

**Boarfish (*Capros aper*) protein hydrolysate has potent insulinotropic and GLP-1 secretory activity
in vitro and acute glucose lowering effects in mice.**

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Abstract:

The antidiabetic actions of a boarfish protein hydrolysate (BPH) were investigated in cultured cells and mice. A boarfish (*Capros aper*) muscle protein hydrolysate was generated using the enzymes Alcalase 2.4L and Flavourzyme 500L. Furthermore, the BPH was subjected to simulated gastrointestinal digestion (SGID). BPH and SGID samples (0.01-2.5 mg/ml) were tested *in vitro* for DPP-IV inhibition and insulin and GLP-1 secretory activity from BRIN-BD11 and GLUTag cells, respectively. The BPH and SGID samples, caused a dose-dependent increase (4.2 to 5.3-fold, $p < 0.001$) in insulin secretion from BRIN-BD11 cells and inhibited DPP-IV activity (IC_{50} 1.18 ± 0.04 and 1.21 ± 0.04 mg/ml), respectively. The SGID sample produced a 1.3-fold ($p < 0.01$) increase in GLP-1 secretion. An oral glucose tolerance test (OGTT) was conducted in healthy mice ($n=8$), with or without BPH (50 mg/kg bodyweight). BPH mediated an increase in plasma insulin levels ($AUC_{(0-120 \text{ min})}$, $p < 0.05$) and a consequent reduction in blood glucose concentration ($p < 0.01$), after OGTT in mice versus controls. The BPH showed potent antidiabetic actions in cells and improved glucose tolerance in mice.

Introduction

Population expansion combined with a reduction in arable farming land, has seen the exploration of natural therapeutics and bioactive food components shift from terrestrial organisms such as dairy, plant and animals to oceanic and coastal marine environments. Thus, bioactive mining of seaweeds, ichthyoids and crustaceous and bivalve organisms has increased (Senevirathne & Kim, 2012). The oceans cover 70% of the earth's surface and is home to only 15% of all living organisms (Grosberg *et al.* 2012), however it is widely accepted that marine proteins have the potential to yield a greater number of uncharacterized biologically active peptides than those from terrestrial protein sources (Wang *et al.*, 2017). Research into marine bioactive peptides has expanded exponentially over the past decade with improved protein extraction and hydrolysis methods and as such, has reaffirmed the importance of utilizing all aspects of marine biomaterials (Senevirathne & Kim, 2012).

Following on from the landmark Diabetes Control and Complications Trial (Nathan *et al.* 1993), classical approaches to treating type-2 diabetes mellitus (T2DM) have been surpassed in favour of a more intensive hyperglycaemia management regime. Various strategies have been applied for the management of T2DM including dietary manipulation, lifestyle changes and medication (Bantle *et al.* 2008; Gibala *et al.* 2012; Inzucchi *et al.* 2012). Development of anti-hyperglycaemic agents from dietary sources has gained impetus as these are generally less expensive to develop, have a reduced side-effects profile and are more accepted by the public (Fayaz *et al.* 2014; Gushiken *et al.* 2016).

Proteins on their own generally only have a purely caloric effect on biological systems, however, hydrolysis of the protein can unlock peptides with clinical or health enhancing significance (Dhaval *et al.* 2016). The discovery of marine bioactive components could contribute to novel strategies

providing therapeutic benefit and better management of many common diseases. To date, many marine bioactive agents have been discovered displaying anti-obesity, anti-diabetic, antimicrobial, antihypertensive and anti-carcinogenic properties (Jensen and Mæhre 2016; Wang *et al.* 2017; Jin *et al.* 2017). Fish protein is a rich but often unexploited source of bioactive peptides (Ryan *et al.* 2011; Kim *et al.* 2012; Urakova *et al.* 2012). *Capros aper* commonly known as boarfish is an underutilized fish species that could provide a sustainable source of marine protein for bioactive peptide discovery for functional food development. Boarfish is a mesopelagic fish species distributed at depths of 40-600 m found abundantly in the Mediterranean and in the Northeast Atlantic stretching from Norway to Senegal (Whitehead *et al.*, 1986). Increased boarfish landings from Irish and Danish fishery fleets have paved the way for long-term storage and exploitation of this non-traditional species (White *et al.* 2011).

Research into marine protein and its subsequent hydrolysates and isolated bioactive peptides targeting diabetes and its complications is limited. Nevertheless, some early studies have shown the beneficial effects of inhibiting dipeptidylpeptidase-4 (DPP-IV) (Silaa *et al.* 2016; Huang *et al.* 2012; Harnedy *et al.* 2015; Harnedy *et al.* 2018a, 2018b), and thus lowering glycated haemoglobin (HbA_{1c}) in human volunteers with T2DM (Zhu *et al.*, 2010). The aims of the present study were firstly to investigate the *in vitro* insulinotropic and GLP-1 stimulatory effects of a BPH, and secondly to investigate the effect of oral consumption of BPH on blood glucose control in healthy mice following an oral glucose challenge.

2. Methods

2.1. Materials

H-Gly-Pro-7-amino-4-methyl coumarin (AMC), Diprotin-A (Ile-Pro-Ile), Leu-Trp-Met-Arg, Asp-Glu and Tyr.HCl were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Alcalase® 2.4L and Flavourzyme® 500L were obtained from Novozymes A/S (Bagsvaerd, Denmark). Corolase PP was provided by AB Enzymes (Darmstadt, Germany) and BC pepsin was provided by Biocatalysts (Cardiff, UK). All other reagents including DPP-IV from porcine kidney (≥ 10 units/mg protein), were supplied by Sigma Chemical Company Ltd. (Wicklow, Ireland). A GLP-1 ELISA assay kit was provided by Millipore (Hertfordshire, UK), lactate dehydrogenase kit by Promega (Madison, WI, USA) and *in vitro* glucose uptake kit was provided by Cayman Chemicals (Ann Arbor, MI, USA).

2.1.1 Generation of a boarfish protein hydrolysate (BPH) and its simulated gastrointestinal digested (SGID) sample.

Samples of minced boarfish (*Capros aper*) meat were kindly provided by Killybegs Fishermen's Organisation, Killybegs, Co Donegal, Ireland, through Bord Iascaigh Mhara (BIM, Ireland) and stored at -20°C . Minced boarfish meat was suspended in distilled water to a final 6.83% (w/v) boarfish protein suspension and homogenised (x4) at 24,000 rpm/min for 15 sec (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany). Hydrolysis was performed at 50°C and pH 7.0 with Alcalase 2.4L and Flavourzyme 500L at an enzyme:substrate (E:S) ratio of 0.67% (v/w) for 4 h. Enzymes were inactivated by heating at 90°C for 20 min. Peptides were then separated by double filtration (Whatman grade 1: 11 μm), freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C until required.

In order to assess the likely retention of bioactivity following gastrointestinal digestion the BPH was subjected to SGID as described by Walsh *et al.*, (2004) with modifications as described below. Briefly, the BPH (2.0% (w/v) protein equivalents) was incubated at 37°C and pH 2 for 90 min with pepsin at an E:S of 2.5% (w/w). The sample was adjusted to pH 7 and heat inactivated at 90°C for

20 min. The sample was incubated for 150 min at 37°C with Corolase PP (E:S of 1% (w/w)) and heat inactivated as before. The sample was subsequently freeze-dried and stored at -20°C (Harnedy *et al.* 2018a).

2.1.2 Kjeldahl nitrogen quantification

The nitrogen content of the minced boarfish meat and BPH were quantified using the macro-Kjeldahl procedure as described previously (Connolly *et al.* 2013). The nitrogen to protein conversion factor used was 6.25 (Kristinsson and Rasco, 2000). All samples were analysed in triplicate (n=3).

2.1.3 Physicochemical characterisation of protein hydrolysates

The peptide profiles of the BPH and SGID samples were determined by reverse-phase ultra-performance liquid chromatography (RP-UPLC (ACQUITY UPLC (Waters, Milford, MA, USA))) as described previously by Nongonierma and FitzGerald (2012) with modifications. In brief, the BPH and SGID samples were reconstituted at 0.5% (w/v) in mobile phase A (0.1% TFA in MS grade H₂O) and separated using an ACQUITY BEH 300 C18 RP column (2.1 x 50 mm, 1.7 µm; Waters, Milford, MA, USA) at 30°C. Mobile phase B was 0.1% TFA in 80% (v/v) MS grade ACN and the flow rate was set at 0.2 mL/min. Peptides and proteins were eluted using a linear gradient: 0–0.28 min: 100% A; 0.28–45 min: 100–20% A; 45–46 min: 20–0% A; 46–48 min 0% A; 48–49 min: 0–100% A; 49–51 min 100% A. The absorbance of the eluent was monitored at 214 nm.

The molecular mass distribution profiles of the samples were determined by gel permeation-high performance liquid chromatography (GP-HPLC) as described by Spellman *et al.* (2005). In brief, the BPH and SGID samples were reconstituted at 0.8% (w/v) in a mobile phase of 0.1% TFA in 30% (v/v) HPLC grade ACN. The samples were separated by isocratic elution using a TSK G2000 SW

separating column (600 x 7.5 mm ID) connected to a TSKGEL SW guard column (75 x 7.5 mm ID) at a flow rate of 1.0 mL min. The detector response was monitored at 214 nm. The molecular mass distribution of the proteins/peptides within the samples were determined from a calibration curve prepared from the average retention times of standard proteins and peptides. These standards include bovine serum albumin (67,500 Da), β -lactoglobulin (36,000 Da), α -lactalbumin (14,200 Da), aprotinin (6500 Da), bacitracin (1400 Da), Leu-Trp-Met-Arg (604.8 Da), Asp-Glu (262.2 Da) and Tyr.HCl (218 Da).

2.1.4 Insulin secretion studies in clonal pancreatic cells

Acute insulin secretory effects of BPH and SGID samples were measured *in vitro* using clonal pancreatic BRIN-BD11 cells (McClenaghan *et al.* 1996). Briefly, BRIN-BD11 cells were incubated for 20 min (acute test) with a range of hydrolysate concentrations (0.039 –2.5 mg/ml) in the presence of 5.6 mM glucose at 37°C. After 20 min the cell supernatant (900 μ l) taken from the acute test wells were frozen at -20°C. Insulin was quantified using a dextran-coated charcoal radioimmunoassay (RIA), using crystalline rat insulin standard, guinea-pig anti-porcine antiserum (1:30,000 dilution) and ¹²⁵I-bovine standard (10,000 cpm), as described by Flatt & Bailey (1981). The concentration of insulin in each sample was determined in duplicate (200 μ l aliquots) from the prepared insulin standard curve ranging from 20 ng/ml stock to 0.039 ng/ml.

2.2 Cytotoxicity assay

To determine the cytotoxicity of the BPH and SGID samples on BRIN-BD11 cells, the release of lactate dehydrogenase (LDH) was measured in cell supernatants obtained from acute insulin-release experiments. LDH activity in the cell supernatants was determined using a CytoTox96 non-

radioactive cytotoxicity assay kit (Promega, Madison, WI, USA), as per manufacturer's instructions. LDH results were compared to cells incubated with KRBB supplemented with 5.6 mmol/L glucose alone.

2.3 *In vitro* GLP-1 secretions from GLUTag cell

In vitro effects of the BPH and the SGID samples on GLP-1 secretion were measured using the murine enteroendocrine GLUTag cell line kindly gifted to Ulster University by Professor Fiona Gribble at University of Cambridge, which originated from the laboratory of Dr Daniel Drucker, Toronto (Drucker *et al.* 1994). Cells were cultured in high glucose (25 mM) Dulbecco's Modified Eagle's Medium as described previously (McLaughlin *et al.* 2016). Cells were seeded into 24 well plates (150,000 cells/ well) and allowed to attach overnight at 37°C. Following a pre-incubation step (1.1 mM glucose solution in KRBB for 40 min at 37°C), cells were incubated with the BPH and SGID samples (2.5 mg/ml) prepared in 2 mM glucose (2 h at 37°C). Thereafter, 800 µl of experimental supernatant was collected and subsequently used to measure total GLP-1 release using an ELISA (Millipore, Hertfordshire, UK) as per manufacturer's protocol.

2.4 *Dipeptidyl peptidase-4 (DPP-IV) inhibition in vitro*

DPP-IV inhibition was determined with porcine kidney DPP-IV using an assay buffer consisting of 100 mM Tris-HCl, pH 8.0 as described previously (Harnedy *et al.* 2015). All assays were performed in triplicate (n=3). For DPP-IV inhibition, activity results were expressed as IC₅₀ values (inhibitory concentration, which inhibited DPP-IV activity by 50%).

2.5 *Glucose uptake study using differentiated adipocytes*

Adipocyte (3T3-L1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cellular glucose uptake studies were carried out as described by O'Harte *et al.* 2018. 3T3-L1 cells were seeded in a 96 blackwalled, clear bottom plate (2×10^4 cell/well; Greiner Bio-one, Germany) and left to attached overnight. Cells were treated with the test sample (100 μ l) or control which were supplemented in glucose-free culture medium containing 150 μ g/ml fluorescently-tagged deoxyglucose analogue (2-NBDG) and incubated for 20 min. After incubation, the plate was then centrifuged for 5 min at $400 \times g$ at room temperature. The supernatant was aspirated and cells were washed with 200 μ l cell-based assay buffer followed by further centrifugation for 5 min. The wash buffer was removed and 100 μ l of cell-based assay buffer was added to all wells and the fluorescence was read immediately at 485 nm with emission measured at 535 nm using the FlexStation scanning fluorimeter (Molecular Devices, USA).

2.6 Intracellular calcium ($[Ca^{2+}]_i$) and membrane potential studies

Monolayers of BRIN-BD11 cells were utilised to measure changes in $[Ca^{2+}]_i$ and membrane potential (Srinivasan *et al.* 2013) using fluorimetric Ca^{2+} and membrane potential assay kits, respectively (Molecular Devices, Sunnyvale, CA, USA) as described by the manufacturer. Briefly, BRIN-BD11 cells were incubated with the BPH (2.5 mg/ml, at $37^\circ C$ for 10 min) in the presence of 5.6 mM glucose. Alanine (10 mM) and KCl (10 mM) were used as positive controls. A Flexstation scanning fluorimeter with integrated fluid transfer was used for data acquisition (Molecular Devices, Rockville, MD, USA).

2.7 In vivo studies in healthy mice

Adult (3 months old) male National Institute of Health Swiss mice (NIH Swiss, Envigo, Bicester, UK) were housed individually in an air-conditioned room ($22 \pm 2^\circ\text{C}$) with a 12 h light: 12 h dark cycle (lights off between 20:00 and 08:00 h). Animals were maintained on a standard rodent chow (Teklad Global 18% Protein Rodent Diet; Harlan, UK; total energy 13.0 kJ/g). All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments and approved by the Ulster University Animal Welfare and Ethical Review Committee. All necessary steps were observed to ameliorate any potential animal suffering. Overnight fasted (16 h) mice ($n=8$) received glucose dissolved in physiological saline (pH 7.4) by oral gavage (18 mmol/kg body weight) or glucose (18 mmol/kg body weight) supplemented with the BPH dissolved in saline (50 mg/kg body weight). Blood samples were collected from the tail at various time points (from $t=0$ to 120 min) as indicated in Fig. 6 and no adverse effects were observed. Blood glucose concentrations were measured using a Bayer Contour glucose monitor (Bayer, Newbury, UK). A small volume of blood (50-100 μl) was collected at each time point in fluoride oxalate coated tubes. Tubes were immediately centrifuged at 13000 rpm for 5 min and plasma stored in low protein binding Eppendorf tubes at -20°C until analysis. Circulating plasma insulin was measured in duplicate (10 μl aliquots) by radioimmunoassay as described previously (Flatt & Bailey, 1981).

2.8 Statistical analysis

All results were analysed using statistical software GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) and data presented as the mean \pm S.E.M. Statistical analyses were performed using the students unpaired t-test. Where appropriate, blood glucose data were

compared using one-way and two-way analysis of variance (ANOVA), followed by Student-Newman-Keul's *post hoc* test. Data were considered to be significantly different when $p < 0.05$.

3 Results

3.1 RP-UPLC and molecular mass distribution profiles

The RP-UPLC profiles of the BPH and SGID samples indicate that further degradation of the hydrolysate occurred during SGID (Fig. 1). Furthermore, the SGID sample has a greater percentage of smaller peptides <1 kDa (91.6%) compared to BPH (73.6%) as determined from molecular mass distribution profiles (Table 1).

3.2 Inhibition of porcine DPP-IV activity

The effect of the BPH and its SGID sample on DPP-IV inhibition were examined. These showed DPP-IV inhibitory IC_{50} values of 1.18 ± 0.04 mg/ml and 1.21 ± 0.04 mg/ml for the hydrolysate and SGID sample, respectively (Table 1). Both samples exhibited potent DPP-IV inhibitory activity. However, no significant difference in DPP-IV inhibition was observed between the BPH and SGID samples.

3.3 Insulin release from BRIN-BD11 cells

The BPH (from 0.078 to 2.5 mg/ml) produced using a combination of the food-grade enzymes Alcalase 2.4L and Flavourzyme 500L, elicited a dose-dependent increase in insulin secretion from 1.5 to 4.2-fold (versus glucose alone) from clonal pancreatic BRIN-BD11 cells cultured at 5.6 mM

glucose (Fig. 2A, $p < 0.001$). The insulinotropic activity of the BPH (2.5 mg/ml) was further enhanced by the SGID process (5.3-fold increase; $p < 0.001$, Fig. 2B) and was more marked when compared to a high concentration of GLP-1 (10^{-6} M) which was the positive control (Fig. 2B). The action of the BPH and SGID samples on insulin secretion was dependent upon their endogenous stimulatory actions and was not due to any cytotoxic cellular effects, as shown by the lack of lactate dehydrogenase (LDH) release, at all of the concentrations tested (Fig. 2 C,D).

3.4 *GLP-1 secretion from GLUTag cells*

Forskolin is a drug which is used to increase adenylyl cyclase activity and enhances intracellular cAMP and was used as a positive control for GLP-1 secretion. GLP-1 secretion from GLUTag cells was increased by 1.9-fold ($p < 0.001$) in the presence of the Forskolin® (10 mM) (Fig. 3) compared to the basal rate (25 mM glucose alone). The BPH at 2.5 mg/ml failed to stimulate GLP-1 secretion from GLUTag cells above the basal rate (1.1-fold, $p > 0.05$, Fig. 3). However, in contrast the BPH subjected to SGID elicited a 1.3-fold ($p < 0.01$, Fig 3) increase over the basal rate. This response was significantly higher than that mediated by the BPH prior to SGID ($p < 0.05$, Fig. 3).

3.5 *Glucose uptake in differentiated adipocytes*

All experimental conditions contained KRBB without D-glucose (glucose free medium) with subsequent addition of 2-deoxyglucose (2-DG) with or without insulin (1 nM). The negative control, apigenin, showed that 2-DG uptake can be blocked in these differentiated 3T3-L1 cells whereas insulin (100 nM) can enhance 2-DG uptake (Fig. 4). The BPH (2.5 mg/ml) increased glucose uptake by 30% in 3T3-L1 adipocytes compared to the 2-DG only control KRBB in glucose free

medium ($p < 0.01$, Fig. 4). This BPH effect was similar to the magnitude of glucose uptake observed with the lower dose of insulin (1 nM). The SGID sample also displayed a similar improved glucose uptake (30%) compared to the glucose free medium control ($p < 0.01$, Fig. 4). Notably the combination of the BPH or SGID sample with low dose insulin (1 nM) did not surpass the effect of either agent alone and thus there were not any statistically significant additive or synergistic effects present ($p > 0.05$; Fig. 4).

3.6 Intracellular calcium concentration and membrane potential studies

The BPH (2.5 mg/ml) when tested on BRIN-BD11 cells resulted in a biphasic increase of $[Ca^{2+}]_i$, including a small sharp increase, followed by gradual sustained increase throughout the 300 s test period, which was significantly greater than the 5.6 mM glucose control ($p < 0.001$, Fig. 5A). In agreement, the integrated area under the curve ($AUC_{(0-300\ s)}$) values were similar to the positive control (10 mM Alanine) and was significantly enhanced (12.5 to 12.8-fold) versus the glucose control ($p < 0.001$, Fig. 5C). Similarly, the BPH produced an initial sharp increase in membrane potential, which by 100 s levelled out to just below basal levels (Fig. 5B). The magnitude of membrane depolarisation was significantly lower compared to the positive control 10 mM KCl ($p < 0.001$, Fig. 5B). Despite this the overall response from $AUC_{(0-300\ s)}$ value was significantly higher for the BPH versus the 5.6 mM glucose control ($p < 0.05$, Fig. 5D).

3.7 Glucose tolerance and insulin release in normal healthy mice

Oral administration of the BPH (50 mg/kg body weight) concomitantly with glucose in NIH Swiss mice resulted in a significant lowering of blood glucose concentrations at 15 min ($p < 0.05$), 30 min

($p < 0.001$) and 60 min ($p < 0.05$) compared to control mice receiving glucose alone (Fig. 6A). This correlated to a significantly decreased integrated $AUC_{(0-120 \text{ min})}$ blood glucose concentration (22% reduction, $p < 0.01$, Fig. 6C). The BPH induced a corresponding increase in plasma insulin concentrations, which were moderately elevated, but failed to reach significance at individual time points (Fig. 6B). Nevertheless the overall integrated $AUC_{(1-120 \text{ min})}$ insulin response was significantly increased (36%, $p < 0.05$, Fig. 6D) compared to the glucose control. Thus, overall oral administration of the BPH showed an enhanced acute insulinotropic response and a corresponding improved glucose tolerance in normal mice challenged with oral glucose.

4 Discussion

In the present study our key findings demonstrated that orally administered BPH (50 mg/ml) showed distinct anti-diabetic actions including *in vivo* insulin releasing responses and associated significantly improved glycaemic control following an oral glucose tolerance test (OGTT) in normal mice. This was in line with their pronounced insulinotropic actions *in vitro*. The present findings compare favourably with results published recently with other fish hydrolysates (Harnedy *et al.* 2018a; 2018b). For example, in the case of blue whiting (*Micromesistius poutassou*) a two-fold higher dose of protein hydrolysate (100 mg/ml) generated using Alcalase 2.4L and Flavourzyme 500L had a similar acute glucose-lowering effect (22% $AUC_{(0-120 \text{ min})}$ reduction) following an OGTT in normal healthy mice (Harnedy *et al.* 2018a). Furthermore, the *in vitro* anti-diabetic effects of protein hydrolysates derived from Atlantic salmon (*Salmo salar*) showed a similar profile to the BPH studied herein (Harnedy *et al.* 2018b). Additionally, Cudennec and colleagues (2015) have demonstrated that a protein hydrolysate from cuttlefish (*Sepia officinalis*) exhibited DPP-IV inhibitory activity and GLP-1 releasing actions on STC-1 cells *in vitro*. Others have demonstrated

that a collagen hydrolysate exhibited DPP-IV inhibitory activity and stimulated glucagon-like-peptide-1 (GLP-1) secretion *in vitro* (Iba *et al.* 2016). Furthermore, they showed that a collagen hydrolysate also improved glucose tolerance in response to oral glucose in normal mice, which was thought to be mediated partially through enhanced GLP-1 secretion as well as inhibition of intestinal glucose uptake (Iba *et al.* 2016; Neves *et al.* 2017).

The wide range of bioactivities contained within the marine environment has sparked much interest in terms of functional food ingredients and the potential for metabolic disease prevention and management (Zhu *et al.* 2010; Lordan *et al.* 2011; Drotningvik *et al.* 2016). To our knowledge, this is the first report of a protein hydrolysate derived from boarfish showing anti-diabetic potential. In the present study, acute incubation of cultured pancreatic BRIN-BD11 cells with a BPH, stimulated insulin secretion in a dose-dependent manner. Interestingly, the insulin releasing action of the hydrolysate was not only retained, but improved following SGID, suggesting the possibility of improved oral efficacy and potency following gastrointestinal digestion. Acute treatment with the BPH or SGID samples did not affect pancreatic β -cell viability, as indicated by LDH assay results, reaffirming that the insulinotropic action was via a regulated physiological signalling pathway rather than simply caused by β -cell damage leading to unregulated insulin release. Modulation of Ca^{2+} handling by clonal beta cells is a key mechanism of the BPH-induced insulin release, although confirmation of this pathway by removing extracellular Ca^{2+} or using a calcium channel blocker such as Verapamil[®] is required to verify the results obtained here (Ojo *et al.* 2014). The membrane potential results revealed a depolarising phase in response to the BPH, which was complemented by a biphasic increase in intracellular Ca^{2+} . The present finding suggests involvement of the β -cell K_{ATP} channel in hydrolysate action.

GLP-1 and CCK-8 secretion from cultured STC-1 cells has been used to screen for bioactivity of fish protein hydrolysate (Cudennec *et al.* 2012) and whey protein hydrolysate (Power-Grant *et al.* 2015). Murine entero-endocrine GLUTag cells, have been previously utilised in the nutritional investigation of bioactive compounds and to screen for potential therapeutic agents affecting GLP-1 secretion as well as for investigating mechanisms of action (Brubaker *et al.* 1998; Brubaker & Anini 2003). The drug Forskolin® is a potent stimulator of cAMP production, which drives GLP-1 secretion (Reimann *et al.* 2008) and was used in the present study for comparison with the BPH related effects. Various nutrient dependant stimuli, including glucose and amino acids such as glutamine can trigger GLUTag membrane depolarization by closure of ATP-sensitive potassium channel and Na⁺-coupled uptake and increased intracellular Ca²⁺ through voltage-gated Ca²⁺ channels (Reimann & Gribble, 2002; Gribble *et al.* 2003). Other agents such as fatty acids and hormones could augment GLP-1 release by acting at points downstream of depolarisation. GLP-1 release from GLUTag cells have been linked to activation of PKA and PKC pathways (Gribble *et al.* 2003). *In vivo* efficacy of peptides and hydrolysates hinge on their capacity to reach the target cells/membrane/receptor without losing this potency. The gastrointestinal (GI) tract is known to be a major barrier, encompassing digestive enzymes and sharp changes in pH which could influence structure and alter functional properties of peptides (Möller *et al.* 2008; Moughan *et al.* 2014). Interestingly, in the present study GLP-1 release from GLUTag cells was enhanced after exposure to SGID, which could be a distinct advantage to incorporating a BPH as a dietary functional food ingredient. This may suggest higher oral bioavailability of the BPH after gastrointestinal digestion, or that perhaps the SGID step liberated further amino acids or small peptides that may exert a greater secretory action on GLUTag cells. The hydrolysate subjected to SGID also showed improved insulin secretion from BRIN-BD11 cells via membrane depolarization

and enhanced intracellular Ca^{2+} fluxes and similar mechanisms could be facilitating its GLP-1 releasing effects, but further detailed mechanistic studies would be required to prove this link.

Recently peptide/protein interaction and inhibition of endogenous enzymes have emerged as a therapeutic approach to treating conditions such as hypertension (Furuta *et al.* 2016) and diabetes (Forbes *et al.* 2013; Harnedy *et al.* 2015). The enzyme DPP-IV is present on cell membranes and in soluble form in blood plasma and is responsible for the rapid inactivation of GLP-1 and GIP (Mentlein *et al.* 1993) and therefore new oral DPP-IV inhibitors such as Vildagliptin[®] and Saxagliptin[®] have been developed to help with glycaemic management in T2DM (Ahren *et al.* 2011; Chen *et al.* 2015). The oral efficacy of BPH-derived peptides, which are absorbed in the small intestine, may play an important role in inhibiting DPP-IV activity. The clinical benefits of peptide and amino acid inhibition of DPP-IV and targeting this as a therapeutic strategy have been previously described (Nongonierma *et al.* 2013). Here we showed that this DPP-IV inhibition was present at least *in vitro* with the BPH and SGID samples having similar IC_{50} values (Table 1). In general, rodent studies have shown that a food protein hydrolysate having *in vitro* DPP-IV inhibitory activity, can also translate to anti-diabetic effects *in vivo* (Nongomierma & FitzGerald 2016). It remains to be seen if the boarfish DPP-IV inhibitory action is due to a single peptide entity, or as seems more likely, a combination of several component peptides and thus further extensive characterization work is required to help identify potent individual bioactive peptide(s) (Harnedy *et al.* 2015).

3T3-L1 adipocyte cells were utilised to examine the ability of the BPH and SGID samples to stimulate glucose transport. 3T3-L1 adipocytes exhibit all the components of insulin receptor and signal transduction cascade and are frequently used to investigate insulin mediated glucose transport (Brubaker *et al.* 1998; Tang *et al.* 2016). The BPH and SGID samples showed improved

glucose uptake in 3T3-L1 cells ($p < 0.01$) which was similar to that of lower dose insulin (10^{-9} M) alone, but it was not as potent as the higher insulin control (10^{-7} M). The action of insulin in adipocytes is known to increase the number of functional glucose transporters, thus increasing the rate of glucose uptake (Shi & Kandror 2008; Simpson *et al.* 1983; Holman and Cushman 1994). The BPH may act through improved activation or redistribution of glucose transporters, although further work is required to investigate their mode of action.

In conclusion, this study set out to display the potential therapeutic utility of a commonly underutilized protein source, namely the pelagic fish boarfish. A BPH displayed positive anti-diabetic like actions in a variety of *in vitro* assays (which was retained following SGID), and these bioactivities were supported from acute glucose tolerance studies in normal mice, demonstrating potent insulinotropic and glucose lowering actions. Taken together our data suggests that a BPH represents a suitable target for development of functional food components for potential treatment of pre-diabetes or T2DM. Further assessment in chronic animal studies and in humans as well as peptide identification studies are required to confirm their potential.

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References

- Ahrén, B., Schweizer, A., Dejager, S., Villhauer, E.B., Dunning, B.E., & Foley J.E. (2011) Mechanisms of action of the dipeptidyl peptidase-4 inhibitor vildagliptin in humans. *Diabetes Obesity and Metabolism*, 13, 775-783.
- Bantle, J.P., Wylie-Rosett, J., Albright, A.L., Apovian, C.M., Clark, N.G., Franz, M.J., Hoogwerf, B.J., Lichtenstein, A.H., Mayer-Davis, .E, Mooradian, A.D., & Wheeler, M.L. (2008) Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association. *Diabetes Care*, 31(Suppl 1), S61-S78.
- Brubaker, P.L., Schloos, J., & Drucker, D.J. (1998) Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. *Endocrinology*, 139(10), 4108-4014.
- Brubaker, P.L., & Anini, Y. (2003) Direct and indirect mechanisms regulating secretion of glucagon-like peptide-1 and glucagon-like peptide-2. *Canadian Journal of Physiology and Pharmacology*, 81, 1005–1012. Review
- Chen, X.W., He, Z.X., Zhou, Z.W., Yang, T., Zhang, X., Yang, Y.X., Duan, W., & Zhou, S.F. (2015) Clinical pharmacology of dipeptidyl peptidase 4 inhibitors indicated for the treatment of type 2 diabetes mellitus. *Clinical & Experimental Pharmacology and Physiology*, 42, 999-1024.
- Connolly, A., Piggott, C.O., & FitzGerald, R.J. (2013) Characterisation of protein-rich isolates and antioxidative phenolic extracts from pale and black brewers spent grain. *International Journal of Food Science and Technology*, 48, 1670–1681.
- Cudennec, B., Caradec, T., Catiau, L., & Ravallec, R. (2012) Upgrading of sea by-products: potential nutraceutical applications. *Advances in Food and Nutrition Research*, 65, 479-494.
- Cudennec, B., Balti, R., Ravallec, R., Caron, J., Bougatef, A., Dhulster, P., & Nedjar N. (2015) In vitro evidence for gut hormone stimulation release and dipeptidyl-peptidase IV inhibitory activity of protein hydrolysate obtained from cuttlefish (*Sepia officinalis*) viscera. *Food Research International*, 78, 238-245.
- Dhaval, A., Yadav, N., & Purwar, S. (2016) Potential applications of food derived bioactive peptides in management of disease. *International Journal of Peptide Research and Therapeutics*, 22, 377-398.
- Drotningvik, A., Mjøs, S.A., Pampanin, D.M., Slizyte, R., Carvajal, A., Remman, T., Høggøy, I., & Gudbrandsen, O.A. (2016) Dietary fish protein hydrolysates containing bioactive motifs affect serum and adipose tissue fatty acid compositions, serum lipids, postprandial glucose regulation and growth in obese Zucker fa/fa rats. *British Journal of Nutrition*, 116(8), 1336-1345.

- Drucker, D.J., Jin, T., Asa, S.L., Young, T.A., & Brubaker, P.L. (1994) Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. *Molecular Endocrinology*, 8, 1646–1655.
- Fayaz, S.M., Kumar, V.S.S., & Rajanikant, K.G. (2014) Finding Needles in a Haystack: Application of Network Analysis and Target Enrichment Studies for the Identification of Potential Anti-Diabetic Phytochemicals. *PLoS One*, 9(11), e112911.
- Flatt, P.R. & Bailey, C.J. (1981) Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia*, 20(5), 573-577.
- Forbes, J.M., Cowan, S.P., Andrikopoulos, S., Morley, A.L., Ward, L.C., Walker, K.Z., Cooper, M.E., & Coughlan, M.T. (2013) Glucose homeostasis can be differentially modulated by varying individual components of a western diet. *Journal of Nutritional Biochemistry*, 24(7), 1251-1257.
- Furuta, T., Miyabe, Y., Yasui, H., Kinoshita, Y., & Kishimura, H. (2016) Angiotensin I Converting Enzyme Inhibitory Peptides Derived from Phycobiliproteins of Dulse *Palmaria palmata*. *Marine Drugs*, 14(2), pii: E32.
- Gibala, M.J., Little, J.P., Macdonald, M.J. & Hawley J.A. (2012) Physiological adaptations to low-volume, high-intensity interval training in health and disease. *Journal of Physiology*, 590(5), 1077-1084.
- Gribble, F.M., Williams, L., Simpson, A.K. & Reimann, F. (2003) A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line. *Diabetes*, 52(5), 1147-1154.
- Grosberg, R.K., Vermeij, G.J. & Wainwright, P.C. Biodiversity in water and on land. *Current Biology*, 22(21), R900-R903.
- Gushiken, L.F., Beserra, F.P., Rozza, A.L., Bérghamo, P.L., Bérghamo, D.A., & Pellizzon, C.H. (2016) Chemical and Biological Aspects of Extracts from Medicinal Plants with Antidiabetic Effects. *The Review of Diabetic Studies*, Summer-Fall, 13(2-3), 96–112.
- Harnedy, P.A., O'Keeffe, M.B., & FitzGerald, R.J. (2015). Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga *Palmaria palmata*. *Food Chemistry*, 172, 400–406.
- Harnedy, P.A., Parthasarathy, V., McLaughlin, C.M., O'Keeffe, M.B., Allsopp, P.J., McSorley, E.M., O'Harte, F.P.M., & FitzGerald, R.J. (2018a) Blue whiting (*Micromesistius poutassou*) muscle protein hydrolysate with in vitro and in vivo antidiabetic properties. *Journal of Functional Foods*, 40,137-145.

- Harnedy, P.A., Parthsarathy, V., McLaughlin, C.M., O'Keeffe, M.B., Allsopp, P.J., McSorley, E.M., O'Harte, F.P.M., & FitzGerald, R.J. (2018b) Atlantic salmon (*Salmo salar*) co-product-derived protein hydrolysates: A source of antidiabetic peptides. *Food Research International*, 106, 598–606.
- Holman, G.D., & Cushman, S.W. (1994) Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. *Bioessays*, 16(10), 753-759.
- Huang, S.L., Jao, C.L., Ho, K.P., & Hsu, K.C. (2012) Dipeptidyl-peptidase IV inhibitory activity of peptides derived from tuna cooking juice hydrolysates. *Peptides*, 35(1), 114-121.
- Iba, Y., Yokoi, K., Eitoku, I., Goto, M., Koizumi, S., Sugihara, F., Oyama, H., & Yoshimoto, T. (2016) Oral Administration of Collagen Hydrolysates Improves Glucose Tolerance in Normal Mice Through GLP-1-Dependent and GLP-1-Independent Mechanisms. *Journal of Medicinal Food*, 19(9), 836-843.
- Inzucchi, S.E., Bergenstal, R.M., Buse, J.B., Diamant, M., Ferrannini, E., Nauck, M., Peters, A., Tsapas, A., Wender, R., & Matthews, D.R. (2012) Management of Hyperglycemia in Type 2 Diabetes: A Patient-Centered Approach. *Diabetes Care*, 35(6), 1364-1379.
- Jensen, I.J., & Mæhre, H.K. (2016) Preclinical and Clinical Studies on Antioxidative, Antihypertensive and Cardioprotective Effect of Marine Proteins and Peptides-A Review. *Marine Drugs*, 14(11) E211
- Jin, Q., Yu, H., & Li, P. (2017) The evaluation and utilization of marine-derived bioactive compounds with anti-obesity effect. *Current Medicinal Chemistry*, doi: 10.2174/0929867324666170602082620. [Epub ahead of print]
- Kim, S.K., Ngo, D.H., & Vo, T.S. (2012) Marine fish-derived bioactive peptides as potential antihypertensive agents. *Advances in Food and Nutrition Research*, 65, 249-260.
- Kristinsson, H.G., & Rasco, B.A. (2000) Biochemical and functional properties of atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agriculture and Food Chemistry*, 48, 657–666.
- Lordan, S., Ross, R.P., & Stanton, C. (2011) Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. *Marine Drugs*, 9(6), 1056-1100.

- McClenaghan, N. H., Barnett, C. R., Ah-Sing, E., Abdel-Wahab, Y. H. A., O'Harte, F. P. M., Yoon, T.W., Swanston-Flatt, S.K., & Flatt, P.R. (1996). Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes*, 45, 1132–1140.
- McLaughlin, C.M., Lampis, S., Mechkarska, M., Coquet, L., Jouenne, T., King, J.D., Mangoni, M.L., Lukic, M.L., Scorciapino, M.A., & Conlon, J.M. (2016) Purification, Conformational Analysis, and Properties of a Family of Tigerinin Peptides from Skin Secretions of the Crowned Bullfrog *Hoplobatrachus occipitalis*. *Journal of Natural Products*, 79(9), 2350-2356.
- Mentlein, R., Gallwitz, B., & Schmidt, W.E. (1993) Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *European Journal of Biochemistry*, 214, 829–835.
- Möller, N.P., Scholz-Ahrens, K.E., Roos, N., & Schrezenmeir, J. (2008) Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition*, 47, 171–182.
- Moughan, P.J., Rutherfurd, S.M., Montoya, C.A., & Dave, L.A. (2014) Food-derived bioactive peptides-a new paradigm. *Nutrition Research and Reviews*, 27(1), 16-20.
- Nathan, D.M., Genuth, S., Lachin, J., Cleary, P., Crofford, O., Davis, M., Rand, L., & Siebert, C. (1993) Diabetes Control and Complications Trial Research Group - The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *The New England Journal of Medicine*, 329(14), 977-986.
- Neves, A. C., Harnedy, P. A., O’Keeffe, M. B., Alashi, M. A., Aluko, R. E., & FitzGerald, R. J. (2017) Peptide identification in a salmon gelatin hydrolysate with antihypertensive, dipeptidyl peptidase IV inhibitory and antioxidant activities. *Food Research International*, 100 (1), 112-120.
- Nongonierma, A.B., & FitzGerald, R.J. (2012) Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase. *Peptides*, 37(2), 263-272.
- Nongonierma, A.B., & FitzGerald, R.J. (2016) Structure activity relationship modelling of milk protein-derived peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. *Peptides*, 79, 1-7.
- Nongonierma, A.B., Mooney, C., Shields, D.C. & Fitzgerald, R.J. (2013) Inhibition of dipeptidyl peptidase IV and xanthine oxidase by amino acids and dipeptides. *Food Chemistry*, 141(1), 644-653.

- O'Harte, F.P.M., Parthsarathy, V., Hogg C., & Flatt P.R. (2018) Apelin-13 analogues show potent *in vitro* and *in vivo* insulinotropic and glucose lowering actions. *Peptides*, 100, 219-228.
- Ojo, O.O., Srinivasan, D.K., Owolabi, B.O., Flatt, P.R., & Abdel-Wahab, Y.H.A. (2014) Magainin-related peptides stimulate insulin-release and improve glucose tolerance in high fat fed mice. *Protein and Peptide Letters*, 22(3), 256-263.
- Power-Grant, O., Bruen, C., Brennan, L., Giblin, L., Jakeman, P., & FitzGerald, R.J. (2015) In vitro bioactive properties of intact and enzymatically hydrolysed whey protein: targeting the enteroinsular axis. *Food and Function*, 6(3), 972-980.
- Reimann, F., & Gribble, F.M. (2002) Glucose-sensing in glucagon-like peptide-1-secreting cells. *Diabetes* 51(9), 2757-2763.
- Reimann, F., Habib, A.M., Tolhurst, G., Parker, H.E., Rogers, G.J., & Gribble, F.M. (2008) Glucose sensing in L cells: a primary cell study. *Cell Metabolism*, 8, 532–539.
- Ryan, J.T., Ross, R.P., Bolton, D., Fitzgerald, G.F., & Stanton, C. (2011) Bioactive Peptides from Muscle Sources: Meat and Fish. *Nutrients*, 3(9), 765–791.
- Senevirathne, M., & Kim, S.K. (2012) Utilization of seafood processing by-products: medicinal applications. *Advances in Food and Nutrition Research*, 65, 495-512.
- Shi, J., & Kandror, K.V. (2008) Study of glucose uptake in adipose cells. *Methods in Molecular Biology*, 456, 307-315.
- Silaa, A., Alvarezc, O.M., Haddar, A., Frikha, F., Dhulster, P., Nedjar-Arroume, N., & Bougatef, A. (2016) Purification, identification and structural modelling of DPP-IV inhibiting peptides from barbel protein hydrolysate. *Journal of Chromatography*, 1008: 260–269.
- Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L.B., & Cushman, S.W. (1983) Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochimica et Biophysica Acta*, 763(4), 393-407.
- Spellman, D., Kenny, P., O'Cuinn, G., & FitzGerald, R. J. (2005). Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities. *Journal of Agricultural and Food Chemistry*, 53(4), 1258-1265.

- Srinivasan, D., Mechkarska, M., Abdel-Wahab, Y.H.A., Flatt, P.R., & Conlon, J.M. (2013) Caerulein precursor fragment (CPF) peptides from the skin secretions of *Xenopus laevis* and *Silurana epittropicalis* are potent insulin-releasing agents. *Biochimie*, 95, 429-435.
- Tang, C., Wu, X.D., Yu, Y.M., Duan, H., Zhou, J., & Xu, L. (2016) Cell extraction combined with off-line HPLC for screening active compounds from *Coptis chinensis*. *Biomedical Chromatography*, 30, 658-662.
- Urakova, N., Pozharitskaya, O.N., Demchenko, D.V., Shikov, A.N., & Makarov, V.G. (2012) The biological activities of fish peptides and methods of their isolation. *Russian Journal of Marine Biology*, 38(6), 417–422
- Walsh, D.J., Bernard, H., Murray, B.A., MacDonald, J., Pentzien, A.K., Wright, G.A., Wal, J.M., Struthers, A.D., Meisel, H., & Fitzgerald, R.J. (2004) In vitro generation and stability of the lactokinin beta-lactoglobulin fragment (142-148). *Journal of Dairy Science*, 87(11), 3845-3857.
- Wang, X., Yu, H., Xing, R., & Li, P. (2017) Characterization, Preparation, and Purification of Marine Bioactive Peptides. *Biomedical Research International*, 2017, 9746720.
- Whitehead, P.J.P., Bauchot, M.L., Hureau, J.C., Nielsen, J., & Tortonese, E. (1986) Fishes of the North-eastern Atlantic and the Mediterranean, vol. 2 London UNESCO
- White, E., Minto, C., Nolan, C.P., King, E., Mullins, E., & Clarke, M. (2011) First estimates of age, growth, and maturity of boarfish (*Capros aper*): A species newly exploited in the Northeast Atlantic. *ICES Journal of Marine Science*, 68(1), 61-66.
- Zhu, C.F., Li, G.Z., Peng, H.B., Zhang, F., Chen, Y., & Li, Y. (2010) Treatment with marine collagen peptides modulates glucose and lipid metabolism in Chinese patients with type 2 diabetes mellitus. *Applied Physiology, Nutrition and Metabolism*, 35(6), 797-804.

Figure 1. Reverse phase ultra-performance liquid chromatography profile of boarfish (*Capros aper*) protein hydrolysate (BPH) and its simulated gastrointestinal digestion (BPH-SGID) sample.

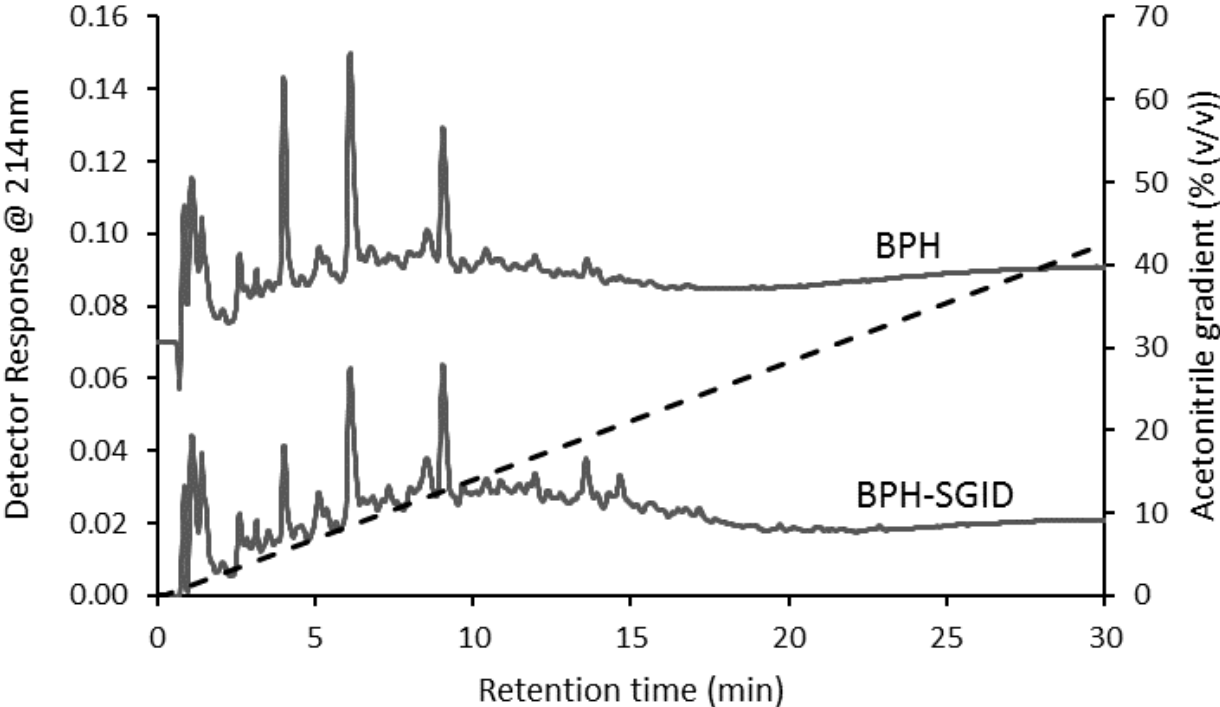


Figure 2: Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on insulin secretion and cytotoxicity.

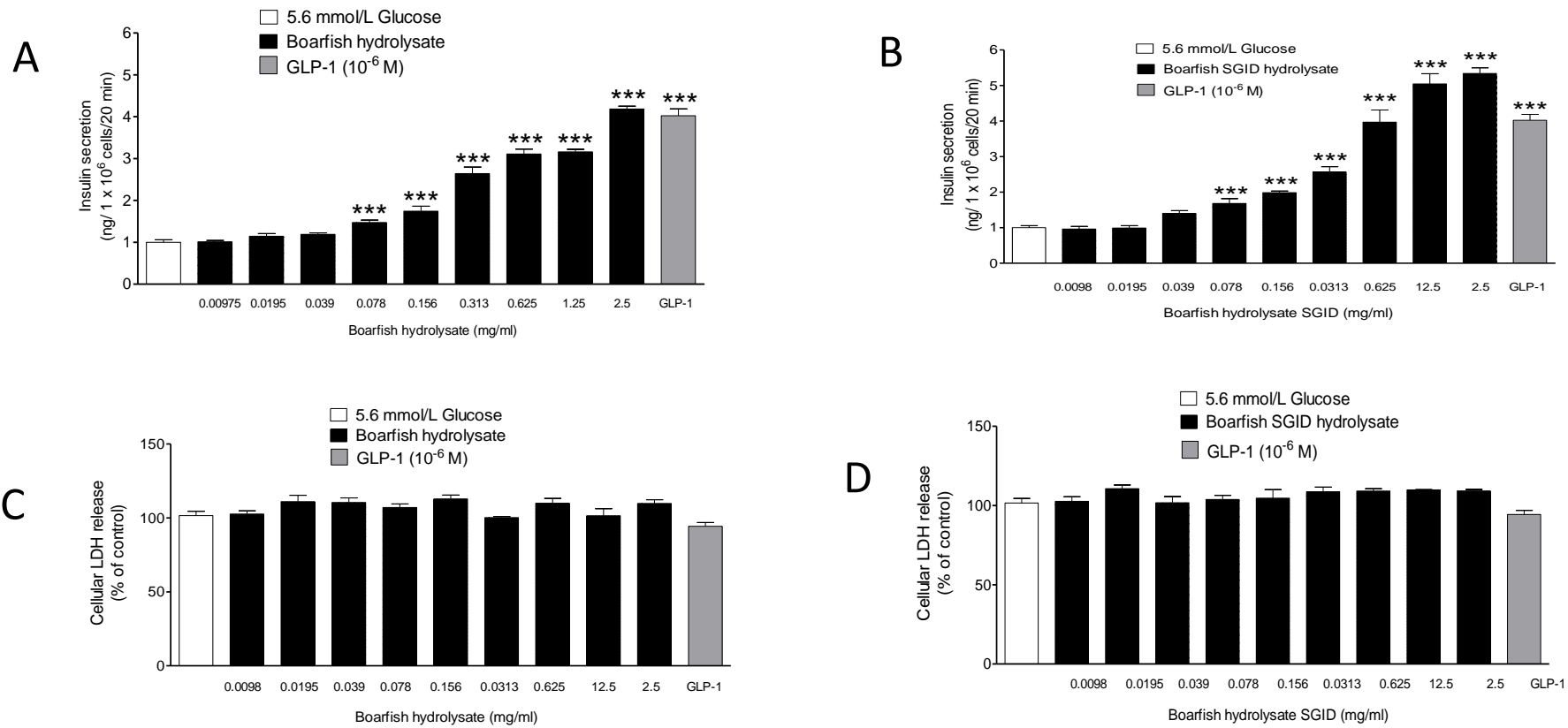


Figure 3: Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on GLP-1 secretion from cultured GLUTag cells

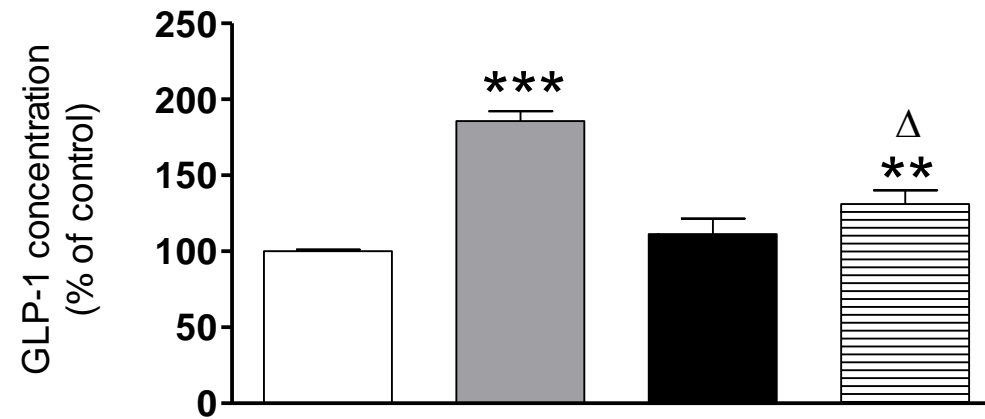


Figure 4. Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on glucose uptake in cultured 3T3-L1 cells.

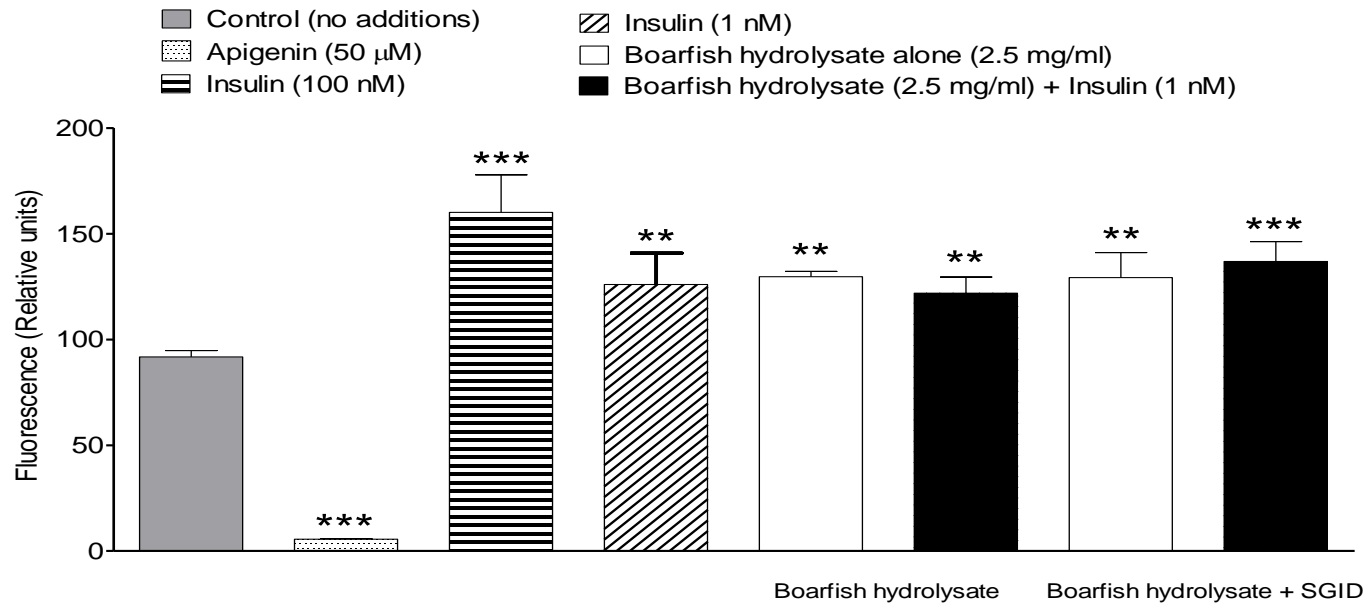


Figure 5: Effects of boarfish hydrolysates on intracellular $[Ca^{2+}]_i$ and membrane potential in pancreatic BRIN-BD11 cells

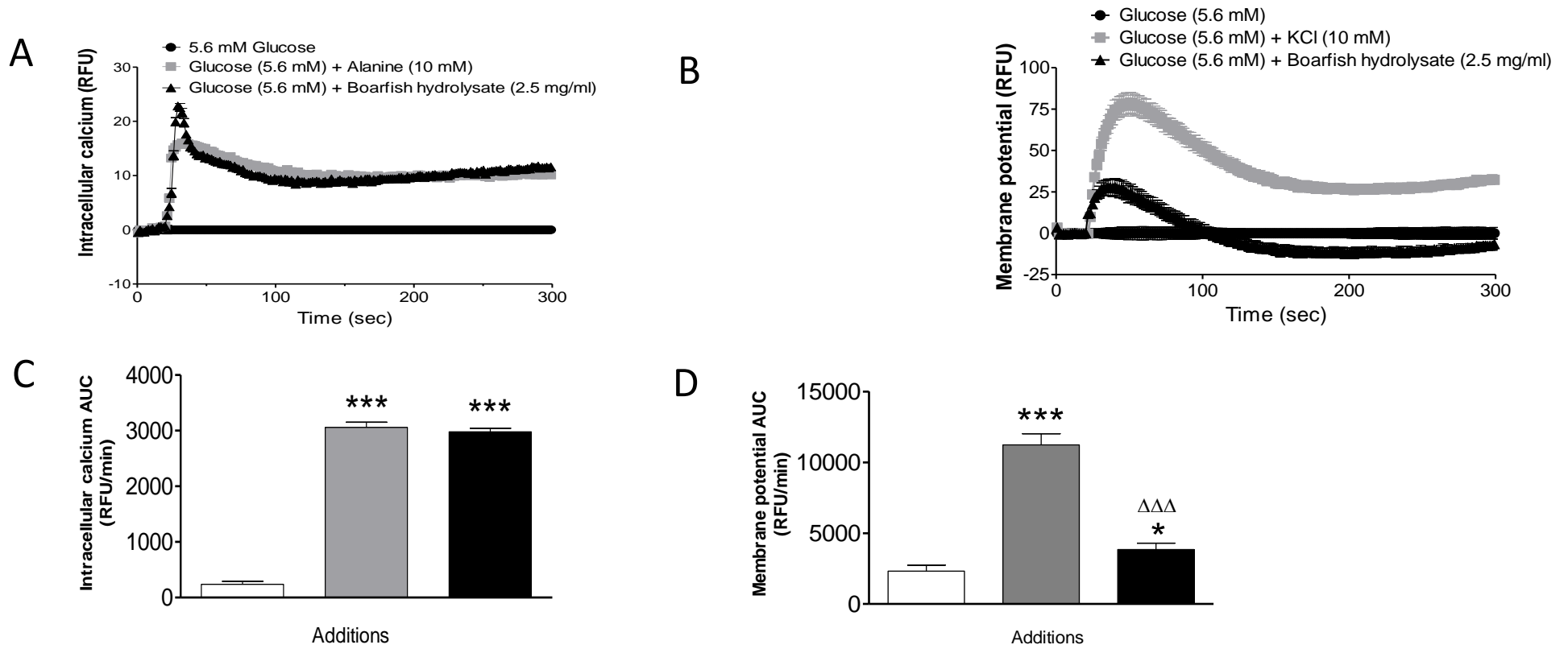


Figure 6: Acute effects of a boarfish (*Capros aper*) protein hydrolysate on glucose tolerance in normal mice.

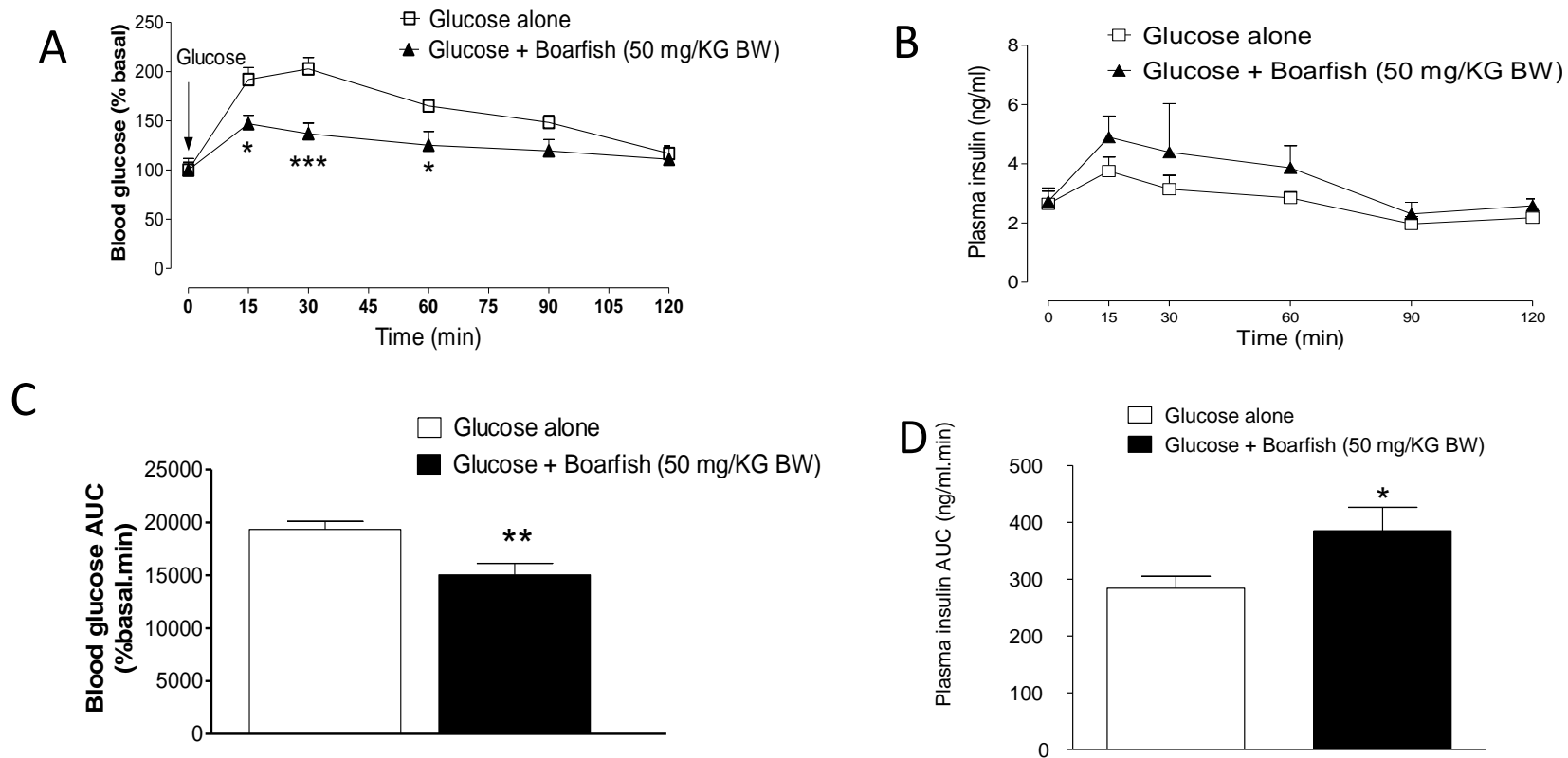


Table 1. Molecular mass (M_w) distribution of a boarfish (*Capros aper*) protein hydrolysate (BPH) generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (BPH:SGID) sample and effects on dipeptidyl peptidase (DPP-IV) inhibition.

Test Sample	Molecular mass distribution (% area)					DPP-IV inhibition
	>10 kDa	5-10 kDa	2-5 kDa	1-2 kDa	<1 kDa	IC ₅₀ (mg/ml)
BPH	0.15	0.80	7.11	18.38	73.56	1.18 ± 0.04 ^a
BPH:SGID	0	0	0.8	7.63	91.57	1.21 ± 0.04 ^a

Mean ± SEM (n=3), IC₅₀: inhibitory concentration that inhibits enzyme activity by 50%, DPP-IV inhibition values with different letters are significantly different at $p < 0.05$.

Legends to Figures

Figure 1. Reverse phase ultra-performance liquid chromatography profile of boarfish (*Capros aper*) protein hydrolysate (BPH) and its simulated gastrointestinal digestion (BPH-SGID) sample.

Separation of the BPH and BPH-SGID was carried out at 30°C, using a 2.1 × 100 mm, 1.7 µm Acquity UPLC C18 BEH column. The flow rate was set at 0.2 mL min⁻¹. Solvent A was 0.1% (v/v) TFA in HPLC grade water and solvent B was 0.1% (v/v) TFA in 80% HPLC grade acetonitrile. Peptides were eluted using a linear gradient: 0–30 min, 0-53% B and the absorbance of the eluent was monitored at 214 nm.

Figure 2. Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on insulin secretion and cytotoxicity.

Concentration-dependent effects of boarfish hydrolysate and SGID hydrolysate generated from Alcalase + Flavourzyme on insulin secretion (A + B) and LDH release (C + D) from BRIN-BD11 cells at 5.6 mM glucose. White bars, glucose alone; grey bars, GLP-1 (10⁻⁶ M); black bars, boarfish hydrolysate (0.009 to 2.5 mg/ml). Values are expressed as mean ± S.E.M. (n=6). ***p<0.001 compared to respective glucose control.

Figure 3: Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on GLP-1 secretion from cultured GLUTag cells.

The white bar represents glucose alone; grey bar is Forskolin® (10 mM); black bar is boarfish hydrolysate (BPH) and horizontal striped bar is the boarfish hydrolysate after SGID. Values are expressed as mean \pm SEM (n=4). **p<0.01, ***p<0.001 compared to glucose control and Δ p<0.05 versus the BPH.

Figure 4. Effect of a boarfish (*Capros aper*) hydrolysate generated with Alcalase 2.4L and Flavourzyme 500L and its simulated gastrointestinal digestion (SGID) sample on glucose uptake in cultured 3T3-L1 cells.

Glucose uptake was measured in differentiated 3T3-L1 adipocytes using a fluorescent assay. Apigenin was used as a negative control and a high insulin 100 nM was used as a positive control. Boarfish hydrolysate and the SGID sample were analysed in the presence and absence of a low basal insulin (1 nM). Values are mean \pm SEM (n=3) **p<0.01, ***p<0.001 compared to the control (deoxyglucose in glucose free KRBB buffer). No significant differences were found between either BPH versus BPH + insulin or between SGID versus SGID + insulin.

Figure 5: Effects of boarfish hydrolysates on intracellular $[Ca^{2+}]_i$ and membrane potential in pancreatic BRIN-BD11 cells

Effects of boarfish hydrolysate on (A) intracellular calcium and (B) membrane potential in BRIN-BD11 cells expressed as RFU and area under the curve (C, D). Panel (C) shows the area under the curve ($AUC_{1-120 \text{ min}}$) data for the blood glucose response in panel A. Panel (D) shows the $AUC_{(0-120 \text{ min})}$ data relating to the plasma insulin responses in panel B. Values represent means \pm SEM for 8 mice. * $p < 0.05$, *** $p < 0.001$ compared to 5.6 mM glucose control. $\Delta\Delta\Delta p < 0.001$ compared to positive control (KCl).

Figure 6: Acute effects of a boarfish (*Capros aper*) protein hydrolysate on glucose tolerance in normal mice.

Acute glucose homeostatic effects of boarfish in 16 h fasted normal mice after standardisation. (A) Blood glucose and (B) plasma insulin concentrations were measured prior to and after oral gavage of glucose alone (18 mmol/kg bw) as a control, or in combination with boarfish extract (50 mg/kg bw). Area under the curve ($AUC_{(0-120 \text{ min})}$) for (C) blood glucose and (D) Plasma insulin post-gavage is also shown. Values represent means \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with glucose alone.