diakogiannaki et al., 2013). However, given that certain amino acids promote insulin and/or GLP-1 secretory activity, it is possible that short peptides containing such residues would also have similar effects. For example, Gly-Pro which has been shown to enhance GLP-1 release, forms part of the 3 amino acid repeat sequence in gelatin and, as shown in Supplementary Table S1 and S2, was found in abundance in peptide sequences identified in the salmon gelatin hydrolysate. Furthermore, this dipeptide was also found in peptide sequences identified in the salmon trimmings hydrolysate (Supplementary Table S3 and S4). Gln has to date been identified as being the most potent GLP-1 secretagogue (Reimann et al., 2004). Numerous short peptides containing Gln in addition to other known GLP-1 secretory residues were present in both hydrolysates and may contribute to the GLP-1 secretory activity observed. These peptides include Gln-Glu, Glu-Gln, Gln-Met, Gln-Ser, Leu-Gln, Gln-Leu, Gly-Gly-Leu-Gln, Gly-Pro-Ser-Gln, Gln-Pro-Pro-Glu, Gln-Glu, Glu-Gln, Gln-Ser, Phe-Gln, Ala-Leu-Gln, and Glu-Leu-Gln. Interestingly, peptides such as Leu-Gln, Gln-Leu, Phe-Gln, and Ala-Leu-Gln which contain two/three amino acids with known insulinotropic activity and peptides such as Leu-Ala, Ile-Ala, Ala-Leu-Leu, Ala-Leu/Ile and Leu-Leu may contribute to both insulin and GLP-1 stimulatory activity. Dipeptides such as Gln-Arg, Arg-Gln, Gln-Val, Arg-Leu, Arg-Val and Lys-Ala, Lys-Phe,
Phe-Gln, Val-Gln possess residues with known insulinotropic activity and these may have contributed to the insulin releasing activity observed. Furthermore, a known DPP-IV inhibitory peptide Gly-Pro-Ala-Gly (Hsu, Tung, Huang, & Jao, 2013) was present in the gelatin hydrolysate, while the trimmings hydrolysate contained a peptide Gly-Pro-Ala-Gly-Leu containing this sequence. The gelatin hydrolysate derived peptides Ala-Val-Leu-Gly-Pro-Lys and Ala-Val-Leu-Gly-Pro-Gln also contain the tetra peptide sequence Val-Leu-Gly-Pro which has previously been reported to exhibit DPP-IV inhibitory activity (Nongonierma & FitzGerald, 2013).

The antidiabetic activity observed with the hydrolysate generated herein may also be due to a synergistic effect mediated by a combination of a number of peptides and/or amino acids and further research is required to identify the most potent peptide and/or amino acids responsible for the observed activity.

3.6. In vitro antidiabetic activity of salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L following simulated gastrointestinal digestion (SGID)

SGID was performed in an attempt to determine if the salmon co-product hydrolysates generated with Alcalase 2.4L and Flavourzyme 500L may be resistant to the enzymes that are present in the gastrointestinal tract of humans. The insulinotropic potential of both hydrolysates increased following SGID (data not shown). The salmon skin gelatin hydrolysate showed an 8.0-fold increase in the concentration of insulin released from BRIN-BD11 cells relative to the glucose control when tested at 2.5 mg/mL following SGID compared to a 3.2-fold increase prior to SGID. The salmon trimmings hydrolysate showed a 4.2-fold increase relative to the glucose control following SGID compared to a 3.0-fold increase for the hydrolysate prior to SGID. Furthermore, the trimmings derived hydrolysate retained GLP-1 stimulatory, (2.4- and 2.3-fold increase pre- and post-SGID, respectively, relative to the glucose control) and DPP-IV inhibitory (IC_{50} values: 0.84 ± 0.04 and 1.00 ± 0.01 mg/mL for pre- and post-SGID, respectively) activity following
SGID. However, the gelatin hydrolysate lost its GLP-1 stimulatory activity following SGID with only a 0.4-fold increase in activity over the glucose control compared to a 3.2-fold increase prior to SGID. The DPP-IV inhibitory activity was also shown to significantly decrease ($p<0.05$) following SGID where IC$_{50}$ values of 0.90 ± 0.05 and 1.19 ± 0.03 mg/mL were obtained for the gelatin hydrolysates pre- and post-SGID, respectively.

An increase in the DH (16.52 to 20.58%) and the quantity of peptides < 1 kDa (67.06 to 84.21%) and differences in the RP-UPLC profiles (data not shown) as seen for the gelatin hydrolysate following SGID indicate that peptides present in the hydrolysate pre-SGID were degraded and that lower molecular weight peptides were generated during the SGID process. It is possible that the observed increase in lower molecular weight peptides mediated a more potent insulinotropic response, while peptides exhibiting GLP-1 secretory and DPP-IV inhibitory activity in the original hydrolysate were degraded during SGID. An increase in DH from 34.07 to 41.77% and peptides < 1 kDa from 69.09 to 87.96% would indicate that further hydrolysis of the trimmings-derived hydrolysate took place during SGID (data not shown). However, the RP-UPLC profiles of the hydrolysate pre- and post-SGID were similar (data not shown). Since the GLP-1 stimulatory and DPP-IV inhibitory activity were retained following SGID and an improvement was seen in the insulin secretory activity, it is possible that peptides in the original hydrolysate mediating these activities were either resistant to degradation during SGID and/or additional lower molecular weight peptides were generated with similar or more potent activity.

Additional small animal studies using the most appropriate diabetic animal models (for example obese diabetic ob/ob or db/db mice), need to be performed to confirm the in vivo efficacy of the hydrolysates.

4. Conclusion
This study shows that salmon co-product-derived protein hydrolysates, in particular those generated using a combination of Alcalase 2.4L and Flavourzyme 500L, exhibit significant antidiabetic (insulin and GLP-1 secretory and DPP-IV inhibitory) activity in vitro. The specific biochemical pathways through which both co-product hydrolysates may mediate their insulinotropic activity were identified. The outcomes from SGID indicate the potential for translation of the activity seen in vitro to in vivo. However, investigations with diabetic animal models need to be performed to confirm these findings. Furthermore, the efficacy of the hydrolysates needs to be confirmed in human studies and the sequence of the peptides and/or identity of the amino acids responsible for the observed antidiabetic activity should to be determined. This study has identified the potential of low-value protein rich co-products, such as salmon trimmings and skin as rich sources of biologically active hydrolysates/peptides, which, following further valorisation can provide the marine industry with an opportunity to add value to existing salmon processing co-products.

Acknowledgments

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References


Table 1. Degree of hydrolysis, molecular mass distribution and dipeptidyl peptidase (DPP)-IV inhibitory activity of Atlantic salmon (Salmo salar) co-product hydrolysates

<table>
<thead>
<tr>
<th>Co-product</th>
<th>Enzyme</th>
<th>Degree of hydrolysis (%)</th>
<th>Molecular mass distribution (%)</th>
<th>DPP-IV inhibitory activity (IC&lt;sub&gt;50&lt;/sub&gt; (mg/mL))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 1kDa</td>
<td>1-5 kDa</td>
</tr>
<tr>
<td>Salmon skin gelatin</td>
<td>Alcalase 2.4L</td>
<td>10.56 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.70</td>
<td>38.17</td>
</tr>
<tr>
<td></td>
<td>Alcalase 2.4L + Flavourzyme 500L</td>
<td>16.52 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.06</td>
<td>32.34</td>
</tr>
<tr>
<td></td>
<td>Promod 144MG</td>
<td>5.77 ± 0.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.66</td>
<td>52.59</td>
</tr>
<tr>
<td>Salmon trimmings</td>
<td>Alcalase 2.4L</td>
<td>26.60 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.99</td>
<td>35.09</td>
</tr>
<tr>
<td></td>
<td>Alcalase 2.4L + Flavourzyme 500L</td>
<td>34.07 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.09</td>
<td>27.74</td>
</tr>
<tr>
<td></td>
<td>Promod 144MG</td>
<td>27.48 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.44</td>
<td>38.60</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=3). For each co-product values with different superscript letters are significantly different (P<0.05). IC<sub>50</sub>: inhibitory concentration that inhibits enzyme activity by 50%
Table 2. Total and free amino acid composition of Atlantic salmon (*Salmo salar*) co-product hydrolysates

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Salmon skin gelatin</th>
<th></th>
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<tr>
<td></td>
<td>Total amino acids</td>
<td>Free amino acids</td>
<td>Total amino acids</td>
<td>Free amino acids</td>
<td></td>
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<tr>
<td></td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
<td>(g/100 g)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Met</td>
<td>2.46</td>
<td>0.26</td>
<td>2.66</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Thr</td>
<td>2.03</td>
<td>0.39</td>
<td>3.23</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Val</td>
<td>1.71</td>
<td>0.25</td>
<td>3.83</td>
<td>0.78</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ile</td>
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<td>0.44</td>
<td>2.98</td>
<td>0.44</td>
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<td></td>
<td></td>
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<td>Leu</td>
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<td>Phe</td>
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<tr>
<td>Asx</td>
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<tr>
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<td>0.05</td>
<td>11.07</td>
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<tr>
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</tbody>
</table>

All amino acids are referred by three letter code. -: not determined, nd: not detected. Glx: Glu + Gln and Asx: Asp + Asn.
Highlights

- Salmon co-product protein hydrolysates displayed antidiabetic activity in vitro
- Insulin secretory activity was observed in cultured pancreatic BRIN-BD11 cells
- Mechanisms of insulin release from pancreatic β-cells were identified
- The salmon co-product hydrolysates enhanced GLP-1 release from GLUTag cells
- Salmon co-product hydrolysates may have potential as antidiabetic food ingredients
Salmon co-products
- Trimmings protein
- Skin gelatin

hydrolysis with:
- Alcalase 2.4L
- Alcalase 2.4L + Flavourzyme 500L
- Promod 144MG

Cytotoxicity: LDH cell viability assay
- Insulin secretion from BRIN BD11 cells
- GLP-1 secretion from GLUTag cells
- DPP-IV inhibition
- Physicochemical characterisation

Alcalase 2.4L + Flavourzyme 500L salmon trimmings and gelatin hydrolysates

- Mechanistic studies
- Simulated gastrointestinal digestion
- Amino acid analysis and peptide identification

Graphics Abstract
Figure 1
Figure 2
Figure 3

(a) 5.6 mM glucose
- 5.6 mM glucose + KCl (10 mM)
- 5.6 mM glucose + salmon skin gelatin hydrolysate (2.5 mg/mL)
- 5.6 mM glucose + salmon trimmings hydrolysate (2.5 mg/mL)

Membrane Potential AUC (RFU/min)

(b) 5.6 mM glucose
- 5.6 mM glucose + Alanine (10 mM)
- 5.6 mM glucose + salmon skin gelatin hydrolysate (2.5 mg/mL)
- 5.6 mM glucose + salmon trimmings hydrolysate (2.5 mg/mL)

Intracellular Calcium AUC (RFU/min)