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Assessment of blue whiting protein hydrolysate bioactivities using cell culture models.

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents.

Signed: _____

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Publications and Awards

First author publications

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2. Heffernan, S., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., Giblin, L., Fitzgerald, R. J., & O'Brien, N. M. (2021). Blue Whiting Protein Hydrolysates Exhibit Antioxidant and Immunomodulatory Activities in Stimulated Murine RAW264. 7 Cells. *Applied Sciences*, *11*(20), 9762. https://doi.org/10.3390/app11209762

3. Heffernan, S., Nunn, L., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., Giblin, L., ... & O'Brien, N. M. (2022). Blue Whiting (Micromesistius poutassou) Protein Hydrolysates Increase GLP-1 Secretion and Proglucagon Production in STC-1 Cells Whilst Maintaining Caco-2/HT29-MTX Co-Culture Integrity. *Marine Drugs*, *20*(2), 112. http://dx.doi.org/10.3390/md20020112

4. Heffernan S, Harnedy-Rothwell PA, Gite S, Whooley J, Giblin L, Fitzgerald RJ, O'Brien NM. Blue whiting protein hydrolysates post simulated gastrointestinal digestion and following simulated intestinal absorption exhibit anti-obesity activities in 3T3-L1 cells. In Preparation.

Other publications

1. Kondrashina, A., Heffernan, S., O'Brien, N., & Giblin, L. (2021). Application in medicine: obesity and satiety control. In *Biologically Active Peptides* (pp. 629-664). Academic Press. https://doi.org/10.1016/B978-0-12-821389-6.00005-4

Online newspaper/magazine articles

1. 'Marine Protein Hydrolysates for Health Enhancement' in UCC Research E-Compilation 'Linking the Food Chain'
2. 'Fish as a source of protein for health' in RTE Brainstorm

Abstracts

1. Heffernan, S., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., FitzGerald, R. J., & O'Brien, N. M. (2019). Bioactive potential assessed using cell culture model systems, of blue whiting protein hydrolysates produced at laboratory scale. *44th Annual Food Science and Technology Conference.* P17.

2. Heffernan, S., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., FitzGerald, R. J., & O'Brien, N. M. (2019). In vitro satiety, anti-obesity and antioxidant potential of blue whiting protein hydrolysates produced at industrial scale. *44th Annual Food Science and Technology Conference.* P8.

3. Heffernan, S., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., FitzGerald, R. J., & O'Brien, N. M. (2021). Antioxidant activity of blue whiting protein hydrolysates–an in vitro study. *Proceedings of the Nutrition Society*, *80*(OCE2).

4. Heffernan, S., Harnedy-Rothwell, P. A., Gite, S., Whooley, J., Giblin, L., FitzGerald, R. J., & O'Brien, N. M. (2021). Investigation of the antioxidant potential of blue whiting protein hydrolysates in oxidatively-stressed 3T3-L1 adipocytes. *Proceedings of the Nutrition Society*, *80*(OCE3).

5. Heffernan, S., Harnedy-Rothwell, P., Gite, S., Whooley, J., Giblin, L., FitzGerald, R., & O'Brien, N. (2022). Blue whiting protein hydrolysates stimulate GLP-1 secretion from STC-1 cells. *Proceedings of the Nutrition Society, 81*(OCE1), E6.

Award

1. UCC School of Food and Nutritional Sciences best review paper of the year award for the review article entitled "Assessment of the biological activity of fish muscle protein hydrolysates using in vitro model systems" published in *Food Chemistry*.

Abstract

Low-value underutilised blue whiting fish represents a potential profitable source of protein for the generation of high-value, health-enhancing fractions. The research described in this thesis assessed the bioactive potential of blue whiting soluble protein hydrolysates (BWSPHs) using cellular model systems.

Minced, deboned blue whiting was initially hydrolysed at laboratory scale to develop a protocol for the reproducible generation of eleven different BWSPHs. Cellular bioactivity analysis of these eleven hydrolysates indicated that none of the BWSPHs tested exhibited satiating activity, antioxidant activity, immunomodulatory activity or anti-obesity activity as measured using specific cellular models. However, six BWSPHs did exhibit anti-diabetic activity in vitro, therefore, these six BWSPHs were prepared at semi-pilot scale and all of the above bioactivities were reassessed in greater detail.

The antioxidant and immunomodulatory activities of the six industrial-scale BWSPHs before simulated gastrointestinal digestion (SGID) (BW-SPH-A to BW-SPH-F) and after SGID (BW-SPH-A-GI to BW-SPH-F-GI) were assessed in stimulated murine RAW264.7 macrophage. Hydrolysate BW-SPH-A (0.5%, w/v dw), both pre- and post-SGID, increased the endogenous antioxidant, reduced glutathione (GSH), in *tert*-butylhydroperoxide (*t*BOOH)-treated cells and reduced reactive oxygen species (ROS) in H_2O_2-challenged RAW264.7 cells compared with stimulated controls ($p<0.05$). In vitro digested hydrolysate BW-SPH-F-GI (0.5%, w/v dw) induced immunostimulating effects in lipopolysaccharide (LPS)-activated RAW264.7 macrophages though increasing pro-inflammatory cytokine interleukin (IL)-6 and tumour necrosis factor (TNF)-α levels compared with the LPS-stimulated control ($p<0.05$).

The satiating potential of the six BWSPHs was then assessed in murine enteroendocrine STC-1 cells. The ability of BWSPHs and SGID BWSPHs to modulate the secretion and/or production of satiety hormones glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) in STC-1 cells was studied as well as the signalling pathway activated by BWSPHs to modulate the secretion of these hormones. All BWSPHs (BW-SPH-A to BW-SPH-F) (1.0%, w/v dw) increased active GLP-1 secretion and proglucagon production in STC-1 cells compared to the basal control (Krebs-Ringer buffer) ($p<0.05$), possibly via intracellular calcium

signalling, however this activity was lost following SGID. In addition, neither pre- nor post-SGID hydrolysates affected epithelial barrier integrity or stimulated IL-6 secretion in differentiated Caco-2/HT-29MTX co-cultured cells.

The anti-obesity effects of BWSPHs and SGID BWSPHs was investigated using the murine 3T3-L1 cell line. Before SGID, hydrolysates BW-SPH-A, -B, -C and -F (1.0%, w/v dw) reduced triglyceride accumulation during preadipocyte differentiation (p<0.05), however none of the hydrolysates hydrolysed triglycerides in fully mature adipocytes. Interestingly, after SGID, all hydrolysates reduced triglyceride accumulation during differentiation and all except one BWSPH increased glycerol levels in mature adipocytes compared with the differentiated controls (p<0.05). Two anti-adipogenic hydrolysates, BW-SPH-A and BW-SPH-F, and their corresponding in vitro digests were observed to modulate triglyceride accumulation during preadipocyte differentiation via down-regulating the expression of key adipogenic transcription factors (peroxisome proliferator activated receptor (PPAR)-γ and CAAT (controlled amino acid therapy)/ enhancer binding protein (C/EBP)-α) compared with the differentiated controls (p<0.05). These SGID hydrolysates also exhibited anti-obesity activities following simulated intestinal permeation. After a 4 h exposure of specific SGID BWSPHs to 21-day differentiated Caco-2/HT-29MTX co-cultured cells, cell basolateral was subsequently observed to exhibit anti-adipogenic and adipolytic activities in 3T3-L1 cellular models.

In addition, exposure of 3T3-L1 preadipocytes to hydrolysate BW-SPH-A during differentiation also increased GSH concentration upon stimulation with antioxidant tBOOH compared with the tBOOH control (p<0.05). Specific BWSPHs were also observed to reduced adiponectin production in LPS-stimulated cells compared with the LPS control (p<0.05).

To conclude, certain BWSPHs exhibited significant bioactivities before SGID, after SGID, and after simulated intestinal absorption, therefore may have potential as health-enhancing functional food ingredients.

Abbreviations

%	Percent
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
µg	Microgram
µL	Microlitre
µM	Micromolar
°C	Degrees Celsius
AA	Amino acid
AAPH	2,2-azobis-(2-amidino- propane) dihydrochloride
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-1-converting enzyme
ADP	Adenosine triphosphate
ANOVA	One-way analysis of variance
aP2	Adipocyte protein 2
ATP	Adenosine diphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
BWPH	Blue whiting protein hydrolysate
BWSPH	Blue whiting soluble protein hydrolysate
C/EBP	CCATT/enhancer binding protein
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCK	Cholecystokinin
CFU	Colony forming unit
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
d	Days

DCF	2′,7′-dichlorofluorescein
DCFH	2′,7′-dichlorodihydrofluorescein
DCFH-DA	2′,7′-dichlorodihydrofluorescein-diacetate
DH	Degree of hydrolysis
DMEM	Dulbecco's Modified Eagles' Medium
DPPH	2,2-diphenyl-1- picrylhydrazyl
DPP-IV	Dipeptidyl peptidase IV
dw	Dry weight
E/S	Enzyme/ substrate
EC_{50}	Concentration corresponding to half-maximal activity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FOSHU	Foods for Specific Health Use
FPH	Fish protein hydrolysate
FRAP	Ferric reducing antioxidant power
FSK	Forskolin
g	Gram
g	Centrifugal force
GLP-1	Glucagon-like peptide-1
GPH	Goby protein hydrolysate
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
h	Hours
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HFD	High-fat diet
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Half maximum inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin

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IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Kg	Kilogram
L	Litre
LPH	Lanternfish protein hydrolysates
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDI	Differentiating media
mg	Milligram
MIC	Minimum inhibition concentration
min	Minute
mM	Millimolar
MTT	3 -(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MW	Molecular weight
NED	Napthylethylenediamine dihydrochloride
NF	Nuclear factor
NO	Nitric oxide
O_2^-	Superoxide anion
OH	Hydroxyl
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
PGE_2	Prostaglandin E_2
PPAR	Peroxisome proliferator-activated receptor
PRR	Pathogen-recognition receptors
PYY	Peptide YY
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPH	Ray protein hydrolysate
RP-HPLC	Reversed-phase high-performance liquid chromatography
rpm	Revolutions per minute

rt-PCR	Real-time polymerase chain reaction
s	Second
SEM	Standard error of the mean
SGID	Simulated gastrointestinal digestion
S-IgA	Secretory immunoglobulin A
SOD	Superoxide dismutase
tBOOH	Tert-butyl hydroperoxide
TE	Trolox equivalents
TEER	Transepithelial electrical resistance
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

Literature Review

Assessment of the biological activity of fish muscle protein hydrolysates using *in vitro* model system

Abstract

The generation of biologically active fish protein hydrolysates (FPH) is a useful technique to produce value-added products with potential application in the functional food and nutraceutical industries. Fish muscle is an attractive substrate for the production of protein hydrolysates due to its rich protein content, containing 15-25% of total fish protein. This paper reviews the production of protein hydrolysates from fish muscle, most commonly via enzymatic hydrolysis, and their subsequent bioactivities including anti-obesity, immunomodulatory, antioxidant, angiotensin I-converting enzyme (ACE)-inhibitory, anti-microbial, and anti-cancer activities as measured by *in vitro* testing methods. Disease prevention with FPH potentially offers a safe and natural alternative to synthetic drugs. Small molecular weight (MW) FPHs generally exhibit favourable bioactivity than large MW fractions via enhanced absorption through the gastrointestinal tract. This review also discusses the relationship between amino acid (AA) composition and AA sequence of FPH and peptides and their exhibited *in vitro* bioactivity.

Keywords: fish protein hydrolysates; enzymatic hydrolysis; *in vitro*; bioactivity

1.1 Introduction

Fish is a rich source of protein, ranging from 10-25% depending on species, with 15-25% of total protein located in fish muscle (Petricorena, 2015). Fish muscle proteins can be divided into 3 groups, namely structural protein (approximately 70-80% of total protein content), myofibrillar protein and sarcoplasmic protein. The three groups contain all essential amino acids (AA); specifically, Lys accounts for 8.8%, Trp 1.0%, His 2.0%, Phe 3.9%, Leu 8.4%, Ile 6.0%, Thr 4.6%, Met-Cys 4.0% and Val at 6.0% (Hayes & Flower, 2013). Marine organisms are reported to produce a variety of potent bioactive compounds as they are forced to live in a complex environment which is exposed to extreme conditions of salinity, pressure, temperature and illumination (Hamed et al., 2015). Bioactive peptides purified from fish sources have garnered considerable interest in recent times with potential applications in both food and pharmaceutical industries. Bioactive fish protein hydrolysates (FPH) and peptides are desirable functional food ingredients due to their natural availability, relatively low-cost extraction methods and their ability to exert a beneficial effect on human health by exhibiting antioxidant, anti-inflammatory, anti-proliferative, anti-hypertensive, and cardio-protective bioactivities (Suleria et al, 2016). The introduction of the 'landing obligation' policy by the European Commission in 2019 has maximized protein harvest from low-value fish species, thereby presenting a profitable source of bioactive peptides.

Bioactive peptides which are usually inactive in the parent protein molecules can be released via enzymatic hydrolysis, chemical hydrolysis or fermentation. These biofunctional peptides generally range in size from 2-20 AA residues. However, the molecular weight (MW) and size of the peptides and the AA composition and sequence of the peptide ultimately influences their bioactive properties.

In vitro testing is often utilized for preliminary research, prior to testing via *in vivo* model systems, due to its cost-efficiency and ability to yield rapid and reproducible data. Although non-cellular bioassays are used to investigate some bioactivities including angiotensin-1-converting enzyme (ACE) inhibition and anti-microbial activity, cellular model systems are useful for investigating various bioactivities as well as unravelling the biological pathways activated upon contact of the bioactive compound with the target cells. Although *in vitro* studies provide a

controlled environment for experimentation, cellular bioassays involve maintaining cells outside of the living organism; therefore, results must be interpreted carefully due to the innate complexity of organ systems *in vivo* (Jain et al., 2018). Bioactive FPH for oral consumption face the challenge of surviving the hydrolytic conditions of the GI tract, so that absorption through the gut barrier and contact with target cells is achieved.

This paper will critically review current knowledge emerging from *in vitro* model systems on the bioactive potential of protein hydrolysates and peptides isolated from various fish muscle sources (Figure 1.1). We highlight limitations of studies, as well identify gaps in the existing knowledge of bioactive fish peptides which has enabled us to recommend future research opportunities.
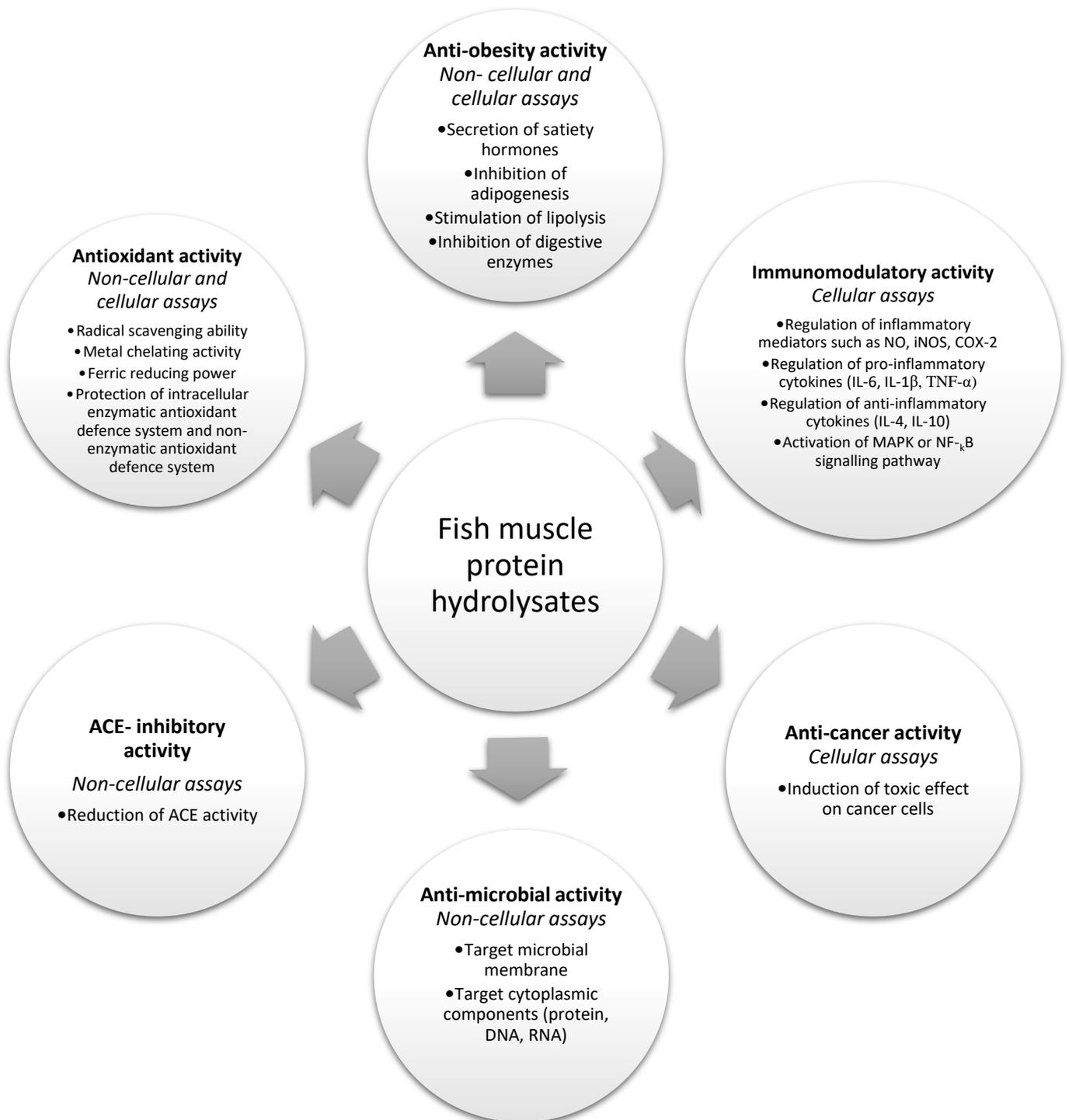
Figure 1.1: Biological activities of fish muscle protein hydrolysates and possible mechanisms of action discussed in this this review

1.2 Production of FPH

The bioactivity of food-derived protein hydrolysate ultimately depends on peptide and AA composition. However, the composition of the resulting fraction is highly influenced by the protein source, method of hydrolysis, hydrolysis conditions and degree of hydrolysis (DH). Protein hydrolysates can be produced via (a) enzymatic hydrolysis with proteases sourced from various commercial animal, microbial and plant sources, (b) fermentation with proteolytic microorganisms, or (c) chemical hydrolysis with either alkali or acid. Chemical hydrolysis is the least common method of hydrolysis due to non-specific cleavage of peptide bonds resulting in high variability in hydrolysate bioactivity. It is also known to yield products with reduced nutritional value due to destruction of Cys, Arg, Thr, Ser, and Lys residues (Nasri, 2017; Provansal et al., 1975). However, it does play a role in bioactive peptide release during gastrointestinal (GI) digestion of dietary protein (Dallas et al., 2017). Fermentation with proteolytic microorganisms utilises starter and non-starter cultures available commercially within the fermented food sector. Although *Bacillus* species are most commonly used for fermentation of FPHs (Godinho et al., 2016; Jemil et al., 2014), Bkhairia and collegaues (2016) reported poor efficiency of proteases from *Pseudomonas aeruginosa* A2 on hydrolysis of golden grey mullet protein. Fermentation is the cheapest proteolysis process; however, enzymatic hydrolysis is the most common method for producing bioactive protein hydrolysates as specific proteases and conditions can be selected to produce hydrolysates of desired size, sequence and bioactivity (Bhandari et al., 2020). The various proteolytic enzymes and hydrolysis conditions employed for generation of bioactive FPH are presented in Table 1.1.

1.2.1 Microbial enzymes

Microbial enzymes commonly used for the release of bioactive protein hydrolysates from fish include Flavourzyme® (*Aspergillus oryzae*), Alcalase® (*Bacillus licheniformis*), Neutrase® (*Bacillus amyloliquefaciens*), and Protamex® (Bacillus *licheniformis and* Bacillus *amyloliquefaciens*). Fish muscle of *Collichthys niveatus* was rinsed, ground, freeze-dried and sieved through a 120-mesh screen (125 micron) prior to hydrolysis with a commercial microbial enzyme, either

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Flavourzyme®, Alcalase®, Neutrase® or Protamex® at the same activity levels (10.103 U). Alcalase® hydrolysis induced the highest DH (17.03%) compared with Neutrase® (15.04%), Protamex® (12.98%) and Flavourzyme® (5.82%) (Table 1.1) (Shen et al., 2012). DH is defined as the percentage of the number of peptide bonds cleaved divided by the total number of peptide bonds in a protein. Seniman et al. (2014) also reported catfish (*C.batrachus*) protein to be more susceptible to hydrolysis with Alcalase® than plant-derived enzyme papain, demonstrating a direct correlation between DH and peptide content (Table 1.1). In contrast, Fonseca et al. (2016) reported Cobia *(Rachycentron canadum)* meat protein showed greater susceptibility to Protamex® hydrolysis exhibiting a DH value of 25.94% after 760 min compared to Alcalase® (10% after 300 min) and Flavourzyme® (12% after 420 min).

1.2.2 Animal-derived enzymes

Digestive enzymes from bovine and porcine GI tracts such as pepsin, trypsin, and chymotrypsin are also commonly used for production of biologically active protein hydrolysates from various fish sources (Bkhairia et al. 2016; Chi et al. 2014; Darewics et al., 2014; Jiang et al., 2014; Kim & Byun, 2012; Ko et al., 2013; Naqash & Nazeer, 2010; Sung et al., 2012). DH values of bioactive hydrolysates were not always reported. Peptic rainbow trout muscle protein hydrolysate exhibited a higher DH value (49.12%) and subsequent ACE inhibitory activity than hydrolysates prepared with trypsin (DH 30.52%), or α-chymotrypsin (DH 28.75%). However, all rainbow trout muscle protein hydrolysates showed greater susceptibility to digestive proteases than Alcalase®, Neutrase®, or papain (Kim & Byun, 2012). Chi et al. (2014) reported that the most influential hydrolysis parameters on DH of trypsin-prepared monkfish protein hydrolysates were temperature, pH, enzyme/substrate (E/S) ratio and time, respectively. The maximum DH ($19.83 \pm 0.82\%$) of monkfish protein hydrolysate was obtained when hydrolysis conditions for trypsin were 40°C, pH 8.0, E/S 2% with 4 h hydrolysis period (Table 1.1). Under-utilised skipjack tuna (*Katsuwonus pelamis*) protein was highly susceptible to hydrolysis with either trypsin or Protamex® exhibiting DH values of 71.68% and 78.33%, respectively, however, trypsin was chosen for future hydrolysis of skipjack tuna protein due to its more attractive price point (Liu et al., 2015). A study by Darewicz et al. (2014) compared *in vitro* and *ex vivo* hydrolysis of salmon myofibrillar and sarcoplasmic proteins and

reported *in vitro* hydrolysis with porcine pepsin or pepsin and Corolase PP was more efficient than human gastric or gastric and duodenal juices, respectively, as less intact protein was measured in *in vitro* hydrolysed fractions, indicating further hydrolysis and production of small MW peptides. This study demonstrates the complexity of the human digestive system and confirms that *in vitro* results may not always predict *in vivo* results.

1.2.3 Plant-derived enzymes

Some studies found plant-derived proteases induced a higher DH in FPHs than alternatively sourced proteolytic enzymes. Catfish (*Pangasius hypothalamus*) meat protein hydrolysed with papain and bromelain exhibited DH values of 31.16% and 29.36%, respectively, compared to a DH of 13.3% for the hydrolysate produced with the microbial enzyme Neutrase® (Table 1.1) (Ha et al., 2017). Hydrolysis with bromelain increased the free AA content most effectively from 28.00 g/kg protein to 58.02 g/kg protein compared with papain and Neutrase® hydrolysis, however, bromelain hydrolysis had no effect on the antioxidant activity of catfish protein. Both papain and Neutrase® hydrolysates demonstrated increased radical scavenging activity compared with the non-hydrolysed control, potentially owing to the reduced hydrolysis of bioactive peptides.

1.2.4 Production challenges

The shortcomings associated with enzymatic hydrolysis of food proteins include solubility and bitterness, both of which are highly influenced by DH, proteolytic enzyme and substrate employed. Bitterness and solubility issues impose sensory and processing challenges, respectively, and must be managed for the practical application of protein hydrolysates. Hydrolysis of *Collichthys niveatus* protein with Neutrase® generated a hydrolysate with a higher content of sweet and umami taste AAs (116.07 μg/ mL) namely Ala, Asp, and Glu and less hydrophobic, bitter AA, Phe, than Alcalase® hydrolysis (Shen et al., 2012). Hydrophobic peptides are associated with bitter taste due to the presence of two functional units, the binding unit and the stimulating unit, responsible for binding with the bitter taste receptor and

determining site for bitterness, respectively (Ishibashi et al., 1988). Dauksas and colleagues (2004) reported that hydrolysates obtained by use of Alcalase$^{®}$ were more bitter than hydrolysates obtained using Flavourzyme$^{®}$ as measured by sensory analysis. However, a secondary treatment with Flavourzyme$^{®}$ did not further reduce bitterness of the fraction, whereas treatment of the FPH with n-butanol and cholestyramine resin did indeed reduce bitterness via extraction of bitter bile compounds (Dauksas et al., 2004).

Although it is reported that a high DH is related to improved solubility due to changes in MW, hydrophobicity and polar groups (Leni et al., 2020), Liu et al. (2015) reported hydrolysis of under-utilised skipjack tuna protein for 5 h completely degraded small MW peptides to AA and the highest soluble protein content (80%) was observed after 2.5 hr hydrolysis with trypsin or Protamex. Glycation of the trypsin hydrolysate with alginate significantly improved solubility (p<0.05) compared with the non-glycated fraction in the pH range of 2-10 possibly due to the glycation-induced shift of isoelectric point towards a more acidic pH. It is well known that hydrolysates generally show low solubility at their isoelectric points, therefore it is probable that the basic pH of the trypsin hydrolysate influenced its solubility.

Table 1.1. Proteases employed for production of fish muscle protein hydrolysates

Fish species	Protease	Protease type	Cleavage site of protease	Hydrolysis conditions	Peptides/ AAs in fraction	Reference
Monkfish	Trypsin	Serine endopeptidase	Lys or Arg residues	40°C, pH 8.0, E/S 2% with 4 h	EWPAQ, FLHRP, LMGQW	Chi et al. (2014)
Salmon	Pepsin Corolase PP	Endopeptidase Endopeptidase (Metalloprotease and serine protease)	Between hydrophobic AA residues Broad substrate specificity	15 U/g protein, 37°C, 120 min 31.2 U/g protein, 37°C, 60 min	IVY, VW, IY, IW, VY, TVY, VFPS, VTVNPYKWLP, IWHHT, YALPHA, ALPHA	Darewicz et al. (2014)
Cod (*Gadus morhua*)	Flavourzyme® Alcalase®	Mixture of endo- and exoproteases Serine endopeptidase	Broad substrate specificity Broad substrate specificity	50°C, 0.1% Flavourzyme® or Alcalase®	Total AA content (52-92% of the total crude proteins) Free AA (28-62% of the total AAs)	Dauksas et al. (2004)
Cobia (*Rachycentron canadum)*	Alcalase® Flavorase Protamex®	Serine endopeptidase Mixture of endo- and exoproteases Serine endopeptidase	Hydrophobic AA Broad substrate specificity Broad substrate specificity	Alcalase® (pH 8.0, 50°C, 99.75 U/g) Flavourzyme® (pH 7.0, 50°C, 2.07 U/g) Protamex® (pH 7.0, 40°C, 8.41 U/g)	-	Fonseca et al. (2016)
Catfish (*Pangasius*	Papain	Cysteine endopeptidase	After Arg or Lys preceded by a hydrophobic AA not followed by Val	Papain (55°C, pH 7.5, 180 min, 36.2 g/L),	Papain: Arg (12.64 ± 0.06 g/kg protein)	Ha et al. (2017)

hypophthalmus)	Bromelain Neutrase®	Cysteine endopeptidase Metalloprotease	After Lys, Ala or Tyr Hydrophobic AA	Bromelain (55°C, pH 6.5, 180 min, 135.8 g/L) Neutrase® (50°C, pH 6.5, 180 min, 108.4 g/L)	Bromelain: Lys (22.46 ± 0.10 g/kg protein) Neutrase®: Lys (18.86 ± 0.07 g/kg protein)	
Rainbow trout	Pepsin

Trypsin α-chymotrypsin

Alcalase® Neutrase® Papain | Endopeptidase

Serine endopeptidase Serine endopeptidase

Serine endopeptidase Metalloprotease Cysteine endopeptidase | Between hydrophobic AA residues Lys or Arg residues Medium to large sized hydrophobic AA (Typ, Phe, Trp) Broad substrate specificity Hydrophobic AA.   After Arg or Lys preceded by a hydrophobic AA not followed by Val | Pepsin (37°C, pH 2.0), Trypsin (37°C, pH 7.0), α-chymotrypsin (37°C, pH 7.0), Alcalase (50°C, pH 7.0), Neutrase (50°C, pH 7.0), papain (37°C, pH 7.0), E/S 1/100 (w/w) for 6 h | Peptide purified from peptic fraction: LVNGPAMSPNAN | Kim and Byun (2012) |
| Under-utilised skipjack tuna (*Katsuwonus pelamis*) | Alcalase® Flavourase Neutrase® Trypsin Protamex® | Serine endopeptidase Metalloprotease Metalloprotease Serine endopeptidase Serine endopeptidase | Broad substrate specificity - Hydrophobic AA Lys or Arg residues Hydrophobic AA | Alcalase® (pH 8.0, 55°C), Flavourase (pH 7.0, 55°C), Neutrase® (pH 7.0, 50°C), Trypsin (pH 8.0, 50°C), | All fractions rich in His (489.14 - 1337.90 mg/100g) and Pro (144.36 - 170.50 mg/100g) Trypsin and Protamex®: 80% soluble protein at 2.5h | Liu et al. (2015) |

				Protamex® (pH 7.0, 50°C), 20,000 U/g, 5h		
Bighead croaker (*Collichthys niveatus*)	Flavourzyme® Alcalase® Neutrase® Protamex®	Mixture of endo- and exoproteases Serine endopeptidase Metalloprotease Serine endopeptidase	Broad substrate specificity Broad substrate specificity Hydrophobic AA Hydrophobic AA	Protease activity 10.103 U, 4h hydrolysis, pH and temperature not reported.	Neutrase®: SUA (88.259 μg/ mL) Alcalase®: SUA (65.129 μg/ mL) Protamex®: SUA (40.796 μg/ mL) Flavourzyme®: SUA (49.172 μg/ mL)	Shen et al. (2012)
Catfish (*C.batrachus*)	Alcalase® Papain	Serine endopeptidase Cysteine endopeptidase	Broad substrate specificity After Arg or Lys preceded by a hydrophobic AA not followed by Val	Alcalase® (pH 8, 55°C), Papain (pH 7, 55°C). E/S ratio 1:100 (w/w), 30-180 min.	Alcalase®: Glu (15.66 ± 0.22%), Lys (10.35 ± 0.09%), Asp (9.86 ± 0.01%) Papain: Glu (16.40 ± 0.22%), Lys (10.96 ± 0.39%), Asp (10.21 ± 0.25%)	Seniman et al. (2014)

AA, amino acid; E/S, enzyme/substrate; SUA, sweet and umami taste amino acids (Ala, Asp and Glu).

1.3 Biological activity of fish muscle protein hydrolysates and peptides as reported in vitro model systems

1.3.1 Inflammation modulatory activity

Table 1.2 details studies investigating the ability of fish muscle hydrolysates to modulate signals within inflammatory response pathways. The human body initiates inflammation in response to various stimuli including infections, injury, and toxins in an attempt to heal itself. Activation of macrophages is essential for initiation and continuation of defensive reactions as macrophages release various pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β and inflammatory mediators such as prostaglandin E_2 (PGE_2) and nitric oxide (NO) which improve tissue repair (Je & Kim, 2012). However, prolonged or excessive inflammation is associated with a wide range of diseases, including chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, and cancer.

Kangsanant et al. (2015) identified a novel anti-inflammatory peptide from Flavourzyme® hydrolysed tilapia muscle protein via gel filtration chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) with an AA sequence of AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (MW: 6309.49 Da) (Table 1.2). The peptide (20 mg protein/mL) reduced NO production by $40.9 \pm 0.2\%$ in the murine macrophage cell line RAW264.7 stimulated with lipopolysaccharide (LPS) for 48h; which was 100-fold higher than that of the crude hydrolysate. The presence of hydrophobic AAs residues in the purified peptide, namely, Ala, Phe, Leu, Val and Ile was hypothesized to play a significant role in its NO-inhibitory activity. Although further explanation regarding the relationship between hydrophobic AAs and their function in inflammatory modulation is required, it has been reported that the presence of hydrophobic AAs enhance attraction and reactivity with the cell membrane and possibly promote downstream signalling pathways with anti-inflammatory effects (Sangtanoo et al., 2020). In a previous study, Kangsanant et al. (2014) demonstrated that ultrasonic pre-treatment of tilapia protein hydrolysate prior to Flavourzyme® hydrolysis demonstrated superior NO inhibitory

activity than non- pre-treated hydrolysates (p<0.05). It was suggested that ultrasonic pre-treatment promoted protein unfolding, thereby increasing enzyme accessibility to its cleavage sites. Anti-inflammatory protein hydrolysates purified from Argentine croaker were reported to be rich in AAs Glu, Asp, Lys, Leu, Arg, and Ala (Da Rocha et al., 2018). These hydrolysates were produced from Argentine croaker isolate and Argentine croaker myofibrillar protein with varying DH (DH: 10-20%) using either Alcalase® or Protamex® (Table 1.2). Interestingly, as DH increased from 10 to 20%, MW distribution decreased, and the content of hydrophobic AAs increased (p<0.05). The hydrolysate produced from Argentine croaker myofibrillar via Protamex® hydrolysis with DH 10% at 5.0 mg/mL exhibited greater NO inhibitory activity in LPS- activated RAW264.7 cells (24h incubation) than all other hydrolysates tested (p<0.05) suggesting a role for peptides in its bioactivity rather than just free AA content. Hydrolysates prepared from Argentine croaker isolate and myofibrillar also demonstrated in vitro antioxidant activity and microbial-inhibitory activity in *Brochothrix thermosphacta, Listeria innocua*, and *Staphylococcus aureus*.

However, the ability of FPHs to regulate NO should not be used as the only indicator of anti-inflammatory activity. The effects of hydrolysates on cytokine and immunoglobulin levels should be included to shed light on the specific biochemical interaction through which the hydrolysate induced its immunomodulatory effect. Sturgeon protein-derived peptides Lys-Ile-Trp-His-His-Thr-Phe, Val-His-Tyr-Ala-Thr-Val-Asp-Tyr, and His-Leu-Asp-Asp-Ala-Leu-Arg-Gly-Gln-Glu which reduced NO concentration in LPS-stimulated RAW264.7 cells (p<0.05), also inhibited the production of cytokine IL-1β at all concentrations tested (12.5-50.0 µM) (Gao et al., 2020). Treatment of LPS-induced RAW264.7 cells with peptides Val-His-Tyr-Ala-Thr-Val-Asp-Tyr (25.0 µM and 50.0 µM), and His-Leu-Asp-Asp-Ala-Leu-Arg-Gly-Gln-Glu (12.5 µM and 25 µM) also inhibited generation of IL-6 (p<0.05). Further investigation revealed that it is probable that these peptides induced anti-inflammatory activity via suppression of the mitogen-activated protein kinase (MAPK) signalling pathway through down-regulation of phosphorylation of the biomarkers JNK and p38. The activation of inflammatory factors is closely associated with the generation of intracellular reactive oxygen species (ROS). Interestingly, sturgeon peptides also increased antioxidant enzyme superoxide dismutase (SOD) activity in LPS-stimulated RAW264.7 cells compared with the LPS control (p<0.01).

Sweetfish protein hydrolysates (200 μg/ mL) prepared with pepsin, trypsin, or α- chymotrypsin for 12h significantly reduced NO production in LPS-challenged RAW264.7 cells after 24h exposure compared with the LPS-control ($p<0.05$) (Sung et al., 2012) (Table 1.2). However, only trypsin and α-chymotrypsin hydrolysates successfully inhibited production of pro-inflammatory cytokines TNF-α and IL-6, and inflammation mediator PGE$_2$. Both hydrolysates effectively attenuated mRNA expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) via downregulation of nuclear factor (NF)-$_k$B ($p<0.05$), thereby implicating the MAPK pathway. The α- chymotrypsin hydrolysate appeared to suppress the phosphorylation signal from ERK-1/2, although no statistical analysis was reported (Sung et al., 2012). Ko and Jeon (2015) also investigated the NO-inhibitory effect of club tunicate (*Styela clava*) protein hydrolysates prepared with digestive proteases, however, reported superior NO inhibiting activity for Protamex® hydrolysed club tunicate protein. The study was expanded to include information on the anti-inflammatory potential of three individual fractions with various MWs: >10 kDa (SFTPH-I), 5-10 kD-0a (SFTPH-II) and <5 kDa (SFTPH-III). SFTPH-I (200 μg/ mL) significantly reduced production levels of inflammation mediators NO and PGE$_2$ ($p<0.05$) and pro-inflammatory cytokines IL-6, IL-1β and TNF-α ($p<0.01$) after 24h exposure to LPS-challenged RAW264.7 compared with macrophage exposed to LPS alone. It was determined that SFTPH-I inhibited production of pro-inflammatory mediators via reducing protein expression levels of iNOS and COX-2 and attenuating phosphorylation of MAPKs (ERK, p38 and JNK) in activated macrophages.

FPHs have also demonstrated pro-inflammatory activity *in vitro*. Activation of pro-inflammatory cytokines can enhance host defence against infection in immunodeficiency patients. Tilapia mince protein hydrolysate (100 and 800 μg/ mL) produced by purified enzyme from *V.halodenitificans* SKI-3-7 significantly increased gene expression of IL-1β and COX-2 in the human monocyte leukaemia cell line THP-1 stimulated with LPS, after 6h incubation ($p<0.05$) (Toopcham et al., 2017) (Table 1.2). Similarly, a low MW fraction (<1 kDa), labelled NJP, isolated from papain hydrolysed *Nibea Japonica* protein (200 μg/ mL) significantly upregulated protein expression of iNOS and production of NO in LPS-activated RAW264.7 cells ($p<0.01$) (Zhang, Hu, et al., 2019). At this concentration, it also increased production of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β and activated the NF-κB signalling

pathway by upregulating NF-κB pathway-related proteins including IκB kinase (Ikk)-α and IKK-β. The neutral red internalization model was employed to demonstrate that NJP increased phagocytosis rate in RAW264.7 cells in a concentration-dependent manner; this initiated the innate immune response via clearance of apoptotic cells or cellular debris.

Although the majority of *in vitro* immunomodulatory studies focus on regulation of pro-inflammatory cytokines IL-6 and TNF-α in LPS-stimulated macrophage, additional possible mechanisms are elucidated in *in vivo* studies, including the promotion of natural killer cells, stimulation of lymphocytes such as T cells and B cells, and stimulation of secretory immunoglobulin A (S-IgA), thereby enhancing levels of mucosal immunity in the gut. Additional clinical trials are necessary to understand the true effect of specific FPHs on the immune system. A study on the immunomodulatory potential of FPHs in humans reported salmon protein hydrolysate (Amizate) orally administered (3 or 6g/ day, 4 months) to malnourished Indian school children induced no effect on serum immunoglobulins IgG, IgM or IgA or CD4/ CD8 lymphocyte ratio (Nesse et al., 2011).

Table 1.2. Assessment of inflammation modulatory activity of fish muscle protein hydrolysates *in vitro*

Fish sources	Protease and hydrolysis conditions	Hydrolysate/ peptide	Inflammation modulatory activity	Reference
Argentine croaker	Alcalase® (pH 8.0, 50°C), Protamex® (pH 7.0, 50°C) at 30 U/g (enzyme/protein ratio)	Argentine croaker isolate hydrolysate Argentine croaker myofibrillar protein hydrolysate	Exposure (24h) at 5.0 mg/mL reduced NO production in LPS-stimulated RAW264.7 compared with LPS control.	Da Rocha et al. (2018)
Sturgeon	Pepsin (pH 2.0, 37°C, 6 h, E/S ratio 1:100 (w/w))	KIWHHTF, VHYAGTVDY, HLDDALRGQE	All peptides reduced production of NO, IL-6 and IL-1β in LPS-stimulated RAW264.7 cells compared with the LPS control via suppression of the MAPK signalling pathway.	Gao et al. (2020)
Tilapia	Flavourzyme® (pH 7.0, 55°C, 20 U/ g protein, 1h)	AFAVIDQDKSGFIEEDELKL FLQNFSAGARAGDSDGDG KIGVDEFAALVK	Exposure (48h) at 100 mg/mL reduced NO production by 40.9 ± 0.2% in LPS-stimulated RAW264.7 compared with LPS control.	Kangsanant et al. (2015)
Club tunicate	Protamex® (pH 6.0, 40°C, 12h, E/S ratio 1:100 (w/w))	>10 kDa fraction (SFTPH-I)	SFTPH-I (200 μg/mL) reduced production levels of NO and PGE$_2$ (p<0.05) and pro-inflammatory cytokines IL-6, IL-1β and TNF-α (p<0.01) after 24 h exposure to LPS-challenged RAW264.7 compared with macrophage exposed to LPS alone, reduced expression levels of iNOS and COX-2, reduced phosphorylation of MAPKS (ERK, p38 and JNK) in activated macrophage.	Ko and Jeon (2015)
Sweetfish	Pepsin (pH 2.0, 37°C), trypsin (pH 8.0, 37°C), α-chymotrypsin (pH 8.0, 37°C), E/S ratio 10:1, 12h	Whole hydrolysate	Hydrolysates (200 μg/mL, 24 h) reduced NO production in LPS-challenged RAW264.7 cells compared with the LPS-control (p<0.05), trypsin and α- chymotrypsin hydrolysates inhibited production of pro-inflammatory cytokines TNF-α and IL-6, and	Sung et al. (2012)

			inflammation mediator PGE$_2$ and reduced mRNA expression levels of iNOS and COX-2.	
Tilapia	*V.halodenitificans* SKI-3-7 (pH 9.0, 60°C, 8h, final unit activity of 1.6 U)	Whole hydrolysate	Hydrolysates (100 and 800 μg/ mL) increased expression levels of IL-1β and COX-2 in LPS-stimulated THP-1 macrophage after 6 h incubation (p<0.05) compared with LPS control.	Toopcham et al. (2017)
Giant croaker *Nibea Japonica*	Papain (pH 6.0, 59°C, 2000 U/ g, 5.4h)	<1 kDa fraction (NJP)	Increased production of NO and iNOS in LPS-activated RAW264.7 cells (p<0.01) after 24h exposure to fraction (200 μg/ mL), increased production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β compared with LPS control.	Zhang et al. (2019)

COX-2, cyclooxygenase-2; E/S, enzyme/ substrate; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PGE$_2$, Prostaglandin E$_2$; TNF-α, tumour necrosis factor-α

1.3.2 ACE inhibitory activity

 ACE inhibition by fish-derived protein hydrolysates represents a safer alternative than synthetic hypertensive drugs (i.e., captopril; IC_{50} value 0.004 mg/mL) for the therapeutic management and treatment of cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke. Suetsuna and Osajima (1986) were the first to confirm the presence of ACE inhibitory protein hydrolysates in fish which they purified from sardine and hairtail muscle via enzymatic hydrolysis with denazyme (Table 1.3). Wijesekara and colleagues (2011) compared the effect of various proteases on ACE-inhibitory activity of hydrolyzed seaweed pipefish muscle protein and reported Alcalase® hydrolyzed seaweed pipefish muscle hydrolysates induced the greatest inhibitory effect on ACE followed by trypsin, papain, pepsin, Neutrase® and pronase (Wijesekara et al., 2011). Peptides Thr-Phe-Pro-His-Gly-Pro (MW: 744 Da) and His-Trp-Thr-Gln-Arg (MW: 917 Da) subsequently purified from the Alcalase® hydrolysate via chromatographic methods exhibited IC_{50} values of 0.62 and 1.44 mg/mL, respectively (Table 1.3). A study by Jiang et al. (2019) offered insight into the molecular binding of ACE-inhibitory seaweed pipefish peptides to ACE protein. Both peptides effectively interacted with ACE through hydrogen bonding and hydrophobic interactions with AAs at the active site of ACE, thereby inhibiting the catalytic activity of ACE. The authors proposed that the superior ACE-inhibitory activity of peptide Thr-Phe-Pro-His-Gly-Pro over His-Trp-Thr-Gln-Arg, owed to the formation of hydrophobic interactions with key ACE AAs Glu384 and Glu41. Additionally, the ACE-Thr-Phe-Pro-His-Gly-Pro complex showed favourable total binding energy (-167.599 ± 49.637 kJ/mol), as well as van der Waals and electrostatic energy than the ACE-His-Trp-Thr-Gln-Arg complex (total binding energy (-141.342 ± 41.245 kJ/mol), indicating formation of stronger complexes with ACE. The peptides are also reported to act via non-competitive inhibition, therefore both peptide and substrate can both be bound to the enzyme at any given time, forming enzyme-substrate-inhibitor and enzyme-inhibitor complexes to inhibit ACE activity. Other fish sources of non-competitive ACE inhibitors include seaweed pipefish, bigeye tuna, upstream chum salmon (Balti et al., 2015; Qian et al., 2007; Ono et al., 2006).

Peptic rainbow trout muscle hydrolysate exhibited superior ACE inhibitory activity (IC_{50} value of 0.61 mg/mL) compared with hydrolysates prepared with trypsin (IC_{50} value of 1.09 mg/mL) and α-chymotrypsin (IC_{50} value of 1.51 mg/mL) (Kim & Byun, 2012) (Table 1.3). Fraction A with AA sequence Lys-Val-Asn-Gly-Pro-Ala-Met-Ser-Pro-Asn-Ala-Asn (1220 Da) purified from the peptic hydrolysate inhibited ACE activity by 50% at a concentration of 63.9 μM. However, this peptide was demonstrated via Lineweaver-Burk plots to act as a competitive inhibitor against ACE, i.e., competed with substrate Hippuryl-Histidyl-Leucine for the binding sites of ACE. Competitive ACE inhibitory peptides were also found in grass carp, snakehead fish and upstream chum salmon (Chen et al., 2012; Ghassem et al., 2014; Ono et al., 2006; Samaranayaka et al., 2010). Nakajima et al. (2009) also employed digestive proteases for the hydrolysis of FPHs and compared the ACE-inhibitory activities of resulting fractions. Atlantic salmon and Coho salmon hydrolysed with thermolysin demonstrated enhanced ACE inhibitory activity (IC_{50} values of 47.3 and 86.6 μg protein/mL, respectively) than pepsin plus pancreatin hydrolysates (IC_{50} values of 791 and 466 μg protein/mL, respectively) (Nakajima et al., 2009). Subsequent ultrafiltration of the thermolysin hydrolysates followed by size exclusion chromatograms deemed 380-920 Da fractions responsible for exhibited ACE-inhibitory activity, possibly due to the presence of ACE inhibiting di- to penta-peptides rich in AAs Ala, Met, Leu, Tyr, Phe and Trp.

In general, low MW fractions of FPHs demonstrate superior ACE-inhibitory activity than large MW fractions. Goby muscle protein hydrolysate produced with crude protease extract from smooth hound intestines demonstrated increased ACE-inhibitory activity with increasing DH ($p<0.05$) due to the generation of small MW peptides (Nasri et al., 2014). Furthermore, fractionation of ACE-inhibiting Pacific hake FPH prepared via autolysis (10^7 *K. paniformis* spores/g fish mince) generated a low MW fraction (1-3 kDa) which exhibited superior ACE inhibition ($66.91 \pm 4.38\%$ at 0.286 mg/mL) than the intact hydrolysate ($55.06 \pm 0.66\%$ at the same concentration) (Samaranayaka et al., 2010) (Table 1.3). Most inhibitory peptides in the fraction were reported to be short-chained, polar and containing few hydrophobic AAs in their sequence. Chen et al. (2012) observed similar results with the <3 kDa fraction of Alcalase® grass carp meat hydrolysate inducing the greatest inhibitory effect on ACE and the >10 kDa fraction showing the lowest anti-ACE activity. A single tripeptide

Val-Ala-Pro purified from the <3 kDa fraction was subsequently observed to exhibit remarkable ACE inhibitory activity inducing 50% inhibition at 0.00534 ± 0.00003 mg/mL. Interestingly, the tripeptide Val-Ala-Pro was also purified from an enzymatic hydrolysate of bovine casein (IC_{50} value of 2.0 μM) (Maruyama et al., 1987). Various ACE inhibitory di- and tripeptides were also purified from salmon muscle tissue prepared with pepsin and Corolase PP and fermented (*Bacillus* sp. SM98011) shark meat protein hydrolysates (Darewicz et al., 2014; Wu et al., 2008) (Table 1.3). Although the 3-5 kDa fraction purified from red lionfish protein exhibited the highest ACE inhibitory activity (43.57%) of the five fractions tested (>10, 5-10, 3-5, 1-3, <1 kDa), it was proposed that the superior anti-ACE activity of the 3-5 kDa fraction was related to a higher content of hydrophobic AAs (40.33%) compared to the other fractions (Chel-Guerrero et al., 2020). Indeed, several of these peptides have been shown to survive gut transit via simulated gastrointestinal digestion (SGID) suggesting ACE-inhibiting ability will be maintained when administered orally (Balti et al., 2015; Chen et al., 2012; Elavarasan et al., 2016; Ghassem et al., 2014).

It is well known that not only peptide size and chain length influences ACE-inhibitory activity, but also type and order of AAs in the sequence. Potent anti-ACE peptides produced from cuttlefish (Sepia officinalis) muscle proteins via hydrolysis using crude enzymes from *B. mojavensis* A2 and cuttlefish hepatopancreas, gel filtration chromatography and RP- HPLC were identified as Val-Glu-Leu-Tyr-Pro, Ala-Phe-Val-Gly-Tyr-Val-Leu-Pro and Glu-Lys-Ser-Tyr-Glu-Leu-Pro via tandem mass spectrometry with corresponding IC_{50} values of 5.22, 18.02 and 14.41 μM, respectively (Balti et al., 2015) (Table 1.3). It was proposed that the presence of hydrophobic AAs and Pro at the C-terminal may influence the ACE inhibitory activity of the peptide. ACE-inhibitory peptides containing Pro at the C-terminal were also observed in seaweed pipefish muscle hydrolysates (Wijesekara et al., 2011) and Alcalase® protein hydrolysates from snakehead fish sarcoplasmic extract (Ghassem et al., 2014). Gómez-Ruiz and colleagues (2006) reported that the rigid structure of Pro can maintain the conformation of the carboxyl group at the C-terminal in a way that favours ACE-inhibitory activity (Gómez-Ruiz et al., 2006). Peptides rich in Pro are also resistant to GI digestion increasing the likelihood of efficacy *in vivo* (Segura-Campos et al., 2011).

The position of Trp residues is also important for ACE-inhibition and inhibition mechanism of the peptides. Peptides with Trp at the C-terminal residue,

namely Ala-Trp, Val-Trp, Met-Trp, Ile-Trp, Leu-Trp with IC_{50} values of 6.4, 2.5, 9.8 and 17.4 μM, respectively, showed non-competitive inhibition (Table 1.3). Whereas, reversed sequence peptides with Trp at the N terminal including Trp-Ala, Trp-Leu, Trp-Met showed reduced ACE inhibitory activity and acted via competitive inhibition (Ono et al., 2006). Similarly, Phe-Leu showed non-competitive ACE inhibitory activity with an IC_{50} value of 13.6 μM. However, Leu-Phe showed competitive ACE inhibitory activity with an IC_{50} value of 383.3 μM. A study by Enari et al. (2008) purified 20 active di- and tripeptides from salmon muscle papain hydrolysate and demonstrated the strongest ACE inhibition by Ile-Trp with an IC_{50} value of 1.2 μM.

Overall, the evidence for ACE-inhibiting peptides derived from fish muscle is strong although the inhibitory mechanism is yet to be fully established owing to its complexity and/ or multi-target nature (Manzanares et al., 2019). Although there is evidence of fish-derived ACE-inhibiting peptides inducing anti-hypertensive effects *in vivo*, the majority of anti-hypertensive peptides are derived from animal products and plants (Lee & Hur, 2017).

Table 1.3. Assessment of the ACE inhibitory activity of fish muscle protein hydrolysates *in vitro*

Fish sources	Protease and hydrolysis conditions	Hydrolysate/ peptide	ACE inhibitory activity (IC$_{50}$ value & % ACE inhibition)	Reference
Cuttlefish	Crude enzymes from B. mojavensis A21 (pH 10.0; 50°C, 3:1 U/mg enzyme/protein ratio, 4h)	SFHPYFSY AFVGYVLP KNGDGY SYHGVW KNGDGY GS	IC$_{50}$ 82.71 μM IC$_{50}$ 18.02 μM IC$_{50}$ 51.63 μM IC$_{50}$ 19.30 μM IC$_{50}$ 32.74 μM IC$_{50}$ 1156.3 μM	Balti et al. (2015)
Cuttlefish	Crude enzymes from cuttlefish hepatopancreas (pH 8.0; 50°C, 3:1 U/mg enzyme/protein ratio, 4h)	GIHETTY EKSYELP VELYP	IC$_{50}$ 25.66μM IC$_{50}$ 14.41 μM IC$_{50}$ 5.22 μM	Balti et al. (2015)
Red lionfish	Alcalase® (pH 8.0, 50°C, 90 min, 0.3 AU/g protein)	10-5, F 5-3, F 3-1, and F <1 kDa fractions	Peptide fractions (equivalent to 500 μg protein) inhibited ACE by 15.03 ± 1.71 to 34.57 ± 0.97%	Chel-Guerrero et al. (2020)
Grass carp	Alcalase® (48 AU/ Kg protein, 50°C, pH 9 until DH of 17.25% was achieved)	<3 kDa fraction VAP	IC$_{50}$ 0.308 mg/mL IC$_{50}$ 0.00534 ± 0.00003 mg/mL	Chen et al. (2012)
Salmon	Pepsin (37°C, 15 U/g of hydrolysed protein, 120 min) Corolase PP (31.2 U/g of hydrolysed protein, 37°C, pH 7.0, 60 min)	Myofibrillar hydrolysate Sarcoplasmic hydrolysate	IC$_{50}$ 0.91 mg/mL IC$_{50}$ 1.04 mg/mL	Darewics et al. (2014)
Freshwater carp	Papain (E/S ratio 0.26%, 50°C, pH 6.5, 60 min)	Oven dried FPH Freeze dried FPH	IC$_{50}$ 1.15 mg protein/mL IC$_{50}$ 1.53 mg protein/mL	Elavarasan et al. (2016)
Salmon	Papain (50°C, 10 g/ kg, 6h)	VL	3.4% at 50 μM	Enari et al. (2008)

		IL	1.2% at 50 μM	
		LI	4.0% at 240 μM	
		VF	66% at 50 μM	
		IF	86% at 50 μM	
		LF	16% at 50 μM	
		IW	49% at 1.0 μM	
		LW	58% at 50 μM	
		FY	63% at 50 μM	
		YF	42% at 50 μM	
		IVL	7.5 at 180 μM	
		VIL	9.2% at 180 μM	
		LVL	33% at 180 μM	
		FIA	24% at 180 μM	
		AFL	9.2% at 50 μM	
		IVF	43% at 50 μM	
		FVL	7.6% at 50 μM	
		VIF	59% at 50 μM	
		TLV	19% at 160 μM	
		IVW	35% at 1.0 μM	
Snakehead fish	Proteinase K (37°C, pH 7.4), Neutrase® (50°C, pH 6.0) papain (55°C, pH 6.5 Alcalase® (50°C, pH 8.0), flavorzyme (50°C, pH 7.0), enzyme/protein ratio 1:100 w/w, 30 min	Proteinase K hydrolysate	IC_{50} 0.137 ± 0.052 mg/mL	Ghassem et al. (2014)
		Neutrase® hydrolysate	IC_{50} 0.152 ± 0.019 mg/mL	
		Papain hydrolysate	IC_{50} 0.182 ± 0.051 mg/m.	
		Alcalase® hydrolysate	IC_{50} 0.038 ± 0.003 mg/mL	
		Flavorzyme hydrolysate	IC_{50} 0.149 ± 0.018 mg/mL	
		Crude protein	IC_{50} 3.34 ± 0.032 mg/mL	
		LYPPP	IC_{50} 1.3 μM	

		YSMYPP	IC$_{50}$ 2.8 μM	
Rainbow trout	Alcalase® (pH 7.0, 50°C), α-Chymotrypsin (pH 7.0, 37°C), Neutrase® (pH 7.0, 50°C), Papain (pH 7.0, 50°C), Pepsin (pH 2.0, 37°C), Trypsin (pH 7.0, 37°C), E/S ratio of 1/100 (w/w), 6h	Pepsin hydrolysate Trypsin hydrolysate α-Chymotrypsin hydrolysate KVNGPAMSPNAN	IC$_{50}$ 0.61 mg/mL IC$_{50}$ 1.09 mg/mL IC$_{50}$ 1.51 mg/mL. IC$_{50}$ 63.9 μM	Kim and Byun (2012)
Atlantic salmon Coho salmon Alaska pollack Southern blue whiting	Pepsin (pH 2.0, 37°C, 2h, E/S ratio 1:250), pancreatin (pH 7.0, 37°C, 3 h, E/S ratio 1:100)	Atlantic salmon hydrolysate Coho salmon hydrolysate Alaska pollack hydrolysate Southern blue whiting hydrolysate	IC$_{50}$ 791 μg protein/mL IC$_{50}$ 466 μg protein/mL IC$_{50}$ 341 μg protein/mL IC$_{50}$ 362 μg protein/mL	Nakajima et al. (2009)
Atlantic salmon Coho salmon	Thermolysin (E/S ratio 50:1, pH 7.0, 37°C, 5h)	Atlantic salmon hydrolysate Coho salmon hydrolysate IVY VW IY IW VY TVY VFPS VTVNPYKWLP IWHHT YALPHA ALPHA	IC$_{50}$ 47.3 μg protein/mL IC$_{50}$ 86.6 μg protein/mL 0.48 μM 1.4 μM 2.1 μM 4.7 μM 7.1 μM 15 μM 0.46 μM 5.5 μM 5.8 μM 9.8 μM 10 μM	Nakajima et al. (2009)

Goby	Crude protease extract from smooth hound intestines (3:1 (U/mg) enzyme/ protein ratio, pH 8.0, 50°C, 250 min)	Whole hydrolysate (DH 25%)	82.8% at 2 mg/mL	Nasri et al. (2014)
Chum salmon	Thermolysin (E/S ratio 20:1 (w/w), 37°C, 5h)	Whole hydrolysate AW VW MW IW LW WA WL WM FL LF	IC_{50} 27.9 μg protein/mL IC_{50} 6.4 μM IC_{50} 2.5 μM IC_{50} 9.8 μM IC_{50} 4.7 μM IC_{50} 17.4 μM, IC_{50} 277.3 μM IC_{50} 500.5 μM IC_{50} 98.6 μM IC_{50} 13.6 μM IC_{50} 383.3 μM	Ono et al. (2003) Ono et al. (2006)
Bigeye Tuna Dark Muscle	Alcalase® (pH 7.0, 50°C), α-Chymotrypsin (pH 8.0, 37°C), Neutrase® (pH 8.0, 50°C), Papain (pH 6.0, 37°C), Pepsin (pH 2.0, 37°C), Trypsin (pH 8.0, 37°C), E/S ratio 1:100 (w/w), 8h	Alcalase, α-Chymotrypsin, Neutrase®, Papain, Pepsin, Trypsin WPEAAELMMEVDP	47.84 ± 3.4% at 2 mg/mL 57.49 ± 2.6% at 2 mg/mL 63.81 ± 3.2% at 2 mg/mL 20.39 ± 2.8% at 2 mg/mL 80.69 ± 3.4% at 2 mg/mL 36.30 ± 2.6% at 2 mg/mL IC_{50} 21.6 μM	Qian et al. (2007)
Sardine Hairtail	Denazyme	Sardine hydrolysate Hairtail hydrolysate	IC_{50} 39.7 mg/100mL IC_{50} 90.1 mg/100mL	Suetsuna and Osajima (1986)

Pacific hake fish	Autolysis (pH 5.50, 52°C, 1h, 10^7 *K. paniformis* spores per gram fish mince)	Whole hydrolysate >10 kDa fraction 3-10 kDa fraction 1-3 kDa fraction <1 kDa fraction	$55.06 \pm 0.66\%$ at 0.286 mg/mL $18.05 \pm 3.88\%$ at 0.286 mg/mL $43.40 \pm 6.70\%$ at 0.286 mg/mL $66.91 \pm 4.38\%$ at 0.286 mg/mL $55.92 \pm 3.30\%$ at 0.286 mg/mL	Samaranayaka et al. (2010)
Shark	*Bacillus* sp. SM98011 (4000 U/mL with E/S ratio 1:5 (w/v), pH 7.0, 50°C, 5h)	EY FE MF CF	IC_{50} 1.96 μM IC_{50} 2.68 μM IC_{50} 0.92 μM IC_{50} 1.45 μM	Wu et al. (2008)
Seaweed pipefish	Alcalase®, Neutrase®, pepsin, papain, pronase and trypsin (E/S ratio 1/100 (w/w), 8h, pH and temperature not given)	TFPHGP HWTQR	IC_{50} 0.62 mg/mL IC_{50} 1.44 mg/mL	Wijesekara et al. (2011)
Tilapia	Enzyme from *Virgibacillus halodenitrificans* SK1-3-7 (60°C, 8 h at a final unit activity of 1.6 U)	LLP AHL PQP MCS GTY ALSC	IC_{50} 0.41 μM IC_{50} 1.23 μM IC_{50} 0.47 μM IC_{50} 0.29 μM IC_{50} 9.77 μM IC_{50} 11.0 μM	Toopcham et al. (2017)

E/S, enzyme/substrate; DH, degree of hydrolysis; IC_{50}, half maximum inhibitory concentration; SGID, simulated gastrointestinal digestion.

1.3.3 Antioxidant activity

Antioxidant FPHs serve as valuable ingredients in functional foods considering their ability to extend shelf-life, as well as induce health benefits via promoting cellular redox balance. Several studies reported the antioxidant activity of FPHs and their ability to modulate oxidative stress pathways *in vitro* (Table 1.4). Oxidative stress is associated with many diseases including cancer, diabetes, rheumatoid arthritis, chronic inflammation, and numerous neurodegenerative diseases (Kumar et al., 2017). The antioxidant activity of marine hydrolysates and peptides has mainly been assessed via scavenging activity of free radicals and ROS in non-cellular *in vitro* assays, namely, 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), oxygen radical absorbance capacity (ORAC), hydroxyl (OH) radical scavenging activity and superoxide anion (O_2) radical scavenging activity. Other common non-cellular *in vitro* antioxidant assays include metal chelating activity and ferric reducing antioxidant power (FRAP).

It is well known that protease and proteolysis conditions employed ultimately determines the size and AA composition of the resulting fraction, and thereby, plays a crucial role in the generation of antioxidant hydrolysates. Bashir et al. (2018) prepared a number of hydrolysates from red muscle and white muscle of Pacific chub mackerel *(Scomber japonicus)* using varying proteolytic enzymes (Protamex® or Neutrase®) and observed significant differences between antioxidant activities of resulting hydrolysates. Pacific chub mackerel red muscle protein hydrolysed by Protamex® (50°C, 120 min), white muscle protein hydrolysed by Protamex® (50°C, 120 min), and white muscle protein hydrolysed by Neutrase® (50°C, 30 min) exhibited the highest DPPH radical scavenging activity (71.69%), SOD-like activity (32.84%) and ABTS radical scavenging activity (95.39%), respectively (Table 1.4). Optimum hydrolysis conditions for generation of antioxidant protein hydrolysates from small spotted catshark and stonefish were determined via response surface methodology (RSM), a statistical method that optimises processes involving many variables (Vázquez et al., 2017; Auwal et al., 2017). Under optimal hydrolysis conditions predicted by RSM, bromelain stonefish protein hydrolysates scavenged DPPH radical and chelated Fe^{2+} by 48.94% and 25.12%, respectively (Auwal et al.,

2017). However, radical scavenging activities were much lower for small spotted catfish protein hydrolysates produced with Alcalase® (DPPH 12.4%, ABTS 5.1%) or esparase (DPPH 16.0%, ABTS 7.3%) (Vázquez et al., 2017) (Table 1.4). Free AA compositional analysis, MW size distribution, DH% or peptide sequence identification were not reported in these studies.

Bkhairia et al. (2016) also investigated the effects of various proteases on antioxidant activity on golden grey mullet (*Liza aurata*) protein hydrolysates and expanded the study to include AA analysis of resulting fractions. Golden grey mullet hydrolysates were prepared with enzymatic preparations from *P. aeruginosa* A2 and *Bacillus subtilis* A26, crude enzyme from *L. aurata*, trypsin or esperase and subsequent antioxidant assays deemed the hydrolysate prepared with *B. subtilis* A26 proteases (rich in Glx, Gly, and Phe) exhibited the highest DPPH and ABTS radical scavenging activity (IC_{50} values of 3.80 mg/mL and 0.47 mg/mL, respectively) and the hydrolysate prepared with *P. aeruginosa* A2 proteases (rich in Arg) induced the highest reducing power as determined by its ability to react with potassium ferricyanide and ultimately ferric chloride to form ferric–ferrous complex (absorbance of 1.061 ± 0.11 at 5 mg/mL). These hydrolysates (0.1 to 10 mg/mL) did not induce haemolysis of human erythrocytes, indicating their non-toxic effect and thereby, may be suitable for nutraceutical application. Washing and membrane removal pre-treatments and ultrasonic treatment of alkaline proteinase prepared grass carp hydrolysates did indeed improve antioxidant capacity as measured by radical scavenging activities (DPPH and ABTS) as well as FRAP, without diminishing its nutritive value (Zhang, Yang, et al., 2018). The removal of oxidized compounds by washing and membrane removal pre-treatments also modified the colour of the lyophilized hydrolysate from a slight yellowish to white, thereby broadening its potential application in food.

Numerous studies have also reported the relationships between DH and antioxidant activity of fish muscle hydrolysates. Although Li et al. (2012) and Klompong et al. (2007) reported antioxidant hydrolysates prepared from grass carp protein and yellow stripe trevally to exhibit reduced radical scavenging activity and reducing power and higher Fe^{2+}-chelating activity with increasing DH, Rabiei et al. (2019) and Da Rocha et al. (2018) reported an increase in radical scavenging activity of hydrolysates produced from Klunzinger's mullet and Argentine croaker muscles with increasing DH. Alcalase® hydrolysed Argentine croaker isolate (DH 20%) rich

in aromatic AA Tyr and charged acidic AA Asp demonstrated higher ABTS radical scavenging activity and metal chelating activity than Protamex® Argentine croaker isolate and Argentine croaker myofibrillar protein hydrolysates produced with Alcalase® or Protamex® (p<0.05). Protamex® Argentine croaker myofibrillar protein hydrolysate (DH 20%) rich in the aromatic AA Phe exhibited the highest FRAP (p<0.05) (Da Rocha et al., 2018) (Table 1.4). Aromatic AAs are reported to improve the radical scavenging activity of peptides via hydrogen donation to electron-deficient radicals (Wang et al., 2014).

Relative to DH, low MW fractions generally exhibit enhanced antioxidant properties than large MW fractions. Low MW fractions, fraction 2 (985-2379 Da) and fraction 3 (658-923 Da), prepared from Flavourzyme® freshwater carp *Catla catla* showed highest DPPH scavenging activity and FRAP, respectively (p<0.05) (Elavarasan & Shamasundar, 2017). Subsequent AA analysis identified fraction 2 to be rich in AAs Gly, Pro and Tyr with a final ratio of total hydrophobic AAs to total AA content of 42.31:1 (Table 1.4). A high proportion of hydrophobic AAs has been reported in peptides/hydrolysates with high antioxidant activity; including monkfish pentapeptides Glu-Trp-Pro-Ala-Gln, Phe-Leu-His-Arg-Pro and Leu-Met-Gly-Gln-Trp (Chi et al., 2014), and peptides Gly-Ala-Ala, Gly-Phe-Val-Gly, Gly-Ile-Ile-Ser-His-Arg, Glu-Leu-Leu-Ile, and Lys-Phe-Pro-Glu purified from spotless smoothhound (*Mustelus griseus*) muscle (Wang et al., 2014), as well as pentapeptides Phe-Trp-Lys-Val-Val and Phe-Met-Pro-Leu-His isolated from papain hydrolysed miiuy croaker muscle (He et al., 2019) (Table 1.4). AA analysis also determined antioxidant whitemouth croaker muscle hydrolysates to be rich in hydrophobic AAs Ala, Pro, Tyr, Val, Met, Ile, Leu and Phe (Lima et al., 2019). Hydrophobic AAs are well known to act as protein donors or electron/lipid radical scavengers.

Low MW fractions from round scad protein hydrolysate (<5 kDa) and cod protein hydrolysate (<3 kDa) exhibited higher radical scavenging activity and reducing power than larger fractions (>10 kDa and >5 kDa, respectively) (Jiang et al., 2014; Sabeena Farvin et al., 2014) (Table 1.4). Peptides His-Asp-His-Pro-Val-Cys and His-Glu-Lys-Val-Cys were purified from the <5 kDa fraction of round scad muscle protein hydrolysate and effectively scavenged DPPH radicals (EC_{50} values of 0.068 ± 0.001 and 0.031 ± 0.001 mM, respectively) and O_2^- radicals (EC_{50} values of 0.374 ± 0.002 and 0.382 ± 0.002 mM, respectively) (Jiang et al., 2014). The antioxidative activities of the peptides were proposed to be enhanced by the

participation of hydrophobic AAs and one or more residues of His, Pro and Cys. The imidazole group and thiol group of His and Cys residues, respectively, promotes proton donation, thereby stabilising ROS. In particular, Cys is one of 3 AA in glutathione (GSH), a potent endogenous antioxidant in mammalian cells. Hydrolysates that are rich in Cys are likely to boost GSH cellular pathways. It is possible that antioxidant activity not only depends on the presence of a specific AA but also its quantity and position within the peptide sequence. The presence of His in the centre of the His-Asp-His-Pro-Val-Cys sequence may have been responsible for its superior antioxidant activity over His-Glu-Lys-Val-Cys (Jiang et al., 2014). Low MW fractions (5-10, 1-3, and <1 kDa) rich in His residues were also purified from Alcalase$^{®}$ hydrolysed red lionfish protein and demonstrated high antioxidant activity, with copper-chelating activity of approximately 88%, as well as inhibiting hydrophobic β-carotene discolouration by 80% versus the negative control. His residues are indeed reported to have a strong binding affinity for copper ions, thereby preventing copper toxicity (Chel-Guerrero et al., 2020).

Interestingly, goby FPH which exhibited significant antioxidant activity as measured by DPPH radical scavenging activity, lipid peroxidation inhibition, β-carotene bleaching inhibition, and metal chelating activity also effectively inhibited lipid peroxidation of turkey meat sausage by 50% by storage day 3 compared with the control (Nasri et al., 2013). As a result, it is possible that incorporation of goby protein hydrolysate as powder with turkey meat sausage may prevent oxidative deterioration and increase shelf life as a result.

FPHs have also been shown to enhance antioxidant defence systems in various cell model systems. Protease N hydrolysed lanternfish protein hydrolysates (LPH) were demonstrated to prevent hydrogen peroxide (H_2O_2)-induced oxidative cell damage in human neuroblastoma cells (SHSY5Y) (Chai et al., 2013). MTT assay demonstrated that the viability of cells exposed to H_2O_2 (400 μM) increased (67.2-82.3%) in a concentration-dependent manner upon addition of LPH (0.10-1.44 mg/mL) over 24h. DNA fragmentation of H_2O_2-treated SHSY5Y was also reduced dose-dependently when exposed to LPHs (0.37–0.73 mg/mL) for 24h as measured by agarose gel electrophoresis (Table 1.4). Tripeptide Phe-Tyr-Tyr and dipeptide Asp-Trp were subsequently identified as the antioxidant peptide fractions from LPH. The position of Tyr and Trp at the C-terminus was also reported in antioxidant tripeptides

derived from canola protein hydrolysate indicating Tyr and Trp positioning is important for bioactivity (Cumby et al., 2008). Antioxidant peptides Val-Cys-Ser-Val and Cys-Ala-Ala-Pro purified from flounder fish muscle protein hydrolysates also demonstrated dose-dependent (12.5–100 μg/ mL) cytoprotective effects against 2,2-azobis-(2-amidino- propane) dihydrochloride (AAPH)-induced oxidative stress in kidney epithelial Vero cells after 24h incubation (Ko et al., 2013). Both peptides dose-dependently decreased DNA fragmentation and total ROS (Table 1.4). Similarly, but at a higher concentration of 0.5 mg/mL, Alcalase® hydrolysed European seabass protein hydrolysate reduced AAPH-induced oxidation in canine kidney MDCK1 cells by $12.8 \pm 4.5\%$ compared with cells treated with AAPH alone (Altinelataman et al., 2019). In the same study, Alcalase® hydrolysed gilthead seabream muscle hydrolysates also reduced AAPH-induced oxidation but only to 91.60% compared with treated control (100%) albeit statistical analysis was not performed.

Large yellow croaker (*Pseudosciaena crocea*) protein hydrolysate (MW <3 kDa) which effectively scavenged DPPH and O_2^- radicals, also regulated the antioxidant enzyme defence system via dose-dependently (50–300 μg/mL) increasing levels of glutathione peroxidase (GSH-Px), SOD and catalase (CAT) in H_2O_2-treated liver HepG2 cells (Zhang et al., 2016) (Table 1.4). Antioxidant peptides Ser-Arg-Cys-His-Val and Pro-Glu-His-Trp were subsequently isolated via ion exchange chromatography, gel chromatography and RP-HPLC. Peptides Glu-Asp-Ile-Val-Cys-Trp, Met-Glu-Pro-Val,Trp and Tyr-Trp-Asp-Ala-Trp (50 μM) isolated from monkfish protein hydrolysate prepared via *in vitro* GI digestion with pepsin and trypsin protected antioxidant enzymes SOD, CAT, and GSH-Px in in H_2O_2-stressed Hep-G2 cells (Hu et al., 2020). Again, it is possible that the high content of hydrophobic AA and presence of Trp at the C-terminal of peptide sequences played a role in the antioxidant capacities of these peptides.

Of note is that immortalised cells are routinely used in these *in vitro* assays but may be inherently oxidatively stressed due to their cancerous origins, compromising any antioxidant readouts. Overall, it is evident that the antioxidant activity of fish muscle protein hydrolysates is mostly studied via non-cellular *in vitro* testing, whereas studies which employed cellular models are limited. Although numerous antioxidant fish muscle protein hydrolysates have been identified via *in vitro* testing, very few

studies exist investigating the antioxidant activity of fish muscle protein hydrolysates *in vivo* (Nazeer, Kumar, & Ganesh, 2012, Bashir et al., 2018).

Table 1.4. Assessment of antioxidant activity of fish muscle protein hydrolysates *in vitro*

Fish sources	Protease and hydrolysis conditions	Peptide	Antioxidant activities	Reference
European seabass	Alcalase® (pH 7.5, 60°C), chymotrypsin (pH 8.0, 45°C), 2 h, 0.5 to 2% of the muscle protein content	-	ABTS radical scavenging activity (848.11 ± 60.78 μmol TE/g protein), reduced AAPH-induced oxidation of canine kidney MDCK1 cells by 12.8% at 0.5 mg/ml compared with cells treated with AAPH alone	Altinelataman et al. (2019)
Stone fish	Bromelain (pH 6.5, 54°C, E/S ratio 1.5% w/w, 360 min))	-	DPPH radical scavenging activity (48.94%) and Fe^{2+}-chelating activity (25.12%) at 1% w/v	Auwal et al. (2017)
Pacific chub mackerel (*Scomber japonicus*)	Alcalase® (pH 8.0, 50°C), Protamex® (pH 8.0, 50°C) and Neutrase® (pH 7.0, 50°C), 10h at 2% of working volume	-	DPPH radical scavenging activity (71.69%), ABTS radical scavenging activity (95.39%), SOD-like activity (32.84%)	Bashir et al. (2018)
Golden grey mullet (L. aurata)	Crude enzyme extract from golden grey mullet (pH 8.0, 45°C), esperase (pH 8.0, 50°C), trypsin (pH 8.0, 40°C), *B. subtilis* A26 and *P. aeruginosa* A2 proteases (pH 8.0, 40°C), enzyme/protein ratio 3:1	-	DPPH (IC_{50} 3.80 mg/mL) and ABTS radical scavenging activity (0.47 mg/mL), reducing power (absorbance of 1.061 ± 0.11 at 5 mg/mL).	Bkhairia et al. (2016)

Lanternfish	Protease A, Protease N, Protemex and Prozyme 6 (pH 6.5-7.0, 50°C, 2h, 1500 U/ mL, E/S ratio 0.1% (v/w))	FYYDY	Prevented H_2O_2-induced cellular damage of SHSY5Y cells, Prevented DNA fragmentation of H_2O_2-treated SHSY5Y cells in a concentration-dependent manner (0.37–0.73 mg/mL)	Chai et al. (2013)
Red lionfish	Alcalase® (pH 8.0, 50°C, 90 min, 0.3 AU/g protein)	10-5, F 5-3, F 3-1, and F <1 kDa fractions	Cu^{2+}-chelating activity, Fe^{2+}-chelating activity, inhibited β-carotene discoloration.	Chel-Guerrero et al. (2020)
Monkfish	Trypsin (pH 8.0, 40°C, E/S ratio 2%, 4h)	EWPAQ, FLHRP, LMGQW	OH radical (EC_{50} 0.269, 0.114 and 0.040 mg/mL), DPPH radical (EC_{50} 2.408, 3.751, and 1.399 mg/mL), and O_2^- radical (EC_{50} 0.624, 0.101, and 0.042 mg/mL) scavenging activity	Chi et al. (2014)
Argentine croaker	Alcalase® (pH 8, 50°C), Protamex® (pH 7.0, 50°C) at 30 U/g (enzyme/protein ratio)	-	DPPH radical scavenging activity, ABTS radical scavenging activity, reducing power, metal chelating activity	Da Rocha et al. (2018)
Freshwater carps (*Catla, Labeo rohita,* and *Cirrhinus mrigala*)	Flavourzyme® (pH 6.5, 50°C, 60 min, concentration not reported)	-	DPPH scavenging activity (50–82% at 10 mg protein/mL), linoleic acid peroxidation inhibition (71-91% at 10 mg protein/mL)	Elavarasan and Shamasundar (2017)
Sturgeon	Pepsin (pH 2.0, 37°C, 6 h, E/S ratio 1:100 (w/w))	KIWHHTF, VHYAGTVDY, HLDDALRGQE	Peptides increased SOD activity in LPS-stimulated RAW264.7 cells compared to LPS-stimulated control (p<0.01)	Gao et al. (2020)
Cod	*Bacillus* sp. from the Manila clam (*Venerupis philippinarum*) (10^4 cfu/ mL, 30°C, 24-72h)	-	DPPH (0.7-1.4 mg protein/ mL) and ABTS radical scavenging activity (1.0-1.3 mg protein/ mL), Cu^{+2}-chelating activity (40-70% at 1 mg/mL), Fe^{+2}-chelating activity (50-90% at 1 mg/mL)	Godino et al. (2016)

Miiuy croaker	Trypsin (pH 8.0, 50°C), Neutrase® (pH 7.0, 60°C), pepsin (pH 2.0, 37°C), Alcalase® (pH 8.0, 50°C), and papain (pH 7.5, 50°C), total enzyme dose of 1.5%, 5h.	YASVV, NFWWP, FWKVV, TWKVV, FMPLH, YFLWP, VIAPW, WVWWW, MWKVW, IRWWW	DPPH radical scavenging activity (0.48 - >5.00 mg/mL), OH radical scavenging activity (0.97->5.00 mg/mL), O_2^- radical scavenging activity (0.15->5.00 mg/mL)	He et al. (2019)
Monkfish	Pepsin (pH 1.5, 37 °C), trypsin (pH 7.0, 37 °C), enzyme doses of 1.0% (w/w)	EDIVCW, MEPVW, YWDAW	DPPH radical (EC_{50} 0.39, 0.62, and 0.51 mg/mL, respectively), OH radical (EC_{50} 0.61, 0.38, and 0.32 mg/mL, respectively), and O_2^- radical (EC_{50} 0.76, 0.94, 0.48 mg/mL, respectively), decreased levels of ROS and MDA in H_2O_2-treated HepG2 cells and increased levels of GSH-Px, SOD and CAT in H_2O_2-treated HepG2 cells (p<0.001) at 100 μM	Hu et al. (2020)
Sardinella, Zebra blenny, Goby, Ray	*Bacillus subtilis* A26 (37°C, pH 8.0, 24h)	-	DPPH radical scavenging activity (65-75% at 6 mg/ml), reducing power, prevented bleaching of β-carotene and protection against hydroxyl radical induced DNA breakage	Jemil et al. (2014)
Round scad	Alcalase® (50°C, pH 9.5), neutral protease (50°C, pH 7.0), papain (55°C, pH 7.0), pepsin (37.5°C, pH 2.0), trypsin (37.5°C, pH 7.8). E/S ratio of 1:100 (w/w)	HDHPVC, HEKVC	DPPH radical scavenging activity (EC_{50} 0.0677 ± 0.0012 and 0.0310 ± 0.0011 mM, respectively), O_2^- radical scavenging activity (EC_{50} 0.3744 ± 0.0021 and 0.3817 ± 0.0017 mM, respectively).	Jiang et al. (2014)

Solitary tunicate	Alcalase® (pH 8.0, 55°C), Thermoase (pH 7.5, 67°C), and pepsin (pH 2.0, 37°C). E/S ratio 1-3%, 1-7h.	-	DPPH radical scavenging activity (IC$_{50}$ values (370.9 μg/ mL–1690.9 μg/mL), ABTS radical scavenging activity (64.0-94.1%, 1 mg/mL), OH radical scavenging activity (IC$_{50}$ values 1,699.9-3,704.4 μg/ mL), H$_2$O$_2$ scavenging activity (904.7-2,241.5 μg/mL at 1 mg/mL)	Jumeri and Kim (2011)
Tilapia	Flavourzyme®	-	DPPH scavenging activity, ABTS scavenging activity, reducing power, metal chelating activity	Kangsanant et al. (2014)
Yellow stripe trevally	Alcalase® 2.4L (pH 8.5, 60°C), Flavourzyme® (pH 7.0, 50°C) 0.25 – 10% (w/w), 20 min	-	DPPH radical scavenging activity, reducing power and metal chelating activity (p<0.05 at 40 mg protein/mL)	Klompong et al. (2007)
Flounder fish	Papain (pH 6.2, 37°C), pepsin (pH 2.0, 37°C), trypsin (pH 7.6, 37°C), Neutrase® (pH 6.0, 50°C), Alcalase® (pH 8.0, 50°C), kojizyme (pH 6.0, 40°C), Protamex® (pH 6.0, 40°C), and α-chymotrypsin (pH 7.8, 37°C), 6-24h, E/S ratio 500:1	VCSV, CAAP	DPPH radical scavenging activity (IC$_{50}$ values 111.32 and 26.89 μM, respectively), protected against AAPH-induced cellular damage of Vero cells, reduced DNA fragmentation and total ROS in AAPH- treated Vero cells at 100 μg/mL	Ko et al. (2013)
Grass carp	Alcalase® (pH 8.0, 55°C, E/S ratio 1:50 (w/w), 5 h), Papain (pH 6.5, 50°C, E/S ratio 1:50 (w/w), 5h)	-	DPPH radical scavenging activity (77.63%), ABTS radical scavenging activity (86.12%) reducing power (A$_{700}$ of 0.55), Fe^{2+}-chelating activity (90.53%) at 7 mg protein/ mL	Li et al (2012)

Whitemouth croaker (*Micropogonias furnieri*)	Alcalase® 2.4L (pH 8.0, 50°C, enzyme/protein ratio 2/100 (m/m), 2, 4, 8 h)	-	ABTS radical scavenging activity (97.35%), DPPH radical scavenging activity (54.11%) at 1.25 mg/mL	Lima et al. (2019
Nemipterus japonicus and *Exocoetus volitans*	trypsin, 4 h (pH 6.0, 37°C), pepsin (pH 2.0, 37°C), papain (pH 8.0, 37°C), E/S ratio1:100 (w/w)	-	DPPH, hydroxyl, and O_2^- radicals of 45.3%, 48.4%, and 42.1%, respectively by *N. japonicus*; and 46.2%, 50.1%, and 44.1%, respectively, by *E. volitans*.	Naqash and Nazeer (2010)
Goby	Triggerfish crude protease, 50°C, pH 10.0, 1:3 U/mg, 360 min,	-	DPPH radical scavenging activity ($17.5 \pm 0.36\%$ @ 5 mg/mL), lipid peroxidation inhibition (98% @ day 9), β-carotene bleaching inhibition (64.8% at 5 mg/mL), metal chelating activity (between 90 and 97% @ 5 mg/mL), reduced lipid peroxidation by 50% in turkey sausage by storage day 3.	Nasri et al. (2013)
Goby	Alkaline protease (Enzyme/protein ratio 3:1 (U/mg), pH 8.0, 50°C, 250 min)	-	DPPH radical scavenging activity, ferric reducing activity, prevented bleaching of β-carotene, metal chelating activity	Nasri et al. (2014)
Klunzinger's mullet	Papain (E/S ratio 1:50 and 1:25, 50°C, for 45, 90 and 180 min)	-	ABTS (IC_{50} values 0.60-0.12 mg/mL), DPPH (IC_{50} values 3.18-2.08 mg/mL), and OH (IC_{50} values 4.13-2.07 mg/mL) radicals scavenging activity, Fe^{+2} chelating capacity (IC_{50} values 2.12-12.60 mg/mL, ferric reducing activity (absorbance at 70 nm 0.01-0.15 at 5 mg/mL).	Rabiei et al. (2019)
Cod	Commercial proteases (hydrolysis conditions not reported)	<3 kDa fraction	DPPH radical scavenging activity (74.1%), Fe^{2+} chelating activity (>80% at 1.7 mg protein/mL), reducing power	Sabeena Farvin et al. (2014)

Small spotted catshark	Esperase (60.8°C, pH 8.9), Alcalase® (64.6°C, pH 9.4) 1% w/v of muscle	-	DPPH and ABTS radical scavenging activity of Alcalase® hydrolysates (12.4% and 5.1%, respectively) and esperase hydrolysates (16% and 7.3%, respectively)	Vázquez et al. (2017)
Spotless smoothhound (*Mustelus griseus*)	Papain (50°C, pH 6.0, 2h, 1.2%)	GAA, GFVG, GIISHR, ELLI, KFPE	ABTS radical scavenging activity (IC_{50} values 0.3274-1.7541 mg/mL), OH radical scavenging activity (IC_{50} values 0.0769-1.6337mg/mL), O_2^- radical scavenging activity (IC_{50} 0.1508-0.6714 mg/mL)	Wang et al. (2014)
Large yellow croaker (*Pseudosciaena crocea*)	Neutral protease (pH 7.0, 46°C, 7.2h, E/S ratio 1:25)	-	DPPH radical scavenging activity (IC_{50} 7.68 mg/ m), O_2^- radical scavenging activity (IC_{50} 7.67 mg/mL), increased levels of GSH-Px, SOD and CAT in H_2O_2-treated HepG2 cells at 50-300 μg/mL ($p<0.05$)	Zhang et al. (2017)
Grass carp	Alkaline proteinase (pH 8.0, 55°C, 2 h, E/S ratio 1:100 (w/w)	Hydrolysate with washing and membrane removal pre-treatments + ultrasonic treatment	DPPH radical scavenging activity, ABTS scavenging activity and FRAP	Zang et al. (2018)

AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; E/S, enzyme/substrate; EC_{50}, concentration corresponding to half-maximal activity; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; IC_{50}, half maximum inhibitory concentration; LPS, lipopolysaccharide; MDA, malondialdehyde; O_2^-, superoxide anion; OH, hydroxyl; ROS, reactive oxygen species; SOD, superoxide dismutase.

1.3.4 Anti-microbial activity

Similar to antioxidant activity, this bioactivity widens the uses of hydrolysates beyond health enhancement to extending shelf life of foods. Hydrolysis of fish proteins can produce various small MW peptides some of which have been shown to exert remarkable antimicrobial activity depending on their AA composition and structural characteristics. Most anti-microbial peptides are amphipathic with a positively charged, hydrophobic face and thereby, defend against bacterial activity directly via electrostatic interactions with the anionic bacterial membrane. Anti-microbial peptides can exhibit pore-forming action in bacterial membrane evoking leaking of intracellular contents or infiltrate the cell entrapped in macropinosomes which are subsequently released into the host cytoplasm resulting in bacterial destruction (Valero et al., 2020). Peptide fractions prepared from yellowfin tuna muscle hydrolysed via SGID were subsequently fractionated via either solid-phase extraction on C18 or graphitized carbon black (GCB) sorbent for purification of medium-sized peptide and short-sized peptide fractions, respectively (Cerrato et al., 2020). The C18 digested fraction exhibited greater antibacterial activity against *S. aureus* bacteria (with a minimum inhibition concentration *(*MIC) value of 1.0 ± 0.1 mg/mL) than the GCB fraction (MIC value of 3.5 ± 0.1 mg/mL) (Table 1.5). Although a total of 403 peptides from medium-sized peptide fraction and 572 peptides from the short-sized peptide fraction were identified, none of these peptides pre-existed on BIOPEP or PeptideDB databases. Interestingly, medium-size peptides were mostly hydrophilic with intermediate polarity and small-sized peptide fractions were composed of mainly hydrophobic, less anionic peptides, however, the combination of several anti-microbial peptides in the fraction may induce a synergistic effect, inhibiting *S. aureus* activity more effectively than a single peptide. Fraction 12 (MW not reported) purified from a bromelain hydrolysate of leatherjacket (*Meuchenia* sp.) muscle protein by size using a RP-HPLC C-18 preparative column demonstrated antimicrobial activities against gram-positive bacteria *Bacillus cereus* and *S. aureus* with a MIC of 4.3 mg/mL (Table 1.5) (Salampessy et al., 2010). A study by Da Rocha et al. (2018) included the anti-bacterial effect of Argentine croaker muscle protein hydrolysates on both gram-positive and gram-negative bacteria. Argentine croaker muscle protein hydrolysates produced with either Alcalase® or Protamex® with DH 10% or 20%, all inhibited gram-positive

bacteria *B. thermosphacta, L. innocua*, and *S. aureus.* However, only Alcalase® hydrolysates inhibited gram positive bacteria *Listeria monocytogenes*, and gram-negative bacteria *Yersinia enterecolitica*. Alcalase® treated hydrolysates had a lower MW distribution (<1285 Da), a higher content of hydrophobic AAs and as a result, more pronounced inhibition zones than Protamex® treated hydrolysates. Alcalase® hydrolysates dose-dependently increased inhibition zones in *Aeromonas hydrophila, B. thermospacta, Debaryomysces hanseii,* and *L. innocua* (1.25-7.5 mg/mL), with Alcalase® hydrolysed Argentine croaker protein isolate at 10% DH inducing the greatest inhibition of *D. hanseii* (2.00-2.75 cm) and *L. innocua* (1.25-1.50 cm) at 7.50 mg/mL (Table 1.5). According to Najafian and Babji (2012), antimicrobial peptides are usually chains of less than 50 AAs in length of which nearly half are hydrophobic with MW less than 10 kDa. Low MW fractions from tuna by-products were also found to exhibit superior antimicrobial compared with larger fractions from the same source (Gomez-Guillén et al., 2010, Pezeshk et al., 2019). Jemil et al. (2014) reported enhanced resistance of gram-negative bacteria compared with gram-positive bacteria upon exposure to FPHs. Fermented protein hydrolysates from sardinelle (*Sardinella aurita*) (SPH), zebra blenny (*Salaria basilisca*) (ZPH), goby (*Zosterizessor ophiocephalus*) (GPH), and ray (*Dasyatis pastinaca*) (RPH) and their antimicrobial activity against four gram-positive bacteria (S. *aureus, Microcossus luteus,* B. *cereus* and *Enterococcus faecalis*) and five gram-negative bacteria (*E. coli, P aeruginosa, Klebsiella pneumonia, Salmonella enterica* and *Salmonella typhi*) was evaluated at 200 mg/mL (Table 1.5). SPH induced the greatest antibacterial effect of the 4 hydrolysates with inhibition zones in all gram-positive bacteria and *E. coli* ranging from 10-24 mm. *E. coli* was also inhibited by ZPH, GPH and RPH; however, none of the hydrolysates were successful in inhibiting any of the other four gram-negative bacteria. In general, gram-negative bacteria have enhanced resistance to antimicrobial components due to the presence of an outer membrane.

Antimicrobial FPHs/peptides present potential alternatives to conventional antibiotics due to their broad-spectrum of activity and development of little to no pathogenic resistance (Wang et al., 2016). They may also be suitable as bio-preservatives in food systems with the aim of enhancing shelf life. The peptide Lys-Val-Glu-Ile-Val-Ala-Ile-Asn-Asp-Pro-Phe-Ile-Asp-Leu identified from Protamex® Atlantic mackerel hydrolysate was subsequently synthesised and demonstrated anti-bacterial activity against food spoilage organisms *Listeria ivanovii* and *L.*

monocytogenes (MIC of 0.131 mM for both) (Offret et al., 2019). Although the peptide also had an inhibitory effect on common human organisms *M. luteus*, *Listeria acidophilus*, and *Bacteroides thetaiotaomicron*, MICs were half that for Listeria strains; therefore, at equal concentrations, the peptide can prevent Listeria growth without impacting normal human flora.

Table 1.5. Assessment of the anti-microbial activity of fish muscle protein hydrolysates *in vitro*

Source	Protease and hydrolysis conditions	Hydrolysate/ peptide	Anti-microbial activity	Reference
Yellowfin tuna	Trypsin (incubated overnight 37°C, enzyme/protein ratio 1:20). Pepsin (pH 2, 37 °C, 1 h, enzyme/protein ratio 1:20) + pancreatin (pH 7.5, 37 °C, 2 h, enzyme/protein ratio 1:10)	C18 digested fraction GCB digested fraction	MIC value of 1.0 ± 0.1 mg/mL against *Staphylococcus aureus* MIC value of 3.5 ± 0.1 mg/mL against *Staphylococcus aureus*	Cerrato et al. (2020)
Argentine croaker	Protamex® (pH 7.0, 50°C, enzyme/ protein ratio 30 U/g)	Argentine croaker isolate hydrolysate (DH 10-20%), Argentine croaker myofibrillar protein hydrolysate (DH 10–20%)	Inhibition zones of *Aeromonas hydrophila*, *Staphylococcus aureus*, *Bronchothrix thermosphacta*, *Listeria innocua* and *Debaryomyces hanseii* ranging from 0.25–1.50 cm at 1.25-5.00 mg/mL.	Da Rocha et al. (2018)
Argentine croaker	Alcalase® (pH 8.0, 50 °C, enzyme/ protein ratio 30 U/g)	Argentine croaker isolate hydrolysate (DH 10-20%), Argentine croaker myofibrillar protein hydrolysate (DH 10–20%)	Inhibition zones of *Brochothrix thermosphacta*, *Listeria innocua*, *Listeria monocytogenes*, Staphylococcus. *aureus*, *Aeromonas hydrophila*, *Yersinia enterecolitica* and *Debaryomyces hanseii* ranging from 0.25 2.75 cm at 1.25-7.00 mg/mL.	Da Rocha et al. (2018)
Loach	-	Polypeptide (9800 Da)	Dose dependently increased inhibition zones in *Baccilus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* (1-1000 µg/mL)	Dong et al. (2002)

Sardinelle	*Baciilus subtilis* A26 (37°C, pH 8.0, 24h)	Whole hydrolysate	Inhibition zones in gram-positive bacteria *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis* and *Micrococcus luteus* and gram-negative bacteria *Escherichia coli* ranging from 10–24 mm at 200 mg/mL	Jemil et al. (2014)
Zebra blenny	*Baciilus subtilis* A26 (37°C, pH 8.0, 24h)	Whole hydrolysate	Inhibition zones in gram-positive bacteria *Bacillus cereus* and gram-negative bacteria *Escherichia coli* ranging from 10–11 mm at 200 mg/mL	Jemil et al. (2014)
Goby	*Baciilus subtilis* A26 (37°C, pH 8.0, 24h)	Whole hydrolysate	Inhibition zones in gram-positive bacteria *Bacillus cereus* and gram-negative bacteria *Escherichia coli* of 10 mm at 200 mg/mL	Jemil et al. (2014)
Ray	*Baciilus subtilis* A26 (37°C, pH 8.0, 24h)	Whole hydrolysate	Inhibition zones in gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and gram-negative bacteria *Escherichia coli* ranging from 10–13 mm at 200 mg/mL	Jemil et al. (2014)
Atlantic mackerel	Protamex® (40-43°C, pH 8.0, 150 min)	Lys-Val-Glu-Ile-Val-Ala-Ile-Asn-Asp-Pro-Phe-Ile-Asp-Leu	*Bacillus megaterium, Blautia coccoides, Bifidobacterium infantis, Enterococcus faecium, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus* (MIC values > 1.050 mM), *Listeria ivanovii, L. monocytogenes* (MIC values of 0.131 mM), *Lactobacillus acidophilus, Micrococcus luteus, Bacteroides thetaiotaomicron* (MIC values of 0.263 mM)	Offret et al. (2019)
Leatherjacket	Bromelain (pH 5.95 - 6.41, 50°C, 1% for pellet suspension (insoluble	Fraction	Fraction 12 had a MIC value of 4.3 mg/mL against *Bacillus cereus* and *Staphylococcus aureus*	Salampessy et al. (2010)

	protein) and 0.5% for supernatant (water-soluble protein)), up to 10h)			

DH, degree of hydrolysis; GCB, graphitized carbon black; MIC, minimum inhibition concentration.

1.3.5 Anti-cancer

A limited number of studies have been published on the potential anti-cancer activity of hydrolysates derived from fish muscle protein by assaying for anti-proliferative effect on immortal cell lines. European seabass hydrolysate (1 mg/mL) prepared with chymotrypsin protease reduced cell viability in the human colon adenocarcinoma cell line, HT-29, by $39.6 \pm 12.8\%$ (Altinelataman et al., 2019). Similar antiproliferative activity was observed for blue whiting protein hydrolysate (BWPH) (1mg/ mL) produced via hydrolysis with either Protamex® or Alcalase® which induced a maximum 30% reduction and 27% reduction in proliferation of breast cancer cells MDA-MB-231 and MCF-7/6 cells, respectively, after 72h (Picot et al., 2006). Size exclusion chromatography confirmed the large MW distribution (100 Da-7 kDa) of BWPH indicating the presence of both free AAs and peptides which may have been responsible for the superior anti-proliferative effect of BWPH over hydrolysates prepared from salmon, emperor, pollack or siki. Similarly, solitary tunicate protein hydrolysates (1 mg/mL) inhibited growth of 3 human cancer cell lines; AGS (stomach cancer), DLD-1 (colon cancer), and HeLa (cervical cancer). Solitary tunicate protein hydrolysate prepared with Alcalase® demonstrated superior anti-proliferative activity than hydrolysates prepared with thermoase or pepsin exhibiting IC_{50} values of 1731.4 and 2922.5 μg/mL for AGS cells and HeLa cells, respectively. The Alcalase® hydrolysate was subsequently fractionated with the resulting low MW fraction (fraction F_2: 3.6 kDa, rich in hydrophobic AAs (78.1%)) inhibiting cell growth of AGS, DLD-1 and HeLa cells with IC_{50} values of 577.1, 1163.3, and 887.2 μg/ mL, respectively (Jumeri & Kim, 2011). It is possible that low MW peptides have enhanced interactions with cancer cell components via enhanced cell mobility and diffusivity than large MW components, thereby improving anti-cancer activity. Song and colleagues (2011) also reported that thermal treatment (121°C, 30 min) of pepsin hydrolysate derived from half-fin anchovy increased free AAs Val, Leu, Phe, His and Arg, increased the number of peptides with MW distribution of 3000-5000 Da and <300 Da (p<0.005), and ultimately, increased anti-proliferative activity against DU-145 human prostate cancer cells, 1299 human lung cancer cells, and 109 human oesophagus cancer cells (p<0.05) compared to the non-heat sterilised hydrolysate.

Hydrophobic AA residues are essential for the formation of a hydrophobic tail in the COOH-terminal region, an attribute important for anti-cancer peptides. A recent review discusses the classifications and structure-activity relationship of anti-cancer peptides in more detail (Chiangjong et al., 2020). In short, in contrast to healthy cells, cancer cells possess phosphatidylserine, a negatively charged phospholipid, allowing for electrostatic attraction between cationic peptides and cancer cells. Upon electrostatic interaction with the membrane surface of cancer cells, peptides arrange in either an α-helix or β-sheet, resulting in cell membrane disruption. Anti-cancer peptides can be classified as i) molecularly targeted peptides, ii) binding peptides, or iii) cell-stimulating peptides. Hydrophobic, positively charged Lys- and Arg-rich peptides can induce cancer cell toxicity via disruption and penetration of anionic, hydrophobic cancer cell membranes; a mechanism known as 'snorkeling'. The peptide Tyr-Ala-Leu-Pro-Ala-His was subsequently purified from the heat-treated pepsin hydrolysate of half-fin anchovy. Although this peptide inhibited prostate cancer PC-3 cells by 50% at 11.4 mg/mL, modification of the peptide to Tyr-Ala-Leu-Arg-Ala-His improved its inhibitory activity (IC_{50} value of 8.1 mg/mL). The enhanced cell permeation efficacy of Arg-rich peptides may be due to the hydrogen-bond formation of guanidine moiety in Arg with phosphates, sulphates, and carboxylates on cellular components (Song et al., 2014). Peptides Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr purified from tuna dark muscle byproduct hydrolysates prepared with papain and Protease XXIII induced a dose-dependent anti-proliferative effect on MCF-7 cells with IC_{50} values of 8.1 and 8.8 μM, respectively (Hsu et al., 2011).

Further work on the isolation, identification, and elucidation of mechanism of action of fish derived anti-cancer peptides is required. Furthermore, studies to-date used various cancer cell lines to demonstrate the chemoprotective abilities of fish muscle protein hydrolysates and peptides *in vitro*, however the majority of these studies lacked the inclusion of a non- cancerous cell line controls. *Nemipterus japonicus* and *Exocoetus volitans* muscle hydrolysate fraction rich in Glu, Lys, Gly, and Thr induced a dose-dependent cytotoxic effect in the human hepatoblastoma cell line, HepG2, with IC_{50} values of 48.5 µg/mL and 21.6 µg/mL (Naqash & Nazeer, 2010). Interestingly, neither fraction induced a cytotoxic effect in Vero (kidney epithelial non-cancerous) cells.

If a peptide derived from fish is intended for use as a treatment for cancer, then similar to all other peptide drugs it may face numerous limitations including peptide instability, poor membrane permeability and poor oral bioavailability. Potential solutions to tackle these challenges include conjugation of therapeutic peptides with cell-penetrating peptides to enhance transport across cellular membrane or conjugation with lipids, promoting amphiphilicity, in turn, enhancing bioavailability, selectivity, potency, and membrane penetration of the peptide (Marqus et al., 2017). Peptide engineering via AA substitution or combination of peptides with other anti-cancer drugs may also promote improve efficacy and efficiency of chemotherapy.

1.3.6 Anti-obesity potential

Although enlargement of adipocytes is the main mechanism of weight gain in adults, obese people generally have a higher number of adipocytes than non-obese people. However, adipocyte number is altered during childhood and adolescence and remains constant during adulthood in both obese and lean people (Spalding et al., 2008). Adipogenesis is regulated by various transcription factors including peroxisome proliferator-activated receptor γ (PPARγ), sterol regulatory element binding protein (SREBP)-1 and CCATT/enhancer binding protein α (C/EBPα) (White & Stephans, 2010). After a thorough search of the relevant literature, no adipocyte-modulating hydrolysate from a fish source was found. However, a peptide derived from desalinated boiled tuna extract (Asp-Ile-Val-Asp-Lys-Ile-Glu-Ile) (5 mg/mL) reduced triglyceride accumulation significantly ($p < 0.05$) in differentiated adipocytes (3T3-L1 cell line) compared with differentiated cells treated with media alone (Kim, Choi, Lee, & Nam, 2015). This peptide also reduced expression levels of C/EBPα and PPARγ, and the expression levels of adipogenic and lipogenic genes in differentiated 3T3-L1 cells. Pentapeptides Val-Ile-Asp-Pro-Trp and Ile-Arg-Trp-Trp-Trp (100 μM) purified from papain hydrolysed miiuy croaker muscle (pH 7.5, 50°C, enzyme dose of 1.5%, 5h) significantly reduced oleic acid-induced lipid accumulation in human liver carcinoma cells (HepG2) after 24h exposure ($p < 0.05$ and $p < 0.01$, respectively) compared to the oleic acid model control (Wang et al., 2020). These pentapeptides (100 μM) also reduced intracellular triglyceride levels ($p < 0.01$ and $p < 0.001$, respectively), total cholesterol levels ($p < 0.01$ and $p < 0.001$, respectively), expression of lipogenesis genes (SREBP-1c, SREBP-2, fatty acid synthase (FAS), acetyl-CoA

carboxylase (ACC), and 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (HMGR)) and increased the expression levels of lipolysis genes (PPARα, acyl-CoA oxidase 1 (ACOX-1), and carnitine palmitoyltransferase-1 (CPT-1)) compared with cellular oleic acid model control. A database search using BIOPEP-UWM revealed no sequence homology with known anti-adipogenic peptides (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). In addition, a peptide structure-activity study by Pak et al. (2005) reported that the presence of Pro residues, Glu, Thr, and Tyr side groups and hydrophobic regions promotes hypocholesterolaemia via stabilisation of a 'turn' conformation and formation of hydrogen bonds to the binding site of HMGR, a known rate limiting enzyme in cholesterol biosynthesis. Moreover, peptide hydrophobicity is also correlated to their ability to bind to bile acids which may inhibit the absorption of bile acids in the ileum and ultimately, decrease serum cholesterol levels (Pak et al., 2005).

Alkaline protease hydrolysate (pH 11, 39°C, enzyme dosage 122 U/ mL) from water-soluble protein of crucian carp muscle increased inhibition of porcine pancreas lipase activity *in vitro* as hydrolysis time increased, reaching a maximal value of $53.21 \pm 1.07\%$ at 10h (Liu et al., 2013). Pancreatic lipase is the enzyme responsible for hydrolysis of 50-70% of total dietary fat into monoglycerides, free fatty acids and other small molecules that are easily absorbed by the intestine. Therefore, inhibition of pancreatic lipase activity can potentially control energy intake. The alkaline protease hydrolysate from crucian carp also reduced α-amylase inhibitory activity by $20.07 \pm 0.87\%$.

In the quest to manage weight gain and reduce the incidence of obesity, food-derived compounds have been identified that influence food intake pathways. Satiety hormones such as Cholecystokinin (CCK), Glucagon-like peptide-1 (GLP-1) and Peptide YY (PYY) which are released from enteroendocrine cells in response to food digestion have been shown to suppress appetite and reduce food intake via activation of various signalization pathways. Murine STC-1, murine GLUTag and human NCI-H716 cell lines are commonly used as enteroendocrine models to screen for food components capable of inducing secretion of satiety hormones.

Incubation of STC-1 cells for 2h with BWPH (hydrolysis conditions not reported) at 0.2% (w/v) and 1.0% (w/v) increased levels of CCK to 49.5 and 122.0 pM, respectively, compared with STC-1 basal CCK levels (4.0 pM) (10 mM glucose)

(Cudennec et al., 2008). Although CCK-stimulating peptides were partially purified and characterised via size exclusion chromatography to have an apparent MW ranging from 1000 to 1500 Da, the study did not identify the peptide sequences which may have influenced bioactivity. A follow-up study determined BWPH (1.0% (w/v) for 2h incubation) also induced a 25-fold increase in GLP-1 concentration over basal (10 mM glucose) (Cudennec et al., 2012). In a study investigating the antidiabetic activity of BWPH, Harnedy et al. (2018) also reported the ability of a BWPH (prepared with Alcalase 2.4L and Flavourzyme 500L, pH 7.0, 50 °C, E/S ratio 0.74% (w/w)) and a SGID digest to increase levels of GLP-1 significantly in GLUTag cells compared to the glucose control (2 mM) (p<0.01 and p<0.001, respectively). An observation worth noting, however, is that studies examining the satiety inducing effect of FPHs *in vitro* failed to include assessment of the possible signalling mechanisms involved. Whereas many studies investigating milk and meat protein hydrolysates reported whether satiety hormone secretion was induced via stimulation of the cyclic adenosine 3′,5′-monophosphate (cAMP) pathway or calcium signalling (Kondrashina et al., 2018; O'Halloran et al., 2018; Reimer, 2006). It should also be noted that, to date, no study to the best of our knowledge, has identified and characterised the peptide responsible for the satiating effect of FPHs. This information is prerequisite for elucidating structure-function relationships and determining exact mechanisms of action. In contrast, a number of GLP-1 and/ or CCK stimulating peptides have been identified from milk and meat sources (Domenger et al., 2017; Komatsu et al., 2019; Tulipano et al., 2011).

Albeit the majority of *in vitro* studies published to-date investigating the anti-obesity activity of protein hydrolysates have focused on milk protein, it is difficult to compare the anti-obesity potential of FPHs to other protein sources due to the methodological variation between studies i.e., cell type, cell density, exposure time, hydrolysate concentration. However, a recent review by Sharkey et al. (2020) concluded that many FPHs have potential to reduce body weight and improve body composition *in vivo* and in clinical studies. The Norwegian Tromsø Study is an epidemiological study with the focus of investigating the relationship between fish consumption and the subsequent beneficial effects on metabolic syndrome. Data collected after a 13-year follow-up period (1994-1995, $n = 23,907$ to 2007–2008, $n = 12,981$) revealed that consumption of lean fish once a week or more was associated with decreased future metabolic score, decreased triglyceride content, and increased

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high-density lipoprotein-cholesterol, whereas decreased waist circumference and blood pressure was identified only for men (Tørris et al., 2017).

1.4 Applications, challenges, and future perspectives

FPHs represent desirable functional food ingredients owing to their beneficial impact on both health and food quality. Addition of antioxidant or anti-microbial FPHs to a food system may inhibit lipid peroxidation or growth of food spoilage microorganisms, respectively, thereby potentially extending shelf life of supplemented products. Not only do FPH have use as preservative ingredients, but some FPH are also commercialised functional food ingredients with health promoting claims. BWPH which induced CCK and GLP-1 secretion in STC-1 cells, was subsequently demonstrated to increase plasma concentrations of CCK and GLP-1, improve body composition and reduce body weight upon oral administration (1.4 g) to 120 overweight (25 kg/m^2 ≤ body mass index (BMI) < 30 kg/m^2) adults over 90 days. BWPH is now commercialised and marketed as Slimpro$^®$ (Nobile et al., 2016). In addition, peptides purified from dried bonito (*katsuobushi*) via thermolysin digestion exhibiting ACE-inhibitory activities *in vitro* were also shown to exhibit anti-hypertensive effects in spontaneously hypertensive rats and borderline (high normal) and mildly hypertensive adults (1.4 g/ day orally administered over 5 weeks) (Fujita et al., 2001; Yokoyama et al., 1992). *Katsuobushi* oligopeptide received official approval as Foods for Specific Health Use (FoSHU) in 1999 by the Ministry of Health and Welfare in Japan for prevention of hypertension in at-risk individuals.

Although this review discusses only six potential bioactivities of fish muscle protein hydrolysates *in vitro*, numerous novel bioactivities have emerged in recent years. FPHs have recently been shown to enhance cognitive memory, promote skin repair and regeneration, and increase post-exercise aminoacidemia, as well as the ability to increase bone mass with potential to treat osteoporosis and bone loss (Cordeiro et al., 2020; Lee et al., 2019; Zhang, Zhang, et al., 2018; Zhang, Su, et al., 2019). There are now vast possible applications for bioactive FPHs which are garnering more and more interest from food, pharmaceutical and cosmetic industries.

Albeit bioactive fish hydrolysates and peptides are generally not as potent as synthetic drugs, they could provide a safe and natural alternative for the prevention more than the treatment of disease. However, for bioactive efficacy in a functional food offering, fish hydrolysates, similar to other food hydrolysates, must overcome several hurdles not least of which includes issues of processing, food formulation, sensory acceptance, survival during GI digestion and bioavailability.

A systematic approach for optimization of the numerous parameters which influence the production of bioactive peptides is now advised compared to 'one factor at a time' or 'trial and error' approaches which should now be deemed obsolete (Chakrabarti et al., 2018). The development of bioinformatics analysis in recent years has promoted a highly useful approach for the generation of bioactive peptides via the utilization of computational data to predict peptide sequences likely to induce specific bioactivities and elucidate structure-function relationships. Peptide databases should be exploited to save time and expenses involved in purifying fish peptides and testing various potential bioactivities. These databases also provide information about peptide structure-function relationships, molecular docking, and peptide-receptor interactions, which are essential for the development of therapeutic products.

Although *in vitro* investigations offer great insight into the potential bioactivities of FPH, for FPHs to have use as bioactive agents with the aim to improve human health, more clinical trials are required in order to determine FPH bioavailability and absorption through the GI barrier ensuring eventual contact with target cells. In addition, future studies should elucidate mechanisms of action of bioactive fish muscle protein hydrolysates, as well as identify individual bioactive peptides from fish protein fractions so that peptide structure-function relationships can be further understood.

1.5 Conclusion

This review has discussed a plethora of biologically active protein hydrolysates (and 82 bioactive peptides) prepared from muscle of various fish species and highlighted the relationship between peptide structure and exhibited *in vitro* bioactivity. Croaker

fish (*Sciaenidae* family) muscle appears to be the most common substrate for generation of antioxidant, anti-inflammatory, and anti-microbial protein hydrolysates and peptides, whereas the majority of ACE-inhibitory hydrolysates discussed were prepared from salmon muscle. Microbial proteases including Alcalase®, Protamex® and Flavourzyme® are generally the enzymes of choice to produce potent fish muscle protein hydrolysates. Alcalase® hydrolysed Argentine croaker myofibrillar protein hydrolysate represented the most multifunctional fish muscle hydrolysate demonstrating antioxidant, anti-inflammatory, and anti-microbial activities (Da Rocha et al., 2018). Due to the range of biofunctionalities exhibited, the hydrolysate possesses enhanced potential as a value-added ingredient for application in functional foods and nutraceutical products. However, the peptide responsible for exhibited bioactivities must be identified prior to commercialisation. It is possible that free AAs released during hydrolysis may also influence bioactivity. Short-chain peptides commonly exhibit bioactivity *in vivo* as they are too small to act as a substrate for digestive proteases, thereby have heightened resistance to GI digestion and increased probability of crossing the intestinal barrier to elicit their biological function. However, similar to other food-derived protein hydrolysates, fish hydrolysates have also failed efficacy assessments in follow-up *in vivo* trials (Chai et al., 2016; Giannetto et al., 2020).

This review offers a comparison of studies investigating the cellular *in vitro* bioactivity of fish muscle protein hydrolysates and has collated numerous data informing the reader of suitable protein sources, enzymes, and processing conditions for the generation of bioactive hydrolysates. This review thereby may be a useful data base when designing future studies on fish muscle protein hydrolysates/peptides *in vitro* or *in vivo* as no database listing exclusively bioactive peptides derived from fish sources currently exists.

Overall, ACE inhibition appears to be the most promising bioactivity of fish muscle protein hydrolysates/ peptides of the six bioactivities discussed in this review. Although hydrolysates from other protein sources such as milk, meat and plant show similar inhibitory activity, fish is now an abundant source of protein due to the 'landing obligation' policy which was introduced by the European Commission with the aim of progressive elimination of discards in all EU fisheries. Fish protein now represents an affordable alternative to milk proteins which are currently the main source of bioactive peptides.

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Research Objectives

Objective of thesis

The overall objective of this thesis was to determine the bioactive potential of protein hydrolysates enzymatically extracted from low-value blue whiting fish using cell culture model systems, and to ascertain the effects, if any, that simulated gastrointestinal digestion (SGID) and post simulated intestinal absorption have on hydrolysate bioactivity.

Objectives of each chapter

The objective of Chapter 1 involved collating and comparing the latest research, conducted using in vitro systems, on the bioactive nature of fish protein hydrolysates. Additionally, the importance of hydrolysate structure and composition on bioactive potential was highlighted.

The objective of Chapter 2 was to determine the bioactive effects of eleven protein hydrolysates extracted from blue whiting at laboratory scale using various enzymes/ enzyme combinations. Bioactivities including antioxidant activity, immunomodulatory activity, satiating potential and anti-adipogenic ability were assessed using well-established cellular systems. Results from this chapter informed the production of hydrolysates at industrial scale.

The objective of Chapter 3 was to determine the antioxidant and immunomodulatory potential of six blue whiting soluble protein hydrolysates (BWSPHs) prepared at industrial scale using RAW264.7 macrophage cellular models. An additional objective of this chapter was to determine the impact that SGID has on these bioactivities and to rank the six hydrolysates and their corresponding gastrointestinal digests from most to least bioactive.

The objective of Chapter 4 was to assess the ability of BWSPHs and SGID BWSPHs to modulate the secretion and/or production of satiety hormones glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) in STC-1 cells and to study the mechanism by which the leading hydrolysate mediates hormonal signalling. Another aim of this chapter was also to investigate the effect of SGID BWSPHs on

intestinal barrier integrity and inflammation, using a 21-day-old differentiated Caco-2/HT-29MTX monolayer, as a model of a healthy gut barrier.

The objective of Chapter 5 was to determine the anti-adipogenic and the adipolytic potential of BWSPHs and SGID BWSPHs using the well-established 3T3-L1 pre-adipocyte cell line and to determine the effect, if any, that simulated intestinal absorption has on these bioactivities. The ability of BWSPHs and SGID BWSPHs to protect against obesity-related diseases through determining their antioxidant and immunomodulatory tendencies in stimulated 3T3-L1 cells was also assessed in Chapter 5.

The objective of Chapter 6 was to discuss the findings of the thesis and to present suggestions for future research.

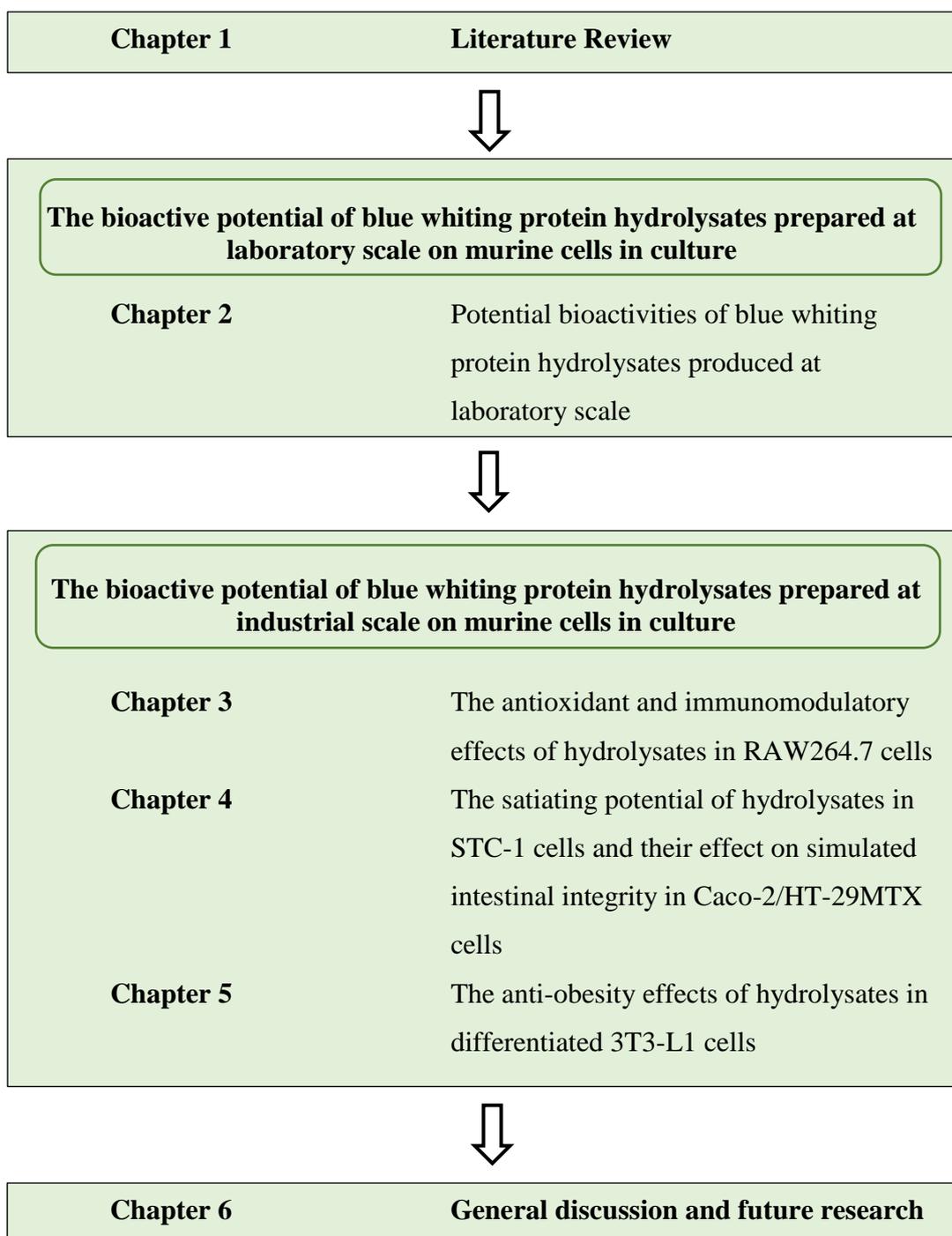A schematic overview of the thesis is provided in Figure 1.2.

Chapter 1	**Literature Review**

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The bioactive potential of blue whiting protein hydrolysates prepared at laboratory scale on murine cells in culture

Chapter 2	Potential bioactivities of blue whiting protein hydrolysates produced at laboratory scale

⇩

The bioactive potential of blue whiting protein hydrolysates prepared at industrial scale on murine cells in culture

Chapter 3	The antioxidant and immunomodulatory effects of hydrolysates in RAW264.7 cells
Chapter 4	The satiating potential of hydrolysates in STC-1 cells and their effect on simulated intestinal integrity in Caco-2/HT-29MTX cells
Chapter 5	The anti-obesity effects of hydrolysates in differentiated 3T3-L1 cells

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Chapter 6	**General discussion and future research**

Figure 1.2. Schematic overview of the thesis structure

Chapter 2

Potential bioactive effects of blue whiting protein hydrolysates produced at laboratory scale on murine cells in culture

Abstract

Blue whiting protein hydrolysates (coded ULPH-MB-01 to ULPH-MB-11) were produced at laboratory scale using various enzymes/enzyme combinations and hydrolysis conditions. The aim of this research was to investigate the potential bioactivities (satiety control, anti-obesity, antioxidant and immunomodulatory activities) of these blue whiting protein hydrolysates using suitable cellular systems. Murine enteroendocrine STC-1 cells were employed to assess the satiety control potential of the eleven hydrolysates. Murine 3T3-L1 preadipocyte cells were used to assess the anti-obesity activity of the hydrolysates. The antioxidant and immunomodulatory tendencies of the hydrolysates was measured using murine RAW264.7 macrophage cells. Following 24 h supplementation, the eleven blue whiting protein hydrolysates (BWPH) (0.05 – 1.0%, w/v dw) were observed to exert varying effects on the viability of STC-1 cells, 3T3-L1 cells and RAW264.7 cells, with IC_{50} values for hydrolysates exposed to STC-1 cells and 3T3-L1 cells ranging from 0.19 - 0.88%, w/v dw and 0.31 - 0.92%, w/v dw, respectively. The hydrolysates were less toxic towards the RAW264.7 cells at the same concentrations, whereby only one of the eleven hydrolysates induced 50% cell death in the RAW264.7 cells with an IC_{50} value of 0.98%, w/v dw. These laboratory scale hydrolysates did not show bioactive potential as treatment with the hydrolysates did not increase satiety hormone active glucagon-like peptide 1 (GLP-1) concentration in STC-1 cells, did not protect against adipogenesis in 3T3-L1 cells, did not protect the antioxidant biomarker, glutathione, in hydrogen peroxide (H_2O_2) stressed RAW264.7 cells, nor did they not prevent nitric oxide (NO) production in lipopolysaccharide (LPS)-challenged RAW264.7 cells.

2.1 Introduction

Fishing has always been economically and socially important to Ireland. Unfortunately, modern fishing vessels and methods pose serious threats to fish stocks, the environment, and jobs in Ireland. Sustainable fishing is now a matter of survival for fish stocks and the fishing communities (Ireland - European Commission, 2017). In recent years, significant improvements in the management of fish waste have been achieved (Prasad & Murugadas, 2019). Efforts have been made by The European Commission to modify the common fishery policy to ensure complete elimination of discards. This push to reduce marine waste has resulted in the use of various technical methods to produce added-value compounds from fish by-products and discards (Morales-Medina et al., 2016).

Although blue whiting is a target species, it is often discarded due to minimum landing size requirements (15cm), fishing quota and high grading practices such as market considerations (García-Moreno et al., 2013). Due to the high protein content of blue whiting fish (18.6%, w/w), their discards can be upgraded via extraction and hydrolysis of proteins. Numerous protein hydrolysates isolated from low-value fish species have shown potential as ingredients for functional foods and nutraceuticals. According to Suleria et al. (2016), marine bioactive compounds are desirable to the food industry due to their natural availability, relatively low-cost extraction methods and health promoting biological activities (Suleria et al., 2016). Marine bioactive protein hydrolysates and peptides have been shown to exhibit various biological functions including antioxidant, anti-inflammatory, anti-proliferative, anti-hypertensive, anti-diabetic and cardio-protective activities (Harnedy & FitzGerald, 2012; Rabiei et al., 2017). There are numerous cases whereby marine protein hydrolysates and peptides have acted as the bioactive ingredient in commercial functional food products advertising these aforementioned health claims (Nirmal et al., 2021). However, the commercialization of functional foods is a challenging process requiring extensive research into the bioactivity, stability, and commercial viability of the bioactive agent. Production of protein hydrolysates at laboratory scale is an important step in the functional food development process, electing various food-grade enzymatic proteases and hydrolysis conditions with the aim of generating multiple bioactive components at a manageable scale. Due to the small scale of production, numerous hydrolysis methods can be employed so that the most suitable

method in respect to product yield and bioactivity is chosen for production at semi-pilot scale. Protein hydrolysates exhibiting multiple biofunctionalities possess greater potential as value-added ingredients for functional food applications.

Many studies have demonstrated the significant role played by gut hormones in response to food digestion. Short term regulatory hormones such as cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) modulate appetite and energy intake in humans and animals (Beglinger & Degen, 2006). Molecules which stimulate the secretion of these satiety hormones act as a potential natural approach to develop weight management strategies (Nobile et al., 2016). GLP-1 is a gut-derived peptide produced upon stimulation with nutrients including carbohydrate, fat and protein (Wang et al., 2015). Albeit GLP-1 in its active form is highly susceptible to the catalytic activity of the dipeptidyl peptidase IV (DPP-IV) enzyme resulting in the generation of inactive forms of GLP-1 which may not act as competitive antagonists at the GLP-1 receptor (Holst & Deacon, 2005). The STC-1 cell line is derived from an endocrine tumour developed in the small intestine of double transgenic mice, representing an appropriate model for the *in vitro* study of the mechanisms involved in the production of GLP-1 (Cordier-Bussat et al., 1997). Intact dietary protein isolated from codfish and protein hydrolysates from blue whiting and brown shrimp have been shown to increase secretion of CCK and GLP-1 *in vitro* in STC-1 cells (Cudennec et al., 2012; Cudennec et al., 2008; Geraedts et al., 2011). Administration of blue whiting protein hydrolysates (BWPHs) to male Wistar rats induced an increase in plasma levels of CCK and GLP-1, followed by a dose dependent decrease in short term food intake and reduced body weight. A follow up study determined that BWPHs also increased serum levels of CCK and GLP-1 and improved body weight and body composition in slightly overweight adults over a 90-day period (Nobile et al., 2016). The BWPHs, enriched with branch chain amino acids, is now commercially available as Slimpro®, a weight management ingredient.

Another potential weight management approach of food components may be their ability to interfere with adipocyte development in cells. Obesity occurs when energy intake exceeds energy expenditure resulting in an energy imbalance. The majority of this excess energy is stored in adipose tissue in the form of triglycerides. Obesity prevention and treatment are crucial to reduce the risk of obesity related diseases such as type 2 diabetes, hypertension and cardiovascular disease (Fingeret et al., 2018). Targeting adipocyte physiology and cell cycle is a potential approach to

reduce obesity. 3T3-L1 cells, a mouse preadipocyte cell line, have acted as an effective *in vitro* model for adipocyte differentiation (Cho et al., 2008). Peptides purified from various fish species have been reported to reduce triglyceride accumulation in 3T3-L1 cells through interfering with major adipogenic transcription factors (Y.-M. Kim et al., 2015; Lee et al., 2017), however to the best of our knowledge, the anti-adipogenic ability of blue whiting protein hydrolysates has yet to be investigated.

Oxidative stress occurs when there are more oxidants in a system than antioxidants. Relatively low levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may induce beneficial effects on cellular responses and immune function, however, high concentrations of ROS and RNS resulting in oxidative stress can potentially damage all cell structures (Di Meo et al., 2016). Oxidative stress is associated with numerous diseases including cancer (Khandrika et al., 2009), gastric ulcers (Das et al., 1997), Alzheimer's (Nunomura et al., 2006) and arthritis (Fonseca et al., 2019). Protein hydrolysates extracted from marine sources such as horse mackerel (Morales-Medina et al., 2017), argentine croaker (Da Rocha et al., 2018), cuttlefish (Ktari et al., 2013), and cod (Farvin et al., 2016) have been shown to exert non-cellular antioxidant activity as measured by the reducing power activity of the hydrolysates via the ferric reducing antioxidant power (FRAP) assay and by determining their ability to act as 'free radical scavengers' as measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay. However, cellular antioxidant assays offer greater physiological relevance than these bench-top assays, enabling the effect of food components on endogenous cellular antioxidant systems to be quantified. There is an abundance of research demonstrating protein hydrolysates and peptides from various sources including fish (Tao et al., 2018; Zhang et al., 2017), oats (Du et al., 2016), brewers spent grain (McCarthy et al., 2013), rice bran (Moritani et al., 2017), casein (Phelan et al., 2009) and whey (Kent et al., 2003) with the ability to regulate these antioxidant enzymatic (catalase and superoxide dismutase) and non-enzymatic (glutathione system) defence systems in cellular models. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is an important biomarker of oxidative stress, with GSH acting as a vital intracellular protective antioxidant.

Inflammation, a defence mechanism induced by tissue injury or invasion of pathogens, involves the activation of macrophage essential for initiation and

continuation of pro-inflammatory cytokines (tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β) and mediators (nitric oxide (NO) and prostaglandin E_2). Although stimulation of macrophage-derived inflammatory mediators is essential for innate immunity, prolonged or excessive inflammation is associated with a wide range of diseases, including chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis and cancer (Medzhitov, 2010). Established cell lines such as the murine RAW264.7 macrophage cell line and the human THP-1 monocyte cell line have been used to explore the immunomodulatory activity of fish-derived protein hydrolysates and peptides. Tilapia protein hydrolysate, sweetfish-derived protein hydrolysates and *Johnius belengerii* frame hydrolysate have been reported to significantly suppress NO production in lipopolysaccharide (LPS)-challenged RAW264.7 cells (Heo et al., 2018; Kangsanant et al., 2014; Sung et al., 2012), presenting anti-inflammatory properties desirable in functional food applications.

The aim of this study was to investigate the potential bioactive effects of eleven different blue whiting protein hydrolysates using three appropriate cell lines. The satiating effect of the hydrolysates was investigated via measuring active GLP-1 secretion in STC-1 cells. The anti-adipogenic potential of the hydrolysates was assessed via measuring triglyceride content in 3T3-L1 preadipocytes which were differentiated in the presence of the hydrolysates over 10 days. To assess their antioxidant and anti-inflammatory potential, the ability of the BWPHs to protect against oxidative stress and inflammation in hydrogen peroxide (H_2O_2) and LPS-challenged RAW264.7 cells, respectively, was assessed.

2.2 Materials and Methods

2.2.1 Materials

Murine STC-1 cells were purchased from the American Tissue Culture Collection (supplied by LGC Standards, Teddington, UK). Murine RAW264.7 cells and murine 3T3-L1 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). 100X Halt Protease and phosphatase inhibitor was purchased from Thermo Fisher Scientific (MSC, Ireland). Mouse Metabolic Magnetic Bead Panel for active GLP-1 analysis (#MMHMAG-44K) was sourced from Millipore (Ireland). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were supplied by Cruinn Diagnostics and Corning incorporated. All other cell culture reagents and chemicals including H_2O_2 and LPS were purchased from Sigma Chemical Co. (Dublin, Ireland) unless otherwise stated. BWPHs were supplied by Bio-Marine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland)

2.2.2 Cell Culture and Sample Preparation

STC-1 cells, 3T3-L1 cells and RAW264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. All cell lines were cultured in an atmosphere of CO_2-air (5:95, v/v) at 37 °C and were maintained in the absence of antibiotics. The BWPHs were diluted directly with DMEM, sterile-filtered using 0.45-micron filters, and stored at −20 °C. Reduced serum media (DMEM supplemented with 2.5% FBS) was used for cell-based experiments.

2.2.3 Cell viability and IC_{50} determination

STC-1 cells (2×10^5 cells/ mL), 3T3-L1 cells (1×10^5 cells/ mL) and RAW264.7 cells (1×10^5 cells/ mL) were seeded in 96 well plates in a final volume of 200 µL/ well and allowed to adhere overnight at 37 °C. After 24 h, well contents were aspirated and cells were either supplemented with increasing concentrations (0.05 - 1.00%, w/v dw) of BWPHs (ULPH-MB-01 to ULPH-MB-11) or DMEM only (control), for 24 h with a final volume of 200 µL. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK), which consisted of a MTT

reagent and a solubilisation solution. The well contents were aspirated and MTT reagent (10 µL) and DMEM (100 µL) were added to the wells and incubated for a further 4 h at 37 °C. The solubilisation solution (100 µL) was added and following overnight incubation, the absorbance was read at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA, USA). For subsequent assays, a nontoxic concentration of each sample was used, which induced greater than 80% cell viability.

2.2.4 STC-1 cell exposure to BWPHs and analysis of active GLP-1 secretion

STC-1 cells were seeded in 12 well plates at a density of 0.5 x 10^6 cells/mL in a final volume of 1.25 mL per well. Following a 24 h incubation period, media was removed, and the cells were washed with Krebs-Ringer buffer, which contains 11mM glucose. The following day, cells were acclimatised in Krebs-Ringer buffer (500 µL) for 1 h. Cells were then exposed for 4 h to either Krebs-Ringer buffer (control) or a concentration of BWPHs which resulted in cell viability above 80%. This concentration was determined by the MTT assay. Sample ULPH-MB-01 was at 0.05% (w/v dw), samples ULPH-MB-02, ULPH-MB-03, and ULPH-MB-07 were at 0.1% (w/v dw). Samples ULPH-MB-04, ULPH-MB-05, ULPH-MB-06, ULPH-MB-08, ULPH-MB-09, ULPH-MB-10 and ULPH-MB-11 were at 0.25% (w/v dw). All hydrolysates were prepared in Krebs-Ringer Buffer. Non-sterile solutions were filter-sterilized prior to cell exposure. After the 4 h incubation period, 5 µL of 100X Halt Protease and Phosphatase Inhibitor were added to inactivate endogenous DPP-IV. Supernatants were then collected and centrifuged (900 g, 4 °C, 5 min) and stored at -80 °C prior to GLP-1 analysis. The Milliplex Map Kit (Mouse Metabolic Magnetic BeadPanel) and MagPix fluorescent detection system (Luminex, The Netherlands) was used to quantify active GLP-1 content in the range of 41 – 30000 pg/mL. Intra-assay and inter-assay variation were <10% and <15%, respectively.

2.2.5 Cell differentiation into adipocytes and triglyceride analysis

3T3-L1 preadipocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) FBS. Cells were cultured in an atmosphere of CO2-air (5:95, v/v) at 37°C and were maintained in the absence of antibiotics. 3T3-

L1 cell passage number did not exceed 20. Preadipocytes were seeded onto 6 well plates at a density of 1×10^5 cells/ mL in media containing 10 % (v/v) FBS (day 0). On day 3, differentiation was initiated with a differentiating media containing 10% (v/v) FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 mM dexamethasone, 1 µg/ mL insulin and 2 µM rosiglitazone and incubated for 72 hours. Three days after the initiation of differentiation, the cell culture medium was replaced with DMEM containing 10% (v/v) FBS and 1 µg/ mL insulin. This step was repeated every other day until 80-90% of cells had differentiated to mature adipocytes. On day 10, media was then removed, cell monolayer was washed twice with PBS buffer and scraped in 1 mL PBS buffer. 500 µL of the cell suspension was centrifuged at 15000 rpm for 5 min at 4 °C, the supernatant was removed, the pellet was resuspended in 100 µL of lipid extraction buffer supplied with the commercial adipogenesis kit (Sigma, MAK040) and heated to 90°C for 30 mins using Eppendorf heater (brand). Total cellular triglyceride contents were determined according to the manufacturer's instructions. The other 500 µL cell suspension was centrifuged at 15000 rpm for 5 min at 4 °C, the supernatant was removed and the pellet was resuspended in 100 ul PBS for protein analysis using the bicinchoninic acid (BCA) method (Smith et al., 1985b) employing bovine serum albumin (BSA) as the calibration standard. Results are represented as the amount of triglyceride in milligram to an equivalent of cellular proteins (in milligram).

2.2.6 Cellular antioxidant status

GSH content was determined in RAW264.7 cells (1×10^5 cells/mL, 200 µL/well). After 24 h incubation at 37 °C, cells were supplemented with BWPHs (ULPH-MB-01 – ULPH-MB-11) in 6-well plates at a final concentration of 0.1% (w/v dw) (2 mL/well). Following 24 h, cells that were treated with BWPHs were incubated in the presence of H_2O_2 prepared in DMEM (0.4 mM, 2 mL/well) for 1 h at 37 °C. The positive control was cells exposed to H_2O_2 only. The negative control was cells exposed to DMEM alone. The ability of the BWPHs to protect against a H_2O_2-induced reduction in cellular GSH was assessed. GSH was determined by the method of (Hissin & Hilf, 1976). Briefly, cells were resuspended in sodium phosphate ethylenediaminetetraacetic acid (EDTA) buffer, sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 14,000 rpm × 30 min at 4 °C to remove

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cellular debris and supernatant was collected. The final GSH assay mixture (2 mL) was made up of 100 µL cell supernatant, 1.8 mL sodium phosphate EDTA buffer, and 100 µL o-phthaldialdehyde (1 mg/mL). Fluorescence was determined at a wavelength of 430 nm followed by excitation at 360 nm (VarioskanFlash, Thermo Scientific). GSH content was expressed relative to total cellular protein content, which was calculated by the BCA method (Smith et al., 1985b). Cell lysates (40 µL) were incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/mL) was calculated using BSA as a standard.

2.2.7 NO production in RAW264.7 cells

NO production was assessed in RAW264.7 cells using the Greiss assay (Kenny et al., 2013). Briefly, cells were seeded in 96 well plates at 1×10^5 cells/mL (200 µL/well) and incubated for 48 h at 37°C. Cells were then simultaneously stimulated using LPS (2 µg/mL) and treated with BWPHs at increasing concentrations of 0.05%, 0.1% and 0.2% (w/v dw) for 24 h. The positive control was cells incubated in the presence of LPS (2 µg/mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. The cultured supernatant (50 µL) was plated on a 96-well plate and 50 µL Griess reagent (1:1 of 1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-1-naphtyl-ethylenediamine dichloride in water) was added. Sodium nitrite was used to generate a standard curve (0–100 µM). The mixture was incubated for 20 min at room temperature in darkness and absorbance was read at 550 nm (VarioskanFlash, Thermo Scientific).

2.2.8 Statistical analysis

All experimental results of this study are expressed as the mean ± standard error of the mean (SEM) and data are from at least three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare significant differences between sample groups and control groups (Prism 5.0, GraphPad Inc. San Diego, CA, USA).

2.3 Results

2.3.1 Effects of BWPHs on cell viability of various cell lines

The hydrolysates exerted varying effects on the viability of the STC-1 cells (Table 2.1), 3T3-L1 cells (Table 2.2) and RAW264.7 cells (Table 2.3) after 24 h at concentrations ranging from 0.05%, w/v dw to 1.00%, w/v dw. In general, exposure to increasing concentrations of the hydrolysates resulted in a gradual decline in cell viability. STC-1 cells were the most susceptible to the cytotoxic effects of the protein hydrolysates. The hydrolysate ULPH-MB-01 had the most toxic effect on the cells, reducing cell proliferation to below 50% at only 0.19%, w/v dw (Table 2.4). All hydrolysates reduced proliferation of 3T3-L1 cells to below 50% at 1.0%, w/v dw. Again, hydrolysate ULPH-MB-01 was the most toxic to the cells with an IC_{50} value of 0.31%, w/v dw. Most of the hydrolysates were non-toxic to the RAW264.7 cells with ULPH-MB-04 being the only hydrolysate to induce 50% cell death (Table 2.4). Concentrations of hydrolysates which induced greater than 80% cell viability were chosen for bioactivity assays involving STC-1 cells: 0.05%, w/v dw for ULPH-MB-01, 0.10%, w/v dw for ULPH-MB-02, ULPH-MB-03 and ULPH-MB-07 and 0.25%, w/v dw for the remaining hydrolysates. Non-toxic concentrations of 0.1%, w/v dw were selected for bioactivity assays involving 3T3-L1 cells and RAW264.7 cell lines.

Table 2.1. The effects of different blue whiting protein hydrolysates (BWPHs) on the viability of murine STC-1 cells

Volume added to cells (%, w/v dw)	Cell Viability (%)										
	ULPH-MB-01	ULPH-MB-02	ULPH-MB-03	ULPH-MB-04	ULPH-MB-05	ULPH-MB-06	ULPH-MB-07	ULPH-MB-08	ULPH-MB-09	ULPH-MB-10	ULPH-MB-11
0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
0.05	83.1 ± 3.4	98.4 ± 1.0	114.6 ± 3.5	113.2 ± 5.0	109.5 ± 4.0	114.8 ± 7.9	112.6 ± 4.5	112.7 ± 7.5	112.1 ± 2.4	121.8 ± 3.1	96.0 ± 4.3
0.1	67.6 ± 7.6*	83.1 ± 1.2*	97.8 ± 5.2	107.6 ± 11.8	93.8 ± 4.2	106.2 ± 5.5	109.3 ± 3.8	107.1 ± 3.2	113.8 ± 3.2	117.7 ± 5.0	94.5 ± 2.4
0.25	39.1 ± 8.8*	40.6 ± 2.2*	62.8 ± 7.8*	103.6 ± 14.5	87.2 ± 6.8	107.4 ± 6.8	105.3 ± 3.4	86.2 ± 14.7	107.3 ± 14.7	121.6 ± 4.9	91.9 ± 4.0
0.5	28.6 ± 3.7*	34.6 ± 8.6*	30.9 ± 7.1*	35.4 ± 5.4*	32.8 ± 6.5*	46.1 ± 12.1*	72.7 ± 7.8*	63.8 ± 21.8*	104.2 ± 7.8	29.6 ± 5.4*	57.3 ± 6.9*
0.75	23.8 ± 4.0*	29.5 ± 6.5*	24.3 ± 4.8*	21.6 ± 3.1*	32.6 ± 7.5*	31.1 ± 7.5*	45.5 ± 14.2*	20.0 ± 8.0*	61.3 ± 13.3*	37.7 ± 7.6*	45.1 ± 12.4*
0.9	18.6 ± 4.4*	16.3 ± 3.2*	22.5 ± 4.8*	21 ± 5.0*	23.9 ± 5.6*	29.3 ± 10.8*	35.2 ± 8.2*	30.8 ± 10.0*	46.7 ± 8.9*	30.6 ± 6.9*	40.2 ± 11.3*
1.0	21.4 ± 6.0*	25.9 ± 5.4*	25.9 ± 6.4*	25.9 ± 9.4*	28.3 ± 4.6*	30.1 ± 15.2*	60.4 ± 21.2*	36.2 ± 10.6*	47.9 ± 7.6*	19.3 ± 6.0*	45.9 ± 9.0*

Murine STC-1 cells (2 x 10^5 cells/ mL, 200 µL/well) were supplemented with increasing concentrations (0.0 -1.0%, w/v dw) of BWPHs (ULPH-MB-01 to ULPH-MB-11) for 24 hours. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only (100% cell viability). Data are expressed as the mean ± SEM of five independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and control ($p < 0.05$).

Table 2.2. The effects of different blue whiting protein hydrolysates (BWPHs) on the viability of murine 3T3-L1 cells

Volume added to cells (%, w/v dw)	Cell viability (%)										
	ULPH-MB-01	ULPH-MB-02	ULPH-MB-03	ULPH-MB-04	ULPH-MB-05	ULPH-MB-06	ULPH-MB-07	ULPH-MB-08	ULPH-MB-09	ULPH-MB-10	ULPH-MB-11
0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
0.05	82.8 ± 1.8*	95.0 ± 4.8	96.6 ± 6.3	113.9 ± 12.9	94.0 ± 7.5	107.9 ± 9.9	103.3 ± 5.1	102.3 ± 7.7	98.6 ± 7.4	92.9 ± 6.4	97.2 ± 3.8
0.1	71.8 ± 4.4*	85.8 ± 4.3	84.8 ± 6.6	111.5 ± 14.9	85.5 ± 10.9	95.8 ± 8.6	102.8 ± 10.0	95.3 ± 6.1	100.8 ± 3.5	90.6 ± 2.5	97.7 ± 4.2
0.25	57.4 ± 5.6*	73.4 ± 5.5*	69.5 ± 4.9*	106.6 ± 16.2	66.5 ± 11.9	85.6 ± 10.7	89.8 ± 8.1	90.3 ± 6.1	95.4 ± 5.7	85.0 ± 5.1	96.3 ± 3.7
0.5	27.9 ± 4.8*	42.0 ± 3.4*	47.7 ± 7.6*	61.7 ± 15.7	38.9 ± 9.7*	73.9 ± 14.7	26.4 ± 10.1*	79.1 ± 8.8	81.2 ± 8.5	66.2 ± 8.2*	81.0 ± 5.8
0.75	18.2 ± 4.4*	33.0 ± 6.9*	25.4 ± 5.0*	37.6 ± 14.8*	30.2 ± 9.6*	56.5 ± 12.8*	14.7 ± 3.1*	58.9 ± 15.6	60.0 ± 13.8*	32.7 ± 11.0*	61.4 ± 17.4*
0.9	12.2 ± 4.4*	19.3 ± 5.6*	18.6 ± 3.2*	25.4 ± 7.5*	33.5 ± 8.1*	35.2 ± 9.2*	20.5 ± 4.8*	52.3 ± 18.0*	47.1 ± 15.1*	27.7 ± 10.2*	45.9 ± 16.2*
1.0	11.1 ± 4.0*	10.4 ± 1.7*	10.7 ± 1.5*	16.1 ± 2.8*	15.6 ± 3.1*	21.1 ± 3.8*	14.3 ± 2.7*	32.0 ± 11.6*	31.6 ± 5.8*	16.8 ± 4.3*	45.9 ± 11.9*

Murine 3T3-L1 cells (1×10^5 cells/ mL, 200 µL/well) were supplemented with increasing concentrations (0.0-1.0%, w/v dw) of BWPHs (ULPH-MB-01 – ULPH-MB-11) for 24 hours. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only (100% cell viability). Data are expressed as the mean ± SEM of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and control ($p < 0.05$).

Table 2.3. The effects of different blue whiting protein hydrolysates (BWPHs) on the viability of murine RAW264.7 cells

Volume added to cells (%, w/v dw)	Cell viability (%)										
	ULPH-MB-01	ULPH-MB-02	ULPH-MB-03	ULPH-MB-04	ULPH-MB-05	ULPH-MB-06	ULPH-MB-07	ULPH-MB-08	ULPH-MB-09	ULPH-MB-10	ULPH-MB-11
0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
0.05	117.8 ± 6.5	123.2 ± 11.4	127.8 ± 13.5	133.4 ± 13.5	111.7 ± 9.6	130.5 ± 16.7	120.3 ± 6.9	137.9 ± 9.5	120.3 ± 10.3	132.0 ± 15.7	117.0 ± 2.0
0.1	111.8 ± 7.9	121.8 ± 14.1	123.0 ± 16.1	121.9 ± 16.1	105.9 ± 9.7	129.0 ± 19.4	108.7 ± 6.7	122.6 ± 13.9	112.8 ± 11.2	120.4 ± 9.0	118.0 ± 1.3
0.25	108.8 ± 9.3	122.4 ± 4.8	117.5 ± 6.4	125.9 ± 6.4	104.0 ± 13.3	134.6 ± 26.5	100.0 ± 12.9	130.9 ± 5.8	115.0 ± 9.1	129.2 ± 14.9	113.9 ± 2.4
0.5	93.9 ± 9.5	102.9 ± 12.0	95.0 ± 10.1	87.2 ± 10.2	94.4 ± 15.9	109.3 ± 8.8	84.4 ± 13.3	117.7 ± 7.5	105.4 ± 4.9	115.3 ± 3.9	105.3 ± 10.7
0.75	82.2 ± 18.9	89.4 ± 15.8	75.0 ± 14.9	61.9 ± 14.9	70.1 ± 12.9	84.7 ± 6.7	63.0 ± 12.4	91.9 ± 14.3	83.6 ± 5.2	100.5 ± 2.6	82.4 ± 19.1
0.9	63.3 ± 22.5	91.2 ± 14.7	72.8 ± 11.0	55.5 ± 11.0*	77.9 ± 25.0	80.5 ± 3.8	64.0 ± 14.0	86.0 ± 15.8	89.4 ± 8.0	93.1 ± 6.4	84.2 ± 15.1
1.0	68.3 ± 10.2	75.5 ± 6.2	59.6 ± 4.4	47.0 ± 4.4*	76.5 ± 12.2	71.7 ± 2.2	56.3 ± 10.9	83.1 ± 3.9	92.2 ± 20.2	66.4 ± 4.0	75.1 ± 11.5

Murine RAW264.7 cells (1 x 10^5 cells/ mL, 200 µL/well) were supplemented with increasing concentrations (0.0 - 1.0%, w/v dw) of BWPHs (ULPH-MB-01 – ULPH-MB-11) for 24 hours. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only (100% cell viability). Data are expressed as the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and control ($p < 0.05$).

Table 2.4. IC$_{50}$ values of BWPHs in various mouse cell lines

BWPH	IC$_{50}$ values		
	STC-1 cells	3T3-L1 cells	RAW264.7 cells
ULPH-MB-01	0.19	0.31	n.d
ULPH-MB-02	0.20	0.43	n.d.
ULPH-MB-03	0.34	0.47	n.d.
ULPH-MB-04	0.44	0.60	0.98
ULPH-MB-05	0.42	0.38	n.d.
ULPH-MB-06	0.49	0.80	n.d.
ULPH-MB-07	0.62	0.39	n.d.
ULPH-MB-08	0.71	0.92	n.d.
ULPH-MB-09	0.88	0.86	n.d.
ULPH-MB-10	0.44	0.63	n.d.
ULPH-MB-11	0.65	0.85	n.d.

Mouse STC-1 cells (4 x 10^4 cells/well), mouse 3T3-L1 cells cells (1 x 10^4 cells/well) and RAW264.7 mouse (1 x 10^4 cells/well) were supplemented with increasing concentrations (0-1.0%, w/v dw) of eleven different BWPHs (ULPH-MB-01 to ULPH-MB-11) for 24 h. Cell viability was determined by the MTT assay. Using these data, IC$_{50}$ values were determined. IC$_{50}$ values are expressed as the concentration of BWPHs which reduce cell viability by 50%. Sample ULPH-MB-09 in STC-1 cells and samples ULPH-MB-01 to ULPH-MB-03 and ULPH-MB-05 to ULPH-MB-11 in RAW264.7 cells did not induce 50% cell death, therefore IC$_{50}$ values could not be determined (n.d.).

2.3.2 Effect of BWPHs on active GLP-1 content

STC-1 cells were exposed to BWPHs at varying concentrations for 4 h and the subsequent levels of active GLP-1 secreted from cells were measured (Figure 2.1). STC-1 cells which were exposed to each of the hydrolysates secreted significantly lower levels of active GLP-1 than STC-1 cells which were exposed to Krebs-Ringer Buffer alone (control) ($p<0.05$) with results ranging from 27.5% to 47.0% of the control.

2.3.3 Effect of BWPHs on triglyceride content

The ability of BWPHs (0.1% w/v dw) to prevent triglyceride accumulation during the differentiation process was assessed. Triglyceride levels in 3T3-L1 cells differentiated in the presence of the hydrolysates were not significantly different compared to those differentiated in the absence of the hydrolysates (differentiated control) (Figure 2.2) ($p>0.05$). Thus, all eleven of the hydrolysates failed to exhibit anti-adipogenic activity in the 3T3-L1 cell model.

2.3.4 Effect of BWPHs on GSH content

RAW264.7 cells exposed to H_2O_2 alone (positive control) significantly reduced GSH levels by 57.4% compared with the untreated control ($P<0.05$) (Figure 2.3). The BWPHs (0.1%, w/v dw) failed to modulate GSH levels in H_2O_2-challenged RAW264.7 cells as similar GSH levels were observed in cells treated with H_2O_2 plus BWPH as those treated with H_2O_2 alone (Figure 2.3). GSH content in cells treated with H_2O_2 plus the hydrolysates ranged from 31.5% to 55.4% of the control (100% GSH).

2.3.5 Effect of BWPHs on NO content

Exposure of RAW264.7 cells with LPS significantly increased NO production compared with cells treated with DMEM alone ($p<0.05$). The levels of NO produced in RAW264.7 cells challenged with LPS were not statistically different to the levels produced in cells treated with LPS plus BWPHs at various increasing concentrations (0.05, 0.1 and 0.2%, w/v dw) (Figure 2.4).
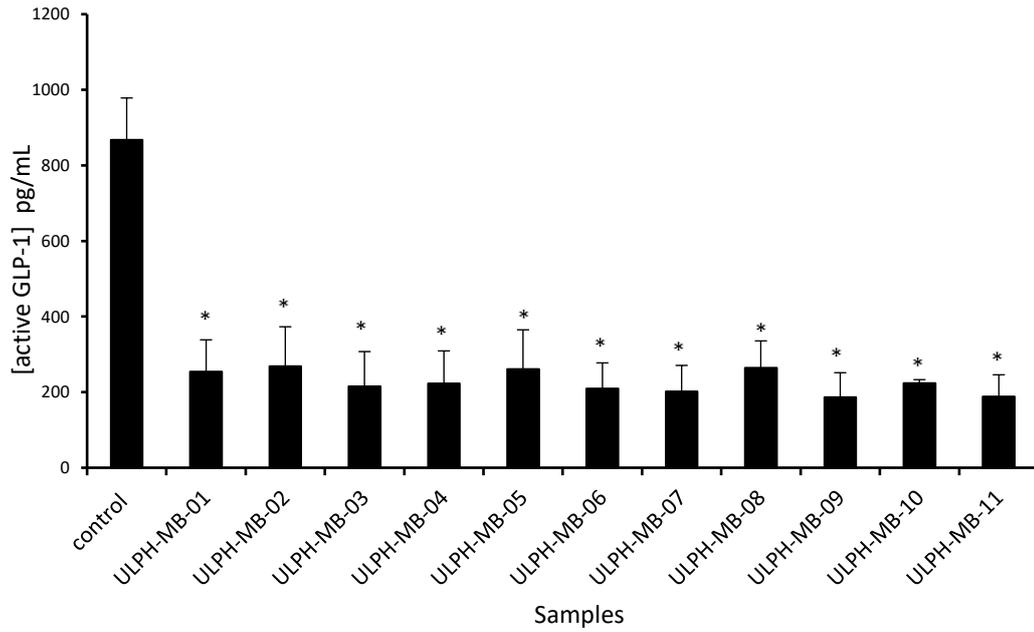
Figure 2.1. Secretion of active glucagon-like peptide-1 (GLP-1) from STC-1 cells
$(0.625 \times 10^6$ cells/well) exposed to Krebs-Ringer buffer (control) or blue whiting
protein hydrolysates (BWPHs) at various concentrations for 4 h. ULPH-MB-01 was
at 0.05%, w/v dw, ULPH-MB-02, ULPH-MB-03 and ULPH-MB-07 were at 0.1%,
w/v dw and ULPH-MB-04, ULPH-MB-05, ULPH-MB-06, ULPH-MB-08, ULPH-
MB-09, ULPH-MB-10 and ULPH-MB-11 were at 0.25%, w/v dw. Active GLP-1
secretion from STC-1 cells was measured using Milliplex Map Kit (Mouse Metabolic
Magnetic Bead Panel) and MagPix fluorescent detection system. Data are expressed
as mean ± SEM of 3 independent experiments. * denotes statistical significance
between individual samples and the control measured using ANOVA followed by
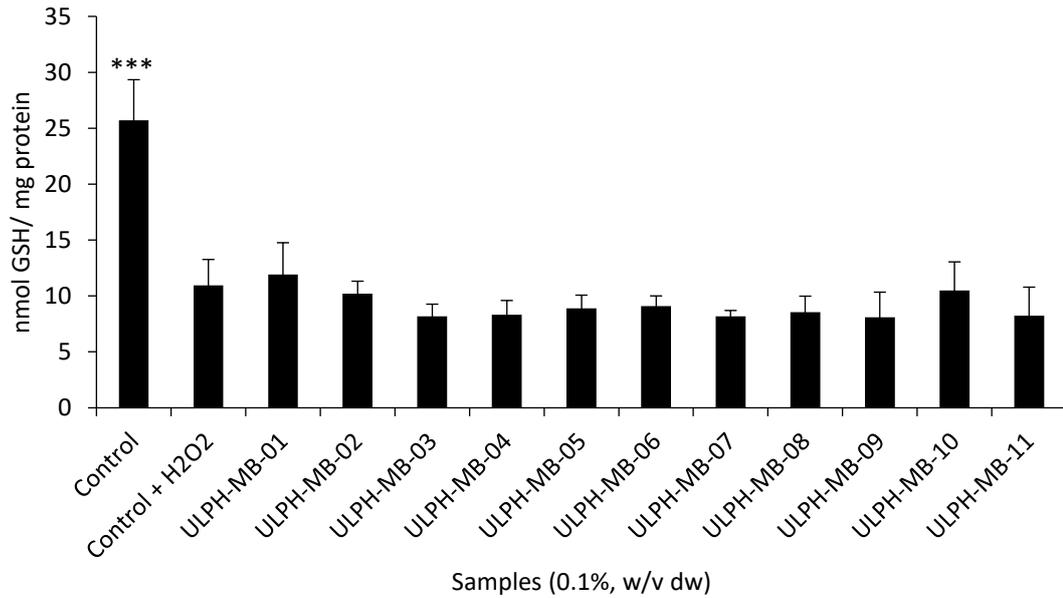Dunnett's test ($p < 0.05$).

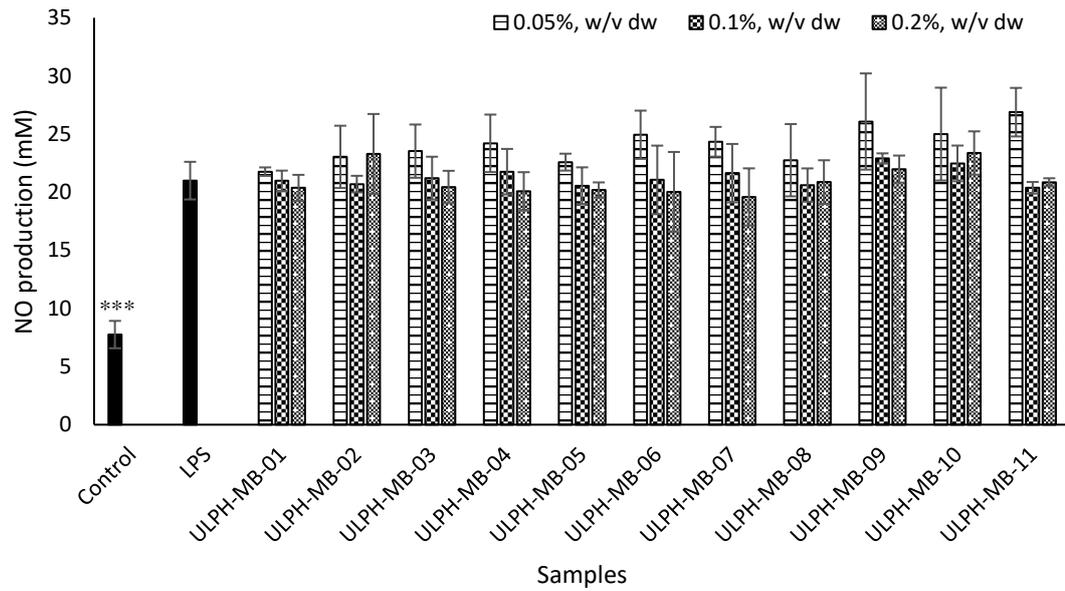**Figure 2.2.** Effect of blue whiting protein hydrolysates (BWPHs) on triglyceride levels in differentiated 3T3-L1 cells. 3T3-L1 preadipocytes (seeded at 1 x 10^5 cells/mL, 2 mL/well of a 6 well plate) were cultured in growth medium until they reached confluency and quiescent cells were incubated in DMEM/FBS (control) or differentiation medium with BWPHs at a final concentration of 0.1% (w/v dw) or without BWPHs (differentiated control) for 10 days. The values are expressed as nanomole of triglyceride per milligram of cellular protein. Data are the mean ± SEM of 3 independent experiments. *** denotes statistical significance between individual samples and the differentiated control measured using ANOVA followed by Dunnett's test ($p < 0.001$).

Figure 2.3. The effects of blue whiting protein hydrolysates (ULPH-MB-01 to ULPH-MB-11) (0.1% w/v dw) on the reduced glutathione (GSH) content in hydrogen peroxide (H_2O_2) stimulated RAW264.7 cells. RAW264.7 cells (1×10^5 cells/mL, 2 mL/well) were exposed to BWPHs for 24 h, followed by a H_2O_2 challenge (0.4 mM, 1 h). Values were expressed as nmol/mg protein. Values represent the mean ± SEM of at least three independent experiments. *** denotes statistical significance between individual samples and the positive control (control + H_2O_2) measured using ANOVA followed by Dunnett's test (p<0.001).

Figure 2.4. Effects of blue whiting protein hydrolysates (ULPH-MB-01 to ULPH-MB-11) at increasing concentrations of 0.05, 0.10 and 0.20%, w/v dw on nitric oxide (NO) production in lipopolysaccharide (LPS)-challenged RAW264.7 mouse macrophage. NO was measured using Griess assay and values represent the mean ± SEM of three independent experiments. *** denotes statistical significance between individual samples and the positive control (LPS) measured using ANOVA followed by Dunnett's test (p<0.001).

2.4 Discussion

In the present study, each of the eleven hydrolysates exerted anti-proliferative activity in a dose dependent manner in both the STC-1 cell line and the 3T3-L1 cell line. The hydrolysate concentration range of 0.05 - 1.0%, w/v dw in this study was chosen based on hydrolysate concentrations used in previous publications (Cudennec et al., 2008; González-Espinosa de los Monteros et al., 2011; O'Sullivan et al., 2017). These aren't the first protein hydrolysates extracted from natural sources to induce cytotoxic effects in these cell lines. Protein hydrolysates isolated from various raw materials including fish and milk also exhibited a growth inhibitory effect in STC-1 cells at 1% (w/v) (Catiau et al., 2011; Cudennec et al., 2008), and black soybean protein hydrolysate also induced anti-proliferative activity in 3T3-L1 cells in the same range as that used for BWPHs in this study (Kim et al., 2007). Hydrolysate ULPB-MB-01 was the most toxic hydrolysate in both the STC-1 cell line and the 3T3-L1 cell line, whereas ULPH-MB-09 and ULPH-MB-08 were the least toxic hydrolysates to the STC-1 cells and the 3T3-L1 cells, respectively. It is possible that molecular weight and/or peptide composition may have an effect on the cytotoxic potential of these hydrolysates. Quah et al. (2018) found that the lower molecular weight peptide fraction (<3 kDa) isolated from marine sponge, *X. testudinaria* papain hydrolysate, induced 7-fold higher toxicity in HeLa cells than that of the larger molecular weight peptide fractions (3-10 kDa) (Quah et al., 2018). The results of this study demonstrate the cell-specific toxicity of food-derived protein hydrolysates, as BWPHs up to a concentration of 1.0%, w/v dw induced limited cytotoxicity towards RAW264.7 cells with only one hydrolysate reducing cell viability by 50%. The cell specific cytotoxic activity displayed in this study validates the fact that generalized toxicological profiles for protein hydrolysates must be avoided and cell viability analysis is essential prior to initiating cellular based assays. Many studies have reported anti-cancer activities of fish protein hydrolysates via their anti-proliferative nature in cells of cancerous origins (Heffernan, Giblin, et al., 2021b). Further research into the effects of these BWPHs towards the viability of specific tumour cell lines may provide more information regarding the potential anti-cancer activity of BWPHs.

This study also investigated the satiety regulatory potential of BWPHs via their ability to stimulate satiety hormone active GLP-1 secretion from STC-1 cells. It was observed that none of the hydrolysates increased active GLP-1 secretion compared

with the Krebs-Ringer buffer control. It is possible that hydrolysate composition played a role in this lack of bioactivity. Caron et al. (2016) isolated specific peptide fractions from bovine haemoglobin and demonstrated that the fractions either reduced GLP-1 secretion or increased GLP-1 secretion from STC-1 cells compared to the control depending on the specific peptide sequences (Caron et al., 2016). The peptide sequences within fractions which reduced GLP-1 secretion were not identified, however, three peptide sequences which were demonstrated to trigger GLP-1 release contained either lysine or asparagine residues. Unfortunately, compositional analysis of hydrolysates in this study remains unknown, making it difficult to speculate the reasons behind observed effects of BWPHs. In contrast to the results of this study, numerous fish-derived protein hydrolysates have demonstrated GLP-1 stimulating ability using cell culture models (Cudennec et al., 2012; Harnedy et al., 2018a). Protein hydrolysates have produced mixed results regarding GLP-1 release in humans. Clinical trials with blue whiting protein hydrolysate resulted in an increase in blood concentrations of GLP-1 and CCK, a decrease in food intake and a reduction in body fat mass in slightly overweight adults (Nobile et al., 2016). However, Dale et al. (2019) found that cod protein hydrolysate did not increase plasma GLP-1 concentrations in healthy, middle-aged adults (Dale et al., 2018).

Differentiation of preadipocytes into mature adipocytes and accumulation of fat are responsible for pathogenesis of coronary heart disease and obesity (Jeon et al., 2004). In this study, the effect of BWPHs on adipogenesis was investigated by measuring triglyceride content in differentiated 3T3-L1 cells. Significantly higher levels of triglyceride accumulation were observed in 3T3-L1 cells treated with a hormonal mixture over a 10-day period. Albeit triglyceride levels in cells differentiated in the presence of hydrolysates were not significantly different to triglyceride levels in cells differentiated in the absence of BWPHs. Another study by González-Espinosa de los Monteros et al. (2011) reported similar finding for a soybean protein hydrolysate at a concentration of 1 mg/mL which failed to modulate adipogenesis, however the length of the differentiated period was not disclosed (González-Espinosa de los Monteros et al., 2011). Furthermore, anti-adipogenic activity is not the only biomarker for anti-obesity potential. Protein hydrolysates have also demonstrated adipolytic activity in 3T3-L1 mature adipocytes which was not analysed for BWPH bioactivity in this study.

Oxidative stress-induced damage can disrupt cell function and membrane integrity, which may result in cell death (Shieh et al., 2010). The endogenous antioxidant enzymatic defence system and the glutathione system are among the principal protective mechanisms against oxidative stress-induced tissue damage (Murphy et al., 1992). The use of synthetic antioxidants is closely regulated in certain countries due to associated health risks, hence, there is an abundance of research focused on the production of natural antioxidants that offer safer alternatives with little to no side effects (Shahidi & Zhong, 2010). In this study, none of the eleven BWPHs protected GSH levels in H_2O_2 challenged RAW264.7 cells. Although BWPHs did not protect against GSH depletion in this study, these results need to be interpreted with caution as, again, only one biomarker of antioxidant activity was investigated. Crowley et al. (2017) demonstrated that a pale phenolic compound isolated from brewers' spent grain (coded PW3) protected against an oxidant-induce decrease in superoxide dismutase and catalase activity in HepG2 cells, however, it did not significantly protect against GSH depletion (Crowley et al., 2017). In general, it is advantageous to assess other antioxidant enzymes with respect to the hydrolysates to determine their true antioxidant potential. Kent et al. (2003) reported that a casein hydrolysate also lacked the ability to protect GSH levels in the human prostate epithelial cell line (RWPE-1) due to the low cysteine content of the casein protein (Kent et al., 2003). Administration of cysteine is believed to increase cellular GSH levels as cysteine is predominantly the limiting substrate in GSH biosynthesis (Meister, 1991). Elucidation of the specific cysteine content in BWPHs may provide insight into the lack of GSH regulating ability as observed herein.

The inflammatory mediator, NO, is produced by macrophages stimulated by cytokines and/or microbial compounds. It is synthesised from L-arginine via the enzymatic activity of inducible nitric oxide synthase (iNOS). In this study, none of the eleven BWPHs significantly reduced NO production in LPS-challenged RAW264.7 cells compared with the LPS-stimulated control, thus potentially lacking immunomodulatory control. O'Sullivan et al. (2017) demonstrated that the immunomodulatory potential of bovine lung hydrolysates was dependant on the enzyme used during the hydrolysis process and as a result, the amino acid composition of the hydrolysates (O'Sullivan et al., 2017). Bovine lung hydrolysates produced with pepsin generated a hydrolysate which failed to significantly reduce NO or pro-

inflammatory cytokine (IL-6 and IL-1β) production in LPS-challenged RAW264.7 cells. On the other hand, hydrolysis of bovine lung using Alcalase or papain generated hydrolysates with anti-inflammatory activity in RAW264.7 cells, significantly decreased NO production and levels of IL-6 and IL-1β in LPS-challenged RAW264.7 cells. A great deal of research regarding the immunomodulatory activity of fish-derived protein hydrolysates has been completed and both anti-inflammatory effects and pro-inflammatory effects of these hydrolysates have been reported (Da Rocha et al., 2018; Heo et al., 2018; Li et al., 2019; Zhang et al., 2019).

These eleven laboratory-scale hydrolysates did not demonstrate bioactive potential in this study; however, this is only the first step of the process to develop a bioactive functional food ingredient. It is possible that further up-scaling of the hydrolysates may alter hydrolysate composition, thus influencing bioactivity. In addition, gastrointestinal transit and intestinal absorption which can be simulated in vitro to mimic physiological digestion may also significantly affect hydrolysate bioactivity.

2.5 Conclusion

In this study, BWPHs prepared at laboratory scale using various enzymes/enzyme combinations and hydrolysis conditions did not exhibit bioactive potential in various murine cell lines. BWPHs did not increase active GLP-1 secretion from STC-1 cells or prevent adipogenesis in 3T3-L1 cells. BWPHs failed to increase GSH levels in H_2O_2-challenged RAW264.7 cells and induced no significant effect on NO levels in LPS-challenged RAW264.7 cells. Further research is required to determine the true bioactivity of the hydrolysates as only one biomarker for each biological activity was assessed in this study

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Chapter 3

Blue Whiting Protein Hydrolysates Exhibit Antioxidant and Immunomodulatory Activities in Stimulated Murine RAW264.7 Cells

Abstract

This study investigated the antioxidant and immunomodulatory potential of six blue whiting soluble protein hydrolysates (BWSPHs, BW-SPH-A to -F) and their simulated gastrointestinal digests (SGID, BW-SPH-A-GI to –F-GI) in murine RAW264.7 macrophages. Hydrolysate BW-SPH-A, both pre- and post-SGID, increased endogenous antioxidant glutathione (GSH) in *tert*-butylhydroperoxide (*t*BOOH)-treated cells and reduced reactive oxygen species (ROS) in H_2O_2-challenged RAW264.7 cells compared with treated controls in the absence of BWSPHs ($p < 0.05$). BW-SPH-A-GI also exhibited higher ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) activities than the other BWSPHs tested ($p < 0.05$). All BWSPHs and SGID BWSPH samples induced immunostimulating effects in lipopolysaccharide (LPS)-activated RAW264.7 macrophages through the upregulation of NO production. BW-SPH-F-GI increased IL-6 and TNF-α levels compared with the LPS controls indicating the liberation of immunomodulatory peptide/amino acids during the SGID process. Therefore, BW-SPH-A and BW-SPH-F may have potential use against oxidative stress and immunosuppression-related diseases, respectively.

3.1 Introduction

Cellular metabolic processes and environmental factors, i.e., atmospheric pollutants, cigarette smoke, and radiation, generate free radicals categorised as either reactive oxygen species (ROS) or reactive nitrogen species (RNS). An excess of free radicals, inducing oxidative stress, is detrimental to cell structures by way of DNA strand damage as well as lipid and protein peroxidation (Tan et al., 2015). Long-term oxidative stress can accelerate the aging process as well as contribute to the development of a range of chronic diseases which include cancer, diabetes, and cardiovascular disease. Lifestyle and diet play an important role in the regulation of oxidative stress and can be modified to promote cellular redox balance, thereby potentially preventing damage and disease. Protection against oxidative stress in the body is provided via intracellular enzymatic antioxidant defence systems namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as non-enzymatic defence systems such as the glutathione system consisting of reduced (GSH) and oxidized (GSSG) forms of glutathione. The antioxidant enzyme, SOD, is the first line of defence against free radicals and converts the superoxide anion ($O_2^-\cdot$) to hydrogen peroxide (H_2O_2) and oxygen. Both CAT and GSH-PX then reduce H_2O_2 to water and oxygen, thereby preventing the formation of free radicals. GSH, which is a substrate for antioxidant enzymes such as GSH-Px also directly scavenges free radicals, regenerates nutrient antioxidants (vitamins A and E) to their active forms and assists the transport of amino acids through the plasma membrane (Valko et al., 2007).

A number of antioxidative fish protein hydrolysates with potential to induce health benefits via promoting cellular redox balance have been identified (Bkhairia et al., 2018; Fernando et al., 2020; Gómez et al., 2019; Hu et al., 2020; Tao et al., 2018; Zhang et al., 2017). However, the generation of antioxidant protein hydrolysates is dependent on hydrolysis conditions (protease source, temperature, pH, and degree of hydrolysis (DH)), which ultimately influences the peptide profile and amino acid composition of the resulting fractions (Heffernan et al., 2021). Small molecular weight (MW) fractions rich in hydrophobic amino acids and hydrophobic di- and tripeptides are generally reported to exhibit effective antioxidant activity owing to the proton donating or electron/lipid radical scavenging ability of hydrophobic amino acids.

Fish protein hydrolysates have also demonstrated both anti-inflammatory activity (Ahn et al., 2015; Da Rocha et al., 2018; Kangsanant et al., 2015; Karnjanapratum et al., 2016; Ko & Jeon, 2015; Sung et al., 2012) and proinflammatory activity (Toopcham et al., 2017; Zhang et al., 2019) via modulation of nitric oxide (NO) and inflammatory cytokines such an interleukin (IL)-6 and tumour necrosis factor-alpha (TNF-α) in macrophage cell lines. The use of macrophage cells has become an increasingly popular approach to investigate the antioxidant potential of natural bioactive compounds. Although macrophages are responsible for the recognition and elimination of microbial pathogens, some virulent bacteria have been reported to induce macrophage apoptosis via stimulating ROS production (Bhaskaran et al., 2012). Due to the adverse effects associated with some immunomodulatory drugs, protein hydrolysates generated from underutilised fish species may offer a sustainable and safe alternative for therapeutic immunomodulation.

Blue whiting (*Micromesistius poutassou*) protein hydrolysates (BWPHs) have previously been demonstrated to exhibit antioxidant activity as measured by DPPH radical scavenging activity, ferrous chelating activity and reducing power (Egerton et al., 2018; García-Moreno et al., 2017). In addition, BWPHs have also displayed cardioprotective and antigenicity activity in vitro, antidiabetic activities in vitro and in vivo and anti-obesity activities in vitro, in vivo, and in clinical trials (Cudennec et al., 2012; García-Moreno et al., 2017; Harnedy et al., 2018; Laroque et al., 2008; Nobile et al., 2016). Identification of bioactive fractions or peptides from blue whiting contributes to the sustainability ethos through reducing waste by converting an underutilised source of high-quality protein, harvested at high volumes into high-value functional food ingredients. To the best of our knowledge, no study to date has investigated the antioxidant potential of BWPHs in cellular systems or their immunomodulatory potential.

In most cases, in order to exhibit effective bioactivity in vivo, bioactive peptides have to survive gut transit and depending on their target cell, may need to permeate the gut membrane. However, due to the large population of macrophages that exist along the entire length of the GI tract (Grainger et al., 2017), it is possible that bioactive food components may interact with and modulate macrophages as they transit the gut. However, gut macrophage populations characteristically do not induce classic inflammatory responses so the ability of BWPHs to modulate inflammation

should initially be assessed in the well-established macrophage cell line RAW264.7, which can generate proinflammatory mediators and cytokines.

Recently our group generated distinct blue whiting soluble protein hydrolysates (BWSPHs) at commercial scale using various food-grade microbial-derived proteolytic enzyme preparations and hydrolysis conditions (enzyme:substrate (E:S) ratios ranging from 0.005% to 0.900% (w/w), 50 °C, 45–120 min, (Harnedy-Rothwell et al., 2021)). Although these hydrolysates differed in their DH and molecular mass distribution, all BWSPHs tested demonstrated in vitro antidiabetic activity through dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulin secretory activity. The objective of this follow-on study was to research additional potential bioactivities of the six BWSPHs and their simulated gastrointestinal (GI) digests through noncellular and cellular antioxidant and immunomodulatory assays. The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays were used to assess noncellular antioxidant activity whilst the ability of the BWSPHs and simulated GI digests to modulate redox balance (GSH, CAT, and ROS) was assessed in oxidatively stressed RAW264.7 cells. The immunomodulatory potential of BWSPHs was also studied via investigating their effect on NO production, and IL-6 and TNF-α cytokine levels in lipopolysaccharide (LPS)-activated RAW264.7 cells.

3.2 Materials and Methods

3.2.1 Materials

RAW264.7 cells were purchased from American Type Culture Collection (Manassas, Virginia). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Corolase® PP was provided by AB Enzymes (Darmstadt, Germany) and BC pepsin was provided by Biocatalysts (Cardiff, United Kingdom). Cell culture plastics were supplied by Cruinn Diagnostics and Corning Incorporated. All other cell culture reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. unless otherwise stated.

3.2.2 Sample Preparation

The six BWSPHs (BW-SPH-A to -F) samples provided by Bio-Marine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland) were generated from minced blue whiting as previously described by (Harnedy-Rothwell et al., 2021). Simulated gastrointestinal digestion (SGID) of the BWSPHs (BW-SPH-A-GI to –F-GI) was performed as described in [26] using pepsin (pH 2, 37 °C, 90 min, E:S of 2.5% w/w) and Coralase PP (pH 7, 37 °C, 150 min, E:S of 1% w/w). Samples were heated at 85 °C for 15 min, freeze-dried and kept at −20 °C until used.

3.2.3 Cell Culture and Sample Preparation

RAW264.7 cells were grown in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% (v/v) FBS. Cells (between passage numbers 15–34) were cultured in an atmosphere of CO_2–air (5:95 (v/v)) at 37 °C and were maintained in the absence of antibiotics. The BWSPHs and SGID BWSPHs were diluted directly with sterile DMEM, unless stated otherwise, sterile-filtered using 0.45-micron filters, and stored at −20 °C. Reduced serum media (DMEM supplemented with 2.5% FBS) was used for cell-based experiments.

3.2.4 DPPH Activity

The DPPH assay was performed according to the method described by (Brand-Williams et al., 1995). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), prepared in methanol with concentrations ranging from 0.04 to 0.40 μM, was used to prepare a standard curve for calibration. Briefly, 100 μL of blank (methanol), standards (0.04 to 0.40 μM Trolox prepared in methanol), and test samples (prepared in DMEM and tested in the range 1.5–4.0 mg/mL), were mixed with 0.06 mM DPPH/methanol solution (3.9 mL) and incubated for 30 min at room temperature (~20 °C). Colour blanks were included consisting of 100 μL DMEM and 3.9 mL methanol. Absorbance at 515 nm of all samples was measured (Lightwave II UV/Visible spectrophotometer, Biochrom Ltd., Cambridge, England). Results were expressed as % DPPH inhibition.

3.2.5 ORAC Activity

The ORAC activity was determined as previously described (Harnedy et al., 2017). In brief, 50 µL of the blank (0.075 M sodium phosphate buffer pH 7.0), standards (0–120 µM Trolox prepared in assay buffers), and test samples (prepared in assay buffers and tested in the range 0.15–0.20 mg/mL), were mixed with 0.78 µM fluorescein (50 µL) and incubated for 15 min at 37 °C in a plate reader (BioTek Synergy HT, VT, USA). The reaction was initiated by the addition of 0.221 M 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) solution (25 µL) and maintained at 37 °C for 120 min. The fluorescence (Ex/Em wavelengths of 485/520 nm) signal was measured every 5 min for 2 h and the ORAC value was calculated with reference to a Trolox standard curve (0–120 µM) and expressed as µmol of Trolox equivalents per gram dry weight (µmol TE/g dw).

3.2.6 FRAP Activity

The FRAP activity was determined as described by (Harnedy & FitzGerald, 2013). In brief, the absorbance (590 nm) of the FRAP reagent ((150 µL) 0.3 M acetate buffer (pH 3.6), 0.01 M 2, 4, 6-tripyridyl-s- triazine (TPTZ), 0.02 M $FeCl_3.6H_2O$, 10:1:1) was determined using a plate reader. A 20 µL aliquot of 0.3 M acetate buffer pH 3.6 (blank), standards (0–200 µM Trolox prepared in assay buffers) and test samples (prepared in assay buffers and tested in the range 15–20 mg/mL) were added, mixed and the absorbance read after 30 min incubation at 37 °C. The FRAP value was expressed as µmol of Trolox equivalents per gram of freeze-dried powder (µmol of TE/g dw).

3.2.7 Cell Viability

RAW264.7 cells were seeded at a density of 1×10^5 cells/mL in DMEM supplemented with 10% FBS with a volume of 200 µL/well in a 96-well plate. After a 24 h incubation at 37 °C, media was aspirated and cells were either supplemented with increasing concentrations of BWSPHs (BW-SPH-A to -F) and SGID BWSPHs (BW-SPH-A-GI to -F-GI) with the final concentrations in the culture medium ranging from 0–1.0% (w/v dw), or DMEM only (control), for 24 h with a final volume of 200 µL. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrezolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK), which consisted of a MTT reagent and a solubilisation solution. The well contents were aspirated and MTT reagent (10 µL) and DMEM (100 µL) were added to the wells and incubated for a further 4 h at 37 °C. The solubilisation solution (100 µL) was added and following overnight incubation, the absorbance was read at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA, USA). For subsequent assays, a nontoxic concentration of each sample was used, which induced greater than 80% cell viability.

3.2.8 ROS Production

Intracellular formation of ROS was determined via the oxidation-sensitive fluorogenic probe 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA). RAW264.7 cells were plated in 96-well plates (1 x 10^5 cells/mL, 200 µL/well) in DMEM supplemented with 10% FBS for 24 h and BWSPHs and SGID BWSPHs were added to the wells for a further 24 h at a final concentration of 0.5% (w/v dw). Cells were then washed with phosphate buffer saline (PBS) and exposed to DCFH-DA prepared in DMEM (20 µM, 200 µL/well) for 40 min at 37 °C in the dark. Intracellular esterases hydrolyse DCFH-DA to nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is then oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) upon reaction with ROS. DCFH-DA was aspirated, and cells were washed with PBS. The cells, except the negative control, were exposed to H_2O_2 (1 mM, 200 µL/well) for 60 min. The positive control was cells treated with H_2O_2. The negative control was cells exposed to DMEM only. Fluorescence of cells was measured at a wavelength of 485 nm followed by excitation at 530 nm every 5 min over the 60 min exposure period (VarioskanFlash, Thermo Scientific). Results were expressed as % of the positive control.

3.2.9 GSH Content

GSH content was determined in RAW264.7 cells (1 × 10^5 cells/mL, 200 µL/well). After 24 h incubation at 37 °C, cells were supplemented with BWSPHs and SGID BWSPHs in 6-well plates at a final concentration of 0.5% (w/v dw) (2 mL/well). Following 24 h, cells that were treated with BWSPHs were incubated in the presence of tBOOH prepared in DMEM (1 mM, 2 mL/well) for 3 h at 37 °C. The positive

control was cells exposed to *t*BOOH only. The negative control was cells exposed to DMEM alone. The ability of the BWSPHs to protect against a *t*BOOH-induced reduction in cellular GSH was assessed. GSH was determined by the method of (Hissin & Hilf, 1976). Briefly, cells were resuspended in sodium phosphate ethylenediaminetetraacetic acid (EDTA) buffer, sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 14,000 rpm $\times$ 30 min at 4 °C to remove cellular debris and supernatant was collected. The final GSH assay mixture (2 mL) was made up of 100 µL cell supernatant, 1.8 mL sodium phosphate EDTA and 100 µL o-phthaldialdehyde (1 mg/mL). Fluorescence was determined at a wavelength of 430 nm followed by excitation at 360 nm (VarioskanFlash, Thermo Scientific). GSH content was expressed relative to total cellular protein content, which was calculated by the bicinchoninic acid (BCA) method (Smith et al., 1985b). Cell lysates (40 µL) were incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/mL) was calculated using bovine serum albumin (BSA) as a standard.

3.2.10 CAT Activity

CAT activity was determined in RAW264.7 cells (1×10^5 cells/mL, 200 µL/well). After 24 h incubation at 37 °C, cells were supplemented with BWSPHs and SGID BWSPHs in 6-well plates at a final concentration of 0.5% (w/v dw) (2 mL/well). Following 24 h, cells that were treated with BWSPHs were incubated in the presence of H_2O_2 prepared in DMEM (1 mM, 2 mL/well) for 3 h at 37 °C. The positive control was cells exposed to H_2O_2 only. The negative control was cells exposed to DMEM alone. Cells were resuspended in buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA), sonicated (13 mA for 10 s, $\times$ 3) (Soniprep 150, MSE), and centrifuged (14,000 rpm $\times$ 30 min at 4 °C) to remove cell pellets. Cell supernatant was collected for assay and stored at −80 °C. CAT activity was measured using a Calbiochem CAT Assay Kit (Merck Chemicals Ltd., Nottingham, U.K.) and performed according to the manufacturer's instructions. One unit of catalase activity was defined as the amount of catalase required to decompose 1 µmol H_2O_2 per minute at pH 7.5 and 25 °C. CAT activity was expressed relative to the total cellular protein content, which was calculated by the BCA method [31]. Cell lysates (40 µL) were

incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/mL) was calculated using BSA as a standard.

3.2.11 NO Secretion

NO secretion was assessed in RAW264.7 cells using the Greiss assay as described in (Kenny et al., 2013). Briefly, cells were seeded in 96-well plates at 1×10^5 cells/mL (200 µL/well) and incubated for 48 h at 37°C. Cells were then simultaneously stimulated using LPS (2 µg/mL prepared in DMEM) and treated with BWSPHs or SGID BWSPHs at a final concentration of 0.5% (*w/v* dw) for 24 h at 37 °C. The positive control was cells incubated in the presence of LPS (2 µg/mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. The cultured supernatant (50 µL) was plated on a 96-well plate and 50 µL Griess reagent (1:1 of 1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-1-naphtyl-ethylenediamine dichloride in water) was added. Sodium nitrite was used to generate a standard curve (0–100 µM). The mixture was incubated for 20 min at room temperature in darkness and absorbance was read at 550 nm (VarioskanFlash, Thermo Scientific).

3.2.12 Cytokine Secretion

RAW 264.7 cells (0.2×10^5 cells/mL, 200 µL/well) were seeded in 96-well plates and incubated for 24 h at 37 °C. Cells were then simultaneously stimulated using LPS (0.1 µg/mL prepared in DMEM) and treated with BWSPHs or SGID BWSPHs at a final concentration of 0.5% (w/v dw) for 24 h at 37 °C. The positive control was cells incubated in the presence of LPS (0.1 µg/mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. After incubation, the culture medium was harvested and the secretion of cytokines IL-6 and TNF-α was measured by enzyme-linked immunosorbent assay (ELISA). Cytokine production was determined using eBioscience ELISA kits (Ready-SET-Go kit purchased from eBioscience, Hatfield, UK). Absorbance was measured at 450 nm on a microplate reader (VarioskanFlash, Thermo Scientific) and cytokine production was expressed as a percentage of LPS-stimulated RAW 264.7 cells (positive control). The ELISA kits

allow the detection of IL-6 and TNF-α with a minimum detection limit of 4.0 and 3.7 pg/mL, respectively, and intra-assay variation <6.5%.

3.2.13 Statistical Analysis

All experimental results of this study are expressed as the mean ± standard error of the mean (SEM) and data are from at least three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare significant differences between sample groups and control groups (Prism 5.0, GraphPad Inc. San Diego, CA, USA). The statistical software programs SPSS (Version 26, IBM Inc., Chicago, IL, USA) was used to perform statistical analyses on the data arising from the ORAC and FRAP analysis and an ANOVA followed by Tukey's and Games–Howell post-hoc tests was used to compare all values. Values before and after SGID were compared using Student's *t*-tests.

3.3 Results and Discussion

In this study, the in vitro antioxidant and immunomodulatory potential of six protein hydrolysates generated at industrial scale from the low-value underutilised species blue whiting, using different hydrolysis conditions, were assessed. All BWSPHs contained high protein contents ranging from 70.37 ± 0.33–73.60 ± 0.53 g/100g (Harnedy-Rothwell et al., 2021). Physicochemical data such as DH, molecular mass distribution, RP-UPLC, and free amino acid profiles demonstrated that the variation in hydrolysis conditions resulted in the generation of BWSPHs with distinctly different characteristics (Harnedy-Rothwell et al., 2021). In summary, BW-SPH-A, BW-SPH-B, BW-SPH-E had significantly higher DH values (43.19%–45.79%, $p < 0.05$) compared to all other samples with an abundance of low MW peptides, i.e., <1 kDa (69.74%–77.86%). BW-SPH-C had the lowest DH value at $27.82\% \pm 1.11\%$ with $55.55\% \pm 0.13\%$ peptides <1 kDa (Harnedy-Rothwell et al., 2021). The DH data, molecular mass distribution and RP-UPLC profiles and free amino acid composition show that all BWSPHs were further degraded during SGID (Harnedy-Rothwell et al., 2021) reaching similar levels at the end of the intestinal phase (57%–65%). Hydrolysate BW-SPH-C which had the lowest DH ($27.82\% \pm 1.11\%$), showed the highest level of hydrolysis during SGID with a resulting DH of $55.37\% \pm 1.83\%$. In vitro GI digestion is useful in predicting the metabolic fate and

bioactive potential of food components during in vivo digestion. The SGID protocol employed herein is an example of a static digestion method whereby gastric enzyme pepsin and intestinal enzyme preparation Corolase PP (containing trypsin, chymotrypsin, and elastase) were used to mimic gastric and intestinal digestion, respectively. While various protocols for static SGID exist (Brodkorb et al., 2019), this method was chosen in order to compare the results of this study to previous works published by our group. No zone of inhibition was observed when SGID-treated samples were added on casein agar plates, which confirmed that there was no residual proteolytic activity associated with SGID-treated samples (data not shown).

3.3.1 Noncellular In Vitro Antioxidant Activity

The ability of food compounds to reduce DPPH radicals is often used as an indicator of their antioxidant potential. The results presented in Table 3.1 show that all BWSPHs demonstrated DPPH radical scavenging activity with half-maximal inhibitory concentration (IC_{50}) values ranging from 2.10 ± 0.12 to 2.47 ± 0.04 mg/mL, indicating all BWSPHs possess electron-donating abilities. The DPPH radical scavenging data obtained for BWSPHs (Table 3.1) are comparable with previous studies investigating the DPPH radical scavenging activity of protein hydrolysates from various fish species such as Klunzinger's mullet ($IC_{50} = 2.08 \pm 0.13$ mg/mL), shortfin scad ($IC_{50} = 1.89$ mg/mL) and yellow-fin tuna waste ($IC_{50} = 1.89$ mg/mL) (Kang et al., 2018; Parvathy et al., 2016; Rabiei et al., 2019). However, although BWSPHs possessed varying physicochemical properties (Harnedy-Rothwell et al., 2021), no significant differences in DPPH scavenging activity were observed ($p >$ 0.05). Published studies have reported a variability in DPPH scavenging activity of BWPHs depending on protease employed for hydrolysis (Egerton et al., 2018; García-Moreno et al., 2017). BWPHs produced with subtilisin, trypsin or a subtilisin-trypsin combination with varying DH (4%–12%) inhibited DPPH by 50% at concentrations ranging from 1.36–2.46 mg protein/mL (Garcia-Monero et al., 2016). Preparation of BWPHs with Flavourzyme[®] 500L resulted in higher DPPH scavenging activity compared with BWPHs generated with Protamex[®] or Savinase[®]16 L ($p < 0.001$) possibly due to the higher proportion of di- and tripeptides and free amino acids (mainly Leu, Phe + Tyr, and Glu) (Egerton et al., 2018). Amino acid residues Trp, Phe, Tyr, Cys, and His can reportedly contribute to antioxidant activity (Corrochano

et al., 2018), however, no relationship was observed between the amino acid composition and the antioxidant activity of BWSPHs in this study.

The ORAC and FRAP activity of the BWSPHs and their simulated GI digests are presented in Table 3.2. Prior to SGID, ORAC values of 330.79 ± 9.76 to 393.32 ± 3.23 μmol TE/g dw were obtained for BWSPHs with BW-SPH-C and BW-SPH-D mediating the lowest and highest activity, respectively. A significant increase ($p <$ 0.05) in ORAC activity was observed with samples BW-SPH-A-GI, -B-GI, -D-GI, and -F-GI following SGID (Table 3.2). As previously stated, the DH data, molecular mass distribution, and RP-UPLC profiles indicate that all BWSPHs were further hydrolysed during SGID, which would indicate that gut enzymes liberated peptides with superior ORAC activity from precursor peptides. Interestingly, the ORAC activity exhibited by BW-SPH-C-GI, the hydrolysate which was hydrolysed by the greatest extent during SGID (from 27.82 ± 1.11 pre-SGID to 55.37 ± 1.83 post-SGID (Harnedy-Rothwell et al., 2021), was similar to that prior to in vitro digestion. It is possible that bioactive peptides were not further hydrolysed by SGID or that peptides were further hydrolysed with the loss and gain of individual bioactivities, resulting in no overall change to the total bioactivity.

Table 3.1. The DPPH radical scavenging activity of blue whiting *(Micromesistius poutassou)* soluble protein hydrolysates (BWSPHs).

	BW-SPH-A	BW-SPH-B	BW-SPH-C	BW-SPH-D	BW-SPH-E	BW-SPH-F
IC_{50} value (mg dw/mL)	2.10 ± 0.12	2.31 ± 0.13	2.14 ± 0.22	2.34 ± 0.30	2.11 ± 0.15	2.47 ± 0.04

Values are expressed as mean $\pm$ SEM of three independent experiments. DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate. IC_{50}: half-maximum inhibitory concentration. No significant difference was observed ($p > 0.05$).

Table 3.2. In vitro ORAC and FRAP antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion (SGID).

Sample Code	ORAC Value (µmol TE/g dw)	FRAP Value (µmol TE/g dw)
BW-SPH-A	387.65 ± 9.97 [ab]	7.41 ± 0.15 [bc]
BW-SPH-A-GI	459.73 ± 8.72 [A*]	5.60 ± 0.03 [A*]
BW-SPH-B	350.65 ± 10.35 [bc]	7.25 ± 0.22 [c]
BW-SPH-B-GI	414.20 ± 4.68 [*]	5.06 ± 0.04 [B*]
BW-SPH-C	330.79 ± 9.76 [c]	7.67 ± 0.14 [bc]
BW-SPH-C-GI	348.49 ± 4.89 [C]	4.64 ± 0.09 [C*]
BW-SPH-D	393.32 ± 3.32 [a]	8.45 ± 0.08 [a]
BW-SPH-D-GI	409.00 ± 2.98 [B*]	4.75 ± 0.06 [BC*]
BW-SPH-E	365.88 ± 8.27 [abc]	7.57 ± 0.10 [bc]
BW-SPH-E-GI	386.50 ± 3.23 [B]	4.74 ± 0.09 [BC*]
BW-SPH-F	345.78 ± 4.26 [c]	8.03 ± 0.23 [ab]
BW-SPH-F-GI	385.01 ± 9.55 [B*]	4.48 ± 0.08 [C*]

Values are expressed as mean $\pm$ SEM of three independent experiments. The samples labelled -GI refer to the samples post-SGID. ORAC: oxygen radical absorbance capacity, FRAP: ferric reducing antioxidant power, TE: Trolox equivalents, dw: dry weight. Different lowercase letters (a,b,c) within a column indicate a significant difference between BWSPHs samples pre-SGID at $p < 0.05$. Different capital letters (A,B,C) within a column indicate a significant difference between BWSPHs post-SGID at $p < 0.05$. * indicates a significant difference at $p < 0.05$ between pre- and post-SGID values.

FRAP values ranging from 7.25 ± 0.22 to 8.45 ± 0.08 µmol TE/g dw were obtained for BWSPHs prior to SGID with BW-SPH-B and BW-SPH-D mediating the lowest and highest activity, respectively (Table 3.2). The FRAP activity of all samples was significantly decreased following SGID (Table 3.2). Similar findings were reported for a whey hydrolysate prepared with Alcalase™, which demonstrated reduced FRAP and ABTS inhibition and increased ORAC activity post-SGID compared to the undigested Alcalase-hydrolysate ($p < 0.05$) (Corrochano et al., 2019). This is not unexpected as the ORAC and FRAP assays are based on different chemical reactions with the ORAC assay measuring the scavenging capacity of test compounds against peroxyl radicals through hydrogen atom transfer (HAT), whereas FRAP is categorized as an electron transfer (ET)-based, nonradical method. Due to the fact that SGID samples demonstrated increased ORAC values but reduced FRAP values compared with the corresponding undigested samples, it is possible that SGID samples scavenge radicals through HAT. The radical scavenging activity of BWSPHs and SGID BWSPHs was also assessed in a cell model (Table 3.4) which provides a better biological predictor for BWSPHs antioxidant ability.

The peptide length and amino acid composition of peptides within the protein hydrolysate, which ultimately influence hydrolysate bioactivity, are dependent on DH. In this case, FRAP results indicate that bioactivity was attenuated during SGID. A correlation between a reduced DPPH scavenging activity and a reducing power of fish protein hydrolysate with increasing DH has been previously observed (Klompong et al., 2007; Li et al., 2012). The highest activity for both ORAC (459.73 ± 8.72 µmol TE/g dw) and FRAP (5.60 ± 0.03 µmol TE/g dw) activity following SGID was observed with sample BW-SPH-A-GI (Table 3.2). The ORAC activity observed herein for the BWSPHs was slightly lower and higher than that reported for protein hydrolysates derived from salmon muscle (587.41 ± 26.50–882.58 ± 105.72 µmol TE/g sample) and mussel meat (66.40 ± 2.27–121.56 ± 3.96 µmol TE/g sample), respectively (Neves et al., 2016; Neves et al., 2017).

3.3.2 Effect of BWSPHs on RAW264.7 Cell Viability

The effect of 24 h incubation with six BWSPHs (BW-SPH-A to -F) and six SGID BWSPHs (BW-SPH-A-GI to -F-GI) with final concentrations ranging from 0%–1.0% (w/v dw), on the viability of RAW264.7 cells was investigated. BW-SPH-A (0.05%

(w/v dw)) was the only BWSPH to induce a significant proliferative effect on RAW264.7 cells ($p < 0.05$) (Table 3.3). In contrast, BW-SPH-D (0.9% (w/v dw)) and BW-SPH-E (1.0% (w/v dw)) reduced the viability of RAW264.7 cells significantly compared with media alone (100%) ($p < 0.05$). SGID samples (up to 1.0% (w/v dw)) did not significantly alter the viability of RAW264.7 cells compared to the control ($p > 0.05$) (Table 3.3). A BWSPHs concentration of 0.5% (w/v dw) was chosen for future cell-based experiments which induced greater than 80% cell viability. The same concentration was chosen for SGID BWSPHs to enable direct comparison between BWSPHs and simulated GI digests.

Table 3.3 The effects of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) on the viability of murine RAW264.7 cells

	Cell viability (%)							
	0%	0.05%	0.1%	0.25%	0.5%	0.75%	0.9%	1.0%
BW-SPH-A	100 ± 0.0	126.9 ± 9.4*	122.6 ± 7.3	125 ± 4.2	117.3 ± 6.5	105.9 ± 9.1	93 ± 6.6	93.2 ± 8.0
BW-SPH-A-GI	100.0 ± 0.0	90.6 ± 4.7	90.4 ± 4.1	99.1 ± 6.1	93.8 ± 8.6	91.7 ± 8.7	86.4 ± 6.9	89.7 ± 4.9
BW-SPH-B	100 ± 0.0	124.5 ± 4.6	126.1 ± 6.0	113.7 ± 1.0	103.5 ± 8.4	98.7 ± 14.0	86.5 ± 12.2	79.8 ± 5.4
BW-SPH-B-GI	100 ± 0.0	100.9 ± 3.2	106.3 ± 0.8	106.8 ± 1.0	108.9 ± 2.7	100.7 ± 1.4	102.2 ± 3.9	91.9 ± 4.2
BW-SPH-C	100 ± 0.0	114.7 ± 8.2	105.4 ± 5.8	93.7 ± 7.6	83 ± 14.6	89.7 ± 15.9	84.4 ± 14.6	71.1 ± 6.8
BW-SPH-B-GI	100 ± 0.0	101 ± 4.4	107.9 ± 4.6	104.6 ± 1.6	109.8 ± 4.1	104.6 ± 1.1	103.6 ± 3.0	94.5 ± 3.2
BW-SPH-D	100 ± 0.0	101.2 ± 9.4	92.4 ± 7.3	84.2 ± 4.2	80.6 ± 6.5	76.7 ± 9.1	70.9 ± 6.6*	71.2 ± 8.0
BW-SPH-D-GI	100 ± 0.0	101.5 ± 6.0	104.9 ± 5.3	106.8 ± 5.9	98.3 ± 3.7	103.7 ± 6.2	103.9 ± 7.4	96 ± 7.9
BW-SPH-E	100 ± 0.0	120.9 ± 12.3	109.2 ± 17.0	99.5 ± 3.0	84.6 ± 6.2	77.1 ± 4.0	75.1 ± 6.8	66.8 ± 4.5*
BW-SPH-E-GI	100 ± 0.0	98.1 ± 5.7	103.5 ± 6.1	102.5 ± 2.8	97.7 ± 4.0	100.7 ± 8.5	91 ± 6.6	88.9 ± 6.0
BW-SPH-F	100 ± 0.0	125.3 ± 15.1	119.1 ± 20.2	106.6 ± 3.4	106.6 ± 3.6	90.7 ± 6.5	84.1 ± 4.6	84.7 ± 2.4
BW-SPH-F-GI	100 ± 0.0	96.8 ± 7.7	96.4 ± 6.1	98.3 ± 6.0	99.1 ± 6.3	99.1 ± 4.7	89.5 ± 5.1	89.4 ± 5.2

Murine RAW264.7 cells (1 x 10^5 cells/ mL, 200 µL/well) were supplemented with increasing concentrations (0.05 - 1.0% w/v dw) of BWSPH (BW-SPH-A to BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) for 24 h. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only (100% cell viability). Data are expressed as the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and control ($p<0.05$).

3.3.3 Cellular Antioxidant Activity

To further evaluate the antioxidant potential of BWSPHs, their ability to affect the endogenous antioxidant defence systems was assessed. The RAW264.7 cell line was selected due to its ability to generate intracellular oxidants as well as express enzymatic and nonenzymatic antioxidants (Lin et al., 2019). Table 3.4 details the effects of BWSPHs and SGID BWSPHs on intracellular GSH concentration, CAT activity, and ROS production. Oxidative stress was induced via treatment with known oxidants tBOOH or H_2O_2. The organic peroxide, tBOOH, was chosen as the oxidative stressor to reduce GSH levels as H_2O_2 did not significantly reduce GSH concentration in RAW264.7 cells (data not shown). Unlike endogenous H_2O_2, the xenobiotic tBOOH, related to industrial air pollution, has no specific detoxifying element in the cell. It has been proposed that the glutathione system catalyses the decomposition of tBOOH to tert-butyl alcohol and glutathione disulphide, resulting in depletion of GSH (Davies, 1989; Lin et al., 2000; Sutherland et al., 1985).

Although BW-SPH-B, -C, -D, -E, and -F did not protect against the suppression of GSH in tBOOH-challenged cells, treatment with BW-SPH-A did increase GSH concentration significantly compared with the control ($p < 0.05$) (Table 3.4). Similarly, BW-SPH-A was the only hydrolysate to increase CAT activity significantly in H_2O_2-challenged RAW264.7 cells compared with the H_2O_2 control ($p < 0.01$) (Table 3.4). Similar to the BWSPHs tested in this study, which contained a high content of low MW peptides, large yellow croaker (*Pseudosciaena crocea*) protein hydrolysate (MW < 3 kDa) exhibited $O_2^-\cdot$ scavenging activity and DPPH scavenging activity in vitro and also effectively and dose-dependently (50–300 μg/mL) increased the activity of antioxidant enzymes GSH-Px, SOD, and CAT in H_2O_2-induced oxidative stress in HepG2 cells ($p < 0.05$) (Zhang et al., 2017). In addition, numerous fish-derived peptides have demonstrated ability to modulate oxidative stress pathways in vitro (Chen et al., 2019; Heffernan et al., 2021; Hu et al., 2020; Mendis et al., 2005; Tao et al., 2018).

The simulated GI digest of BW-SPH-A also significantly increased GSH concentration in oxidatively stressed RAW264.7 cells compared to treated controls ($p < 0.05$), indicating that antioxidant peptides and/or antioxidant free amino acids were released from parent peptides during digestion (Table 3.4). SGID did not alter the ability of BW-SPH-A to increase GSH levels in oxidatively stressed RAW264.7 cells

(Table 3.4) but did inhibit its ability to promote CAT activity (Table 3.4) indicating different components of the hydrolysate mediate GSH and CAT. In contrast, BW-SPH-E had no effect on CAT activity, whereas BW-SPH-E-GI did indeed enhance CAT activity compared with the H_2O_2 control ($p < 0.05$), possibly due to the release of encrypted antioxidant peptides/free amino acids during SGID. Interestingly, BW-SPH-A and BW-SPH-E were observed to have high DH values (43.19 ± 2.16 and 42.97 ± 3.30, respectively) and a high quantity of components <1 kDa (77.86 ± 0.16 and 69.74 ± 0.13, respectively) (Harnedy-Rothwell et al., 2021), therefore it is possible that the generation of short-chain peptides and free amino acids influenced subsequent antioxidant potential. Although BW-SPH-B and its simulated GI digest, BW-SPH-B-GI, did not alter GSH concentration or CAT activity significantly compared with controls, there was a significant difference between activities pre- and post-SGID ($p < 0.05$).

The fluorescence indicator DCFH-DA was used to detect H_2O_2-induced ROS production in RAW264.7 cells, which were pre-incubated with BWSPHs and SGID BWSPHs. RAW264.7 cells treated with H_2O_2 for 60 min significantly increased ROS generation ($p < 0.001$) (data not shown). Interestingly, cells pre-incubated with either BW-SPH-A or its digest BW-SPH-A-GI (0.5% (w/v dw)) significantly attenuated the production of ROS in H_2O_2-challenged RAW264.7 cells ($p < 0.05$). No significant differences in ROS production were observed between the hydrolysates and their corresponding simulated GI digests ($p > 0.05$). In contrast, Zhang et al. (2018) reported that SGID soybean protein hydrolysate prepared with gastric proteases enhanced ROS inhibitory activity compared to the undigested soybean protein hydrolysate in H_2O_2 (1mM, 6 h)-stimulated caco-2 cells (Zhang et al., 2018). The soybean protein fraction obtained post-SGID had a higher DH and a higher content of short chain peptides than the pre-SGID fraction, which may have been responsible for its superior ROS reducing ability, possibly through enhanced cellular absorption. Subsequent isolation and characterisation of antioxidant peptides revealed each peptide sequence contained at least one of the following amino acid residues: Pro, Asp, Leu, Val, Arg, and His. The presence of hydrophobic amino acids is associated with high antioxidant activity through a radical scavenging mechanism. Interestingly, SGID BWSPHs, BW-SPH-A-GI, and BW-SPH-E-GI contained the highest levels of hydrophobic Met (1.01% and

1.03% w/w, respectively) and Phe (1.63% and 1.52% w/w, respectively) (Harnedy-Rothwell et al., 2021).

Arithmetical ranking of BWSPHs and SGID BWSPHs deemed BW-SPH-B, BW-SPH-C-GI, and BW-SPH-D-GI to be the lowest ranking of the hydrolysates tested for overall antioxidant potential, whereas BW-SPH-A and BW-SPH-A-GI were the top rankers (Table 3.5). Results obtained for SGID BWSPHs demonstrate that BW-SPH-A and BW-SPH-E may have potential applications as antioxidant agents in a functional food offering.

Table 3.4. Cellular antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion (SGID).

Sample Code (0.5% w/v dw)	GSH Concentration (% tBOOH)	CAT Activity (% H$_2$O$_2$)	ROS Production (% H$_2$O$_2$)
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
BW-SPH-A	140.3 ± 14.1 *	137.6 ± 7.4 **	86.4 ± 1.8 *
BW-SPH-A-GI	138.5 ± 12.7 *	133.2 ± 4.6	81.0 ± 3.7 *
BW-SPH-B	79.7 ± 11.1	123 ± 1.2	89.9 ± 2.1
BW-SPH-B-GI	124.8 ± 14.4 #	97.8 ± 4.2	90.7 ± 5.7
BW-SPH-C	102.3 ± 8.0	125.7 ± 3.1	89.7 ± 3.2
BW-SPH-C-GI	94.3 ± 13.2	138.3 ± 16.9	90.2 ± 3.0
BW-SPH-D	82.5 ± 12.2	125.6 ± 9.1	92.4 ± 3.0
BW-SPH-D-GI	108.7 ± 13.8	116.6 ± 9.3	104.3 ± 7.6
BW-SPH-E	108.6 ± 8.8	110.6 ± 11.6	94.7 ± 7.3
BW-SPH-E-GI	113.0 ± 9.4	146.4 ± 11.4 *	91.7 ± 3.5
BW-SPH-F	97.2 ± 13.4	128.4 ± 10.4	104.1 ± 6.5
BW-SPH-F-GI	113.7 ± 4.5	128.5 ± 13.8	96.1 ± 4.0

Antioxidant potential of BWSPHs (BW-SPH-A–F) and SGID BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (w/v dw (dry weight)) as assessed by their ability to increase glutathione (GSH) concentration in tertbutyl hydroperoxide (tBOOH)-treated RAW264.7 cells, increase catalase (CAT) activity in hydrogen peroxide (H$_2$O$_2$)-treated RAW264.7 cells and reduce reactive oxygen species (ROS) production in H$_2$O$_2$-treated RAW264.7 cells. For GSH and CAT assays, RAW264.7 cells (1 × 10^5 cells/mL, 2 mL/well) were exposed to BWSPHs and SGID BWSPHs for 24 h, followed by a tBOOH (1 mM, 3 h) or H$_2$O$_2$ challenge (1 mM, 3 h), respectively. ROS production was measured in RAW264.7 cells (1 × 10^5 cells/mL, 200 mL/well) exposed to BWSPHs and SGID BWSPHs for 24 h, followed by a H$_2$O$_2$ challenge (1 mM, 1 h). Values were expressed as a percentage relative to positive control (100%). Values represent the mean ± SEM of at least three independent experiments. Statistical significance between samples and the control was measured using ANOVA followed by Dunnett's test. * and ** denote statistically significant differences between samples and the control at $p < 0.05$ and $p < 0.01$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by t-test.

Table 3.5. Arithmetical ranks of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) pre- and post-simulated gastrointestinal digestion (SGID) with respect to individual antioxidant parameters.

Sample Code	ORAC	FRAP	GSH	CAT	ROS INHIBITION	Sum of Rank	Final Rank
BW-SPH-A	5	5	1	4	3	18	2
BW-SPH-A-GI	1	7	2	2	1	13	1
BW-SPH-B	9	6	12	9	6	42	12
BW-SPH-B-GI	2	8	3	12	4	29	4
BW-SPH-C	12	3	8	7	9	39	9
BW-SPH-C-GI	10	11	10	3	7	41	11
BW-SPH-D	4	1	11	8	11	35	5
BW-SPH-D-GI	3	9	6	10	12	40	10
BW-SPH-E	8	4	7	11	5	35	5
BW-SPH-E-GI	6	10	5	1	2	24	3
BW-SPH-F	11	2	9	6	8	36	7
BW-SPH-F-GI	7	12	4	5	10	38	8

Depending upon the antioxidant potential assessed through antioxidant assays, scores were given to each BWSPH and SGID BWSPH and the pooled score was computed to indicate the cumulative antioxidant potential of each hydrolysate. Values were arranged in descending order for each parameter and arithmetic ranks were given. ORAC: oxygen radical absorbance capacity, FRAP; ferric reducing antioxidant power, GSH; reduced glutathione, CAT; catalase, ROS; reactive oxygen species.

3.3.4 Cellular Immunomodulatory Activity

The activation of macrophages is an important part of initiating defensive reactions ensuring effective innate and adaptive immunity. As observed in Figure 3.1, exposure of RAW264.7 cells to the endotoxin LPS for 24 h upregulated generation of the inflammatory mediator NO, compared with the untreated control ($p < 0.0001$). Figure 3.1 also demonstrates that all BWSPHs and SGID BWSPHs (0.5% (w/v dw)) increased NO production in LPS-induced RAW264.7 cells compared with the LPS control ($p < 0.05$). The NO producing ability of BW-SPH-F was the only hydrolysate which was significantly reduced by SGID ($p < 0.05$).

Upon exposure to invasive species, pathogen-recognition receptors (PRRs) of immune cells stimulate numerous signalling cascades resulting in the upregulation of inflammatory cytokines such as TNF-α and IL-6. TNF-α controls inflammatory response through upregulation of proinflammatory cytokines IL-6 and IL-1β as well as through the upregulation of endothelial cell adhesion molecules inducing leukocyte extravasation (Begue et al., 2006; Zhao et al., 2017). IL-6 plays an important role in immunity through terminal differentiation of B cells into immunoglobulin-secreting cells as well as regulating the balance between regulatory T cells and Th (T helper) 17 cells (Heinrich et al., 1990; Kimura & Kishimoto, 2010). No signal for IL-6 or TNF-α protein expression was detected in untreated controls indicating DMEM and FBS were endotoxin free (data not shown). In addition to increasing NO production, hydrolysates BW-SPH-A, -B, -C and -F also increased IL-6 production in LPS-stimulated cells ($p < 0.05$), with hydrolysates BW-SPH-C, -D, and E increasing levels of TNF-α compared with the LPS control ($p < 0.05$). This immunostimulation is likely to be lost as the hydrolysates transit the gut, as SGID data show that BW-SPH-F-GI was the only digest to enhance IL-6 and TNF-α cytokine levels in LPS-activated macrophage ($p < 0.05$) (Figures 3.2 and 3.3) compared with the LPS control, indicating the release of immunostimulant peptides from BW-SPH-F during the digestion process. Arithmetical ranking demonstrated that BW-SPH-F and BW-SPH-F-GI were the highest-ranking hydrolysates of the BWSPHs and SGID BWSPHs tested with respect to individual immunomodulatory parameters (Table 3.6). No relationship between free amino acid content (Harnedy-Rothwell et al., 2021) and the immunomodulatory effect of BW-SPH-F-GI was observed, suggesting a role for peptides in its bioactivity.
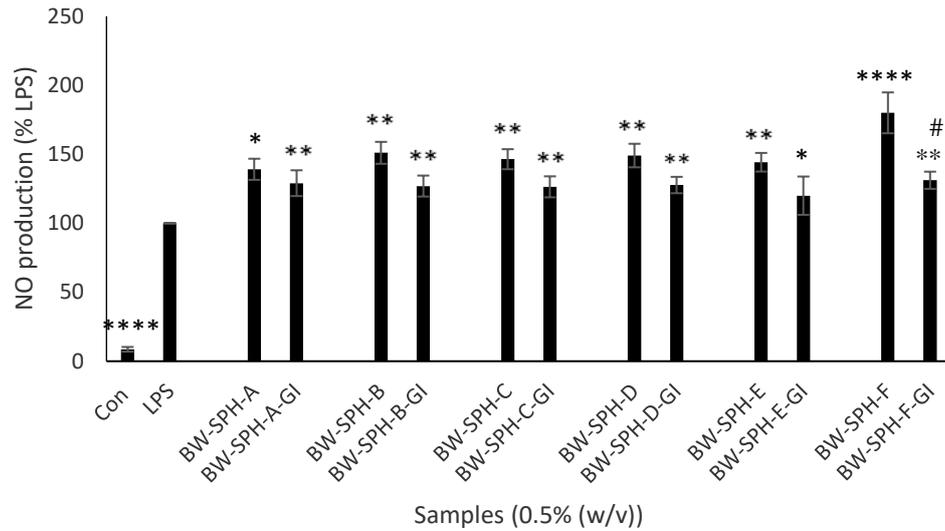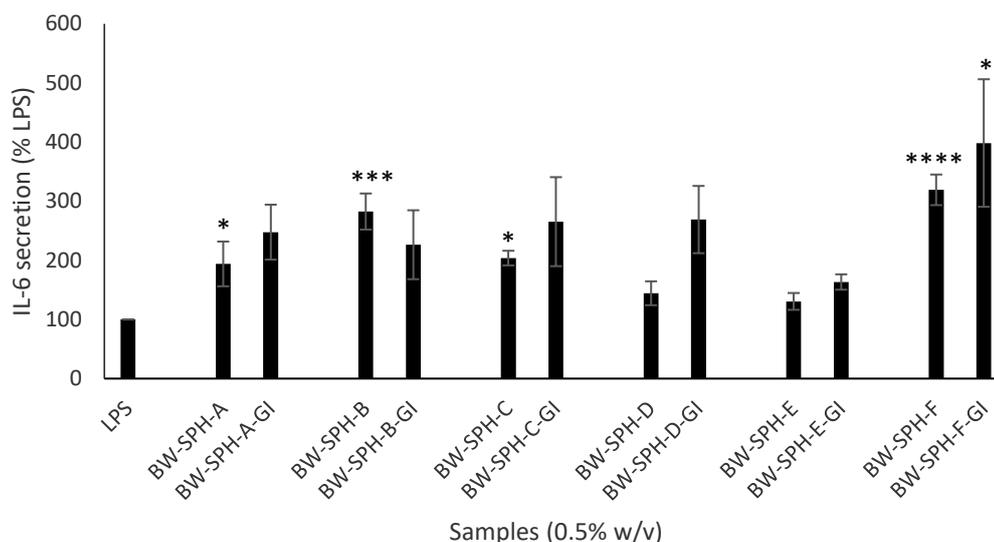
Figure 3.1. Effects of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (w/v dry weight) on nitric oxide (NO) production in lipopolysaccharide (LPS)-challenged RAW264.7 mouse macrophage. RAW264.7 cells (1×10^5 cells/mL, 200 µL/well) were simultaneously exposed to 2 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. NO was measured using Griess assay and values were expressed as a percentage relative to positive control (Con), cells treated with 2 µg/mL LPS alone (100% NO secretion). Values represent the mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. *, **, and **** denote statistically significant differences between sample and LPS control at $p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by t-test.
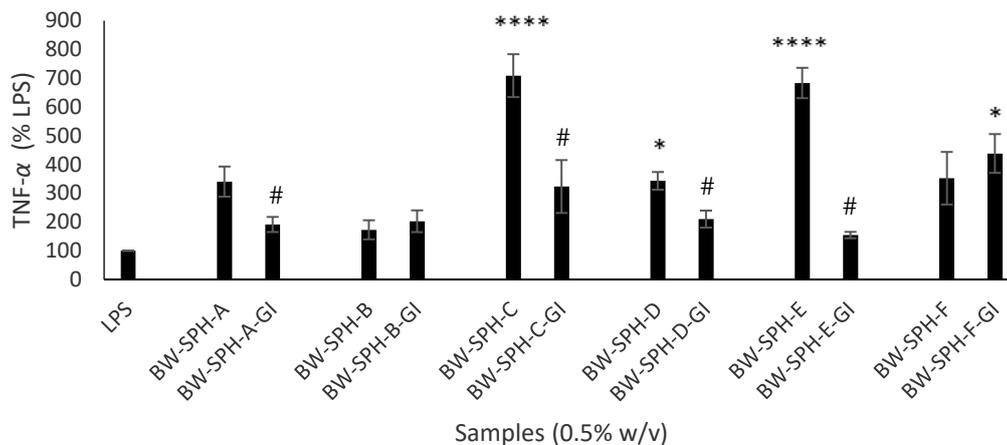
Figure 3.2. The effect of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (w/v dry weight) on IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW264.7 cells (0.2 × 10^5 cells/mL, 200 µL/well) were simultaneously exposed to 0.1 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. Values were expressed as a percentage relative to positive control, cells treated with 0.1 µg/mL LPS alone (100% IL-6 secretion). Values represent the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. *, ***, and **** denote statistically significant difference in IL-6 production between sample and LPS control at $p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively.

Figure 3.3. The effect of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (w/v dry weight) on TNF-α production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW264.7 cells (0.2 $\times$ 10^5 cells/mL, 200 µL/well) were simultaneously exposed to 0.1 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. Values were expressed as a percentage relative to positive control, cells treated with 0.1 µg/mL LPS alone (100% TNF-α secretion). Values represent the mean $\pm$ SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * and **** denote statistically significant difference in TNF-α production between sample and LPS control at $p < 0.05$ and $p < 0.0001$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by t-test.

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Table 3.6. Arithmetical ranks of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) pre- and post-simulated gastrointestinal digestion (SGID) with respect to individual immunomodulatory parameters.

Sample Code	NO	IL-6	TNF- α	Sum of Rank	Final Rank
BW-SPH-A	6	9	6	21	7
BW-SPH-A-GI	8	6	10	24	10
BW-SPH-B	2	3	11	16	4
BW-SPH-B-GI	10	7	9	26	11
BW-SPH-C	4	8	1	13	3
BW-SPH-C-GI	11	5	7	23	9
BW-SPH-D	3	11	5	19	5
BW-SPH-D-GI	9	4	8	21	7
BW-SPH-E	5	12	2	19	5
BW-SPH-E-GI	12	10	12	34	12
BW-SPH-F	1	2	4	7	1
BW-SPH-F-GI	7	1	3	11	2

Depending on the effects of BWSPH and SGID BWSPH on nitric oxide (NO), interleukin (IL)-6 and tumour necrosis factor (TNF)-α, scores were given to each hydrolysate and the pooled score was computed to indicate the cumulative immunomodulatory potential of each hydrolysate. Values were arranged in descending order for each parameter and arithmetic ranks were given.

In a similar cell model, exposure of wheatgrass to LPS-stimulated THP-1 monocytes increased NO production along with increasing levels of inflammatory cytokines TNF-α, IL-6, IL-1β compared with the LPS control ($p < 0.05$) (Rathor et al., 2017). Inflammatory compounds have been identified in numerous food components including fatty acids (Håversen et al., 2009), polysaccharides (Yang et al., 2015), and protein sources (Huang et al., 2014; Yang et al., 2015; Yang et al., 2020). Although the majority of immunomodulatory fish protein hydrolysates have been shown to mitigate proinflammatory mediators and cytokines, proinflammatory hydrolysates have been generated from giant croaker (*Nibea Japonica*) and tilapia fish with the ability to increase inflammation mediators and cytokines in cellular systems (Toopcham et al., 2017; Zhang et al., 2019). Intragastric administration of marine oligopeptide preparation from chum salmon (0, 0.22, 0.45, 1.35 g/kg bodyweight (BW), 4 weeks) did not activate macrophage cells, however innate and adaptive immunities were enhanced via the promotion of natural killer cell activity and stimulation of Th cells, thereby increasing the secretion of Th1 and Th2 cytokines (Yang et al., 2009). In addition, a fermented fish protein concentration prepared from pacific whiting, which has been shown to increase immunoglobulin (Ig)A+ cells, secretory-IgA (S-IgA), and cytokines IL-4, IL-6, IL-10, IFNγ, and TNF-α upon oral administration to BALB/c mice (0.30 mg/mL, 7 days), is now commercially available as Seacure® (Duarte et al., 2006). The findings of the present study demonstrated that BW-SPH-F may act as a nonspecific immunostimulant (i.e., not antigen specific) upon oral administration, with the potential to stimulate immune cells for therapeutic use in chronic infections, immunodeficiency, autoimmunity, and neoplastic diseases. The production of inflammatory mediators and cytokines must be regulated carefully, however, as excess secretion may negatively impact human health (Libby, 2007).

While previous studies have reported fish protein hydrolysates with antioxidant and anti-inflammatory properties (Da Rocha et al., 2018; Qian et al., 2020), we have identified hydrolysate BW-SPH-A herein, which exhibited antioxidant activities in oxidatively stressed RAW264.7 cells as well as proinflammatory effects in LPS-activated RAW264.7 cells. A study concluded that tripeptide glutathione, which decreased LPS-induced ROS generation, also stimulated the production of NO and proinflammatory cytokines in RAW264.7 cells via nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and Notch signal pathways (Kwon et al., 2019). Similarly, bioavailable whey peptides obtained post-SGID inhibited free

radicals in oxidatively stressed muscle and liver cells and increased the secretion of proinflammatory cytokine IL-1β from LPS-stimulated THP-1 macrophages (Corrochano et al., 2019). However, in this study, the immunostimulating activity of BW-SPH-A was lost post-SGID.

3.4 Conclusions

The results presented herein are, to the best of our knowledge, the first evidence of a protein hydrolysate purified from blue whiting exhibiting antioxidant or immunomodulatory potential in a cellular model. Hydrolysate BW-SPH-A, which enhanced cellular redox status pre- and post-SGID, may have potential as an effective natural antioxidant. Hydrolysate BW-SPH-F, which increased the production of proinflammatory mediators and cytokines, also maintained its bioactivity post-SGID; therefore, it may have application as an immunostimulant with potential to improve the quality of life of immunosuppressed patients. The extraction and identification of biofunctional protein hydrolysates presents an opportunity to increase the value of low-value blue whiting through their applications as high-value functional food ingredients. Future experimentation should involve the identification and characterization of peptides responsible for the antioxidant and immunomodulatory activities exhibited by BW-SPH-A and BW-SPH-F, respectively. In vivo interventional studies should be used to assess the biofunctional activities of BWSPHs, as well as to examine the capacity of gut digestive enzymes to functionalise hydrolysates in addition to the study of potential bioactive peptide protection mechanisms, such as microencapsulation, for the fractions that lost activity during simulated gut transit.

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Chapter 4

Blue whiting (*Micromesistius poutassou*) protein hydrolysates increase GLP-1 secretion and proglucagon production in STC-1 cells whilst maintaining Caco-2/HT29-MTX co-culture integrity

Abstract

Inducing the feeling of fullness via the regulation of satiety hormones presents an effective method for reducing excess energy intake and, in turn, preventing the development of obesity. In this study, the ability of blue whiting soluble protein hydrolysates (BWSPHs) and simulated gastrointestinal digested (SGID) BWSPHs, to modulate the secretion and/or production of satiety hormones glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) was assessed in murine enteroendocrine STC-1 cells. All BWSPHs (BW-SPH-A to BW-SPH-F) (1.0% w/v dw) increased active GLP-1 secretion and proglucagon production in STC-1 cells compared to the basal control (Krebs-Ringer buffer) ($p<0.05$). The signalling pathway activated for GLP-1 secretion was also assessed. A significant increase in intracellular calcium levels was observed after incubation with all BWSPHs ($p<0.05$) compared with the control, although none of the BWSPHs altered intracellular cyclic adenosine monophosphate (cAMP) concentrations. The secretagogue effect of the leading hydrolysate was diminished after SGID. Neither pre- nor post-SGID hydrolysates affected epithelial barrier integrity or stimulated interleukin (IL)-6 secretion in differentiated Caco-2/HT-29MTX co-cultured cells. These results suggest a role for BWSPH-derived peptides in satiety activity, however, these peptides may need to be protected by some means to avoid loss of activity during gastrointestinal transit.

4.1 Introduction

Excess energy intake is the main contributor to the increasing prevalence of obesity worldwide with overweight and obesity now taking fifth place in the leading causes of global death (Romieu et al., 2017; WHO, 2021). Researchers are currently investigating various potential obesity prevention strategies in an attempt to manage the global obesity pandemic and to alleviate the pressure that obesity and obesity-related diseases place on our healthcare systems. Inducing the feeling of fullness through the regulation of hormonal signalling presents an effective method for reducing food intake and in turn, preventing the onset of obesity (Cifuentes & Acosta, 2021). Satiety hormones which can be categorized as either long-term or short-term regulators, inform the brain about fluctuations of body mass and communicate the energy available in the gastrointestinal tract, respectively (Pucci & Batterham, 2020). Numerous weight loss drug-therapies, including satiety hormone analogues, are available commercially (Williams et al., 2020), however, identification of non-pharmacological satiating components from food sources may present cost-effective, safe alternatives to synthetic drugs for weight management.

The fish species blue whiting (*Micromesistius poutassou*) has been landed in high volumes in the Northeast Atlantic recently due to the Common Fisheries Policy reform (EU 1380/2013, Article 15) which introduced a landing obligation for all commercial fisheries. As there is a limited number of species that dominate seafood consumption, the majority of blue whiting is processed into fishmeal and oil. The opportunity now exists to up-value this underutilised source of high-quality protein through the identification of health-enhancing protein fractions with potential applications as high-value functional food ingredients. Several marine-derived peptide mixtures and hydrolysates are commercially available worldwide as anti-obesity food supplements, many of which contain a large variety of non-identified or partially identified peptides (Kondrashina et al., 2021). Blue whiting muscle protein hydrolysates have previously been shown to modulate the secretion of satiety hormones *in vitro* and *in vivo*, associated with a subsequent reduction in food intake and body weight gain (Cudennec et al., 2012), however utilization of the whole fish as the starting material for bioactive peptide production would reduce the yield of fish by-products, presenting environmental benefits. There are considerable advantages to identifying bioactive proteins from blue whiting fish for application as functional food

ingredients, such as (i) the discovery of a potential natural therapeutic approach for disease prevention, in place of or in combination with reduced doses of conventional synthetic drugs, (ii) managing environmental sustainability through exploiting abundant, underutilised blue whiting fish stocks, and (iii) promoting commercial sustainability through converting low-value blue whiting to high-value proteinaceous ingredients. However, the development of functional foods is faced with many challenges and is dependent on i) cost-effective generation of bioactive compounds, ii) identification of the level of bioactive compound required to reach target cells to exert their bioactive effect, taking potential degradation during digestion and absorption into account and iii) safety of the final product (Kondrashina et al., 2021).

The gastrointestinal tract is the site of nutrient digestion and absorption and plays a key role in mediating the physiological effects induced by ingested nutrients as it represents the largest endocrine organ in the body (Lu et al., 2021). It is generally accepted that enteroendocrine cells, which are found scattered along the epithelial layer of the gastrointestinal tract, are characterised by the hormones they secrete, i.e., I cells secreted cholecystokinin (CCK) and L cells secreted glucagon-like peptide-1 (GLP-1) and peptide YY (PYY); however, a study by Haber et al. (2017) observed an unexpected degree of heterogeneity in hormone expression from specific enteroendocrine cells (Haber et al., 2017). With that being said, gut hormone expression does vary along the gastrointestinal tract with CCK predominantly expressed in the duodenum and jejunum, GLP-1 in the jejunum, ileum, and colon and PYY in the distal ileum and colon (Psichas et al., 2015). These hormones are secreted basolaterally in the gut barrier upon exposure of enteroendocrine cells to digested nutrients to modulate physiological responses including gastric emptying, gut mobility, and central nervous system signalling. Although a heterogenous cell line, STC-1 cells which are derived from murine enteroendocrine tumours, are capable of secreting satiety hormones including CCK, PYY and GLP-1, thus are a popular cellular model for the assessment of nutrient-stimulating hormone release (Huang et al., 2020).

The GLP-1 hormone, which is generated as a result of post translational modifications during proglucagon processing, has received significant attention due to its classification as both an anorexigenic hormone and an incretin hormone (Watkins et al., 2021). Fish protein hydrolysates which modulated plasma GLP-1 levels have demonstrated anti-hyperglycaemic and satiating effects in clinical trials (Sharkey et

al., 2020) and have been shown to induce GLP-1 secretion *in vitro* through modulating intracellular levels of cyclic adenosine monophosphate (cAMP) and/ or Ca^{2+} (Harnedy et al., 2018). Nutrient-induced GLP-1 secretion may occur in response to increased intracellular levels of cAMP and/ or Ca^{2+} through activation of G-protein coupled receptors or nutrient transporters via enteroendocrine cell membrane depolarization (Nakamura et al., 2020). Although GLP-1 is produced in its active forms (GLP-1$_{(7-36)}$ amide and GLP-1$_{(7-37)}$), the lifespan of active GLP-1 is limited with a circulation half-life of less than 2 minutes due to cleavage by endogenous proteolytic enzyme dipeptidyl peptidase IV (DPP-IV) to its inactive forms (GLP-1$_{(9-36)}$ amide or GLP-1$_{(9-37)}$) (Sharma et al., 2018). Therefore, not only can food components promote GLP-1 hormone signalling through increasing the production/ release of GLP-1, or activating GLP-1 receptors, but also by increasing hormone circulation time via DPP-IV inhibition.

Protein and hydrolysed protein fractions are reported to induce greater satiating effects than carbohydrates and fats (Santos-Hernández et al., 2018). Satiating proteins have been identified in numerous food sources including plants, pulses, eggs, dairy, seafood and meat (Greco et al., 2017), however, their digestion, bioavailability and metabolism have not been studied sufficiently (Kondrashina et al., 2021). In addition to proteases in the gut lumen, proteins/ hydrolysates/ peptides can be further degraded by brush border membrane proteases or blood plasma proteases resulting in physicochemical changes which can alter their bioactive potential. Moreover, it is important that bioactive protein digestion products which target gut cells do not cause inflammation or damage to the gut barrier.

The research presented herein is a continuation of a study by Harnedy-Rothwell et al. (2021) in which blue whiting (*Micromesistius poutasso*) soluble protein hydrolysates (BWSPHs) were prepared using food-grade microbial proteases and assessed in terms of their amino acid profile and physicochemical properties. In addition, all BWSPHs tested mediated DPP-IV inhibitory (IC$_{50}$: 2.12–2.90 mg protein/mL) and insulin secretory activity (2.5 mg/mL; 4.7 to 6.4-fold increase compared to the basal control (5.6 mM glucose alone)) (Harnedy-Rothwell et al., 2021), therefore, the present study was designed to assess the ability of BWSPHs to modulate the secretion and/or production of satiety hormones GLP-1, CCK and PYY in STC-1 cells. Additionally, the impact, if any, simulated gastrointestinal digestion (SGID) has on this activity was studied, as well as the mechanism by which the leading

hydrolysate mediates hormonal signalling. Finally, the effect of SGID BWSPHs on intestinal barrier integrity and inflammation, using a 21-day-old differentiated Caco-2/HT-29MTX monolayer, as a model of a healthy gut barrier was investigated.

4.2 Materials and Methods

4.2.1 Materials

Human Caco-2 cell line were obtained from the European Collection of Authenticated Cell Cultures (Salisbury, Wilts, UK). Murine STC-1 and human HT-29MTX cell lines were kindly gifted by Dr. Giblin (Teagasc Food Research Centre, Moorepark, Fermoy, Ireland). Mouse IL-6 ELISA kit was purchased from ThermoFisher Scientific (BioSciences, Ireland). Halt Protease and phosphatase inhibitor was purchased from ThermoFisher Scientific (MSC, Ireland). Mouse Metabolic Magnetic Bead Panel for active GLP-1 analysis was from Millipore (Ireland). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). RNeasy mini kit, DNase digestion kit and the QuantiTect Reverse Transcription kit were from Qiagen (UK). LightCycler 480 SYBR Green was from Roche (Roche Products Ireland Limited). Forskolin and cAMP enzyme-linked immunosorbent assay (ELISA) kit were from Enzo Life Sciences (UK). Cell culture plastics were supplied by Corning incorporated and Cruinn Diagnostics (Dublin, Ireland). Fluo-4 AM calcium indicator was from Molecular Probes (UK). The remaining chemicals and cell culture reagents were from Sigma Chemical Co. (Dublin, Ireland) unless stated otherwise. Bio-Marine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland) supplied the BWSPHs.

4.2.2 Sample Preparation

Bio-Marine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland) supplied the six BWSPHs which were purified from minced blue whiting as described by Harnedy-Rothwell et al. (2021) (Harnedy-Rothwell et al., 2021). For the generation of hydrolysates BW-SPH-A, BW-SPH-B, BW-SPH-E and BW-SPH-F, fish was solubilized in water at a fish to water ratio of 2:1, whereas a 1.7:1 fish to water ratio was used for the production of hydrolysates BW-SPH-C and BW-

SPH-D (Harnedy-Rothwell et al., 2021). The six hydrolysates were prepared with different food-grade, microbial proteases/ protease combinations with enzyme to substrate ratios (E:S) ranging from 0.005% w/w to 0.900% w/w and hydrolysis time ranging from 45 to 120 mins. The same temperature (50 °C) was used for the generation of the six hydrolysates (Harnedy-Rothwell et al., 2021). Harnedy-Rothwell et al. (2021) completed SGID of the BWSPHs using pepsin (E:S of 2.5% w/w, 37°C, pH 2, 90 min) and Corolase PP (E:S of 1% w/w, 37°C, pH 7, 150 min)(Harnedy-Rothwell et al., 2021). The study by Harnedy-Rothwell et al. (2021) also details the physicochemical properties of BWSPHs pre- and post-SGID including the degree of hydrolysis, molecular mass distribution and total and free amino acid composition (Harnedy-Rothwell et al., 2021). The degree of hydrolysis ranged from 27.82% to 45.78% in undigested hydrolysates and 55.37% to 65.23% in SGID hydrolysates. The BWSPHs and SGID BWSPHs were prepared directly with Krebs-Ringer buffer, sterile-filtered and stored at -20 °C for STC-1 cell viability and satiety hormone secretion/ production analysis. The BWSPHs and SGID BWSPHs were prepared in Hank's Balanced Salt Solution (HBSS), sterile-filtered and stored at -20 °C for Caco-2/HT-29MTX co-culture cell viability, barrier integrity and cytokine secretion analysis.

4.2.3 Cell Culture

STC-1 cells, Caco-2 cells and HT-29MTX cells were grown in 75 cm^2 tissue culture flasks and cultured in antibiotic free Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% (v/v) FBS. STC-1 cells (between passage numbers 23 and 35), Caco-2 cells (between passage numbers 66 and 72) and HT29-MTX cells (between passage numbers 64 and 70) were incubated in an atmosphere of 5% CO_2 at 37°C. Media was refreshed every 48 hours and cells were passaged every 2-3 days (80-90% confluence).

4.2.4 Co-Culture

Caco-2 cells (75%) and HT-29MTX cells (25%) in DMEM with 10% FBS was added to polyester permeable-membrane inserts in 12 well plates at a final density of 6.0×10^4 cells/insert. DMEM with 10% FBS (1.5 mL) was added to the basolateral

compartment. Cells were differentiated over a 21-day period and apical and basolateral compartments received 500 µL and 1.5 mL of fresh culture media, respectively, every 48 h.

4.2.5 TEER Measurements

The co-culture monolayer integrity was analysed using a Millicell ERS-2 electrical-resistance system (Millipore, USA) on the final day of cell differentiation (day 21). TEER values were measured and co-culture monolayers were washed with HBSS two times. Only monolayers with TEER values exceeding 700 Ω.cm2 were used for experiments. BWSPHs were diluted in HBSS to a concentration of 1.0%, w/v dw and added to the apical compartment (500 µL) for 4 h at 37°C. Addition of HBSS alone (500 µL) to co-culture monolayers acted as the control. The basolateral compartments received 1.5 mL HBSS. To monitor the integrity of the monolayer, TEER values were recorded before (0 h) and after incubation (4 h). Finally, apical contents were collected and stored at −20 °C prior to cytokine analysis.

4.2.6 Cell Viability

To test STC-1 cell and Caco-2/HT-29MTX cell viability, STC-1 cells (2×10^5 cells/mL) and a mixture of 75% Caco-2 and 25% HT-29MTX cells (6×10^4 cells/mL) were seeded in 200 µL DMEM supplemented with 10% FBS in 96 well plates for 24 h at 37°C. Well contents were then aspirated and the six BWSPHs (coded BW-SPH-A, -B, -C, -D, -E and -F) and SGID BWSPHs (coded BW-SPH-A-GI, -B-GI, -C-GI, -D-GI, -E-GI and -F-GI) (0-1.0% w/v dw, 200 µL/well) or DMEM only (control), were added to wells for a further 24 h. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) which consisted of the MTT reagent and solubilisation solution was used to assess cell viability. BWSPHs and DMEM was removed from the wells and replaced with the MTT reagent (10 µL) and DMEM (100 µL) for 4 h at 37 °C. Solubilisation solution (100 µL) was then added to all of the wells and incubation at 37 °C continued overnight. Absorbance (570 nm) was read using a microplate reader (Varioskan TM Flash Multimode Reader, Thermoscientific, Waltham, MA, USA).

4.2.7 IL-6 Secretion

Caco-2/HT-29MTX co-culture apical samples were subjected to one freeze thaw cycle and the secretion of IL-6 was measured using eBioscience ELISA kits (Ready-SET-Go kit). This ELISA kit detects IL-6 at a minimum limit of 4.0 pg/mL

4.2.8 RNA Extraction and Real-Time Reverse Phase Polymerase Chain Reaction (rt-PCR)

Following a 4 h incubation period with Krebs-Ringer buffer (negative control) or BWSPHs (1.0% w/v dw), STC-1 cells were washed with Phosphate Buffered Saline (PBS) buffer, lysed in 350 µL of lysis buffer and stored overnight at -80 °C. RNA was extracted from the lysate using the RNeasy mini kit and genomic DNA was eliminated from the RNA preparation via on-column DNase digestion according to manufacturer's instructions. The Nanodrop 1000 (Thermo Fisher Scientific, USA) was used to quantify total RNA with OD 260/280 ratios ranging from 1.93-2.07. RNA (1 µg) was then used to synthesize cDNA. Real-time PCR (LightCycler 96, Roche Diagnostics, Germany) was used to determine proglucagon, CCK and PYY mRNA transcript levels. The various primer sequences, accession codes and annealing temperatures are presented in the table below (Table 4.1). Amplification efficiencies of primers ranged from 1.87–2.15 using cDNA dilutions of 1:1, 1:10, 1:100 and 1:1000. Each PCR reaction contained 1 µL cDNA, 0.5 µL of forward and reverse primers, 3 µL RNase free water and 5 µL of LightCycler 480 SYBR Green, making a final volume of 10 µL/well. For each sample, the relative amount of target was calculated by the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t$ is [C_t (target gene) – C_t (36B4)]test condition – [C_t (target gene) – C_t (36B4)]control condition and C_t is the cycle at which the threshold is crossed.

Table 4.1: Primer names, accession numbers, forward and reverse sequences and annealing temperatures.

Primer	Accession number	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	Annealing temperature
Proglucagon	Z46845.1	CCTTCAAGACACAGAGGAGAAC	GGAGTCGAGGTATTTGCTGTAG	56 °C
CCK	NM_001284508	CTGTCTGCATTTGGCTTGAC	GCCCACTACGATGGGTATTC	55 °C
PYY	NM_145435.1	AACTGCTCTTCACAGACGAC	GTGCCCTCTTCTTAAACCAAAC	55 °C
36B4 (RPLP0)	NM_007475	TGCCACACTCCATCATCAAT	CATCTGATTCCTCCGACTCTT	51°C

4.2.9 Active GLP-1 Secretion

STC-1 cells were seeded in 12 well plates at a density of 0.5×10^6 cells/mL in a final volume of 1.25 mL per well. Following a 24 h incubation period, media was removed, and the cells were washed with Krebs-Ringer buffer, which contains 11mM glucose. Cells were acclimatised in Krebs-Ringer buffer (500 µL) for 1 h. Buffer was then replaced 500 µL of BWSPHs and SGID BWSPHs (1.0% w/v dw) prepared in Krebs-Ringer buffer was added to the appropriate wells. Following a 4 h incubation period with Krebs-Ringer buffer (negative control) or BWSPHs, 100X Halt Protease and Phosphatase Inhibitor (5 µL) was added to inactivate endogenous DPP-IV activity. Supernatants were then collected and centrifuged (900 g, 4 °C, 5 min) and stored at -80 °C prior to GLP-1 analysis. The Milliplex Map Kit (Mouse Metabolic Magnetic BeadPanel) and MagPix fluorescent detection system (Luminex, The Netherlands) was used to quantify active GLP-1content in the range of 41 – 30000 pg/mL. Intra-assay and inter-assay variation were <10% and <15%, respectively.

4.2.10 Intracellular Ca^{2+} Assay

STC-1 cells were seeded in 96 well plates at a density of 0.25×10^6 cells/mL and final volume of 200 µL per well, for 24h. After 4 h exposure to 100 µL of BWSPHs (1.0% w/v dw), SGID BWSPHs (1.0% w/v dw), carbachol (1mM) (positive control) or Krebs Ringer buffer (negative control), Fluo-4 AM intracellular calcium probe prepared in Krebs-Ringer buffer (5 µM) was added and cells were further incubated for 1 h at 37° C. Cell monolayers were then washed 2 times with Krebs-Ringer buffer which was supplemented with sulfinpyrazone (0.1 mM) to inhibit changes in cytosolic calcium levels. Fluorescence (excitation 488 nm and emission 520 nm) was measured using a microplate reader (Varioskan TM Flash Multimode Reader, Thermoscientific, Waltham, MA, USA).

4.2.11 cAMP Accumulation Assay

STC-1 cells were seeded at 0.5×10^6 cells/mL in a final volume of 1.25 mL per well of a 12 well plate. After 24 hours, well contents were removed, and monolayers were washed with Krebs Ringer buffer as described above. 3-isobutyl-1-

methylxanthine (IBMX) (1mM) was added to BWSPH sample wells and control wells (positive control was 10 μM forskolin, negative control was Krebs Ringers buffer) before a 4 h incubation. STC-1 supernatants were then aspirated off and cell lysates were collected after 10 min incubation with 0.2 mL of 0.1 M hydrochloric acid at room temperature. Lysates were then centrifuged (900 × g for 5 min) and stored at −80 °C before cAMP analysis. A direct cAMP ELISA kit with a minimum detection limit of 0.39 pmol/ mL was used to measure intracellular cAMP levels and absorbance was read at 405 nm using a microplate reader (Varioskan TM Flash Multimode Reader, Thermoscientific, Waltham, MA, USA).

4.2.12 Statistical Analysis

The data in this study were collected from at least three independent experiments and all results are expressed as the mean ± standard error of the mean (SEM). Significant differences between sample groups and control groups were analysed using a one-way analysis of variance (ANOVA) followed by Dunnett's test and Student's t-test was used to determine significant differences between hydrolysates before and after SGID (Prism 5.0, GraphPad Inc. San Diego, CA, USA.

4.3 Results

4.3.1 BWSPHs have no effect on cell viability

   The cytotoxicity of six BWSPHs (BW-SPH-A to BW-SPH-F) and six SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) (0 - 1.0% w/v dw) in STC-1 cells (Table 4.1) and a Caco-2/HT-29MTX co-cultured cells (Table 4.2) after 24 h incubation, was studied. There was no significant difference in the viability of cells exposed to BWSPHs (0 – 1.0% w/v dw) compared with the control (DMEM) indicating the non-toxic effect of BWSPHs and their corresponding simulated gastrointestinal digests in STC-1 cells and the co-cultured cells (Tables 4.1 and 4.2). Based on these results, a protein hydrolysate concentration of 1.0% w/v dw was chosen for all cellular assays.

Table 4.1. The effects of blue whiting protein soluble hydrolysates (BWSPHs) pre- and post- simulated gastrointestinal digestion (SGID) on the viability of murine STC-1 cells.

	BWSPH concentration (w/v, dw)							
	0.00%	0.05%	0.10%	0.25%	0.50%	0.75%	0.90%	1.00%
	Cell viability (%)							
BW-SPH-A	100.0 ± 0.0	110.0 ± 6.1	105.9 ± 3.8	99.0 ± 0.5	99.0 ± 4.3	99.9 ± 5.8	97.8 ± 5.0	88.1 ± 7.0
BW-SPH-A-GI	100.0 ± 0.0	105.8 ± 10.3	114.0 ± 11.5	116.4 ± 12.3	118.5 ± 14.7	111.3 ± 10.5	116.4 ± 13.2	96.4 ± 4.8
BW-SPH-B	100.0 ± 0.0	105.5 ± 5.2	101.8 ± 4.1	102.0 ± 2.4	96.8 ± 3.6	103.3 ± 5.7	101.6 ± 5.6	93.3 ± 2.3
BW-SPH-B-GI	100.0 ± 0.0	127.3 ± 4.1	118.6 ± 4.4	119.3 ± 2.4	129.1 ± 5.4	127.0 ± 7.6	123.3 ± 8.8	96.7 ± 5.1
BW-SPH-C	100.0 ± 0.0	107.6 ± 8.0	106.6 ± 5.3	106.3 ± 7.1	99.9 ± 7.3	108.6 ± 8.3	103.8 ± 6.4	92.2 ± 8.5
BW-SPH-C-GI	100.0 ± 0.0	123.3 ± 0.5	117.1 ± 3.5	128.3 ± 2.9	128.2 ± 5.3	129.5 ± 7.8	122.7 ± 9.8	93.0 ± 3.1
BW-SPH-D	100.0 ± 0.0	93.8 ± 3.2	84.8 ± 4.7	86.8 ± 6.1	84.5 ± 7.9	83.0 ± 7.9	82.4 ± 7.2	82.4 ± 9.0
BW-SPH-D-GI	100.0 ± 0.0	122.4 ± 7.5	114.3 ± 5.9	121.1 ± 4.4	124.6 ± 7.5	116.0 ± 7.0	122.6 ± 7.8	94.3 ± 0.8
BW-SPH-E	100.0 ± 0.0	106 ± 5.4	104.4 ± 4.2	100.8 ± 4.1	97.8 ± 5.0	99.2 ± 2.9	99.5 ± 4.6	86.8 ± 0.7
BW-SPH-E-GI	100.0 ± 0.0	122.5 ± 5.2	117.7 ± 5.5	124.8 ± 5.6	124.2 ± 9.4	122.0 ± 8.2	113.8 ± 5.3	94.4 ± 3.7
BW-SPH-F	100.0 ± 0.0	103.7 ± 2.0	100.2 ± 2.4	105.8 ± 2.2	95.7 ± 2.9	98.7 ± 6.3	95.6 ± 1.4	84.4 ± 0.3
BW-SPH-F-GI	100.0 ± 0.0	105.7 ± 8.7	106.4 ± 10.5	104.1 ± 8.4	106.8 ± 10.9	103.1 ± 6.4	116.0 ± 6.6	103.8 ± 5.0

Murine STC-1 cells (2×10^5 cells/mL, 200 µL/well) were supplemented with increasing concentrations (0-1.0%, w/v dw) of BWSPHs (BW-SPH-A to BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) for 24 hours. Cell viability was determined by the MTT assay and expressed as a percentage of the untreated control. Data are the mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

Table 4.2. The effects of blue whiting protein soluble hydrolysates (BWSPHs) pre- and post- simulated gastrointestinal digestion (SGID) on the viability of a Caco-2/HT-29MTX co-culture.

	BWSPH concentration (w/v, dw)							
	0.00%	0.05%	0.10%	0.25%	0.50%	0.75%	0.90%	1.00%
	Cell viability (%)							
BW-SPH-A	100.0 ± 0.0	95.8 ± 8.5	104.4 ± 6.5	111.5 ± 11.1	114.5 ± 12.0	113.2 ± 4.0	111.6 ± 9.0	110.1 ± 6.6
BW-SPH-A-GI	100.0 ± 0.0	102.5 ± 6.1	107.1 ± 9.3	109.0 ± 7.2	107.8 ± 7.6	104.6 ± 1.8	106.1 ± 3.3	102.7 ± 5.7
BW-SPH-B	100.0 ± 0.0	100.3 ± 3.0	102.2 ± 4.6	99.2 ± 4.6	102.9 ± 6.4	100.7 ± 1.5	101.0 ± 3.1	109.0 ± 14.9
BW-SPH-B-GI	100.0 ± 0.0	111.0 ± 4.5	109.7 ± 5.5	104.5 ± 2.6	110.6 ± 3.7	111.0 ± 3.6	114.0 ± 6.2	107.5 ± 7.9
BW-SPH-C	100.0 ± 0.0	104.7 ± 2.8	107.2 ± 7.1	102.5 ± 2.7	103.9 ± 10.6	97.5 ± 8.8	107.3 ± 5.4	102.3 ± 8.3
BW-SPH-C-GI	100.0 ± 0.0	93.4 ± 6.5	103.3 ± 5.9	107.2 ± 4.6	102.0 ± 6.7	105.4 ± 4.4	106.3 ± 3.1	96.6 ± 5.9
BW-SPH-D	100.0 ± 0.0	102.7 ± 8.1	99.1 ± 10.5	106.4 ± 8.6	102.4 ± 12.2	99.9 ± 10.0	102.2 ± 10.3	110.7 ± 2.9
BW-SPH-D-GI	100.0 ± 0.0	93.3 ± 9.1	105.8 ± 1.5	104.2 ± 6.1	106.8 ± 3.1	108.2 ± 5.1	113.9 ± 5.9	98.0 ± 5.5
BW-SPH-E	100.0 ± 0.0	100.2 ± 7.7	103.9 ± 8.9	105.5 ± 6.5	108.6 ± 9.0	100.2 ± 5.1	102.4 ± 4.7	107.7 ± 7.3
BW-SPH-E-GI	100.0 ± 0.0	109.8 ± 4.4	114.9 ± 3.0	109.3 ± 4.7	118.3 ± 7.5	116.9 ± 6.0	122.5 ± 3.8	108.4 ± 9.8
BW-SPH-F	100.0 ± 0.0	96.4 ± 8.9	97.0 ± 6.2	94.4 ± 4.9	99.7 ± 12.0	102.8 ± 7.9	97.8 ± 9.3	112.0 ± 8.0
BW-SPH-F-GI	100.0 ± 0.0	112.3 ± 11.4	124.0 ± 8.0	116.2 ± 7.6	123.5 ± 10.4	117.9 ± 9.9	124.8 ± 4.0	114.2 ± 2.5

Caco-2/HT-29MTX co-culture (6 x 10^4 cells/mL, 200 µL/well) were supplemented with increasing concentrations (0-1.0%, w/v dw) of BWSPHs (BW-SPH-A to BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) in DMEM media for 24 hours. Cell viability was determined by the MTT assay and expressed as a percentage of the untreated control '0%' (DMEM only). Data are the mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

4.3.2 BWSPHs do not induce inflammation or disrupt co-culture integrity

After 21 days, cell monolayers were considered to have reached confluence and were fully polarised with TEER values exceeding 700 Ω.cm^2. As shown in Figure 4.1, BWSPHs and SGID BWSPHs did not alter cell monolayer integrity with TEER values ranging from $774.3 \pm 57.2 - 898.3 \pm 109.3$ Ω.cm^2 and $758.0 \pm 23.7 - 860.0 \pm 94.3$ Ω.cm^2, respectively compared with the HBSS buffer control (787.0 ± 26.2 Ω.cm^2) after 4 h exposure. An inflammatory effect of BWSPHs and their corresponding gastrointestinal digests on human intestinal epithelial cells was investigated by measuring the reputable pro-inflammatory cytokine, interleukin (IL)-6 in the apical compartment of Caco-2/HT-29MTX co-cultured cells which were exposed to the test samples for a 4 h period. IL-6 concentrations in the co-culture apical compartments containing BWSPHs, SGID BWSPHs or HBSS buffer were below the minimum limit of detection (4pg/ml), indicating that these protein hydrolysates could not induce an IL-6 response upon contact with gut epithelial cells.

Figure 4.1. Transepithelial electrical resistance (TEER) values of 21-day-old differentiated Caco-2/HT-29MTX monolayers treated with blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates before (BW-SPH-A to BW-SPH-F) and after simulated gastrointestinal digestion (BW-SPH-A-GI to BW-SPH-F-GI) for 4 h. The control is Hank's Balanced Salt Solution (HBSS) only. Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

4.3.3 BWSPHs increase proglucagon production and GLP-1 secretion from STC-1 cells

To determine the potential of BWSPHs to modulate satiety, their ability to produce satiety hormones at a transcriptional level and increase active GLP-1 secretion in murine enteroendocrine STC-1 cells was assessed. GLP-1 precursor proglucagon mRNA transcript levels were upregulated upon exposure of STC-1 to BWSPHs for 4 h compared with the negative control (Krebs-Ringer buffer) ($p<0.05$) (Figure 4.2). However, post-SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) did not alter proglucagon production significantly ($p>0.05$) compared to the basal control. In addition, no significant differences were observed between proglucagon levels produced by BWSPHs before SGID and after SGID (Figure 4.2).

Hydrolysates BW-SPH-A, BW-SPH-C and BW-SPH-F reduced CCK mRNA transcript levels compared to the basal control ($p<0.05$), whereas BW-SPH-B, BW-SPH-D and BW-SPH-E induced no effect on CCK production after the 4 h incubation period in STC-1 cells ($p>0.05$) (Figure 4.3). In addition, all BWSPHs reduced PYY mRNA transcript levels compared with the Krebs-Ringer buffer control ($p<0.05$) (Figure 4.4). The CCK and PYY mRNA transcript levels observed after STC-1 cell exposure to SGID BWSPHs were not significantly different to the control ($p>0.05$) (Figure 4.3 and Figure 4.4). In addition, no significant differences were observed between CCK and PYY levels produced by BWSPHs before SGID and after SGID ($p>0.05$) (Figure 3 and Figure 4.4).
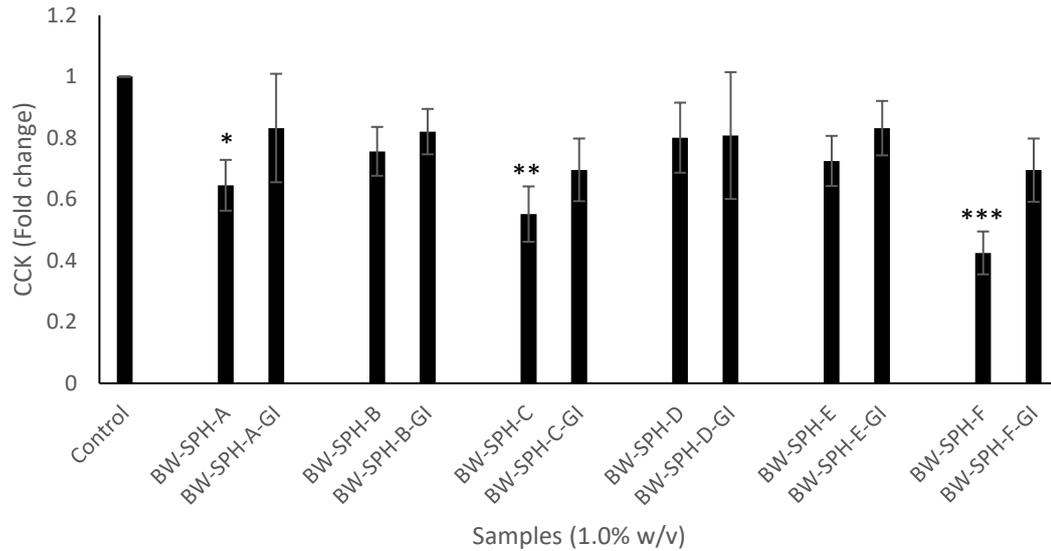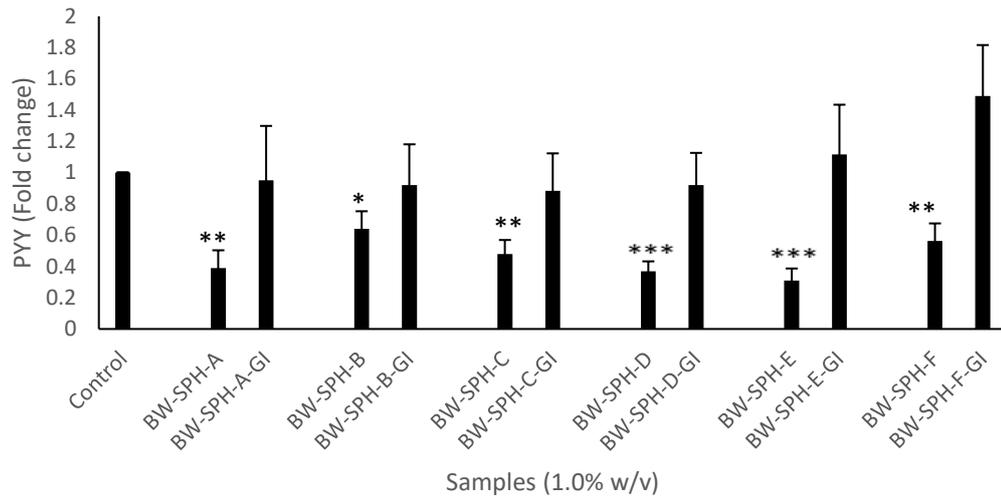
Figure 4.2. Levels of proglucagon mRNA transcripts in STC-1 cell lysates after exposure to blue whiting protein hydrolysates before (BW-SPH-A to BW-SPH-F) and after simulated gastrointestinal digestion (BW-SPH-A-GI to BW-SPH-F-GI) for 4 h in Krebs Ringers buffer. *36B4* (RPLP0) was used as a reference gene. Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. * denotes statistical significance between individual samples and the control (Krebs Ringer buffer) ($p < 0.05$), ** $p < 0.01$, *** $p < 0.001$.

Figure 4.3. Levels of cholecystokinin (CCK) mRNA transcripts in STC-1 cell lysates after exposure to blue whiting protein hydrolysates before (BW-SPH-A to BW-SPH-F) and after simulated gastrointestinal digestion (BW-SPH-A-GI to BW-SPH-F-GI) for 4 h in Krebs Ringers buffer. *36B4* (RPLP0) was used as a reference gene. Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. * denotes statistical significance between individual samples and the control (Krebs Ringer buffer) ($p<0.05$).

Figure 4.4. Levels of peptide YY (PYY) mRNA transcripts in STC-1 cell lysates after exposure to blue whiting protein hydrolysates before (BW-SPH-A to BW-SPH-F) and after simulated gastrointestinal digestion (BW-SPH-A-GI to BW-SPH-F-GI) for 4 h in Krebs Ringers buffer. *36B4* (RPLP0) was used as a reference gene. Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. * denotes statistical significance between individual samples and the control (Krebs Ringer buffer) ($p < 0.05$).

Following a 4 h incubation period, all BWSPHs tested increased the secretion of active GLP-1 in STC-1 cells compared to the basal control (Krebs-Ringer buffer) ($p < 0.05$) (Figure 4.5A). The concentration of active GLP-1 secreted from STC-1 cells exposed to BWSPHs ranged from 2727.6 ± 160.5 pg/mL (BW-SPH-A) to 5163.8 ± 495.6 pg/mL (BW-SPH-F) with Krebs-Ringer buffer only stimulating the secretion of 769.7 ± 63.5 pg/mL active GLP-1 from STC-1 cells (Figure 4.5A). As active GLP-1 concentrations were highest in cells treated with undigested hydrolysate BW-SPH-F, SGID hydrolysate BW-SPH-F-GI was chosen to assess if *in vitro* digestion influenced BWSPH active GLP-1 stimulating ability. The GLP-1 stimulating ability of BW-SPH-F was lost following SGID as active GLP-1 concentrations in STC-1 cells exposed to BW-SPH-F-GI were significantly lower than the Krebs-Ringer buffer control ($p < 0.05$) (Figure 4.5B).

(A)

(B)

Figure 4.5 (A) Secretion of active glucagon-like peptide-1 (GLP-1) from STC-1 cells (0.625×10^6 cells/well) exposed to blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) at 1.0% (w/v dw) or Krebs-Ringer buffer (control) for 4 hrs. * denotes statistical significance between individual samples and the control measured using ANOVA followed by Dunnett's test ($p < 0.05$). **(B)** Secretion of active GLP-1 from STC-1 cells (0.625×10^6 cells/well) exposed to BWSPH BW-SPH-F post simulated gastrointestinal digestion (SGID) (BW-SPH-F-GI) at 1.0% (w/v dw) or Krebs-Ringer buffer (control) for 4 hrs expressed as a percentage of the control. * indicates a significant difference between the control and BW-SPH-F-GI values measured by Student's t-test ($p < 0.05$). Data are expressed as mean ± SEM of three independent experiments.

4.3.4 Effects of BWSPHs on GLP-1 signalling mechanisms

The effect of BWSPHs BW-SPH-A to BW-SPH-F and their simulated gastrointestinal digests (BW-SPH-A-GI to BW-SPH-F-GI) on electrogenic (Ca^{2+}) and electroneutral (cAMP) governed release of GLP-1 was assessed in STC-1 cells. Both the positive control carbachol (1mM) and the pre-SGID BWSPHs (1.0% w/v) increased intracellular Ca^{2+} levels compared with the basal control (Krebs Ringer buffer) after 4 h exposure to STC-1 cells ($p<0.05$) (Figure 4.6). However, post-SGID, all hydrolysates failed to elicit intracellular Ca^{2+} changes in STC-1 cells compared to the control and fluorescence intensities were significantly lower in SGID hydrolysates compared to their undigested forms (Figure 4.6). Neither the pre- nor post-SGID BWSPHs induced any effect on intracellular cAMP levels compared to the control (Krebs Ringer buffer supplemented with 1 mM IBMX) in contrast to the positive control, FSK (10 µM), which increased cAMP levels 4-fold (Figure 4.7). No differences in intracellular cAMP concentration were observed between the pre- and post SGID BWSPHs after statistical analysis with Student's t-test (Figure 4.7).
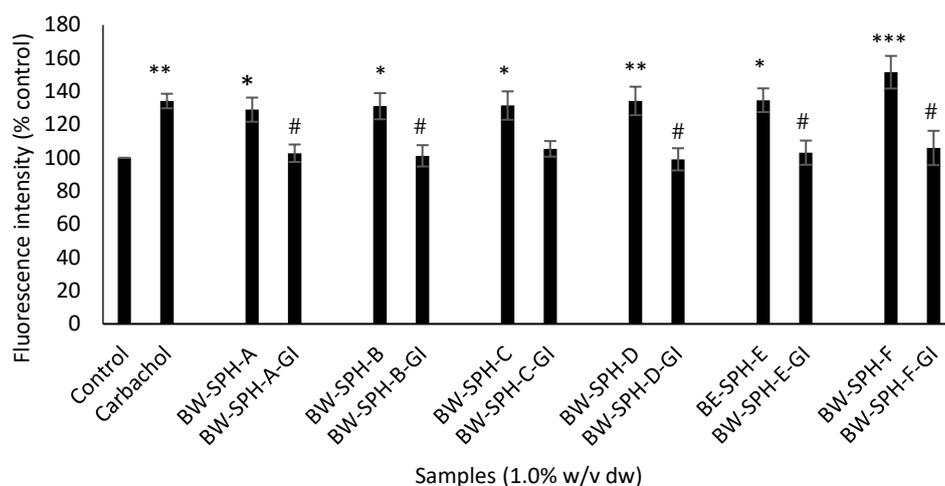
Figure 4.6. Intracellular Ca^{2+} levels in STC-1 cells (0.25 x 10^6 cells/ mL, 200µL/ well) after 4 h exposure to blue whiting soluble protein hydrolysates (BWSPHs, BW-SPH-A to BW-SPH-F) (1.0% w/v dw) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) (1.0% w/v dw) and controls (positive control was carbachol (1 mM) and negative control was Krebs Ringer buffer) followed by 1 h incubation with 5 µM Fluo-4-AM dye. Data is presented as intensity of fluorescence signals at excitation 494 nm and emission 506 nm compared with the control (Krebs Ringer buffer). Significance was measured using ANOVA followed by Dunnett's test. *, ** and *** denotes statistical significance between samples and the control at p<0.05, p<0.01 and p<0.001, respectively. # indicates a significant difference at p<0.05 between pre- and post- SGID values measured by Student's t-test.
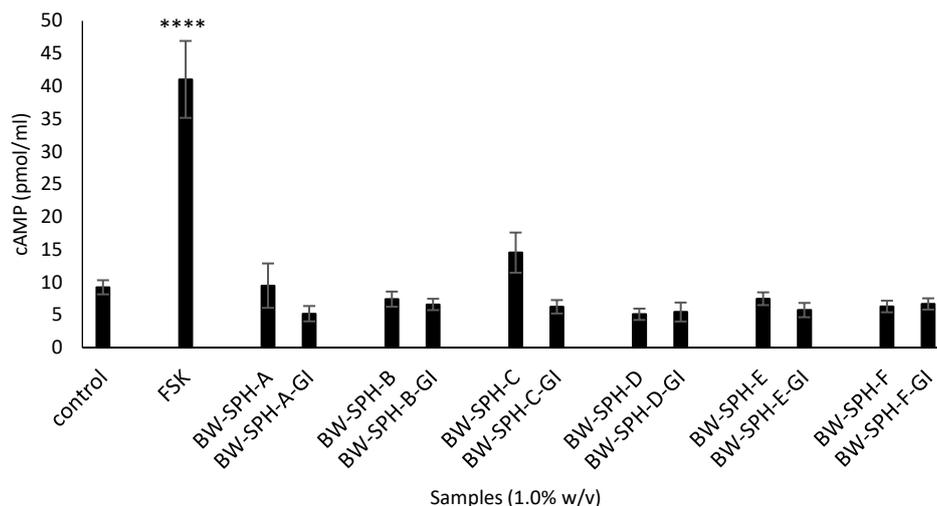
Figure 4.7. Intracellular cyclic adenosine monophosphate (cAMP) levels in STC-1 cells after 4 h exposure to blue whiting soluble protein hydrolysates (BWSPHs, BW-SPH-A to BW-SPH-F) (1.0% w/v dw) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) (1.0% w/v dw), and controls (positive control was forskolin (FSK) (10µM) and negative control was Krebs Ringer buffer). Test samples and controls were supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX). Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. **** denotes statistical significance between samples and the negative control (p<0.0001).

4.4 Discussion

Bioactive peptides identified from low-value fish species such as blue whiting may represent profitable functional food ingredients due to their natural availability, low-cost extraction methods and ability to exert various health-enhancing activities. The shortcomings (time, safety, extraction efficiency) associated with traditional biomolecule extraction techniques such as acid, alkaline, salt and solvent extraction has led to the popular use of safer, more efficient extraction methods including enzymic hydrolysis for the effective recovery of desirable bioactive components (Bruno et al., 2019). The BWSPHs tested herein were generated from the same source material using different food-grade proteolytic enzymes and enzymatic hydrolysis conditions in a biorefinery approach at industrial scale (Harnedy-Rothwell et al., 2021). These BWSPHs have previously demonstrated *in vitro* anti-diabetic activity (Harnedy-Rothwell et al., 2021), and the results of this study show that BWSPHs may also exhibit satiating potential through synthesizing GLP-1 precursor peptide, proglucagon, and stimulating the secretion of active GLP-1 (3.5-fold for BW-SPH-A to 6.7-fold for BW-SPH-F) in STC-1 cells, indicating BWSPHs can regulate GLP-1 at both transcriptional and post-transcriptional levels. However, the GLP-1 stimulating effect of BWSPH subjected to SGID was lost indicating its instability during gut transit. The study by Harnedy-Rothwell et al. (2021) demonstrated that all pre-SGID BWSPHs inhibited the activity of DPP-IV enzyme, an enzyme responsible for inactivating more than 80% of secreted GLP-1, by 50% at concentrations ranging from 2.12 - 2.90 mg protein/ mL (Harnedy-Rothwell et al., 2021). However, a significant reduction in DPP-IV inhibitory activity was observed following SGID of BW-SPH-B, -D, -E and -F ($p<0.05$), possibly relating to the loss of bioactivity demonstrated by SGID BWSPH BW-SPH-F-GI in this study. In addition, BWSPHs subjected to i*n vitro* gastrointestinal digestion (2.5 mg/mL) were shown to stimulate insulin secretion from clonal pancreatic BRIN-BD11 cells compared with the control (buffer/media containing 5.6 mM glucose) ($p<0.05$), although a significant reduction in insulinotropic potency was observed following SGID of BW-SPH-F ($p<0.05$) (Harnedy-Rothwell et al., 2021). As GLP-1 potentiates glucose-stimulated insulin secretion, the loss of the hydrolysates secretagogue ability post-SGID may have been associated with this reduction in insulin secretion. It is well known that due to the crude nature of hydrolysate mixtures, the content of low molecular weight peptides or free

amino acids may directly influence hydrolysate bioactivities. Hydrolysate composition is highly influenced by protein source, method of hydrolysis, hydrolysis conditions and the degree of hydrolysis (Heffernan et al., 2021). It is likely that changes in the physicochemical properties of hydrolysate BW-SPH-F as a result of *in vitro* gastrointestinal digestion were responsible for the variation in GLP-1 secretion, DPP-IV inhibitory activity and insulin secretion. A study by McLaughlin et al. (2021) reported similar findings as SGID of protein hydrolysates purified from the microalgal *Palmaria palmata* exhibited varying effects on DPP-IV inhibition, insulin secretion in BRIN-BD11 cells and total GLP-1 secretion in GLUTag cells, depending on the protease and hydrolysis conditions employed (McLaughlin et al., 2021).

Secretion of satiety hormones from enteroendocrine cells in response to nutrient signals in the gut is essential for energy homeostasis. Protein hydrolysates which stimulated *in vitro* anorexigenic hormone release have also been shown to trigger postprandial physiological responses *in vivo* with reductions in food intake, body weight gain and subjective rate of hunger being reported (Cudennec et al., 2012; Sukkar et al., 2013). Protein hydrolysates purified from fish (Harnedy et al., 2018), dairy (Kondrashina et al., 2020) and plant (Chen et al., 2018) origins have been shown to modulate the secretion of various satiety hormones. Cudennec et al. (2012) reported blue whiting (*Micromesistius poutassou*) muscle protein hydrolysates increased the secretion of both active GLP-1 (25-fold) and CCK (30-fold) from STC-1 cells compared with the basal control (10mM glucose) at a concentration of 1% w/v (p<0.05) (Cudennec et al., 2012). Although we also observed increases in active GLP-1 concentrations in STC-1 cells exposed to BWSPHs, CCK and PYY levels were either unaffected or down-regulated *in vitro*. This lack of connection between PYY, CCK and GLP-1 has been reported previously. A study by O'Halloran et al. (2018) also found that a sodium caseinate hydrolysate (10mg/mL) increased total GLP-1 secretion from STC-1 cells (p<0.05), but with no effect on CCK or PYY mRNA transcript levels (O'Halloran et al., 2018). In order to determine the true physiological effect of BWSPHs-induced satiety hormone secretion, *in vivo* trials investigating satiety hormone plasma levels and subsequent food intake are necessary. Although there are many advantages of utilizing cellular assays for hormone secretion studies, there are also limitations associated with *in vitro* models compared with *in vivo* models, in particular, the lack of physiological relevance of *in vitro* assays (Kuhre et al., 2021). Moreover, a specific limitation of the STC-1 cell line employed herein is it's

heterogeneity which can ultimately induce variability in hormone expression, however as recommended by McCarthy et al. (2015), cells were passaged greater than ten times to improve cellular homogeneity (McCarthy et al., 2015). Additionally, large inter-experimental variability was not observed further indicating homogeneity in cells. To further verify the results of this study, the hormone stimulating effect of BWSPHs could be assessed in other murine and human enteroendocrine cells such as GLUTag cells or NCI-H716 cells, respectively.

It is possible that the differential modulation of satiety hormones is attributed to specific peptide sequences found within the protein fraction. All BWSPHs tested in this study have previously been characterized by Harnedy-Rothwell et al. (2021) (Harnedy-Rothwell et al., 2021). Physicochemical analysis (degree of hydrolysis, molecular mass distribution and reversed-phase ultra-performance liquid chromatography) demonstrated that all BWSPHs were further hydrolysed during SGID, with the degree of hydrolysis of undigested hydrolysates ranging from 27.82% (BW-SPH-C) to 45.78% (BW-SPH-B) and the degree of hydrolysis of SGID BWSPHs ranging from 55.37% (BW-SPH-C-GI) to 65.23% (BW-SPH-E-GI). BWSPHs were rich in Leu and Arg residues (Harnedy-Rothwell et al., 2021), however, the possibility that individual amino acid contents were responsible for the GLP-1 secreting activity of BWSPHs can be discounted as *in vitro* digestion of BWSPHs, which inhibited proglucagon production and GLP-1 secretion, increased <1 kDa components and free amino acid levels of all BWSPHs with the exception of Val, thereby suggesting larger peptides were responsible for BWSPHs bioactivity as opposed to the free amino acid content. As discussed previously, the six BWSPHs analysed in this study were prepared with different protease/ protease combinations, therefore the size and sequence of the peptides generated during hydrolysis differ in each hydrolysate depending on the substrate cleavage site and specificity of the protease employed. In order to identify which peptide(s) are responsible for GLP-1 secretion, it is necessary to identify the peptide sequences in each hydrolysate, thus allowing for peptide sequence homology with known anorectic food-derived peptides listed on peptide databases to be investigated.

Although GLP-1-secreting L cells increase in number along the length of the gastrointestinal tract, hydrolysis of protein hydrolysates by gastrointestinal proteases can reduce bioactivity. Our data indicates loss of bioactivity post upper gut transit. Hydrolysate BW-SPH-F, which induced the greatest increase in active GLP-1

secretion of the six hydrolysates tested, lost its secretagogue ability when subjected to SGID indicating its instability towards gastrointestinal proteases. In agreement, Kondrashina et al. (2018) investigated the GLP-1 secretory ability of a casein hydrolysate (10 mg/mL) in STC-1 cells during simulated gut transit and reported a 39% and 51% reduction in GLP-1 secretory capacity following gastric digestion and duodenal digestion, respectively (Kondrashina et al., 2018). Loss of bioactivity upon digestion may be a result of an increase in the degree of hydrolysis and subsequent generation of free amino acids, which are reportedly less effective GLP-1 stimulants (Cordier-Bussat et al., 1998; Cudennec et al., 2012). Geraedts et al. (2011) also reported that intestinal digestion of intact codfish protein with intestinal protease trypsin reduced GLP-1 secretion from STC-1 cells (p<0.05) (Geraedts et al., 2011). In contrast to the SGID results in this study, Harnedy and colleagues (2018) demonstrated that *in vitro* digestion of a different blue whiting protein hydrolysate, prepared with Alcalase 2.4L and Flavourzyme 500L, increased GLP-1 secretory activity compared with the non-digested hydrolysate in GLUTag cells (p<0.05), indicating that the release of lower molecular weight peptides from precursor peptides during the digestion process were responsible for the hydrolysates secretagogue activity (Harnedy et al., 2018). A significant increase in the degree of hydrolysis of the blue whiting protein hydrolysate following SGID was observed along with an increase in <0.5 kDa components (Harnedy et al., 2018), however, the degree of hydrolysis of the SGID hydrolysate (32.58 ± 0.30%) in the study by Harnedy et al. (2018) was more comparable with the degree of hydrolysis of the undigested BWSPHs in this study (Harnedy-Rothwell et al., 2021), again indicating the role of peptides over free amino acid content in blue whiting protein hydrolysate bioactivity. Cudennec et al. (2012) also showed bioactivity maintenance of blue whiting muscle protein hydrolysate in the gut with increased CCK and active GLP-1 plasma levels upon oral administration to male Wistar rats at concentrations of 100 and 250 mg/mL (p<0.05), correlating with a decrease in short term food intake and a decrease in body weight gain (Cudennec et al., 2012). It is possible that the BWSPHs herein may induce GLP-1 production and secretion prior to intestinal digestion through interaction with L-cells located in the duodenum. Harnedy-Rothwell et al. (2021) reported that little to no hydrolysis of BWSPHs occurred during the pepsin (gastric) phase of SGID, and that degradation of BWSPHs predominantly occurred during the simulated intestinal phase upon exposure to intestinal enzymes such as trypsin, chymotrypsin and/or elastase present in Corolase

PP (Harnedy-Rothwell et al., 2021). Alternatively, for protection of BWSPHs stability through intestinal delivery, protective coatings such as pH sensitive coatings are available (Zhuo et al., 2020).

In vitro analysis of the mechanistic actions of BWSPHs further highlighted their ability to influence GLP-1 release. Open-type enteroendocrine cells, which include L cells, where the microvilli of the apical side are in contact with the luminal contents, act as direct sensors of luminal nutrients and non-nutrients to activate hormonal regulators through activation of G-protein coupled receptors and transporters (ATA2, PEPT1). Activation of G-protein coupled receptors, such as GPR131, stimulates adenylate cyclase, resulting in an increase in the concentration of cAMP within the cells, which is linked to an increase in the intracellular adenosine triphosphate (ATP)/ adenosine diphosphate (ADP) ratio and a subsequent rise in intracellular Ca^{2+} mobilization (Gribble & Reimann, 2017). However, in this study, BWSPHs prior to SGID, were observed to increase intracellular Ca^{2+} concentration, without eliciting any effect on intracellular cAMP concentration. It is not uncommon for food-derived protein fractions to stimulate GLP-1 through activation of calcium pathways (Kato et al., 2017; O'Halloran et al., 2018). The lack of any detectable changes in cellular cAMP levels post BWSPHs incubation in STC-1 cells does not exclude the involvement of a G-protein coupled receptor. Elevation of intracellular Ca^{2+} concentrations can occur through sensing of proteins, peptides and amino acids by enteroendocrine cells which activates calcium sensing receptor and/or triggers membrane depolarization via activation of ion-coupled transporters (Gribble & Reimann, 2017).

As the quest for novel proteins with bioactivities continues at pace, it is important that gut barrier health is tracked in line with bioactivity assessment. A differentiated Caco-2/HT-29MTX co-culture was chosen to represent the human intestinal epithelium as absorptive cells (Caco-2) and goblet cells (HT-29MTX) are major cell types of the intestinal epithelial tract (Ali et al., 2020). Caco-2 cells secrete pro-inflammatory cytokine IL-6 upon exposure to various inflammatory mediators (Van De Walle et al., 2010), which can ultimately increase the permeability of Caco-2 cell monolayers, potentially resulting in homeostatic imbalance of the internal environment (Al-Sadi et al., 2014; Cui et al., 2010). No effect of BWSPHs on the viability of intestinal epithelial co-cultured cells was observed, nor were TEER values altered. TEER values remained greater than 700 $\Omega.cm^2$, indicative of tight junctions

(Bavaro et al., 2021). In addition, BWSPHs present in the apical side of co-cultured cells did not induce IL-6 secretion, indicating BWSPHs are unlikely to elicit a sizable immune response in the gut epithelium (Hoffmann et al., 2021). Proteins and peptides have been shown to maintain high TEER values and increase tight junction proteins such as occludin and claudin1 (Arbizu et al., 2020; Bavaro et al., 2021), however limited research exists on the protective capacity of proteins derived from fish specifically towards gut health. In agreement with our study, Cinq-Mars et al. (2008) observed that a hake fillet hydrolysate (10mg/mL) maintained TEER values in Caco-2 cell monolayers above 900 $\Omega.cm^2$ (Cinq-Mars et al., 2008).

4.5 Conclusion

All BWSPHs upregulated GLP-1 precursor proglucagon mRNA levels and stimulated the secretion of active GLP-1 from STC-1 cells, possibly via intracellular calcium signalling, whilst maintaining the integrity of co-cultured intestinal cells. However, SGID inhibited GLP-1 secretion and proglucagon production indicating that bioactivity is sensitive to the hydrolytic conditions of the upper gut. BWSPH-derived peptides may require encapsulation or another means of protection to avoid loss of activity during gastrointestinal transition in order to have use as a potential functional food ingredient for weight management. Albeit it would be beneficial to study the effect of simulated gastric digestion, without simulating intestinal digestion, on hydrolysate bioactivity to determine whether bioactive peptides regulate satiety hormone levels, by any degree, in the gut prior to complete proteolysis. Future studies should also identify the peptide(s) responsible for the observed bioactivity of BWSPHs in this study and determine the efficacy of the peptide(s) *in vivo*.

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Chapter 5

Blue whiting protein hydrolysates post simulated gastrointestinal digestion and following simulated intestinal absorption exhibit anti-obesity activities in 3T3-L1 cells.

Abstract

The purpose of this study was to investigate the potential anti-obesity activity of six protein hydrolysates (encoded BW-SPH-A to BW-SPH-F) enzymatically derived from blue whiting using 3T3-L1 cellular models. The blue whiting soluble protein hydrolysates (BWSPHs) coded BW-SPH-A, BW-SPH-B, BW-SPH-C and BW-SPH-F significantly reduced triglyceride accumulation during 3T3-L1 preadipocyte accumulation compared with the differentiated control ($p<0.05$) although none of the hydrolysates modulated glycerol content in mature 3T3-L1 adipocytes ($p>0.05$). Interestingly, after simulated gastrointestinal digestion (SGID) of the BWSPHs, all hydrolysates reduced triglyceride levels and all except one SGID BWSPHs significantly increased glycerol release compared with the controls ($p<0.05$). Two anti-adipogenic hydrolysates, BW-SPH-A and BW-SPH-F, and their corresponding in vitro digests, BW-SPH-A-GI and BW-SPH-F-GI, were observed to modulate triglyceride accumulation during preadipocyte differentiation via down-regulating the expression of key adipogenic transcription factors (peroxisome proliferator activated receptor (PPAR)-γ and CAAT (controlled amino acid therapy)/ enhancer binding protein (C/EBP)-α) compared with the differentiated controls ($p<0.05$). These SGID hydrolysates also exhibited anti-obesity activities following simulated intestinal permeation utilising 21-day differentiated Caco-2/HT-29MTX co-cultured cells, indicating their potential to reach the bloodstream in a bioactive form. Exposure of 3T3-L1 preadipocytes to hydrolysate BW-SPH-A during differentiation also increased reduced glutathione (GSH) concentration upon stimulation with antioxidant tert-butyl hydroperoxide (tBOOH) compared with the tBOOH control ($p<0.05$). Specific BWSPHs were also observed to reduced adiponectin production in lipopolysaccharide (LPS)-stimulated cells compared with the LPS control ($p<0.05$). In conclusion, these BWSPHs may have potential as anti-obesity agents in functional food applications.

5.1 Introduction

The prevalence of obesity has increased significantly over the past several decades with the World Health Organisation reporting a 300% increase in obesity rates since 1975 (WHO, 2021). The occurrence and development of obesity are associated with the expansion of white adipose tissue as a result of genetic factors and/ or physical inactivity, unhealthy diet and smoking (Hwang et al., 2017). Hypertrophic expansion of white adipose tissue resulting in lipotoxicity and adipose tissue dysfunctionality can contribute to chronic diseases, such as type 2 diabetes, hypertension, carcinogenesis and cardiovascular diseases (Torres-Fuentes et al., 2015). This expansion in adipose tissue mass may be a result of excessive fat deposition in pre-existing adipocyte cells or the differentiation of preadipocytes to mature adipocytes in a process known as adipogenesis. Researchers in recent years have advised that adipose tissue should become a main drug target for obesity and lipid metabolism dysregulation thus greater focus has been directed on the development of natural products with the ability to interfere with the molecular pathways of adipogenesis (Kersten, 2001; Kim et al., 2017). The process of adipogenesis involves molecular regulation of adipocytes via sequential activation of master transcription factors (peroxisome proliferator activated receptor (PPAR)-γ and CAAT (controlled amino acid therapy)/ enhancer binding protein (C/EBP)-α), and lipogenesis genes (adipocyte protein 2 (aP2), lipoprotein lipase (LPL) and others).

In 2020, blue whiting represented one of the most abundant species landed by Irish fishing vessels in Ireland according to the Central Statistics Office (CSO, 2021), however, due to the low market value and low consumer interest in blue whiting, a profitable method for valorising blue whiting is desirable. One such approach to increase the value of blue whiting fish is the extraction and identification of bioactive compounds with potential use as high-value functional food ingredients. Blue whiting protein hydrolysates have previously demonstrated weight management potential through the regulation of satiety hormones in vitro, in vivo and in clinical trials (Cudennec et al., 2012; Cudennec et al., 2008; Heffernan et al., 2022; Nobile et al., 2016). However, little is known about their effect on preadipocyte differentiation and the formation of mature adipocytes. Protein hydrolysates and peptides from other fish sources including tuna fish have been shown to reduce triglyceride accumulation in

differentiated 3T3-L1 adipocytes through modulation of key transcription factors C-EBPα and PPARγ, and adipogenic and lipogenic gene expression (Kim et al., 2017; Lee et al., 2017).

Hypertrophy is also associated with increasing levels of reactive oxygen species (ROS) and pro-inflammatory adipokines which can result in elevated systemic oxidative stress and low-grade inflammation, respectively (Masschelin et al., 2020; Pratelli et al., 2022). The development of low-grade inflammation can be accompanied by the promotion of pro-inflammatory genes and an influx of immune cells which can amplify inflammation possibly inducing systemic inflammation (Zieger et al., 2018). Hydrolysates and peptides from various food sources have been shown to modulate pro-inflammatory and anti-inflammatory adipokines in adipocyte cellular models (Kwak et al., 2016; Romacho et al., 2015), although research regarding the immunomodulatory potential of fish protein hydrolysates in adipocytes remains limited. Fernández-Sánchez et al. (2011) listed multiple reasons for the generation of ROS during adipogenesis including: 1) the presence of pro-inflammatory cytokines in preadipocytes and adipocytes which are potent stimulators of ROS production, 2) fatty acid oxidation, 3) overconsumption of oxygen, 4) cellular damage due to excessive fat accumulation, 5) high fat diet (Fernández-Sánchez et al., 2011). Although many food-derived bioactive compounds have been shown to prevent the accumulation of triglycerides in 3T3-L1 cells as well as reduce oxidative stress (Choi et al., 2018; Choi et al., 2020; Nam et al., 2019), this is the first study investigating the anti-obesity and antioxidant potential of fish protein hydrolysates in vitro, to the best of our knowledge.

The 3T3-L1 cell line established by Greene and Kehinde (1975) is a well-characterised, reliable model for the in vitro study of adipocyte biology (Green & Kehinde, 1975). Upon reaching confluency, part of the cell can spontaneously differentiate into rounded cells with the formation of triglyceride droplets. However, differentiation can be accelerated by exposure to an adipogenic cocktail consisting of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) resulting in complete differentiation within 12 days (Tung et al., 2017). The aim of this study was four-fold. Firstly, the ability of blue whiting soluble protein hydrolysates (BWSPHs) to inhibit the accumulation of triglycerides in 3T3-L1 preadipocytes and increase glycerol production in 3T3-L1 adipocytes was assessed. Secondly, the impact, if any, of simulated gastrointestinal digestion (SGID) and following simulated intestinal absorption of the BWSPHs on the above cellular effects in preadipocytes and mature

adipocytes was evaluated. Thirdly, the effects of the BWSPHs on the regulation of essential transcription factors involved in the cascade of adipogenesis was assessed. Fourthly, the antioxidant and immunomodulatory effects of BWSPHs in stimulated 3T3-L1 cells was investigated.

5.2 Materials and Methods

5.2.1 Materials

The human Caco-2 cell line were obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Murine 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, Virginia). Human HT-29MTX cell lines were kindly gifted by Dr. Giblin (Teagasc Food Research Centre, Moorepark, Fermoy, Ireland). Mouse interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1) and adiponectin enzyme-linked immunosorbent assay (ELISA) kits were purchased from ThermoFisher Scientific (BioSciences, Dublin, Ireland). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). RNeasy mini kit, DNase digestion kit and the QuantiTect Reverse Transcription kit were from Qiagen (Manchester, UK). LightCycler 480 SYBR Green was from Roche (Roche Products Ireland Limited, Dublin, Ireland). Cell culture plastics were supplied by Cruinn Diagnostics and Corning Incorporated (Dublin, Ireland). The adipogeneis kit, adipolysis kit and all other cell culture reagents and chemicals were purchased from Sigma Chemical Co. (Dublin, Ireland) unless otherwise stated. BWSPHs were provided by Bio-Marine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland).

5.2.2 Sample Preparation

The six BWSPHs (BW-SPH-A to BW-SPH-F) provided by Bio-Marine Ingredients Ireland Ltd. (Monaghan, Ireland) were generated from minced blue whiting (Harnedy-Rothwell et al., 2021). Harnedy-Rothwell et al. (2021) performed SGID of the BWSPHs (BW-SPH-A-GI to BW-SPH-A-F-GI) using pepsin (pH 2, 37°C, 90 min, enzyme: substrate (E:S) of 2.5% w/w) and Coralase PP (pH 7, 37°C,

150 min, E:S of 1% w/w). The study by Harnedy-Rothwell et al. (2021) details the degree of hydrolysis (DH), molecular mass distribution and total and free amino acid composition of the hydrolysates (Harnedy-Rothwell et al., 2021). The BWSPHs and SGID BWSPHs were prepared directly with Dulbecco's Modified Eagle's Medium (DMEM), sterile-filtered and stored at -20 °C.

5.2.3 Cell culture and adipocyte differentiation

3T3-L1 preadipocytes were grown in DMEM supplemented with 10%, v/v FBS. Cells were cultured in an atmosphere of CO_2-air (5:95, v/v) at 37°C and were maintained in the absence of antibiotics. 3T3-L1 cell passage number did not exceed 20. Preadipocytes were seeded onto 6 well plates at a density of 1 x 10^5 cells/ mL in media containing 10%, v/v FBS in a final volume of 2 ml per well. At 2 days post-confluence (day 0), differentiation was initiated with differentiation medium 1 (MDI) which consisted of DMEM with 10%, v/v FBS, 0.5 mM IBMX, 0.25 mM dexamethasone, 1 µg/ mL insulin and 2 µM rosiglitazone and cell were incubated for 48 h. Two days after the initiation of differentiation (day 2), the cell culture medium was replaced with differentiation medium 2, DMEM containing 10%, v/v FBS and 1 µg/ mL insulin. This step was repeated every other day until 80-90% of cells had differentiated to mature adipocytes. On day 8, media was then removed, the cell monolayer was washed twice with phosphate buffer saline (PBS) and scraped in 1 mL PBS buffer. Harvested cells were then sonicated and centrifuged. Supernatant was collected and stored at -80°C prior to analysis. To examine the effects of BWSPHs on the differentiation of preadipocytes to adipocytes, the hydrolysates were added to cells cultured with the MDI on day 0. Alternatively, cells were differentiated in the presence of resveratrol (25 µM) which acted as the positive control for adipogenesis assays. Experiments commenced on day 8.

5.2.4 Cell viability

The cytotoxic effect of BWSPHs (BW-SPH-A to BW-SPH-F) was assessed on 3T3-L1 preadipocytes and mature adipocytes. 3T3-L1 preadipocytes were seeded in 96 well plates at a density of 1 x 10^5 cells/ mL (200 µL/ well) for 24 h and were then

supplemented with increasing concentrations (0.05 - 1.00 %, w/v dw) of BWSPHs and SGID BWSPHs for a further 24 h. To determine the effect of BWSPHs on mature adipocytes, 3T3-L1 preadipocytes were seeded in 96 well plates at a density of 1 x 10^5 cells/ mL (200 µL/ well) and differentiated in the absence of hydrolysates over 8 days as previously described. Mature adipocytes were then supplemented with increasing concentrations (0.05 - 1.00 %, w/v dw) of BWSPHs (BW-SPH-A to BW-SPH-F) for 24 h. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) which consisted of the MTT reagent and solubilisation solution. Following 24 h exposure to test samples, well contents were aspirated and the MTT reagent (10 µL) and DMEM (100 µL) were simultaneously added to the wells and incubated for a further 4 h at 37 °C. Solubilisation solution (100 µL) was then added to the well contents and following overnight incubation, the absorbance was read at 570 nm using a microplate reader (Varioskan TM Flash Multimode Reader, Thermoscientific, Waltham, MA, USA). Hydrolysate concentration that induced greater than 80% cell viability was used for anti-obesity, antioxidant and immunomodulatory assays.

5.2.5 Triglyceride analysis

On day 8 of 3T3-L1 cell differentiation, well contents were aspirated, and cell monolayers were wash twice with PBS and scraped into a homogenizing solution in 1 mL PBS. Cell suspension (500 µL) was centrifuged at 15000 rpm for 5 min at 4 °C, the supernatant was removed, the pellet was resuspended in 100 µL of lipid extraction buffer supplied with the commercial adipogenesis kit and heated to 90°C for 30 mins using an Eppendorf heater (Stuart Scientific SHT100D, Cole-Parmer, Staffordshire, UK). Total cellular triglyceride contents were determined according to the manufacturer's instructions. The other 500 µL cell suspension was sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 15000 rpm for 5 min at 4 °C, the supernatant was removed, and the pellet was resuspended in 100 µL PBS for protein analysis using the bicinchoninic acid (BCA) method employing bovine serum albumin as the calibration standard (Smith et al., 1985). Cell lysates (40 µL) were incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates

and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/ mL) was calculated.

5.2.6 Adipolysis analysis

Murine 3T3-L1 cells were differentiated for 8 days in the absence of BWSPHs. On day 8, media was removed, and cell monolayers were washed twice with PBS. Either DMEM (negative control), isoproterenol (10 µg/mL) (positive control) or BWSPHs (1.0% w/v dw) was added to the appropriate wells with a final volume of 2 mL per well. After 4 h incubation at 37°C, cell supernatants were harvested and used to measure free glycerol levels according to the manufacturer's instructions. Cell monolayers were then washed and collected in 500 µL PBS, sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 15000 rpm for 5 min at 4 °C, and the pellet was resuspended in 100 µL PBS for protein analysis using the BCA method as described above (Smith et al., 1985).
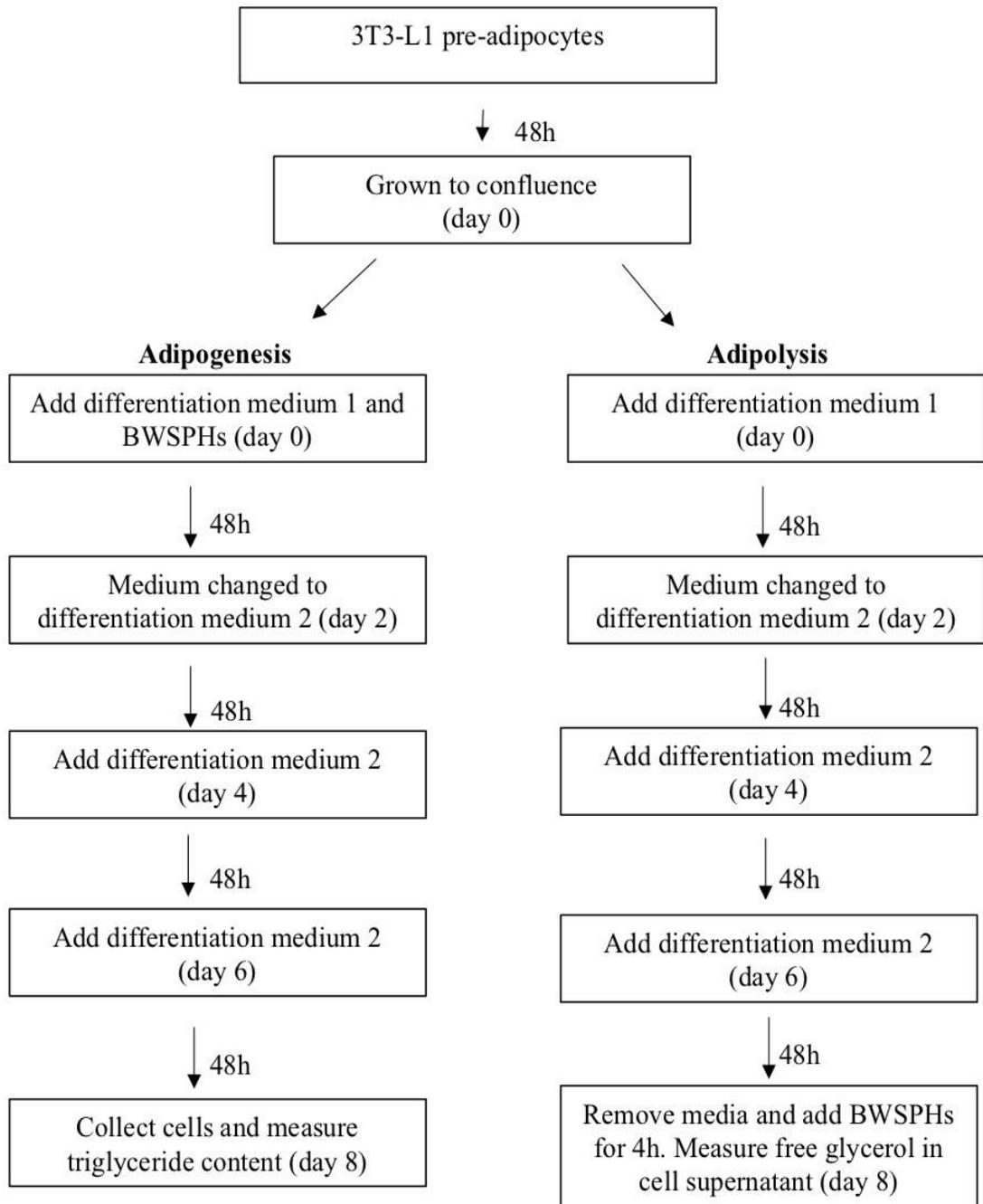
Figure 5.1. 3T3-L1 differentiation protocol and exposure to blue whiting soluble protein hydrolysates (BWSPH) during differentiation for adipogenic and adipolytic analysis. Differentiation medium 1, Dulbecco's Modified Eagle's Medium (DMEM) containing 10%, v/v foetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 mM dexamethasone, 1 µg/ mL insulin and 2 µM rosiglitazone; Differentiation medium 2, DMEM containing 10%, v/v FBS and 1 µg/ mL insulin.

5.2.7 RNA Extraction and Real-Time Reverse Phase Polymerase Chain Reaction (rt-PCR)

After differentiation of 3T3-L1 cells in the presence of BWSPHs (1.0% w/v dw), the cells were washed with PBS buffer, lysed in 700 µL of lysis buffer and stored overnight at −80 °C. RNA extraction was performed with RNeasy Mini Kit, including on-column DNase digestion, following the manufacturer's instructions. Total RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, US) with OD 260/280 ratios ranging from 1.95-2.06. A tetro cDNA synthesis kit was used to prepare cDNA from 1 µg of RNA according to the manufacturer's instructions. Real-time PCR was performed with a LightCycler 96 instrument (Roche Diagnostics, Germany) and LightCycler 480 SYBR Green I Master kit to quantify adipogenic transcription factors PPARγ and C/EBPα. Primers for murine PPARγ (AB644275): forward 5'- TTTTCAAGGGTGCCAGTTTC- 3'; reverse 5'- AATCCTTGGCCCTCTGAGAT- 3', annealing temperature 55 °C. Primers for murine C/EBPα (NM_001287523): forward 5'- TTACAACAGGCCAGGTTTCC- 3'; reverse 5'- GGCTGGCGACATACAGTACA- 3', annealing temperature 56 °C. Primers for murine acidic ribosomal phosphoprotein P0 (*36B4*) (NM_007475): forward 5'- TGCCACACTCCATCATCAAT- 3'; reverse 5'- CATCTGATTCCTCCGACTCTT- 3', annealing temperature 51°C. Primer efficiency was measured by performing PCR for four dilutions of cDNA (1:1, 1:10, 1:100, 1:1000) and was in the range of 1.92 – 2.06. Each PCR reaction contained 1 µL cDNA, 0.5 µL of forward and reverse primers, 3 µL RNase free water and 5 µL of LightCycler 480 SYBR Green, making a final volume of 10 µL/well. For each sample, the relative amount of target was calculated by the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t$ is [C_t (target gene) $- C_t$ (36B4)]test condition $-$ [C_t (target gene) $- C_t$ (36B4)]control condition and C_t is the cycle at which threshold is crossed.

5.2.8 ROS production

Intracellular formation of ROS was determined via the oxidation-sensitive fluorogenic probe 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA). 3T3-L1 cells were seeded in 96 well plates (1 x 10^5 cells/ mL, 200 µL/ well) in DMEM supplemented with 10%, v/v FBS and differentiated over 8 days as discussed above.

3T3-L1 cells were either exposed to BWSPHs (1.0% w/v dw) on day 0 of the 8-day differentiation period or on day 8 of preadipocyte differentiation for a total of 24 h. Cells were then washed with PBS and exposed to DCFH-DA prepared in DMEM (20 µM, 200 µL/well) for 40 min at 37 °C in the dark. Intracellular esterases hydrolyse DCFH-DA to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) which is then oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) upon reaction with ROS. Well contents were then aspirated, and cells were washed with PBS. The cells, except the negative control, were exposed to hydrogen peroxide (H_2O_2) (1 mM, 200 µL/well) for 60 min. The positive control was cells treated with H_2O_2. The negative control was cells exposed to DMEM only. Fluorescence of cells was measured at a wavelength of 485 nm followed by excitation at 530 nm every 5 min over the 60 min exposure period (VarioskanFlash, Thermo Scientific). Results were expressed as % of the positive control.

5.2.9 Reduced Glutathione (GSH) concentration

3T3-L1 cells were seeded in 6 well plates (1 x 10^5 cells/ mL, 2 mL/ well) in DMEM supplemented with 10%, v/v FBS and 3T3-L1 cells were either exposed to BWSPHs (1.0% w/v dw) on day 0 of the 8-day differentiation period or on day 8 of preadipocyte differentiation for a total of 24 h. Treated cells were then incubated in the presence of tert-butyl hydroperoxide (*t*BOOH) prepared in DMEM (1 mM, 2 mL/well) for 3 h at 37°C to induce oxidative stress. The positive control was cells exposed to *t*BOOH alone. The negative control was cells exposed to DMEM alone. The ability of the BWSPHs to protect against a *t*BOOH-induced reduction in cellular GSH was assessed. The method of Hissin and Hilf (1976) was employed for GSH determination (Hissin & Hilf, 1976). Briefly, cells were resuspended in sodium phosphate ethylenediaminetetraacetic acid (EDTA) buffer, sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 14,000 rpm x 30 min at 4 °C to remove cellular debris and supernatant was collected. The final GSH assay mixture (2 mL) was made up of 100 µL cell supernatant, 1.8 mL sodium phosphate EDTA buffer, and 100 µL o-phthaldialdehyde (1 mg/ mL). Fluorescence was determined at a wavelength of 430 nm followed by excitation at 360nm (VarioskanFlash, Thermo Scientific). GSH content was expressed relative to total cellular protein content, which was calculated by the BCA method as described above (Smith et al., 1985).

5.2.10 Cytokine secretion

3T3-L1 cells (1 x 10^5 cells/ mL, 200 µL/well) were seeded in 96 well plates in DMEM supplemented with 10%, v/v FBS and differentiated over 8 days. On day 8, mature adipocytes were exposed to BWSPHs (1.0% w/v dw) for 24 h at 37°C in media containing 2.5%, v/v FBS. After 24 h, stress was induced using lipopolysaccharide (LPS) (0.1 µg/ mL prepared in DMEM) and incubated for a further 24 h. The positive control was cells incubated in the presence of LPS (0.1 µg/ mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. After incubation, the culture medium was harvested and the secretion of cytokines IL-6, adiponectin and MCP-1 was measured by ELISA. Absorbance was measured at 450 nm on a microplate reader (VarioskanFlash, Thermo Scientific) and cytokine production was expressed as a percentage of LPS-stimulated 3T3-L1 cells (positive control). The ELISA kits allow detection of mouse IL-6, adiponectin, and MCP-1 with minimum detection limits of 4.0 pg/mL, 50 pg/mL and 2.2 pg/mL, respectively.

5.2.11 Simulated intestinal absorption

Caco-2 cells and HT-29MTX cells prepared in DMEM with 10%, v/v FBS were seeded at a ratio of 3:1, respectively, in polyester permeable-membrane inserts in 12 well plates at a final density of 6.0×10^4 cells/ insert. The culture media (1.5 mL) was also added to the basolateral compartment. Every 2-3 days, apical and basolateral compartments received 500 µL and 1.5 mL of fresh culture media, respectively, for a 21-day period. On day 21, monolayers with trans-epithelial electrical resistance (TEER) values exceeding 900 Ω.cm^2 were exposed to SGID BWSPHs (500 µL) for 4 h at 37°C. Subsequently, cell monolayers were washed with PBS and the co-culture monolayer integrity was analysed using a Millicell ERS-2 electrical-resistance system (Millipore, USA). The basolateral compartments received 1.5 mL DMEM. Basolateral contents were collected and stored at −20 °C prior to adipogenesis and adipolysis analysis.

5.2.12 Statistical analysis

All experimental results of this study are expressed as the mean ± standard error of the mean (SEM) and data are from at least three independent experiments. Significant differences between sample groups and control groups were analysed using a one-way analysis of variance (ANOVA) followed by Dunnett's test and Student's t-test was used to determine significant differences between hydrolysates before and after SGID (Prism 5.0, GraphPad Inc. San Diego, CA, USA).

5.3 Results

5.3.1. BWSPHs are not cytotoxic towards 3T3-L1 preadipocytes or 3T3-L1 mature adipocyte cells.

The effect of BWSPHs before and after SGID (0.05 - 1.00% w/v dw) on the viability of 3T3-L1 preadipocytes and 3T3-L1 mature adipocytes was investigated. Neither the pre-SGID BWSPHs nor their corresponding gastrointestinal digests, up to a concentration of 1.00% w/v dw, altered the viability of 3T3-L1 preadipocytes or adipocytes compared with the control (DMEM alone, 0.00% w/v dw BWSPH) (Tables 5.1 and 5.2). Based on these results, a protein hydrolysate concentration of 1.00% w/v dw was chosen for future experiments.

Table 5.1. The effects of blue whiting protein soluble hydrolysates (BWSPHs) pre- and post- simulated gastrointestinal digestion (SGID) on the viability of 3T3-L1 preadipocytes

	BWSPH concentration (w/v, dw)							
	0.00%	0.05%	0.10%	0.25%	0.50%	0.75%	0.90%	1.00%
	Cell viability (%)							
BW-SPH-A	100.0 ± 0.0	87.3 ± 11.8	84.4 ± 12.4	77.6 ± 9.7	81.3 ± 7.4	74.2 ± 6.8	77.1 ± 6.1	70.0 ± 5.7
BW-SPH-A-GI	100.0 ± 0.0	92.1 ± 3.3	94.0 ± 3.0	93.5 ± 1.4	89.2 ± 3.8	92.1 ± 9.9	85.9 ± 7.1	87.8 ± 5.4
BW-SPH-B	100.0 ± 0.0	90.1 ± 9.0	87.2 ± 12.9	89.7 ± 11.7	81.4 ± 9.5	80.2 ± 6.6	76.0 ± 7.4	79.1 ± 9.0
BW-SPH-B-GI	100.0 ± 0.0	102.2 ± 1.1	97.6 ± 5.6	94.7 ± 3.7	97.9 ± 7.7	92.7 ± 9.2	94.3 ± 9.8	79.6 ± 0.4
BW-SPH-C	100.0 ± 0.0	86.5 ± 3.3	83.0 ± 1.2	74.2 ± 3.2	77.9 ± 3.7	77.2 ± 6.0	69.9 ± 4.8	67.4 ± 3.9
BW-SPH-C-GI	100.0 ± 0.0	98.8 ± 1.0	94.7 ± 3.8	98.0 ± 3.1	102.0 ± 3.0	96.8 ± 6.9	91.5 ± 12.0	93.5 ± 12.7
BW-SPH-D	100.0 ± 0.0	79.9 ± 3.7	70.7 ± 5.6	70.1 ± 7.0	67.5 ± 8.6	79.0 ± 7.0	60.9 ± 5.0	69.2 ± 10.9
BW-SPH-D-GI	100.0 ± 0.0	97.6 ± 3.6	97.0 ± 6.3	93.5 ± 3.6	89.8 ± 3.9	90.5 ± 3.7	87.6 ± 6.9	89.7 ± 9.7
BW-SPH-E	100.0 ± 0.0	87.6 ± 6.7	85.9 ± 2.5	76.4 ± 4.4	72.9 ± 1.6	83.8 ± 2.9	75.8 ± 5.4	77.4 ± 2.7
BW-SPH-E-GI	100.0 ± 0.0	97.0 ± 4.5	100.4 ± 6.2	92.7 ± 6.3	96.5 ± 4.2	86.4 ± 2.7	94.1 ± 7.5	92.1 ± 11.8
BW-SPH-F	100.0 ± 0.0	97.1 ± 3.1	90.5 ± 2.0	80.9 ± 1.5	76.3 ± 3.0	72.4 ± 8.6	58.1 ± 4.4	71.0 ± 6.7
BW-SPH-F-GI	100.0 ± 0.0	96.7 ± 6.5	96.6 ± 0.9	94.2 ± 2.6	105.2 ± 5.6	102.0 ± 8.4	101.5 ± 10.5	98.9 ± 13.0

Murine 3T3-L1 preadipocyte cells (1×10^5 cells/ mL, 200 µL/well) were supplemented with increasing concentrations (0.05-1.00%, w/v dry weight (dw)) of BWSPHs (BW-SPH-A to BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) for 24 h. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only/ 0.00% w/v dw BWSPH (100% cell viability). Data are expressed as the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test.
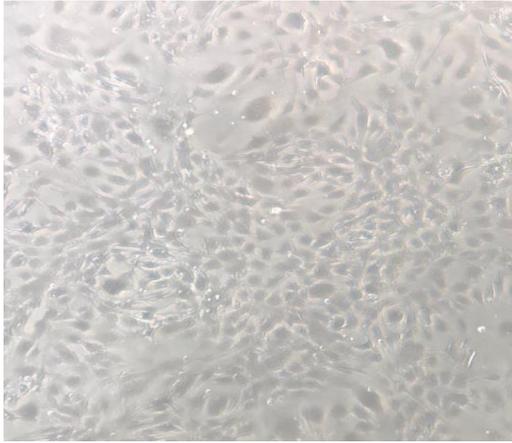
Table 5.2. The effects of blue whiting protein soluble hydrolysates (BWSPHs) pre- and post- simulated gastrointestinal digestion (SGID) on the viability of 3T3-L1 mature adipocytes

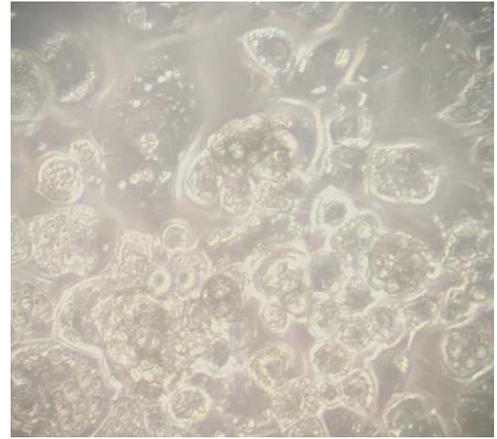|	BWSPH concentration (w/v, dw)							
	0.0%	0.05%	0.10%	0.25%	0.50%	0.75%	0.90%	1.00%
	Cell viability (%)							
BW-SPH-A	100.0 ± 0.0	90.4 ± 3.2	89.8 ± 3.6	90.6 ± 4.2	93.2 ± 6.1	94.3 ± 7.7	98.9 ± 9.6	92.7 ± 10.3
BW-SPH-A-GI	100.0 ± 0.0	103.4 ± 6.8	101.2 ± 5.0	110.9 ± 9.1	104.1 ± 7.8	103.2 ± 11.8	112.5 ± 7.5	121.9 ± 6.7
BW-SPH-B	100.0 ± 0.0	120.3 ± 4.8	111.9 ± 1.5	117.1 ± 5.9	125.5 ± 2.9	113.9 ± 2.0	112.7 ± 5.2	118.9 ± 2.4
BW-SPH-B-GI	100.0 ± 0.0	101.8 ± 0.4	106.3 ± 4.0	103.7 ± 9	102.2 ± 5.4	104.0 ± 11.0	113.5 ± 8.7	83.7 ± 11.0
BW-SPH-C	100.0 ± 0.0	101.3 ± 6.3	106.3 ± 7.7	114.0 ± 5.7	105.2 ± 8.6	117.1 ± 10.5	118.1 ± 8.7	114.0 ± 7.4
BW-SPH-C-GI	100.0 ± 0.0	98.9 ± 2.6	91.4 ± 3.1	90.1 ± 1.7	89.8 ± 0.5	81.7 ± 2.0	104.2 ± 5.1	91.8 ± 2.6
BW-SPH-D	100.0 ± 0.0	104.9 ± 5.1	95.8 ± 3.1	101.3 ± 4.7	94.4 ± 6.1	107.6 ± 6.8	118.8 ± 8.5	122.5 ± 8.6
BW-SPH-D-GI	100.0 ± 0.0	113.0 ± 4.4	105.7 ± 9.9	98.2 ± 4.7	99.7 ± 10.3	104.9 ± 8.6	97.1 ± 3.0	96.5 ± 2.0
BW-SPH-E	100.0 ± 0.0	103.6 ± 6.2	96.2 ± 11.9	97.9 ± 4.3	104.9 ± 8.0	112.3 ± 8.5	117.8 ± 8.5	120.3 ± 4.3
BW-SPH-E-GI	100.0 ± 0.0	101.9 ± 0.9	103.7 ± 4.0	97.0 ± 3.3	99.3 ± 12.0	112.0 ± 10.9	99.5 ± 8.2	109.9 ± 11.2
BW-SPH-F	100.0 ± 0.0	101.7 ± 1.4	107.2 ± 8.1	102.4 ± 1.0	102.8 ± 7.2	109.6 ± 2.6	113.1 ± 4.5	114.1 ± 10.4
BW-SPH-F-GI	100.0 ± 0.0	104.8 ± 10.9	106.6 ± 5.5	108.0 ± 7.4	107.8 ± 3.5	105.2 ± 8.4	101.5 ± 5.6	110.0 ± 4.4

Murine 3T3-L1 preadipocyte cells (1 x 10^5 cells/ mL, 200 µL/well) were differentiated over a 8-day period and then mature adipocytes were supplemented with increasing concentrations (0.05-1.00%, w/v dry weight (dw)) of BWSPHs (BW-SPH-A to BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI to BW-SPH-F-GI) for 24 h. Cell viability was determined by the MTT assay and expressed as a percentage of the control, DMEM only/ 0.00% w/v dw BWSPH (100% cell viability). Data are expressed as the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test.

5.3.2. BWSPHs and in vitro BWSPHs digests reduced adipogenesis in 3T3-L1 cells

The anti-adipogenic effect of BWSPHs and in vitro digested BWSPHs on the differentiation of 3T3-L1 preadipocytes to adipocytes was examined through comparing the triglyceride levels in 3T3-L1 adipocytes which were differentiated in the presence of BWSPHs to 3T3-L1 adipocytes differentiated in the absence of BWSPHs. Differentiation of 3T3-L1 preadipocytes into adipocytes induced morphological changes due to the accumulation of lipid droplets in the cytoplasm (Figure 5.2). Figure 5.3 shows that triglyceride accumulation was largely increased in adipocyte cells which were exposed to differentiating media over an 8-day period compared with cells which were treated with DMEM/FBS media over the same period ($p<0.001$). Cells which were differentiated in the presence of BWSPHs or SGID BWSPHs showed significantly reduced intracellular triglyceride accumulation compared with the differentiated control (MDI) ($p<0.05$) except for hydrolysates BW-SPH-D and BW-SPH-E. The anti-adipogenic ability of BW-SPH-D was the only hydrolysate which was significantly altered by SGID ($p<0.05$). All hydrolysates post SGID significantly reduced triglyceride accumulation in the range of 36.9 % (BW-SPH-A-GI) to 58.9 % (BW-SPH-C-GI), similar to that of positive control resveratrol (25 µM) which reduced triglyceride accumulation by 44.4%, compared to the differentiated control (100% triglyceride accumulation).

<div align="center">(A)   (B)</div>

Figure 5.2 (A). Morphology of 3T3-L1 preadipocyte cells at day 0. (B). Morphology of 3T3-L1 mature adipocyte cells at day 8 after induction with differentiation medium (MDI). The differentiated cells show characteristics of lipid droplets.
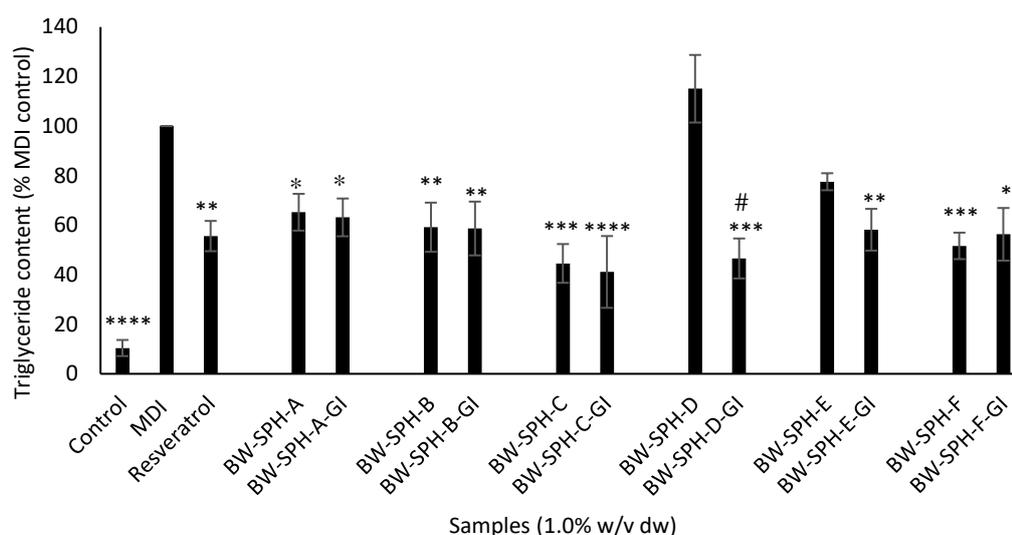
Figure 5.3. The anti-adipogenic effect of blue whiting soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion (SGID) as measured by triglyceride content. 3T3-L1 preadipocytes (seeded at 1 x 10^5 cells/ mL, 2 mL/ well of a 6 well plate) were cultured in growth medium until they reached confluency and quiescent cells were incubated in DMEM/FBS (control) or differentiation medium (MDI) with BWSPHs at a final concentration of 1.0%, w/v dw or without BWSPHs (MDI control) for 8 days. Resveratrol (25 μM) acted as the positive control. Triglyceride content is expressed as % MDI control and data are mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and MDI control ($p<0.05$). ** $p<0.01$ and *** $p<0.001$. # indicates a significant difference at $p<0.05$ between pre- and post- SGID values measured by Student's t-test.

5.3.3 SGID BWSPHs increased glycerol levels in differentiated 3T3-L1 cells.

Figure 5.4 demonstrates that prior to SGID, incubation of 3T3-L1 adipocytes with BWSPHs (1.0% w/v dw) for 4 h induced no significant adipolytic effect as glycerol contents were not altered compared with the differentiated (MDI) control (p>0.05), in contrast to the positive control, isoproterenol (10 µg/mL), which increased glycerol content 5-fold (p<0.05). However, post-SGID, all hydrolysates, except BW-SPH-B-GI, at a concentration of 1.0% w/v dw, significantly increased glycerol content after 4 h exposure to mature adipocytes compared with the MDI control which was treated with DMEM alone for the 4 h period (p<0.05). Except for hydrolysate BW-SPH-B and its in vitro digest, all SGID BWSPHs demonstrated increased glycerol contents in 3T3-L1 adipocytes compared to the pre-digested forms (p<0.05) (Figure 5.4). In vitro digests BW-SPH-A-GI and BW-SPH-F-GI induced the highest levels of glycerol release of the six SGID hydrolysate tested with glycerol contents increasing from 100% (MDI control) to 675.1 % and 590.0 % for BW-SPH-A-GI and BW-SPH-F-GI, respectively (Figure 5.4).
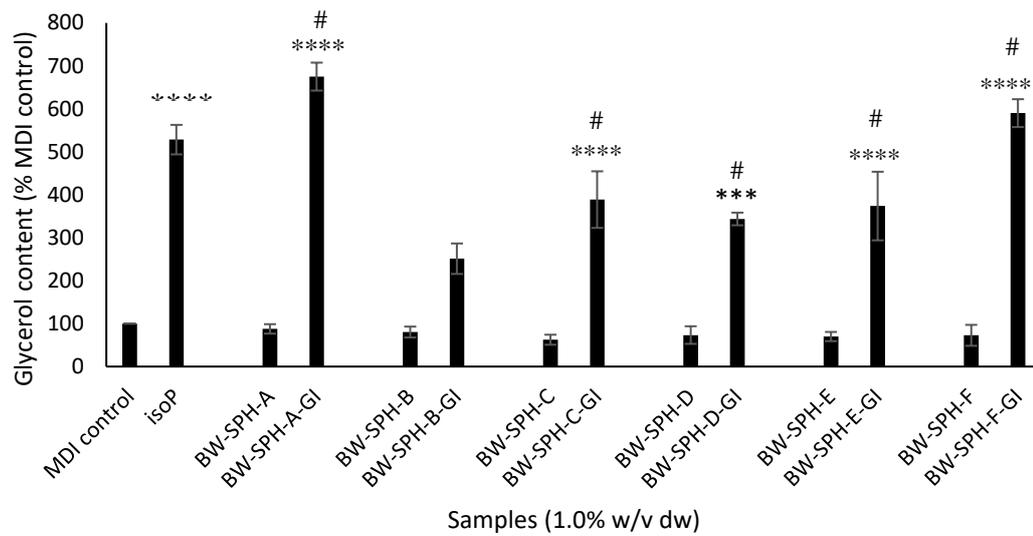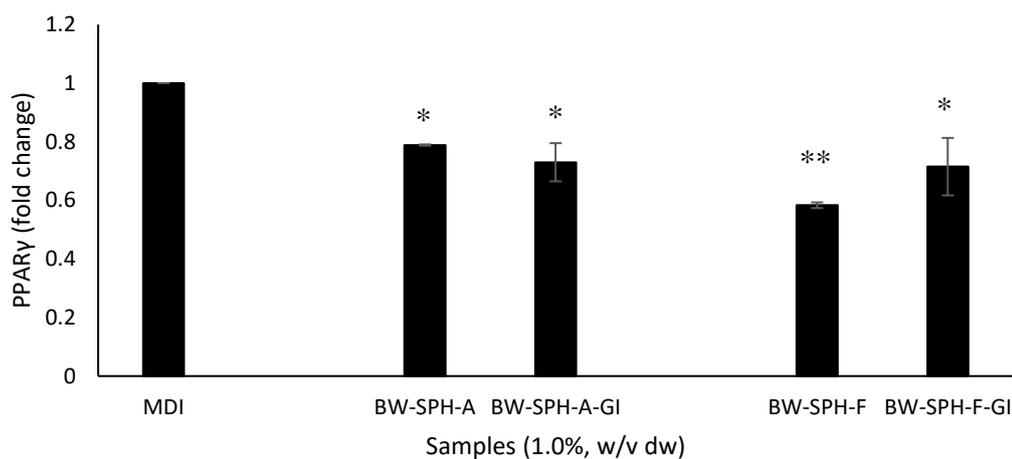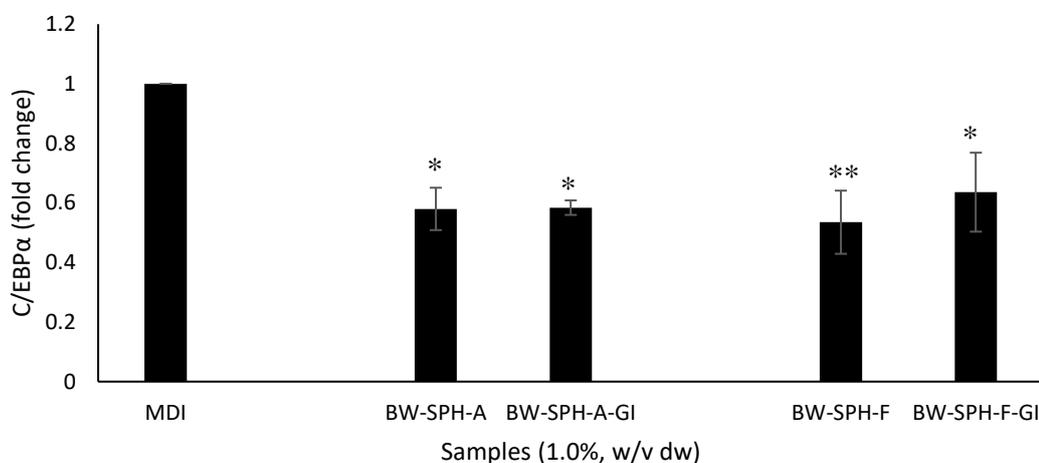
Figure 5.4. The adipolytic effect of blue whiting soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion as measured by glycerol content in 3T3-L1 adipocytes. 3T3-L1 preadipocytes (seeded at 1×10^5 cells/ mL, 2 mL/ well of a 6 well plate) were cultured in growth medium until they reached confluency and quiescent cells were incubated differentiation medium (MDI) for 8 days. Mature 3T3-L1 adipocytes were either exposed to the BWSPHs at a final concentration of 1.0% w/v dw, the positive control isoproterenol (isoP) (10 µg/mL), or the negative control DMEM alone (MDI control) for 4 h at a final volume of 2 mL per well. Glycerol content is expressed as % MDI control and data are mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * denotes statistically significant difference between the sample and MDI control ($p<0.05$). *** $p<0.001$ and **** $p<0.0001$. # indicates a significant difference at $p<0.05$ between pre- and post- SGID values measured by Student's t-test.

5.3.3 BWSPHs regulated adipogenic transcription factors

Two anti-adipogenic hydrolysates and their in vitro digests were chosen for assessment from this point forward. After incubation with BWSPHs BW-SPH-A and BW-SPH-F and their in vitro digests encoded BW-SPH-A-GI and BW-SPH-F-GI (1.0% w/v dw) for 8 days during 3T3-L1 preadipocyte differentiation, cell lysates were collected, RNA was extracted, cDNA was synthesised and the expression of adipogenesis-related transcription factors, PPARγ and C/EBPα, was analysed using RT-qPCR. As depicted in Figure 5.5 (A) and Figure 5.5 (B), the expression of both PPARγ and C/EBPα, respectively, was reduced following treatment with the BWSPHs and SGID BWSPHs compared with the differentiated control (MDI) ($p<0.05$) so that reductions in PPARγ were 0.21 to 0.42 -fold and reductions in C/EBPα were 0.36 to 0.46 -fold. These results indicate that 1.0% w/v dw BWSPHs suppressed adipogenesis via downregulation of C/EBPα and PPARγ expression.
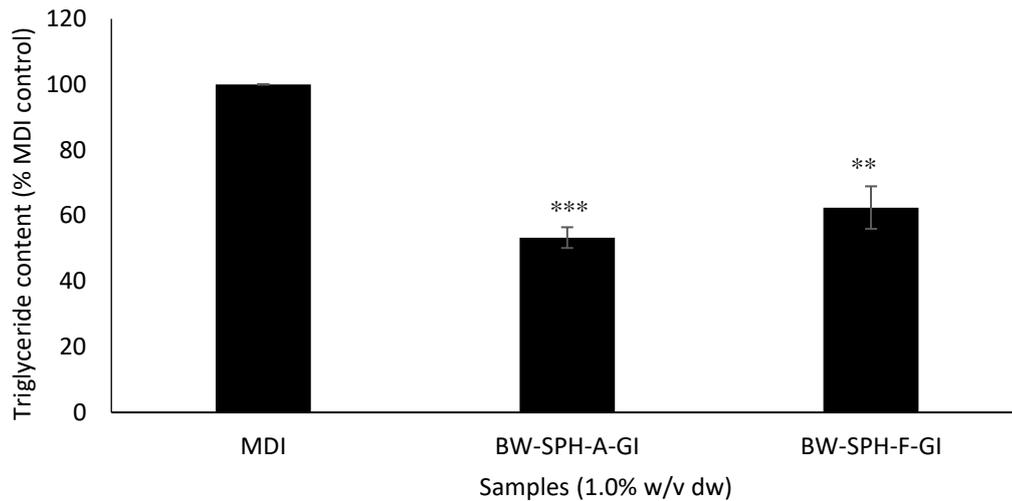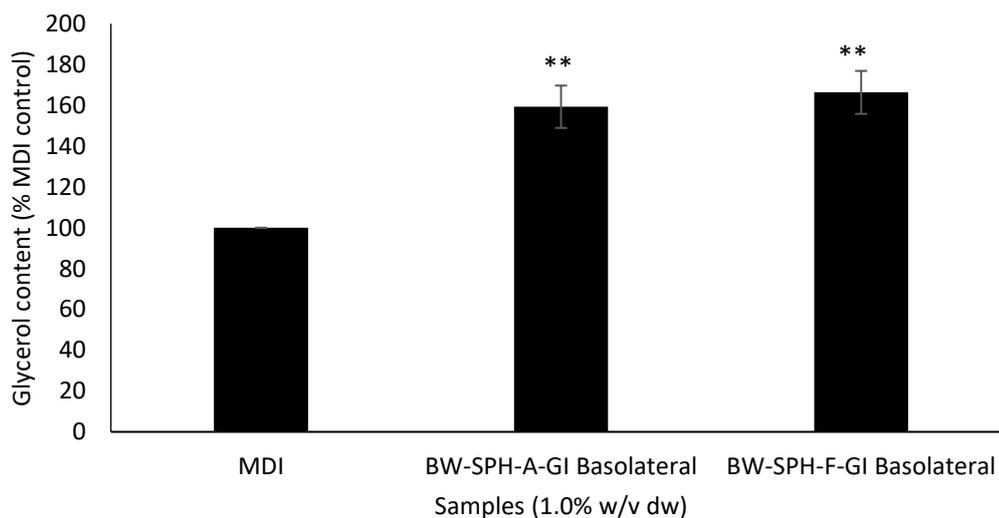
(A)

(B)

Figure 5.5. The effect of blue whiting soluble protein hydrolysates (BWSPHs) on adipogenic transcription factors. (A) The levels of PPARγ mRNA transcripts in 3T3-L1 cell lysates after exposure to BWSPHs (BW-SPH-A and BW-SPH-F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI and BW-SPH-F-GI) during 3T3-L1 cell differentiation compared with the differentiated control (MDI). (B) The levels of C/EBPα mRNA transcripts in 3T3-L1 cell lysates after exposure to BWSPHs (BW-SPH-A and BW-SPH-F) and SGID BWSPHs (BW-SPH-A-GI and BW-SPH-F-GI) during 3T3-L1 cell differentiation compared with the differentiated control (MDI). *36B4* (RPLP0) was used as a reference gene. Data are expressed as mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. * denotes statistical significance between individual samples and the MDI control (p<0.05), ** p<0.01.

5.3.4. BWSPHs post-SGID and following simulated intestinal absorption exhibited anti-obesity activities in 3T3-L1 cells

To test if hydrolysate bioactivity could cross the gut barrier, basolateral samples were collected from differentiated Caco2-HT29MTX monolayers after 4 h exposure to leading SGID hydrolysates BW-SPH-A-GI and BW-SPH-F-GI and 3T3-L1 cells were subsequently differentiated in the presence of MDI plus the co-culture cell basolateral. Figure 5.6 (A and B) demonstrates that following SGID and simulated intestinal permeation, hydrolysates BW-SPH-A-GI and BW-SPH-F-GI significantly reduced triglyceride accumulation during 3T3-L1 differentiation and increased glycerol production in fully mature 3T3-L1 adipocyte compared with the MDI controls (p<0.05).

(A)

(B)

Figure 5.6. The anti-obesity activities of blue whiting soluble protein hydrolysates (BWSPHs) post-simulated gastrointestinal digestion (SGID) and following simulated intestinal absorption in 3T3-L1 cellular model systems. (A). The anti-adipogenic effect of BWSPHs post-SGID and simulated intestinal absorption as measured by quantifying triglyceride contents in 3T3-L1 adipocytes. Basolateral samples were collected from differentiated Caco2-HT29MTX cells after 4 h exposure to SGID BWSPHs BW-SPH-A-GI and BW-SPH-F-GI. 3T3-L1 preadipocytes (seeded at 1 x 10^5 cells/mL, 2 mL/well of a 6 well plate) were subsequently differentiated in

differentiation medium (MDI) plus the BWSPHs following SGID and simulated intestinal permeation at a final concentration of 1.0% w/v dw or without BWSPHs (MDI control) for 8 days. (B) The adipolytic effect of BWSPHs post-SGID and simulated intestinal absorption as measured by glycerol contents in 3T3-L1 adipocytes. After 4 h exposure of SGID BWSPHs to differentiated Caco2-HT29MTX cells, basolateral samples were collected and fully mature 3T3-L1 adipocytes were either exposed to BWSPHs basolateral (final concentration of 1.0% w/v dw) or not (MDI control) for 4 h. Triglyceride content and glycerol content are expressed as % MDI control and data are mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. ** and *** denotes statistically significant difference between sample and MDI control at $p<0.01$ and $p<0.001$, respectively.

5.3.4 The antioxidant activity of BWSPHs

Two 3T3-L1 cellular models were employed to assess the antioxidant potential of BWSPHs and SGID BWSPHs. In one model, 3T3-L1 preadipocytes were differentiated in the presence of the BWSPHs over 8 days and the ability of the BWSPHs to protect 3T3-L1 cells against oxidative stress was assessed. In the alternative model, the antioxidant effect of 24 h incubation of fully mature adipocytes with BWSPHs (1.0% w/v dw) against an oxidative challenge was assessed. As observed in Table 5.3, preincubation with BW-SPH-A significantly increased GSH concentration in tBOOH-challenged 3T3-L1 adipocytes compared with tBOOH-stimulated cells which were differentiated with MDI alone (control) ($p < 0.05$). However, this antioxidant response was lost following SGID of the hydrolysate as no significant effect of BW-SPH-A-GI on GSH content was observed in tBOOH-challenged 3T3-L1 cells compared to the tBOOH control ($p > 0.05$). Preincubation with hydrolysate BW-SPH-F, neither pre-SGID nor post-SGID, significantly influenced GSH levels in tBOOH-challenged 3T3-L1 cells compared with the stimulated control ($p > 0.05$). In addition, none of the hydrolysates before or after SGID significantly altered ROS production compared with the H_2O_2-stressed control (Table 5.3). In the alternative cellular model, it was observed that 24 h incubation of fully mature adipocytes with BW-SPH-A, BW-SPH-A-GI, BW-SPH-F or BW-SPH-F-GI did not induce any effect on antioxidant GSH or ROS levels after stimulation with tBOOH or H_2O_2 ($p > 0.05$) (Table 5.4). Therefore, BWSPHs may induce antioxidant effects when exposed to immature adipocytes but not in fully formed adipocyte cells.

Table 5.3. The cellular antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) during 3T3-L1 preadipocyte differentiation

Sample Code (1.0% w/v dw)	ROS production (% H_2O_2)	GSH content (% tBOOH)
Control	100.0 ± 0.0	100.0 ± 0.0
BW-SPH-A	92.1 ± 1.4	145.0 ± 4.1*
BW-SPH-A-GI	94.4 ± 7.5	87.9 ± 5.6
BW-SPH-F	110.5 ± 6.6	122.4 ± 8.0
BW-SPH-F-GI	90.0 ± 4.2	98.4 ± 0.9

The antioxidant potential of BWSPHs before simulated gastrointestinal digestion (SGID) (BW-SPH-A and BW-SPH-F) and after SGID (BW-SPH-A-GI and BW-SPH-F-GI) at a concentration of 1.0%, w/v dry weight (dw) as assessed by their ability to reduce reactive oxygen species (ROS) production in hydrogen peroxide (H_2O_2)-treated 3T3-L1 cells and increase glutathione (GSH) concentration in tertbutyl hydroperoxide (tBOOH)-treated 3T3-L1 cells. For GSH assays, 3T3-L1 cells (1×10^5 cells/ mL, 2 mL/ well) were exposed to BWSPHs on day 0 of the differentiation period and fully differentiated 3T3-L1 cells were stimulated with tBOOH (1 mM, 3 h) on day 8. ROS production was measured in 3T3-L1 cells (1×10^5 cells/ mL, 200 mL/ well) which were differentiated in the presence of BWSPHs (1.0% w/v dw) over 8 days, followed by a H_2O_2 challenge (1 mM, 1 h) on day 8. Values were expressed as a percentage relative to positive control (100%). Values represent the mean $\pm$ SEM of at least three independent experiments. Statistical significance between samples and the control was measured using ANOVA followed by Dunnett's test. * denotes statistically significant difference between sample and the control at $p<0.05$.

Table 5.4. Cellular antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) after 3T3-L1 cell differentiation

Sample Code (1.0% w/v dw)	ROS production (% H_2O_2)	GSH activity (% tBOOH)
Control	100.0 ± 0.0	100.0 ± 0.0
BW-SPH-A	98.3 ± 1.2	109.4 ± 13.4
BW-SPH-F	104.1 ± 3.4	84.1 ± 19.2

The antioxidant potential of BWSPHs (BW-SPH-A and BW-SPH-F) at 1.0% w/v dry weight (dw) as assessed by their ability to increase glutathione (GSH) concentration in tertbutyl hydroperoxide (tBOOH)-treated 3T3-L1 cells and reduce reactive oxygen species (ROS) production in hydrogen peroxide (H_2O_2)-treated 3T3-L1 cells. For GSH assays, 3T3-L1 cells (1×10^5 cells/mL, 2 mL/well) were exposed to BWSPHs on day 8 of the differentiation period for 24 h followed by a 3 h exposure to tBOOH challenge (1 mM). For the ROS assay, 3T3-L1 cells (1×10^5 cells/mL, 200 mL/well) were exposed to BWSPHs on day 8 of the differentiation period for 24 h followed by a 1 h exposure to H_2O_2 challenge (1 mM). Values were expressed as a percentage relative to positive control (100%). Values represent the mean $\pm$ SEM of at least three independent experiments. Statistical significance between samples and the control was measured using ANOVA followed by Dunnett's test.

5.3.5 The immunomodulatory activity of BWSPHs

Mature adipocytes were pretreated with BWSPH prior to SGID (BW-SPH-A and BW-SPH-F) and after SGID (BW-SPH-A-GI and BW-SPH-F-GI) (1.0% w/v dw) for 24 h followed by exposure to LPS for a further 24 h period to determine the effect that BWSPHs have on pro-inflammatory and anti-inflammatory cytokine production in 3T3-L1 adipocyte cells (Table 5.5). Pre-SGID hydrolysate BW-SPH-F reduced pro-inflammatory cytokine IL-6 levels in LPS-stimulated 3T3-L1 adipocytes compared with the LPS control (p<0.05), however, this activity was lost after SGID of the hydrolysate. Contradictory, both hydrolysates BW-SPH-A and BW-SPH-F before and after SGID reduced anti-inflammatory cytokine adiponectin production in LPS-stimulated cells (p<0.05). No effect on the levels of pro-inflammatory cytokine MCP-1 was observed following exposure of 3T3-L1 adipocytes to hydrolysates plus LPS compared to cells treated with LPS alone (p>0.05).

Table 5.5. The effect of blue whiting soluble protein hydrolysates (BWSPHs) before simulated gastrointestinal digestion (SGID) (BW-SPH-A and BW-SPH-F) and after SGID (BW-SPH-A-GI and BW-SPH-F-GI) on cytokine production in lipopolysaccharide (LPS)-stimulated 3T3-L1 adipocyte cells.

Sample Code (1.0% w/v dw)	IL-6 (% LPS)	MCP-1 (% LPS)	Adiponectin (% LPS)
Control	100.0 ± 0.0	100.0 ± 0	100.0 ± 0.0
BW-SPH-A	95.9 ± 3.2	106.1 ± 5	61.4 ± 6.4 **
BW-SPH-A-GI	125.0 ± 5.6	94.6 ± 6.0	74.6 ± 2.4 ***
BW-SPH-F	80.4 ± 6.3 *	100.4 ± 8.7	67.3 ± 8.3 **
BW-SPH-F-GI	109.3 ± 10.4	88.8 ± 8.6	74.7 ± 4.8 ***

Mature 3T3-L1 adipocytes were incubated in the presence of BWSPHs and SGID BWSPHs for 24 h, followed by stimulation with lipopolysaccharide (LPS) at a concentration of 0.1 µg/mL for a further 24 h. The levels of interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1) and adiponectin produced were then analysed using commercial enzyme-linked immunosorbent assay (ELISA) kits. Values were expressed as a percentage relative to positive control, cells treated with 0.1 µg/mL LPS alone (Control, 100% cytokine secretion). Values represent the mean ± SEM of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * and ** denotes statistically significant difference in cytokine production between sample and LPS control at $p<0.05$ and $p<0.01$, respectively.

5.4 Discussion

The results presented in this study demonstrate that hydrolysates generated from blue whiting fish exhibit anti-obesity, antioxidant and immunomodulatory activities in 3T3-L1 cells. The hydrolysates tested herein have previously been shown to demonstrate weight management potential through in vitro promotion of satiety hormones (Heffernan et al., 2022), as well as antioxidant and immunomodulatory activities through various chemical and cellular assays (Heffernan et al., 2021). Bioactive compounds have been shown to exert anti-obesity activities via numerous cellular modifications such as inducing cytotoxic effects on preadipocyte cells, inhibiting adipogenesis or hydrolysing triglycerides in adipocyte cellular model systems. The results of this study show that BWSPHs neither pre- nor post- SGID affected preadipocyte or adipocyte cell proliferation up to a concentration of 1.0% w/v dw (Tables 5.1 and 5.2). Nevertheless, some studies have demonstrated the relationship between adipocyte cell proliferation and differentiation (Fan et al., 2018; Henda et al., 2015). Peptides (2 mg/mL) purified from *Spirulina platensis* were observed to inhibit 3T3-L1 preadipocyte proliferation by up to 60.02% after incubation for 48 h, without affecting normal liver cells L-O2. It is expected that this inhibitory effect on preadipocyte proliferation is related to the subsequent reduction in triglyceride accumulation after 48 h exposure to differentiated preadipocytes compared with the control (buffer only) (Fan et al., 2018). In addition, numerous natural products including phenolic compounds have been shown to modulate adipocyte tissue mass through inhibition of cell division and/or induction of cell death by apoptosis (Rayalam et al., 2008).

Moreover, BWSPHs tested in this study did demonstrate anti-obesity potential through anti-adipopgenic and adipolytic activities in 3T3-L1 cells. Although BWSPHs BW-SPH-A, BW-SPH-B, BW-SPH-C and BW-SPH-F reduced triglyceride accumulation in 3T3-L1 preadipocytes compared with the differentiated control (p<0.05), all SGID BWSPHs, including BW-SPH-D-GI and BW-SPH-E-GI which exerted no anti-adipogenic effect prior to SGID, reduced triglyceride levels in 3T3-L1 preadipocytes compared with the differentiated control (p<0.05). These results indicate that the hydrolysate bioactive components were either resistant to degradation during SGID and/or additional lower molecular weight peptides were generated with similar activities for hydrolysates BW-SPH-A, BW-SPH-B, BW-SPH-C and BW-

SPH-F or more potent peptides/ amino acids were released upon digestion with gastrointestinal proteases in the case of hydrolysates BW-SPH-D and BW-SPH-E.

To determine the mechanism by which BWSPHs reduced adipogenesis in 3T3-L1 cells, their effect on key transcription factors (PPARγ and C/EBPα) associated with the induction of adipocyte phenotypes was assessed. Transcription factors C/EBPβ and C/EBPδ are expressed at the early stages of differentiation and together stimulate the expression of PPARγ and C/EBPα in the later stages of differentiation to induce the activation of adipocyte specific mRNAs necessary for adipocyte formation (Ahmad et al., 2020). Results of RT-qPCR analysis demonstrated that SGID hydrolysates significantly down-regulated PPARγ and C/EBPα mRNA transcript levels compared with the MDI control (p<0.05), thereby elucidating the mechanism by which BWSPHs BW-SPH-A and BW-SPH-F and their in vitro digests regulated the differentiation of 3T3-L1 preadipocytes to adipocytes. Many other marine-derived peptides have been shown to module lipid accumulation in adipocyte cellular models through the regulation of these major transcription factors as well as adipogenic and lipolytic genes. Treatments with peptides derived from boiled tuna (0.5 and 1.0 mg/ mL, 48 h) and subcritical water-hydrolysed fish collagen (0.5 and 1.0 mg/ mL, 8 d) were shown to reduced triglyceride accumulation in differentiated 3T3-L1 cells through suppressing the activation of PPARγ and C/EBPα (Kim et al., 2015a; Lee et al., 2017). A follow up study by Kim et al., (2015b) reported that the boiled tuna peptide also downregulated the expression of key adipogenic and lipogenic genes whilst expression levels of Wnt signaling pathway-related proteins were upregulated in differentiated 3T3-L1 adipocytes, thereby activating the Wnt/β-catenin signalling pathway involved in adipogenesis inhibition (Kim et al., 2015b).

The induction of adipolysis is another potential therapeutic target for obesity albeit research investigating the adipolytic potential of food-derived protein hydrolysates is limited. In the study herein, prior to SGID, all BWSPHs failed to modulate glycerol levels in fully mature 3T3-L1 adipocytes compared with the differentiated control (p>0.05). Interestingly, upon in vitro digestion of the hydrolysates, adipolytic activity was significantly improved. All SGID BWSPHs, with the exception of BW-SPH-B-GI, increased glycerol release after 4 h exposure to fully differentiated 3T3-L1 adipocytes compared with the differentiated control (p<0.05). It is possible that the increased DH as a result of SGID of the BWSPHs improved their adipolytic activity as a result of the generation of more bioactive peptides and/or amino

acids, or even through the removal of conflicting fractions which may have been negating hydrolysate adipolytic ability.

A previous study by Harnedy-Rothwell et al. (2021) analysed the physicochemical properties of the BWSPHs tested herein including DH, molecular mass distribution and reversed-phase ultra-performance liquid chromatography (Harnedy-Rothwell et al., 2021). These data demonstrated that SGID further hydrolysed BWSPHs with the DH of pre-SGID hydrolysates ranging from $27.82 \pm 1.11\%$ (BW-SPH-C) to $45.78 \pm 2.91\%$ (BW-SPH-B) and the DH of SGID hydrolysates ranging from $55.37 \pm 1.83\%$ (BW-SPH-C-GI) to $65.23 \pm 1.05\%$ (BW-SPH-E-GI), correlating with an increase in <1 kDa components. After SGID, the hydrolysates were observed to have high levels of amino acids Arg, Leu and Lys (Harnedy-Rothwell et al., 2021). Interestingly, studies have demonstrated Arg, Leu and Lys to interfere with adipocyte metabolism in adipocyte tissue and in vitro cultured cells (Bi'e Tan et al., 2012; Kyoya et al., 2011; Zhang et al., 2020), therefore the amino acid content may have played a role in the adipolytic activity of SGID BWSPHs. Future studies should investigate the underlying mechanism which mediate SGID hydrolysates adipolytic activity as protein hydrolysates purified from ask shell were observed to modulate glycerol levels in mouse mesenchymal stem cells through down-regulation of LPL and fatty acid synthase expression (Hyung et al., 2017).

In addition to proteases such as pepsin, trypsin and chymotrypsin located along the gastrointestinal tract, hydrolysing enzymes can also be found at the brush border membrane of intestinal epithelial cells which can influence the physicochemical and bioactive properties of food components (Karaś, 2019). In order for BWSPHs to exert anti-obesity effects in vivo, including weight management and blood lipid profile enhancement, hydrolysate bioactivity must survive epithelial permeation. A co-cultivation of Caco-2 cells and HT-29MTX cells provides a cellular model constituting the two cell types that are most represented in normal epithelium, enterocytes and goblet cells, thereby expressing the brush border with typical small intestinal enzymes (Kleiveland, 2015). The bioavailability, i.e., the ability of a peptide or hydrolysate to reach the systemic circulation intact whilst retaining its bioactivity, was assessed through exposing the lead anti-obesity SGID BWSPHs to 21-day differentiated Caco-2/HT-29MTX monolayers and subsequently measuring the anti-adipogenic and adipolytic effects of the sample basolateral in 3T3-L1 cells. The basolateral media of lead hydrolysates BW-SPH-A-GI and BW-SPH-F-GI significantly reduced

triglyceride accumulation during the differentiation of 3T3-L1 preadipocytes and increased glycerol release in mature adipocytes compared with the MDI controls ($p < 0.05$). These hydrolysates may resist complete degradation by gastrointestinal proteases and brush-border peptidases, be absorbed through the intestinal epithelium, and reach the bloodstream in an active form, although in vivo studies are necessary to validate the results of this in vitro study.

It is commonly reported that oxidative stress and obesity are closely linked. Excessive fat accumulation may result in the development of cellular damage which in turn, induces pro-inflammatory cytokines production, generating ROS and increasing lipid peroxidation rate in tissue (Fernández-Sánchez et al., 2011). The overproduction of ROS is associated with cellular oxidative stress and impairment of cell function. BWSPHs tested herein have previously demonstrated 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} values ranging from 2.10 ± 0.12 to 2.47 ± 0.04 mg/mL), Oxygen Radical Absorbance Capacity (ORAC, 330.79 ± 9.76 to 393.32 ± 3.23 µM TE/g dw) and exhibited Ferric Reducing Antioxidant Power (FRAP) values ranging from 7.25 ± 0.22 to 8.45 ± 0.08 µM TE/g dw (Heffernan et al., 2021). In addition, when exposed to oxidatively stressed RAW264.7 cells for 24 h at a concentration of 0.5% w/v dw, hydrolysate BW-SPH-A significantly reduced ROS production and increased GSH concentration compared with the stimulated controls ($p < 0.05$) (Heffernan et al., 2021). Therefore, this study was designed to assess the protective effects of BWSPHs against oxidative stress during and after the differentiation of 3T3-L1 preadipocytes.

Although many studies have reported a relationship between lipid accumulation and ROS production (Choi et al., 2018), a significant increase in ROS production during 3T3-L1 cell differentiation was not observed in this study (data not shown), therefore 3T3-L1 adipocytes were stimulated with oxidants (either tBOOH or H_2O_2) to induce oxidative stress. When added simultaneously with MDI on day 0 of 3T3-L1 preadipocyte differentiation, BW-SPH-A significantly increased cellular GSH content in tBOOH-stimulated 3T3-L1 adipocytes compared with the tBOOH control ($p < 0.05$). Studies have reported the involvement of the cellular antioxidant defence system in the regulation of the adipogenic differentiation response. For example, Kim and Lee (2017) reported that the coumarin derivative and polyphenol compound, esculetin, at concentrations ranging from 25 to 100 µM, reduced lipid accumulation and ROS production and increased GSH levels in 3T3-L1 adipocyte cells after 6 days

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of differentiation (p<0.05) (Kim & Lee, 2017). In this study, it is less likely that GSH promotion induced by hydrolysate BW-SPH-A during the differentiation of 3T3-L1 preadipocytes played a role in reducing triglyceride accumulation and more likely that the regulation of adipogenic transcription factors were responsible for this observed anti-adipogenic effect. Moreover, a relatively low increase in GSH levels alone as observed in this study may not have important physiological relevance and sustained stimulation of GSH metabolism is necessary to physiologically reduce oxidative stress in adipose tissue. In vivo studies may be necessary to assess the physiological meaning of these antioxidant results however gastrointestinal protective coatings may be required as BWSPHs post-SGID did not exhibit antioxidant activity in vitro.

It is proposed that the antioxidant activity of fish protein hydrolysates is substantially affected by processing factors and the structural properties of resulting peptides (Ishak & Sarbon, 2018). Hydrolysates BW-SPH-A and BW-SPH-F were hydrolysed from blue whiting with different proteolytic preparations and E:S ratios (Harnedy-Rothwell et al., 2021) which can play a major role in the DH % and the composition of the resulting fractions due to protease specificity. It may be the case that the specific peptide(s) in BW-SPH-A were responsible for hydrolysate antioxidant activity and that these peptides were degraded during SGID, resulting in the loss of this antioxidant potential. Future studies should identify the peptide(s) responsible for the GSH promotion induced by BW-SPH-A in tBOOH-stimulated 3T3-L1 adipocytes.

High fat diets have been shown to induce metabolic endotoxemia in murine studies resulting in increased body weight, fasted glycemia, and inflammation similar to that of LPS infused normal diet-fed mice (Cani et al., 2007). LPS has been shown to trigger inflammation in adipose tissue through activation of Toll-like receptor-4 which upregulates IL-6 and MCP-1 expression via mitogen-activated protein kinase (MAPKs) signalling pathways (Kim et al., 2012; Kopp et al., 2009). An innate immune response induced by the gram-negative bacterial LPS has been observed in differentiated 3T3-L1 adipocytes (Chirumbolo et al., 2014). 3T3-L1 preadipocytes fully differentiated in vitro and challenged with LPS have been utilised as a useful model to identify anti-inflammatory food components which can potentially regulate inflammation in adipose tissue (Zoico et al., 2010). To study the effect of BWSPHs on stimulated inflammation in adipocyte cells, the levels of pro-inflammatory cytokines, IL-6 and MCP-1, and anti-inflammatory cytokine, adiponectin, were measured after 24 h incubation of 3T3-L1 adipocytes with BWSPHs followed by stimulation with

LPS for a further 24 h. In this study, varying effects on pro-inflammatory and anti-inflammatory cytokine levels in 3T3-L1 cells after BWSPH and LPS treatments were observed. Although hydrolysate BW-SPH-F reduced pro-inflammatory cytokine IL-6 levels in LPS-stimulated 3T3-L1 adipocytes compared with the LPS control (p<0.05), this relatively low reduction may not be sufficient to induce anti-inflammatory effects physiologically. Moreover, this activity was lost following SGID of the hydrolysate indicating the instability of the hydrolysates anti-inflammatory activity during gastrointestinal transit. Furthermore, after SGID, both hydrolysates reduced protein levels of anti-inflammatory cytokine adiponectin in LPS-stimulated 3T3-L1 adipocytes compared with the LPS control (p<0.05) indicating the possibility that these hydrolysates may induce an inflammatory response in adipocyte tissue.

Although, to the best of our knowledge, there are no other studies reporting pro-inflammatory effects of food components in similar cell models, numerous food components with anti-inflammatory activities in LPS-stimulated 3T3-L1 adipocyte models have been identified. Alliin, a garlic compound, prevented LPS-induced inflammation in 3T3-L1 adipocytes. After 24 h pre-treatment of fully differentiated 3T3-L1 adipocytes with alliin (100 µmol/L) followed by 1 h stimulation with LPS (100 ng/mL), mRNA transcript levels of pro-inflammatory genes IL-6, MCP-1 and early growth receptor 1 (Egr-1) were significantly reduced compared with the LPS control (p<0.05) (Quintero-Fabián et al., 2013). Similar anti-inflammatory results were observed for peanut sprout, tart cherry, saccharin, catechin (Cheng et al., 2019; Jayarathne et al., 2018; Kim et al., 2020; Seo et al., 2021).

The outcome regarding the inflammatory effects of BWSPHs towards LPS-stimulated 3T3-L1 adipocytes in this study is consistent with the data obtained in our previous study which examined the immunomodulatory effect of BWSPHs and their corresponding gastrointestinal digests in LPS-stimulated RAW264.7 cells. These SGID BWSPHs were also observed to induce pro-inflammatory effects in LPS-stimulated RAW264.7 macrophages through increasing inflammatory cytokine IL-6 and TNF-a protein levels compared with the LPS control (p<0.05). The therapeutic use of anti-obesity compounds which possess pro-inflammatory activity is unlikely however murine studies investigating the levels of pro-inflammatory and anti-inflammatory cytokines in adipose tissue following BWSPHs treatment should be conducted to assess the physiological immunomodulatory effects of these hydrolysates.

5.5 Conclusion

In conclusion, the present study demonstrated that specific BWSPHs post SGID and following simulated intestinal absorption reduced lipid accumulation during 3T3-L1 differentiation and increased glycerol content in mature 3T3-L1 adipocyte cells, indicative of anti-adipogenic and adipolytic activities, respectively. The mechanism of action of these anti-adipogenic BWSPHs was elucidated, and it was revealed that BWSPHs downregulated the expression of key adipogenic transcription factors, PPARγ and C/EBPα, during preadipocyte differentiation. In addition, these BWSPHs were observed to induce antioxidant and immunomodulatory effects in stimulated 3T3-L1 cells, however further in vivo studies are recommended to assess the physiological relevance of these results.

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Chapter 6

General Discussion

6.1 Main findings

A rapid increase in the number of commercial functional foods has been observed in recent years with the functional food market estimated to be worth over €250 billion by 2023 (Bordbia, 2019). This growth is correlated with the increase in consumer awareness of the relationship between diet and health (La Barbera et al., 2016). Recent scientific and global protein demand, along with EU fish quota changes, clearly indicate that marine species have potential as a viable source of nutritional and functional ingredients. Blue whiting *(Micromesistius poutassou)* is landed in large quantities with very few profitable applications; thus, the opportunity exists to increase its value through the identification of bioactive components which can be advantageously used as high-value ingredients in functional foods. The aim of this thesis was to identify a bioactive, bioavailable protein hydrolysate enzymatically purified from blue whiting for potential functional food development. With modern aqua-cultural businesses producing large amounts of waste material, academic and industrial collaborative projects, such as this study, can symbiotically exploit bioactive peptides from waste to assist the development of value-added products. Interdisciplinary expertise is required to solve the many challenges associated with functional food development, including production issues, gastrointestinal stability, bioavailability, sensory issues, and food grade formulations.

Generally, hydrolysis of proteins enhances their bioactivity through the exposure of active sites embedded within the parent protein matrix and via the release of bioactive peptides which are more bioavailable than larger molecular weight proteins. Many factors affect the resulting peptides composition including protein source, the choice of protease, the degree of hydrolysis and the hydrolysis conditions employed. Blue whiting soluble protein hydrolysates (BWSPHs) used in the studies described in this thesis were prepared using singular or combinations of food-grade microbial proteolytic enzymes with various hydrolytic conditions. The generation of BWSPHs initially began at laboratory scale to create a consistent and reproducible protocol. Production was upscaled and subsequent assessment of industrial scale BWSPHs demonstrated that certain bioactivities were in fact enhanced, thus these hydrolysates are potentially suitable for economically viable commercial production.

The aim of the initial study (Chapter 2) was to screen, using cellular in vitro assays, eleven protein hydrolysates from blue whiting produced with various proteases

at laboratory scale. This study assessed the antioxidant, immunomodulatory, satiating and anti-adipogenic abilities of these protein hydrolysates using appropriate cell lines. All of the laboratory scale hydrolysates failed to modulate biomarkers indicative of the specific bioactivities. Laboratory scale BWSPHs neither stimulated active glucagon-like peptide-1 (GLP-1) secretion from murine enteroendocrine STC-1 cells, nor prevented triglyceride accumulation in murine 3T3-L1 preadipocytes. In addition, BWSPHs did not modulate reduced glutathione (GSH) and nitric oxide (NO) levels in hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS) challenged murine RAW264.7 macrophage cells, respectively. However, this study only measured one biomarker of each bioactivity, therefore no definitive conclusions regarding the potential bioactivities of BWSPHs can be taken from these results. With that being said, laboratory-generated BWSPHs induced cytotoxic effects on STC-1 cells, 3T3-L1 cells and RAW264.7 cells as measured using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Many studies have reported anti-cancer activities of fish protein hydrolysates via their anti-proliferative nature in cells of cancerous origins (Shaik & Sarbon, 2020). Although cells such as MDA-MB-231 and MCF-7/6 cells (breast cancer cell lines), AGS cells (stomach cancer cell line), or DLD-1 cells (colon cancer cell line) are commonly used to measure anti-cancer activity, the STC-1 and RAW264.7 cell lines used in Chapter 2 are also immortalised cell lines of cancerous origins. If time allowed, it would have been an interesting addition to the thesis to study the effect of these hydrolysates on the viability of the aforementioned breast cancer, stomach cancer or colon cancer cells; however bioactive assessment of industrially prepared hydrolysates took precedence.

Our collaborators in the University of Limerick, who generated the BWSPHs, chose six laboratory scale hydrolysates to produce at industrial scale based on physicochemical (molecular mass distribution and hydrophobic/hydrophilic profiles) and biofunctional (dipeptidyl peptidase IV (DPP-IV) inhibitory activity and Oxygen Radical Absorbance Capacity (ORAC)) properties. The remainder of the thesis (Chapters 3-5) investigated the six BWSPHs prepared at industrial scale for their bioactivity, gastrointestinal stability and bioavailability. Cellular oxidative stress can be induced through cellular metabolic processes and environmental factors potentially resulting in the development of non-communicable diseases including cancer, diabetes and cardiovascular disease. The majority of protein hydrolysates purified from marine sources have demonstrated antioxidant activities in non-cellular models. In this study,

in vitro results demonstrated that the BWSPHs exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibitory activity comparable with that of other fish protein hydrolysates (Chapter 3). The results of further in vitro antioxidant assays were obtained from the University of Limerick who demonstrated the ORAC activity and Ferric Reducing Antioxidant Power (FRAP) exhibited by the BWSPHs. In addition, the effect of simulated gastrointestinal digestion (SGID) on hydrolysate antioxidant activity was assessed. Antioxidant activity is largely influenced by hydrolysate composition which can be significantly altered upon further hydrolysis with gastrointestinal proteolytic enzymes. As both pre- and post- SGID BWSPHs demonstrated ORAC and FRAP activities, cellular models were employed to determine the ability of BWSPHs to modulate endogenous cellular antioxidant defence systems. The hydrolysate designated BW-SPH-A exhibited the greatest antioxidant potential of the six hydrolysates tested, increasing the levels of non-enzymatic antioxidant GSH and enzymatic antioxidant catalase (CAT) as well as reducing reactive oxygen species (ROS) production in oxidatively-stressed RAW264.7 cells. In addition, SGID of BW-SPH-A did not affect its ability to increase GSH content or reduce ROS production in stimulated cells, however SGID did inhibit BW-SPH-A from significantly increasing CAT activity compared with the H_2O_2 -stimulated control. In contrast, SGID hydrolysate BW-SPH-E-GI modulated CAT activity in H_2O_2-stimulated cells whereas prior to in vitro digestion, the hydrolysate displayed no antioxidant activity across the three cellular antioxidant biomarkers assessed, thereby presenting the various effects that SGID can impose on hydrolysate bioactivity. Hydrolysates BW-SPH-A and BW-SPH-A-GI were observed as the top rankers for overall antioxidant potential.

The immunomodulatory potential of BWSPHs was also assessed (Chapter 3). Surprisingly, all BWSPHs and SGID BWSPHs were observed to increase nitric oxide (NO), an inflammatory mediator, and certain BWSPHs and SGID BWSPHs were observed to increase inflammatory cytokines IL-6 and TNF-α. SGID hydrolysate BW-SPH-F-GI significantly increased both IL-6 and TNF-α levels in LPS-stimulated RAW264.7 cells. This immunostimulating property may have use in specific applications, potentially targeting immunosuppressed patients. Future studies should identify peptide sequences in hydrolysates which demonstrated varying bioactive potential before and after SGID to determine which peptide sequences were responsible for the observed bioactivity. In addition, identification of peptide

sequences within antioxidant protein hydrolysate BW-SPH-A and immunomodulatory hydrolysate BW-SPH-F would allow for comparisons to be made with known bioactive peptides listed in online peptide databases.

In relation to the satiating potential of BWSPHs (Chapter 4), BWSPHs were demonstrated to significantly increase active GLP-1 secretion and proglucagon mRNA transcript levels in STC-1 cells compared with the Krebs-Ringer buffer control. No effect on cholecystokinin (CCK) or peptide YY (PYY) mRNA transcript levels were observed. The physiological effect of active GLP-1 promotion without CCK or PYY enhancement requires further studies with in vivo models to determine the actual relationship between hormone plasma levels and food intake analysis. However, the in vitro digestion of hydrolysates inhibited this hormone stimulating ability, possibly indicating the role of peptides rather than amino acids in hydrolysate satiating activity. The signalling pathway activated for GLP-1 secretion was subsequently assessed. BWSPHs prior to SGID were observed to promote intracellular calcium accumulation without inducing any effect on cyclic adenosine monophosphate (cAMP) indicating either the involvement of calcium sensing receptors or ion-coupled transporters.

The loss of satiety-inducing effects upon digestion of the BWSPHs could restrict their use as functional food ingredients unless some means of hydrolysate protection was applied. Encapsulation with pH coatings or alternative protective coatings may protect the hydrolysate structure during gastrointestinal transit, thereby maintaining their satiating potential. Other protein hydrolysates derived from blue whiting fish have demonstrated satiating potential in vitro, in vivo and in clinical trials and a satiety-inducing functional food product is now available commercially with the active ingredient being a branched chain amino acid enriched blue whiting hydrolysate. The exact composition of this hydrolysate is not available; it would have been interesting to compare the composition of our BWSPHs with the composition of the blue whiting ingredient in the commercial product.

This study also aimed to determine the effects of BWSPHs on gut health by assessing simulated intestinal barrier integrity and inflammation using the well-established Caco-2/HT-29MTX co-culture model (Chapter 4). Transepithelial electrical resistance (TEER) values were monitored before and after BWSPH exposure to the intestinal epithelial cell lines and TEER values remained greater than 900 $\Omega.cm^2$, indicative of tight junctions and a healthy barrier. In addition, BWSPHs did not stimulate IL-6 production in differentiated Caco-2/HT-29MTX cells, indicating

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BWSPHs may not induce an inflammatory response in the gut, which can potentially lead to the development of inflammatory bowel disease.

The ability of BWSPHs to modulate triglyceride and glycerol levels in differentiated 3T3-L1 preadipocytes was assessed to determine their potential anti-obesity activity (Chapter 5). Interestingly, hydrolysates demonstrated varying anti-obesity activities with some hydrolysates including BW-SPH-A, BW-SPH-B, BW-SPH-C and BW-SPH-F significantly reducing triglyceride accumulation during the cell differentiation process, whereas none of the hydrolysates increased glycerol levels after 4 h exposure to fully mature adipocytes. Further in vitro hydrolysis of BWSPHs with gastric and intestinal proteases enhanced hydrolysate anti-obesity activities, with all SGID hydrolysates inducing anti-adipogenic effects and all except one SGID hydrolysates exhibiting adipolytic effects in 3T3-L1 adipocyte cell models, potentially as a result of the release of bioactive peptides and/or amino acids upon digestion. Two anti-adipogenic hydrolysates, BW-SPH-A and BW-SPH-F, and their corresponding in vitro digests, BW-SPH-A-GI and BW-SPH-F-GI, were chosen to investigate the mechanism by which these hydrolysates reduced triglyceride accumulation during preadipocyte differentiation. Both hydrolysates significantly down-regulated the expression of key adipogenic transcription factors (peroxisome proliferator activated receptor (PPAR)-γ and CAAT (controlled amino acid therapy)/ enhancer binding protein (C/EBP)-α) compared with the MDI control ($p < 0.05$), thereby elucidating the mechanism by which BWSPHs BW-SPH-A and BW-SPH-F and their in vitro digests regulated the differentiation of preadipocytes to adipocytes.

In order for these hydrolysates to induce anti-obesity effects in vivo, it is essential that bioactivity is also maintained during intestinal epithelial absorption. Upon absorption of luminal nutrients via intestinal epithelial cells, brush border proteases may hydrolyse these components further, possibly altering their composition and in turn, influencing bioactivity. Therefore, further investigations into the anti-obesity effects of BW-SPH-A-GI and BW-SPH-F-GI after simulated intestinal absorption were carried out. Caco-2/HT-29MTX co-cultured cells were employed to simulate the permeation of BWSPHs across the human intestinal barrier. After 4 h exposure of fully differentiated Caco-2/HT-29MTX cells to the BWSPHs, the basolateral was collected and the anti-obesity potential of these basolateral samples was assessed to determine if hydrolysate bioactivity is maintained after absorption through the gut barrier. The results of these assays demonstrated that BW-SPH-A-GI

and BW-SPH-F-GI after simulated intestinal absorption still significantly reduced triglyceride levels and enhanced glycerol levels in 3T3-L1 adipocytes compared with MDI controls indicating that these hydrolysates may resist complete degradation by gastrointestinal proteases and brush-border peptidases, be absorbed through the intestinal epithelium, and reach the bloodstream in an active form. Future studies should also assess the bioavailability of BWSPHs in vivo. Therefore, these BWSPHs may have potential use as obesity alleviating functional food ingredients without the need for protective measures against gastrointestinal and intestinal membrane proteases.

6.2 SGID and Bioavailability

In order to develop a functional food ingredient, it is essential that the component remains bioactive post ingestion, gastrointestinal tract transit and intestinal absorption. Food-derived protein hydrolysate bioactivity is a popular area of research; however, many studies fail to include assessment of hydrolysate bioactivity post-SGID and post-intestinal absorption. It can be considered redundant to study the health benefits of protein hydrolysates without investigating the effects of SGID on their bioactivity due to the major effect that enzymes present throughout the gastrointestinal tract can have on hydrolysate structure and amino acid composition, thereby influencing bioactivity.

In this study, in vitro digestion induced interesting effects on BWSPH bioactivities with three main outcomes; 1) no significant differences in hydrolysate bioactivity before and after SGID, 2) loss of hydrolysate bioactivity post-SGID and 3) promotion of bioactivity after simulated digestion with gastrointestinal proteases. As examples, in Chapter 3, hydrolysates BW-SPH-A and BW-SPH-F demonstrated antioxidant and immunomodulatory activities, respectively, both before and after SGID. In Chapter 4, the ability of BWSPHs to regulate active GLP-1 secretion and proglucagon expression was lost upon digestion of all BWSPHs and in Chapter 5, adipolytic activity was observed in BWSPHs post-SGID but not beforehand. These results highlight the substantial effects of digestion on hydrolysate composition and the ultimate effect that this can have on hydrolysate bioactivity.

Digestion can also occur during intestinal absorption via peptidases in the brush border membrane (including aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases). In order for BWSPHs to exert anti-obesity effects

in vivo, including weight management and blood lipid profile enhancement, hydrolysate bioactivity must survive epithelial permeation. Although the anti-obesity activities of SGID BWSPHs were reduced following simulated intestinal absorption, both hydrolysates BW-SPH-A-GI and BW-SPH-F-GI still significantly reduced triglyceride levels and increased glycerol production in 3T3-L1 adipocytes. Therefore, BWSPHs in anti-obesity functional food products may not require bioavailability enhancers such as surfactants and chelators, albeit gastrointestinal protective coatings may be required for BWSPHs in satiety regulating products. Future bioactivity studies will be required for BWSPH-incorporated foods to ensure that food matrices don't interfere with hydrolysate bioactivity.

6.3 Optimisation of Cellular Models

In addition to the cost-effective, rapid and reproducibility characteristics associated with in vitro chemical assays, cell culture models also provide important information relating to cytotoxicity, cellular interactions and activation mechanisms. When designing the cellular models, it was imperative that suitable cell lines were chosen depending on the intended evaluation of the hydrolysates, taking cell model validity and its limitations into account. In the studies outlined in this thesis, cytokine secreting RAW264.7 cells, hormone-secreting STC-1 cells and adipocyte resembling 3T3-L1 cells were chosen to assess immunomodulatory, satiating and anti-obesity activities of BWSPHs, respectively. Although all three cell lines are popular for their respective applications, in the studies described in this thesis some alterations were made to cellular models for their optimisation. Cell culture models are generally easier and less costly than utilizing freshly isolated cells, however the formation of 3T3-L1 adipocytes can be a cumbersome process due to the considerable length of time required to complete 3T3-L1 differentiation. A reduction in differentiation time whilst still promoting adipocyte development would aid bioactive compound discovery. Therefore, varying from some studies which supplement differentiating media with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin reporting adipocyte phenotype identification at around 13 days (Hsieh et al., 2017; Vishwanath et al., 2013), the differentiation medium employed in this study (Chapter 5) included insulin sensitizer rosiglitazone to promote fat accumulation in less time (8 days after addition). Also observed throughout data collection for Chapter 5 was that 3T3-L1 preadipocytes

failed to differentiate once passaged 20 times and over-confluency of seeded 3T3-L1 preadipocytes in culture inhibited preadipocyte differentiation, therefore awareness of these adipogenesis inhibiting factors enhanced cellular models for adipogenesis studies. STC-1 cells have also been reported to switch to different states of differentiation during proliferation which can result in varying levels of hormone secretion, thus variability between experimental tests. In this study (Chapter 3), only cells above passage number 10 were employed for hormone stimulating and mechanistic action studies, in an attempt to improve homogeneity (McCarthy et al., 2015). In addition, suitable controls were included where possible and little interassay and intraassay variation was observed indicating validity of the cellular model. It is also recommended that the degree of subculturing of RAW264.7 cells should be included in published studies utilising the cell model due to the effect that passage number has on RAW264.7 cell phenotypic and functional characteristics (Taciak et al., 2018). Taciak et al. (2018) reported that the level of NO production in RAW264.7 cells stimulated with LPS varied significantly between 30 and 50 passages with cells passaged 50 times producing greater levels of NO than those at passage 30 (Taciak et al., 2018). Although in this study (Chapter 3), RAW264.7 cells between passages 15 and 34 were employed for bioactivity assessment and high levels of NO and inflammatory cytokines were generated upon stimulation with LPS with little variation between independent experiments, indicating that this cellular model was effective for immunological assessment of BWSPHs. In addition, an improved intestinal barrier model was utilised in this study (Chapters 4 and 5) employing a Caco-2/HT-29MTX co-culture which created a more physiological model of the small intestine than the well-established Caco-2 cell line alone for intestinal absorption analysis. The presence of mucous-producing goblet HT-29MTX cells modulate tight junctions and yield a mucous layer over the cell monolayer, thus mimicking physiological conditions (Hilgendorf et al., 2000).

6.4 Conclusion

With the functional food market currently booming, the generation and identification of bioactive protein hydrolysates derived from currently underutilised protein sources may promote environmental sustainability as well as support food industries. Results from this thesis suggest that hydrolysates purified from blue

whiting demonstrated bioactivity in cellular models. Hydrolysate BW-SPH-A represented the leading hydrolysate for antioxidant activity, displaying antioxidant tendencies in both murine RAW264.7 macrophage and 3T3-L1 preadipocyte cells. Hydrolysate BW-SPH-F induced pro-inflammatory effects in both the RAW264.7 cells and 3T3-L1 adipocytes and stimulated the highest levels of satiety hormone active GLP-1 in STC-1 cells. Both BW-SPH-A and BW-SPH-F also exhibited significant anti-obesity activities in 3T3-L1 cells after SGID and following simulated intestinal absorption. Taking this on board, it is recommended that future studies should incorporate these hydrolysates, or fractions of these hydrolysates, into suitable food systems and reassess their bioactivity, gastrointestinal stability and bioavailability in vitro, in vivo and in clinical trials prior to functional food commercialisation.

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Appendix

Publications in international peer-reviewed journals and books from this PhD project