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In vitro bioactive properties of intact and enzymatically hydrolysed whey protein: targeting the enteroinsular axis

O. Power-Grant,[a,d,e] C. Bruen,[b,e] L. Brennan,[c,e] L. Giblin,[b,e] P. Jakeman[d,e] and R. J. FitzGerald*[a,e]

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Enzymatically hydrolysed milk proteins have a variety of biofunctional effects some of which may be beneficial in the management of type 2 diabetes mellitus. The purpose of this study was to evaluate the effect of commercially available intact and hydrolysed whey protein ingredients (DH 32, DH 45) on markers of the enteroinsular axis (glucagon like peptide-1 secretion, dipeptidyl peptidase IV inhibition, insulin secretion and antioxidant activity) before and after simulated gastrointestinal digestion (SGID). A whey protein hydrolysate, DH32, significantly enhanced ($P < 0.05$) insulin secretion from BRIN BD11 β-cells compared to the positive control (16.7 mM glucose and 10 mM Ala). The whey protein hydrolysates inhibited dipeptidyl peptidase IV activity, yielding half maximal inhibitory concentration values (IC_{50}) of 1.5 ± 0.1 and 1.1 ± 0.1 mg mL^{-1} for the DH 32 and DH 45, samples respectively, and were significantly more potent than the intact whey ($P < 0.05$). Enzymatic hydrolysis of whey protein significantly enhanced ($P < 0.05$) its antioxidant activity compared to intact whey, as measured by the oxygen radical absorbance capacity assay (ORAC). This antioxidant activity was maintained (DH 32, $P > 0.05$) or enhanced (DH 45, $P < 0.05$) following SGID. Intact whey stimulated GLP-1 secretion from enteroendocrine cells compared to vehicle control ($P < 0.05$). This data confirm that whey proteins and peptides can act through multiple targets within the enteroinsular axis and as such may have glucoregulatory potential.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder which imposes a substantial personal and economic burden.[1] There is an increasing prevalence of T2DM and the number of cases worldwide are predicted to reach 550 million by 2030.[2] Changes in diet[3] and increasing physical activity[4] are key strategies to prevent and manage T2DM. The health enhancing properties of dietary proteins and their constituent peptides are being increasingly recognised.[5,6] In particular, emerging evidence suggests that ingestion of whey protein and its enzymatic hydrolysates may have a role in the management of T2DM.[7]

Whey proteins account for ~20% of bovine milk protein. The globular proteins in whey consist of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), immunoglobulins and a number of minor proteins such as glycomacropeptide, lactoferrin and lactoperoxidase.[8] Whey proteins are a rich source of essential amino acids. In addition each individual whey protein contains bioactive peptides within their primary structures which may be released by enzymatic hydrolysis.[5] The profile of peptides generated is dependent on the enzyme, the hydrolysis conditions and the whey protein substrate. Bioactive peptides are typically short (5–11 amino acids) sequences that can act *via* intestinal receptors or systemic targets.[9]

Incretin hormones secreted from enteroendocrine cells are natural, post-prandial hormones that augment the insulinotropic response.[10] The incretin hormone, glucagon-like peptide-1 (GLP-1) is an enteroendocrine L-cell derived peptide. Plasma concentrations of biologically active GLP-1, GLP-1(7–36), increase within 10–15 min of food ingestion, peak between 30–45 min and persist for 1–2 hours in a nutrient dependent manner.[11–13] It has been demonstrated *in vitro*, using different cell lines, that intact food proteins,[14,15] food protein hydrolysates[14,16,17] and the amino acids Leu, Ile and Gln[15,18] can stimulate the release of GLP-1 from intestinal cells. Once in the circulation GLP-1 travels to pancreatic β-cells where it binds to the GLP-1 receptor resulting in insulin production

[a]*Department of Life Sciences, University of Limerick, Limerick, Ireland. E-mail: dick.fitzgerald@ul.ie; Fax: +00353-61-331490*
[b]*Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland*
[c]*School of Agriculture and Food Science, University College Dublin, Ireland*
[d]*Centre for Interventions in Inflammation, Infection and Immunity (4i), University of Limerick, Castletroy, Limerick, Ireland*
[e]*Food for Health Ireland, Ireland*

and secretion. Therefore, natural, food derived compounds that stimulate the release of GLP-1 may be an alternative to synthetic drugs in the control of glycaemic function in man.[19] GLP-1 has a short circulating half-life. It is rapidly cleaved by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) resulting in loss of its insulinotropic activity.[20] Therefore, a potential strategy to increase incretin half-life is to inhibit DPP-IV activity.[21] Recently, whey protein derived peptides with DPP-IV inhibitory activity have been identified.[22–26] Further investigation of the DPP-IV inhibitory properties of food derived peptides may help to identify natural compounds that may be used in addition to, or in replacement of, synthetic drug inhibitors.[27]

The mechanism of protein-stimulated insulin secretion is not fully elucidated, however, a change in circulating essential amino acids[28] and the release of incretin hormones[29] are proposed as the primary regulators. Amino acid residues including Ala, Leu, Arg have been shown to stimulate acute insulin secretion *in vitro*.[30,31] Recently, whey protein hydrolysates have been shown to induce insulin secretion in BRIN BD11 cells.[32,33] In humans, acute ingestion of intact and hydrolysed whey proteins were shown to act as insulin secretagogues.[34–36]

Development of T2DM compromises the body's natural antioxidant defence and leads to an increase in oxidative stress.[37] Reactive oxygen species damage vital cellular components such as proteins, lipids and DNA ultimately compromising cellular function. The antioxidant activity of whey protein hydrolysates has been highlighted.[38,39] Whey protein hydrolysates have been shown to act as radical scavengers,[24,26,40–42] reducing agents and inhibitors of lipid peroxidation.[43] More recently, whey protein hydrolysates were shown to increase the production of antioxidant enzymes and to enhance antioxidant gene expression in human umbilical vein endothelial cells.[44]

Emerging evidence of the multiple bioactive properties of whey protein hydrolysates support their use as potential regulators of glucose homeostasis. These natural, food derived, multifunctional compounds could remove or decrease reliance on pharmacological therapy and may prove effective in the dietary management of T2DM. The aim of this study was to evaluate the effect of intact and hydrolysed whey protein on components of the enteroinsular axis. *In vitro* model systems were used to evaluate the effect of two whey protein hydrolysates on GLP-1 secretion from intestinal STC-1 cells, DPP-IV inhibition, insulin secretion from pancreatic β-cells and antioxidant activity. In addition, the stability of each bioactivity following simulated gastrointestinal digestion was evaluated.

2. Methods and materials

2.1 Materials

Intact whey protein concentrate (WPC, Carbelac WPC80 Carbery Ingredients, Ballineen, Ireland, 79.0% (w/w) protein, Table 1), WPC hydrolysate degree of hydrolysis (DH) 32% (Optipep®, Carbery Ingredients, 78.0% (w/w) protein, Table 1), whey protein isolate hydrolysate DH 45% (Glanbia Nutritionals, Kilkenny, Ireland, 84.0% (w/w) protein Table 1) were obtained from the manufacturers. Protein hydrolysates were obtained by enzymatic hydrolysis of the native proteins. An aqueous solution was prepared and equilibrated to the hydrolysis reaction temperature and pH. Following this microbial

Table 1 Protein content (% dry weight (dw)), degree of hydrolysis (%), total and free amino acid composition (g/100 g powder) for intact whey protein concentrate (WPC), whey protein hydrolysate degree of hydrolysis (DH) 32% (DH 32) and whey protein hydrolysate DH 45% (DH 45)[a]

Test Sample	WPC	DH32		DH45	
Protein source	Whey protein concentrate	Whey protein concentrate		Whey protein isolate	
Protein Nitrogen (% dw)	79.0	78.0		84.0	
Degree of Hydrolysis (%)	NA	32		45	
Amino Acid	g/100 g powder				
	Total amino acids	Total amino acids	Free amino acids	Total amino acids	Free amino acids
Ala	4.3	3.5	*0.3*	5.5	*0.6*
Arg	2.4	2.0	*0.8*	1.2	*0.2*
Asp	9.5	5.5	*0.1*	3.9	*0.1*
Cys	2.1	2.1	*ND*	0.2	*0.3*
Glu	15.2	11.9	*0.4*	6.3	*0.1*
Gly	1.7	1.5	*ND*	1.0	ND
His	1.6	1.5	*0.4*	2.3	*3.5*
Ile	5.1	4.4	*0.9*	ND	*2.3*
Leu	9.4	7.7	*2.3*	15.2	*8.1*
Lys	8.1	6.5	*2.3*	3.7	*0.7*
Met	1.6	1.7	*ND*	3.2	*3.0*
Phe	2.8	2.6	*0.8*	3.2	*ND*
Pro	5.1	4.3	*0.1*	0.9	*0.1*
Ser	4.7	5.1	*0.4*	3.2	*0.5*
Thr	6.4	5.5	*0.6*	6.3	*2.9*
Try	1.2	1.4	*ND*	ND	*ND*
Tyr	2.7	2.5	*0.6*	2.0	*1.2*
Val	5.1	4.3	*0.8*	6.6	*1.7*

[a] NA: Not applicable, ND: Not detected.

enzymes which are Halal, Kosher and non-GMO were added to the solution. The aqueous solution was held at optimal conditions for the enzymes in order to achieve the desired DH. Following hydrolysis the solution was heat treated to inactivate the enzyme. This solution was spray dried. Krebs ringer bicarbonate buffer pH 7.4, Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 tissue culture medium, Leu, Gln, fetal bovine serum, penicillin, streptomycin, sodium pyruvate, trifluoroacetic acid (TFA), Tris(hydroxymethyl)aminomethane hydrochloride (Trizma® base), ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA), DPP-IV (EC 3.4.14.5, human recombinant; 8 mU mL^{-1}), Tyr-hydrochloric acid (Tyr-HCl), bacitracin, aprotinin, cytochrome *c*, α-lactalbumin (α-La), β-lactoglobulin (β-Lg), BSA, phosphate buffered saline, sodium phosphate dibasic, fluorescein sodium salt, 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH), Trolox™ (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) were all obtained from Sigma-Aldrich (Dublin, Ireland). Pepsin (BC pepsin, Biocatalysts, Cardiff, United Kingdom) and Corolase PP (CorPP; AB Enzymes, Darmstadt, Germany) were obtained from the manufacturers. 7-Amino-4-methylcoumarin (AMC) standard, H-Gly-Pro-AMC, Diprotin A (Ile-Pro-Ile), Asp-Glu and Leu-Trp-Met-Arg were obtained from Bachem (Bubendorf, Switzerland). High performance liquid chromatography (HPLC) grade acetonitrile (ACN), sodium hydroxide (NaOH) and HPLC grade water were obtained from VWR (Dublin, Ireland). HPLC grade methanol was obtained from Lennox (Dublin, Ireland). Murine secretin tumour cell line (STC-1) (ATCC code: SD5482) was purchased from the American Tissue Culture Collection (LGC Standards, Teddington, UK). Halt Protease and Phosphatase Inhibitor was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The insulin enzyme linked immunosorbent assay (ELISA) kit was obtained from Mercodia (Uppsala, Sweden).

2.2 Reverse phase ultra performance liquid chromatography

Intact and hydrolysed whey protein samples were analysed by reverse phase ultra performance liquid chromatography (RP-UPLC) as previously described by Nongonierma & FitzGerald.[45] The UPLC system (Acquity UPLC®, Waters, Milford, MA, USA), comprising of binary solvent and auto sample manager, a heated column compartment and TUV absorbance detector. The pump was operated at a flow rate of 0.3 mL min^{-1} and 1 μL of each sample was injected onto the column. Separation of proteins and peptides was carried out at 30 °C using a 2.1 × 50 mm, 1.7 μm Acquity UPLC C18 BEH column (Waters) fitted with a pre-column security guard (VanGuard, Waters). The system was interfaced with Empower 2 (Waters) data handling software. Mobile phase A consisted of 0.1% (v/v) TFA in HPLC grade water. Mobile phase B was 0.1% (v/v) TFA in 80% HPLC grade ACN in HPLC grade water. Freeze dried intact and hydrolysed protein material were diluted to a concentration of 0.8% (w/v) in mobile phase A and were filtered through 0.2 μm filters (Phenomenex, Phenex RC, Cheshire, UK) prior to injection. The gradient elution program used to separate the proteins and peptides consisted of a linear gradient 0–0.3 min 0% B; 0.3–45 min 0–80% B; 45–46 min 80–100% B; 46–48 min 100% B; 48–49 min 100–0% B, 49–51 min 0% B. The absorbance of the eluent was monitored at 214 nm.

2.3 Gel permeation chromatography

Molecular mass distribution profiles of the intact and hydrolysed whey proteins were obtained as per the methodology described by Spellman *et al.*[46] Briefly, a gel permeation chromatography (GPC) system (Waters) comprising of a binary pump (Waters, Model 1525), dual absorbance detector (Waters, 2487) and an autosampler (Waters 717 Plus) was utilised. Separation was by isocratic elution with 0.1% TFA in 30% HPLC grade ACN at a flow rate of 0.5 mL min^{-1} and 20 μL of sample was injected. Each sample was prepared at a concentration of 0.25% (w/v) in 0.1% TFA, 30% (v/v) HPLC grade ACN and pre-filtered through 0.2 μm polytetrafluoroethylene filters (VWR, Dublin, Ireland). Separation of proteins and peptides were carried on a TSK-Gel G2000SW column (10 μm Particle size, 600 mm × 7.5 mm, ID; Tosoh Biosciences, Tokyo, Japan) connected to TSK-Gel G2000SW guard column (10 μm, 50 mm × 7.5 mm ID; Tosoh Biosciences). The detector response was monitored at 214 nm and the total run time was 60 min. The system was calibrated using protein, peptide and amino acid standards with a molecular mass between 67 500 and 218 Da including BSA (67 500 Da), β-Lg (36 000 Da), α-La (14 200 Da), cytochrome *c* (12 300 Da), aprotinin (6500 Da), bacitracin (1400), Leu-Trp-Met-Arg (604 Da), Asp-Glu (262 Da) and Tyr-HCl (218 Da). The calibration curve was prepared from the average retention time (n = 3) of each standard plotted against the Log of the molecular mass of each standard. The system was interfaced with Breeze Software (Waters) for data analysis. Data for each hydrolysate sample was expressed as percentage area within a defined molecular mass range for each chromatogram obtained at 214 nm.

2.4 Simulated gastrointestinal digestion of intact and hydrolysed whey protein

Test samples were subjected to a simulated gastrointestinal digestion (SGID) process.[47] Briefly, freeze dried samples were diluted to 2.0% (w/w) protein and resuspended in distilled water at 37 °C for 30 min with overhead stirring at pH 2. Pepsin was then added at an enzyme to substrate ratio (E : S) of 1 : 40 (w/w). After 90 min, a sub-sample was removed and heat inactivated at 90 °C for 20 min in a waterbath. The pH of the remaining reaction mixture was adjusted to 7.5. CorPP was added at an E : S of 1 : 100 (w/w). After 150 min incubation at 37 °C, the hydrolysis reaction was terminated by heat inactivation in a waterbath at 90 °C for 20 min. SGID samples and undigested control material were cooled to room temperature, frozen at −20 °C and freeze dried (Freezone 181, Labconco, Kansas, USA). All samples were stored at −20 °C until further analysis.

2.5 DPP-IV inhibition assay

DPP-IV inhibitory activity was evaluated using a fluorescence based assay as per the methodology of Power *et al.*[26] Briefly,

the assay was performed in a 96 well microplate (Fisher Scientific, Dublin, Ireland). An AMC standard curve was generated by assaying 100 μL of AMC standards (1–8 μM). Diprotin A was employed as a reference inhibitor (positive control). The DPP-IV half maximal inhibitory concentration (IC_{50}) value of Diprotin A was calculated by assaying at concentrations between 1.25–100.00 μM. Test hydrolysates or positive controls were added (10 μL) to each well and pre-incubated with 30 μL of 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl and 1 mM EDTA and 50 μL 200 μM H-Gly-Pro-AMC at 37 °C for 5 min in a microplate reader (Biotek Synergy HT, Winooski, USA). The reaction was initiated by addition of 10 μL of human DPP-IV (8 mU mL^{-1}) to the wells. The microplate was incubated at 37 °C for 30 min after which fluorescence was measured at an excitation of 360 nm and an emission 460 nm. One unit of DPP-IV activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of H-Gly-Pro-AMC per minute at 37 °C. The DPP-IV IC_{50} value, the concentration of peptide required to inhibit 50% of the enzyme activity, for each hydrolysate was determined by plotting DPP-IV inhibition as a function of hydrolysate concentration. The logarithmic regression equation generated from this plot was then used to calculate the IC_{50} value. DPP-IV inhibition (%) and IC_{50} values for each hydrolysate were expressed as the mean ± SD of independent triplicate analyses.

2.6 *In vitro* GLP-1 secretion in STC-1 cells

The ability of the test samples to stimulate GLP-1 secretion was evaluated using the murine enteroendocrine cell line (STC-1). Cells were cultured in DMEM media containing 4.5 g L^{-1} glucose and L-Gln supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. Cells were passaged upon reaching confluence and all cells used in these studies were between passages 15–25.

Test sample stock solutions were prepared at a concentration of 50 mg mL^{-1} in HPLC grade water. The pH of each was adjusted to pH 7.4 using 1 M NaOH and the solution was hydrated overnight at 4 °C. Following hydration, the solution was centrifuged (Beckman Coulter, Allegra X-22R) at 1257 g, for 5 min at room temperature and filter sterilised using a sterile syringe filter (0.45 μm Filtropur, Sarstedt, Wexford, Ireland). All test samples were assayed at 10 mg mL^{-1} prepared in modified Krebs buffer (Krebs ringer bicarbonate buffer pH 7.4 containing 1% BSA).

STC-1 cells were seeded into 6-well plates (Fisher Scientific, Dublin, Ireland) at a density of 1.5 × 106 cells per well and placed in an incubator (Forma Scientific, Marietta, USA) at 37 °C in 5% carbon dioxide for 18 h prior to test sample addition. Media was aspirated and the cell monolayers in each well were washed with 1 mL of modified Krebs buffer. Cells were pre-incubated for 1 h in 500 μL of modified Krebs buffer. Modified Krebs buffer was aspirated off and replaced with 1 mL of the 10 mg mL^{-1} test sample. A monosaccharide solution (40 mm glucose and 40 mM fructose) was used as the positive control to induce GLP-1 secretion from STC-1 cells. Plates were incubated (Forma Scientific) for 4 h at 37 °C in 5% carbon dioxide. The heterogeneous nature of the STC-1 cells results in varying levels of hormone secretion from flask to flask. Therefore, test samples were assayed in quadruplicate on a single day and modified Krebs buffer acted as the vehicle control. In addition, test samples were assayed in duplicate on separate days. Following the 4 h incubation period, 10 μL of 10 × Halt Protease and Phosphatase Inhibitor was added to each well to inactivate endogenous DPP-IV activity. Cellular supernatants were collected by aspiration and stored at −80 °C prior to GLP-1 analysis.

Cellular supernatant levels of total GLP-1 were assayed using a GLP-1 assay kit (Meso Scale Discovery (MSD®), Rockville, MD, USA) according to the manufacturer's instructions. GLP-1 concentration (pM) in the samples was quantified by interpolating the intensity of emitted light from a GLP-1 standard curve generated under the assay conditions. Each sample was assayed in duplicate and plates were read using a MSD® Sector Imager 2400 instrument (Meso Scale Discovery).

2.7 *In vitro* insulin secretion by pancreatic β-cells

Pancreatic BRIN BD11 β-cells were used to measure acute insulin secretion.[48] Cells were maintained in RPMI-1640 tissue culture medium supplemented with 10% (v/v) fetal bovine serum, 0.1% antibiotics (100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin) containing 11.1 mM glucose and 0.1% Gln pH 7.4. Cells were seeded into a 6-well microplate (Sarstedt, Wexford, Ireland), incubated (Forma Scientific, Marietta, USA) with 5% carbon dioxide and 95% air at 37 °C and allowed to adhere overnight. Cells were then washed with phosphate buffered saline before being incubated in Krebs-Ringer bicarbonate buffer at pH 7.4 containing 1.1 mM glucose. After 40 min of incubation the buffer was removed. Test samples were incubated with the cells at a concentration of 1 mg mL^{-1} in Krebs ringer buffer containing 16.7 mM glucose for 20 min. The supernatant was then removed and acute insulin secretion was measured by ELISA. The positive control for induction of insulin secretion was 16.7 mM glucose and 10 mM Ala. Insulin secretion data is presented as the mean ± SD of four independent experiments.

2.8 Oxygen radical absorbance capacity (ORAC) assay

Antioxidant capacity was evaluated using the fluorescence based ORAC assay as per the methodology of Power *et al.*[26] Briefly, the assay was performed in a 96 well microplate (Fisher Scientific, Dublin, Ireland). A Trolox standard curve was generated by assaying Trolox standards at concentrations between 10 and 200 μM. Test samples, blank (assay buffer) and Trolox standards were dissolved in 75 mM sodium phosphate buffer, pH 7.0 and were added (50 μL) to the appropriate wells and pre-incubated with 50 μL of 0.312 μM fluorescein (final concentration) at 37 °C for 10 min in a microplate reader (Biotek Synergy HT, Winooski, USA). Baseline fluorescence was measured at excitation (485 nm) and emission (520 nm) wavelengths after 1 min. The reaction was initiated by addition of 25 μL of 44.2 mM AAPH (final concentration) to each well. The microplate was incubated at 37 °C for 120 min

during which fluorescence was measured every 5 min. For each sample, the reaction was deemed to be complete if final fluorescence intensity (FI_n) was less than 5% of initial fluorescence (FI_0). Final results were presented as μmol TE per 100 g of dry weight (μmol TE/100 g dw). All data are presented as the mean ± SD of independent triplicate analyses (n = 3).

2.9 Statistical analysis

All analyses were performed at least in triplicate and presented as the mean ± SD. Data were tested for normality (Shapiro-Wilk) and evaluated by analysis of variance (ANOVA; one-way) followed by Tukey's test and a significance level of $P < 0.05$ was employed. All analysis was performed using SPSS (SPSS, version 19, IBM Inc., Armonk, USA).

3 Results

3.1 Physicochemical characterisation of the test samples

The physicochemical properties of the different milk protein hydrolysates were evaluated by determination of the RP-UPLC and the molecular mass distribution profiles. The RP-UPLC profiles highlight differences in the intact protein and the hydrolysates (Fig. 1a–c). Enzymatic hydrolysis resulted in significant degradation of the intact whey proteins. However, the DH 32 sample still contained some intact β-Lg (Fig. 1b). The peptide profiles of the DH 32 and DH 45 hydrolysates contained distinct peaks which may be attributed to the specificity of the enzymatic cleavage and differences in the hydrolysis parameters. The RP-UPLC profiles (Fig. 1d–f) also highlight the degradation of the protein substrates that occurred following SGID. In all cases there was an increase in abundance of the peptide peaks particularly those in the more hydrophilic region of the chromatogram. SGID of WPC resulted in a reduction in the intact whey proteins and an increase in abundance of peptide peaks (Fig. 1a and d). For the DH 32 hydrolysate there was an increase in abundance of the main peptide peaks at 2.7, 4.7 and 7.4 min (Fig. 1e). SGID of the DH 45 hydrolysate resulted in an increase in abundance of the peptide peaks at 4.8 and 7.7 min and a general reduction in the apparent complexity of the peptide profile (Fig. 1f).

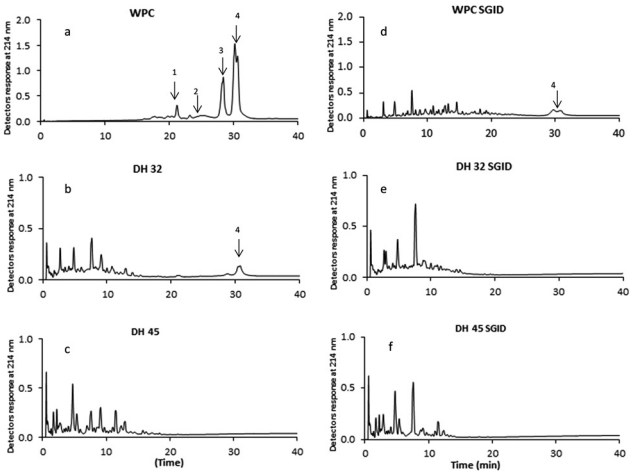

Fig. 1 Reverse phase ultra performance liquid chromatography profile for (a) intact whey protein concentrate (WPC), (b) whey protein hydrolysate degree of hydrolysis 32% (DH 32) and (c) whey protein hydrolysate DH 45% (DH 45) and associated simulated gastrointestinal digested (SGID) samples (d–f). Freeze dried intact and hydrolysed protein material was diluted to a concentration of 0.8% (w/v) in 0.1% (v/v) TFA in high performance liquid chromatography grade water. Individual whey protein peaks, glycomacropeptide (1), bovine serum albumin (2), α-lactalbumin (3) and β-lactoglobulin a and b (4) are labelled.

Table 2 Molecular mass distribution profile for intact whey protein concentrate (WPC), whey protein hydrolysate degree of hydrolysis (DH) 32% (DH 32), whey protein hydrolysate DH 45% (DH 45) and associated simulated gastro-intestinal (SGID) digested samples. Freeze dried samples were prepared as 0.25% (w/v) in 0.1% TFA, 30% (v/v) high performance liquid chromatography grade acetonitrile

	Molecular mass distribution (% area)[a]			
Test sample	>5 kDa	5-1 kDa	1-0.5 kDa	<0.5 kDa
WPC	86.9	12.6	0.4	0
WPC SGID	17.4	28.9	20.0	33.7
DH 32	21.8	10.7	15.4	52.1
DH 32 SGID	0	12.5	19.8	67.8
DH 45	0	12.7	15.4	71.9
DH 45 SGID	0	0	3.6	96.4

[a] Values expressed as % area within a defined molecular mass range for a gel permeation chromatogram obtained at 214 nm.

In line with the RP-UPLC profiles, 86.9% of the material in the intact WPC had a mass >5 kDa (Table 2). Within the DH 32 hydrolysate, 21.8% of the material had a mass >5 kDa and this hydrolysate also contained a large proportion of low molecular mass material, 52.1% of the area was <0.5 kDa. The DH 45 hydrolysate contained the highest proportion of low molecular mass material, 71.9% <0.5 kDa (Table 2). SGID increased the proportion of peptide material within the low molecular mass range (<0.5 kDa; Table 2) for all test samples. However, there was large variability in the proportion of low molecular weight peptides within the digested samples ranging from 33.7 to 96.4% for WPC SGID and DH 45 SGID, respectively.

3.2 GLP-1 secretion *in vitro* by STC-1 cells

The ability of WPC and the hydrolysates (DH 45 and DH 32) to stimulate total GLP-1 secretion from enteroendocrine cells was evaluated. Preliminary experiments indicated that although modified Krebs buffer contained 10 mM glucose, it was not sufficiently stimulatory to distort results. It was therefore a suitable vehicle control as it maintains viable STC-1 cells during 4 hour exposures. Intact WPC prepared in modified Krebs buffer resulted in increased GLP-1 secretion compared to modified Krebs buffer alone (189.8 ± 18.7 *vs.* 81.4 ± 3.5 pM; $P < 0.05$; Table 3). Whey protein hydrolysates (DH 32 and DH 45) prepared in modified Krebs buffer did not increase the level of secreted GLP-1 above levels observed for buffer alone, 90.7 ± 32.1 *vs.* 127.4 ± 38.6 pM ($P > 0.05$, Table 3) for the DH

Table 3 *In vitro* glucagon-like peptide-1 (GLP-1) secretion from murine secretin tumour cells (STC-1), *in vitro* insulin secretion from BRIN BD11 β-cells, dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration (IC_{50}) and oxygen radical absorbance capacity (ORAC) values for intact whey protein concentrate (WPC), whey protein hydrolysate degree of hydrolysis (DH) 32% (DH 32) and whey protein hydrolysate DH 45% (DH 45), associated simulated gastrointestinal digested (SGID) samples and associated assay controls. Values are expressed as the mean ± SD, $n \geq 3$. Within each column values with different letters indicate significant differences determined by ANOVA ($P < 0.05$)[a]

Sample	GLP-1* (pM)	DPP-IV IC_{50} (mg mL^{-1})	Insulin** (ng mg^{-1} protein)	ORAC (μmol TE/100 g dw)
WPC	189.8 ± 18.7[b]	>4	20.95 ± 6.45[a,c]	13 662 ± 1018[a]
SGID WPC	119.8 ± 19.7[a]	3.3 ± 0.3[a]	15.79 ± 4.71[a]	36 605 ± 3390[b]
DH 32	90.7 ± 32.1[a]	1.5 ± 0.1[b]	31.51 ± 5.42[b,c]	37 391 ± 2298[b]
SGID DH 32	118.9 ± 67.2[a,b]	0.9 ± 0.2[b,c]	33.93 ± 8.45[b]	44 489 ± 2064[b]
DH 45	127.4 ± 38.6[a,b]	1.1 ± 0.1[c]	17.78 ± 3.33[a]	77 692 ± 1464[c]
SGID DH 45	87.2 ± 2.7[a]	1.3 ± 0.1[b,c]	17.74 ± 3.72[a]	60 613 ± 4540[d]
16.7 mM Glucose + 10 mM Ala	NA	NA	18.03 ± 5.7[a]	NA
Modified Krebs buffer	81.4 ± 3.5[a]	NA	NA	NA
Diprotin A	NA	0.002 ± 0.0001[d]	NA	NA

[a] NA: Not applicable, TE: Trolox equivalents, dw dry weight of powder, *assayed at 10 mg mL^{-1}, **assayed at 1 mg mL^{-1}.

32 and DH 45, respectively. SGID of intact WPC led to a 37% reduction in GLP-1 secretion, 189.8 ± 46.4 *vs.* 119.8 ± 19.7 pM for WPC and SGID WPC, respectively ($P < 0.05$, Table 3). There was no significant difference in GLP-1 secretion for the DH 32 and DH 45 hydrolysates following SGID.

3.3 *In vitro* DPP-IV inhibition

The ability of the three test compounds to inhibit DPP-IV activity was evaluated *in vitro*. Table 3 compares the IC_{50} values for all compounds before and after SGID. The DPP-IV IC_{50} values of both hydrolysates were significantly lower than for the intact WPC. However, these IC_{50} values were 647–882 fold higher than the positive control, Diprotin A. There was a significant reduction in the DPP-IV IC_{50} value for the intact WPC following SGID (Table 3). The DH 32 SGID hydrolysate gave a 37% reduction in the DPP-IV IC_{50} value, 1.5 ± 0.1 *vs.* 0.9 ± 0.2 mg mL^{-1} ($P > 0.05$; Table 3). In contrast, the DH 45 SGID hydrolysate resulted in a 14% increase in the DPP-IV IC_{50} value, 1.1 ± 0.1 *vs.* 1.3 ± 0.1 mg mL^{-1} ($P > 0.05$; Table 3).

3.4 Insulin secretion by pancreatic β-cells

The effect of intact and hydrolysed whey proteins on acute insulin secretion was evaluated in BRIN BD11 β-cells. The intact WPC produced a similar response to the positive control, 16 mM glucose and 10 mM Ala (Table 3). However, the DH32 hydrolysate produced an insulin response (31.5 ± 5.4 ng mg^{-1} protein) significantly higher ($P < 0.05$) than the positive control, (18.03 ± 5.7 ng mg^{-1} protein). Intact protein and hydrolysates were subjected to SGID and in all cases there was no significant change ($P < 0.05$) in insulin secretion compared to non SGID treated samples (Table 3).

3.5 Antioxidant activity

The *in vitro* antioxidant activity was measured using the ORAC assay which measures the ability of an antioxidant to scavenge a peroxyl radical. Both intact and hydrolysed whey proteins displayed antioxidant activity (Table 3). There was a 3–6 fold increase ($P < 0.05$) in the antioxidant activity due to hydrolysis, 37 391 ± 2298 and 77 692 ± 1464 μmol Trolox equivalents (TE) /100 g dw of powder for the DH 32 and DH 45, respectively. Following SGID of WPC there was a 2.5 fold increase in antioxidant activity ($P < 0.05$; Table 3). There was no change in the antioxidant activity for the DH 32 following SGID ($P > 0.05$; Table 3). In contrast, there was a 22% reduction ($P < 0.05$) in the antioxidant activity for the DH 45 following SGID (Table 3).

4. Discussion

There is increasing interest in understanding the biofunctional properties of food and in utilizing food-derived bioactive components in the management of diet related diseases. Whey proteins in particular are a rich source of bioactive peptides.[49] A number of human studies have highlighted the potential for intact and hydrolysed whey proteins to regulate plasma glucose in healthy and T2DM subjects.[50,51] Furthermore, it has been suggested that much of this regulation takes place *via* targets within the enteroinsular axis.[52] However, the mechanisms responsible are yet to be fully explored. In this study, we investigated the potential regulatory effects of intact and hydrolysed whey protein *via* a number of mechanisms using different *in vitro* test systems.

GLP-1 is a 30 amino acid, L cell derived, polypeptide with a variety of physiological functions including incretin activity.[10] The combined action of the incretin hormones (GLP-1 and glucose dependent insulinotropic polypeptide) account for 50–70% of the postprandial insulin response.[53] However, the magnitude of this response may be nutrient specific.[12] There is considerable evidence *in vivo* that ingestion of protein,[54,55] peptides[56] and individual amino acids[57] elevate plasma GLP-1. The relative potency of each compound and the mechanism(s) responsible, however, remain to be established. STC-1 cells are a heterogeneous intestinal epithelial cell population derived from the intestinal endocrine tumor of double transgenic mice[58] and an accepted model of enteroendocrine cells.[53] Fol-

lowing exposure of STC-1 cells to intact and hydrolysed whey proteins only intact WPC enhanced GLP-1 secretion above the vehicle control. This is in agreement with previous data reporting the stimulation of GLP-1 secretion by intact whey proteins.[14] Interestingly, further degradation of the intact protein during SGID reduced the GLP-1 secretory properties of WPC suggesting that it may be important to protect (encapsulate or enteric coat) the components responsible to prevent gastric and intestinal degradation.

DPP-IV is the principal enzyme that rapidly degrades active incretins following their secretion.[20] Whey protein-derived peptides have been shown to act as DPP-IV inhibitors.[22,24–26,59] In addition, peptides can also act as substrate-like DPP-IV inhibitors.[60,61] Recently, individual amino acids (Met, Leu and Trp) have also shown moderate DPP-IV inhibition.[62] Therefore, peptides or amino acids present in, or released from, intact whey proteins could contribute to increasing the half-life of active incretins. The hydrolysed whey proteins evaluated herein acted as DPP-IV inhibitors and the IC_{50} obtained (Table 3) compare well with previously published values (0.075–1.51 mg mL^{-1}) for whey protein hydrolysates.[26,27,63] A variety of factors contribute to the DPP-IV inhibitory properties of a peptide including amino acid composition,[64] sequence[24] and physicochemical characteristics.[65] However, the relative contribution of each parameter is still unclear. Milk protein derived DPP-IV inhibitory peptides are typically short sequences containing 2–7 amino acids.[66] The DH 45 hydrolysate had a significantly lower DPP-IV IC_{50} than DH 32. The DH 45 hydrolysate contained a higher proportion of short peptides (Table 2) which may have contributed to the greater potency of this hydrolysate.

Some amino acids (Arg, Leu, Gln, Ala, Lys) and protein hydrolysates can directly stimulate insulin secretion by pancreatic β-cells *in vitro*.[30–33,48] In this study, only the DH 32 hydrolysate and its SGID form had a significantly higher insulinotropic response than the positive control (glucose + Ala, Table 3). The DH 32 hydrolysate also had a 1.7 fold greater ($P < 0.05$) insulinotropic potency than the DH 45 hydrolysate (Table 3). Interestingly, the DH 45 hydrolysate contained a higher proportion of low molecular mass peptides than the DH 32 sample (Table 2) which suggests that the insulinotropic response is not solely dependent on the DH or the abundance of low molecular mass peptides. Independent of carbohydrate, the insulinotropic properties of milk protein hydrolysates has been demonstrated in a small number of human studies.[34–36] The mechanism(s) responsible are not yet fully elucidated but changes in the concentration of key insulinotropic amino acids[28,67] or peptides[36] have been implicated. The DH 32 hydrolysate had a higher concentration of free Arg and Lys, than the DH 45 hydrolysate, two highly potent insulinotropic amino acids which may have contributed to the potency of this hydrolysate. Short peptides containing branched chain amino acids were reported to contribute to the insulinotropic action of whey proteins.[36] The specific peptide profile within the DH 32 hydrolysate (Fig. 1b) may be responsible for the insulinotropic response; however, the peptides therein have yet to be identified. If bioactive peptides act *via* systemic targets then gastrointestinal stability of the peptides may be necessary to ensure translation of the bioactive effect *in vivo*. Although there was further degradation of the protein substrates during SGID (Fig. 1 & Table 2) this did not alter the bioactivity and, in all cases, the insulinotropic action was retained (Table 3). We have previously shown that the DH 32 hydrolysate has potent insulinotropic effects in humans[34] thus confirming, in this instance, the ability of the present *in vitro* model system to be predictive of *in vivo* bioactivity.

There is a well-established link between an increase in oxidative stress and the development of T2DM.[68] Therefore, ingestion of multifunctional peptides that act *via* the enteroinsular axis and also possess antioxidant activity could be a novel and beneficial dietary strategy in the management of T2DM. Enzymatic hydrolysis significantly enhanced antioxidant activity (Table 3). This is not surprising given that amino acid side chains and polar or charged residues contributing to the redox potential become exposed during hydrolysis.[69,70] Antioxidant activity increased with increasing DH and consistent with the higher DH, the DH 45 hydrolysate contained a greater abundance of low molecular mass peptides. Peptides with higher peroxyl radical scavenging activity typically contain 4–20 amino acid residues.[71] Specific individual amino acids have been highlighted for their antioxidant capability include His,[69] Cys,[72] hydrophobic[73] (Leu, Val and Tyr) and aromatic residues[69] (Trp, Phe and Tyr). Interestingly, the DH 45 hydrolysate contains a higher proportion of Leu, His and Phe (Table 1) than DH 32 which may have contributed to the antioxidant activity reported here. Quantitative structure–activity modeling has shown that the location of the amino acid within the peptide sequence is an important determinant of antioxidant activity. In particular, peroxyl radical scavenging activity is increased if bulky hydrophobic amino acid residues are located at the C terminal and polar amino acids are located at the N terminal of a peptide.[71] Therefore, the antioxidant activity reported here may be due to the combined action of antioxidant peptides and amino acids release during enzymatic hydrolysis. There was a notable decrease in the antioxidant activity of the DH 45 hydrolysate following SGID (Table 3). Peptides present in the DH 45 hydrolysate were further degraded by the gastrointestinal enzymes resulting in a 25% increase in the proportion of small peptides (Table 2). Pepsin preferentially cleaves hydrophobic amino acids. The observed loss of activity following peptic digestion may be explained if the DH 45 hydrolysate contained a high proportion of hydrophobic amino acid residues.

5. Conclusions

To our knowledge this is the first time that these four bioactive effects (GLP-1 secretion, DPP-IV inhibition, insulinotropic and antioxidant activity) have been studied in parallel. This work confirms that intact whey can induce GLP-1 secretion. More importantly we have identified two extensively hydrolysed

whey protein hydrolysates that regulate insulin secretion, inhibit DPP-IV and have an antioxidant activity *in vitro*. In most instances, these bioactive effects were maintained or enhanced following SGID. The *in vitro* models employed here suggest these bioactive effects may be retained *in vivo*, however, this requires further validation through human studies. In summary, several novel findings from the present work support a potential glucoregulatory capacity/capability of whey protein hydrolysates acting *via* targets within the entero-insular axis indicating that the whey protein hydrolysates evaluated herein may be a suitable multifunctional nutritional therapy for the management of T2DM.

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Notes and references

1 American Diabetes Association, *Diabetes Care*, 2013, **36**, 1033–1046.
2 World Health Organisation, Diabetes programme, country and regional data, http://www.who.int/diabetes/facts/world_figures/en/, Accessed Sep 12, 2012.
3 A. D. Association, *Diabetes Care*, 2009, **32**, S13–S61.
4 S. R. Colberg, R. J. Sigal, B. Fernhall, J. G. Regensteiner, B. J. Blissmer, R. R. Rubin, L. Chasan-Taber, A. L. Albright and B. Braun, *Diabetes Care*, 2010, **33**, 2692–2696.
5 R. J. FitzGerald and H. Meisel, in *Advanced Dairy Chemistry 1: Proteins*, ed. P. F. Fox and P. L. H. Mc Sweeney, Kluwer Academic/Plenum Publishers, New York, 3rd edn, 2003, pp. 675–698.
6 R. Nagpal, P. Behare, R. Rana, A. Kumar, M. Kumar, S. Arora, F. Morotta, S. Jain and H. Yadav, *Food Funct.*, 2011, **2**, 18–27.
7 D. Jakubowicz and O. Froy, *J. Nutr. Biochem.*, 2012, **24**, 1–5.
8 P. Fox and P. McSweeney, in *Advanced dairy chemistry*, ed. P. Fox and P. McSweeney, Kluwer Academic/Plenum Publishers, New York, 3rd edn, 2003, pp. 146–238.
9 A. R. Madureira, T. Tavares, A. M. P. Gomes, M. E. Pintado and F. X. Malcata, *J. Diary Sci.*, 2010, **93**, 437–455.
10 D. J. Drucker, *Cell Metab.*, 2006, **3**, 153–165.
11 J. J. Holst, *Physiol. Rev.*, 2007, **87**, 1409–1439.
12 A. Mansour, S. Hosseini, B. Larijani, M. Pajouhi and M. R. Mohajeri-Tehrani, *Nutrition*, 2013, **29**, 813–820.
13 C. Herrmann, R. Göke, G. Richter, H. C. Fehmann, R. Arnold and B. Göke, *Digestion*, 1995, **56**, 117–126.
14 M. C. P. Geraedts, F. J. Troost, M. A. J. G. Fischer, L. Edens and W. H. M. Saris, *Mol. Nutr. Food Res.*, 2011, **55**, 476–484.
15 Q. Chen and R. A. Reimer, *Nutrition*, 2009, **25**, 340–349.
16 T. Hira, T. Mochida, K. Miyashita and H. Hara, *Am. J. Physio. GastrL.*, 2009, **297**, G663–G671.
17 R. A. Reimer, C. Darimont, S. Gremlich, V. Nicolas-Métral, U. T. Rüegg and K. Macé, *Endocrinology*, 2001, **142**, 4522–4528.
18 F. Reimann, L. Williams, G. Silva-Xavier, G. A. Rutter and F. M. Gribble, *Diabetologia*, 2004, **47**, 1592–1601.
19 M. A. Nauck, T. Vilsbøll, B. Gallwitz, A. Garber and S. Madsbad, *Diabetes Care*, 2009, **32**, S223–S231.
20 R. Mentlein, *Regul Pept*, 1999, **85**, 9–24.
21 B. Ahrén, M. Landin-Olsson, P.-A. Jansson, M. Svensson, D. Holmes and A. Schweizer, *J. Clin. Endocrinol. Metab.*, 2004, **89**, 2078–2084.
22 M. Uchida, Y. Ohshiba and O. Mogami, *J. Pharmacol. Sci.*, 2011, **117**, 63–66.
23 H. Uenishi, T. Kabuki, Y. Seto, A. Serizawa and H. Nakajima, *Int. Dairy J.*, 2012, **22**, 24–30.
24 A. B. Nongonierma and R. J. FitzGerald, *Peptides*, 2013, **39**, 157–163.
25 S. T. Silveira, D. Martínez-Maqueda, I. Recio and B. Hernández-Ledesma, *Food Chem.*, 2013, **141**, 1072–1077.
26 O. Power, A. Fernández, R. Norris, F. A. Riera and R. J. FitzGerald, *J. Funct. Foods*, 2014, **9**, 38–47.
27 A. B. Nongonierma and R. J. FitzGerald, *Int. Dairy J.*, 2013, **32**, 33–39.
28 J. Floyd, S. Fajans, S. Pek, C. A. Thiffault and R. Knopf, *Diabetes*, 1970, **19**, 102–108.
29 J. J. Holst and J. Gromada, *Am. J. Phys. Endo M*, 2004, **287**, E199–E206.
30 P. Newsholme, L. Brennan and K. Bender, *Diabetes*, 2006, **55**, S39–S47.
31 G. Dixon, J. Nolan, N. McClenaghan, P. Flatt and P. Newsholme, *J. Endocrinol.*, 2003, **179**, 447–454.
32 C. Gaudel, A. B. Nongonierma, S. Maher, S. Flynn, M. Krause, B. A. Murray, P. M. Kelly, A. W. Baird, R. J. FitzGerald and P. Newsholme, *J. Nutr.*, 2013, **143**, 1109–1114.
33 A. B. Nongonierma, C. Gaudel, B. A. Murray, S. Flynn, P. M. Kelly, P. Newsholme and R. J. FitzGerald, *Int. Dairy J.*, 2013, **32**, 163–168.
34 O. Power, A. Hallihan and P. Jakeman, *Amino Acids*, 2009, **37**, 333–339.
35 M. Claessens, W. H. M. Saris and M. A. van Baak, *Br. J. Nutr.*, 2008, **100**, 61–69.
36 M. Morifuji, M. Ishizaka, S. Baba, K. Fukuda, H. Matsumoto, J. Koga, M. Kanegae and M. Higuchi, *J. Agric. Food Chem.*, 2010, **58**, 8788–8797.
37 C. A. Rice-Evans and A. T. Diplock, *Free Radical Biol. Med.*, 1993, **15**, 77–96.
38 A. Pihlanto, *Int. Dairy J.*, 2006, **16**, 1306–1314.
39 O. Power, P. Jakeman and R. J. FitzGerald, *Amino Acids*, 2013, **44**, 797–820.
40 M. Salami, A. A. Moosavi-Movahedi, M. R. Ehsani, R. Yousefi, T. Haertlé, J.-M. Chobert, S. H. Razavi,

R. Henrich, S. Balalaie, S. A. Ebadi, S. Pourtakdoost and A. Niasari-Naslaji, *J. Agric. Food Chem.*, 2010, **58**, 3297–3302.
41 A. Dryáková, A. Pihlanto, P. Marnila, L. Čurda and H. Korhonen, *Eur. Food Res. Technol.*, 2010, **230**, 865–874.
42 B. Hernandez-Ledesma, A. Davalos, B. Bartolome and L. Amigo, *J. Agric. Food Chem.*, 2005, **53**, 588–593.
43 X. Peng, B. Kong, X. Xia and Q. Liu, *Int. Dairy J.*, 2010, **20**, 360–365.
44 M. B. O'Keeffe and R. J. FitzGerald, *Int. Dairy J.*, 2014, **36**, 128–135.
45 A. B. Nongonierma and R. J. FitzGerald, *Peptides*, 2012, **37**, 263–272.
46 D. Spellman, G. O. Cuinn and R. FitzGerald, *J. Dairy Res.*, 2005, **72**, 138–143.
47 D. J. Walsh, H. Bernard, B. A. Murray, J. MacDonald, A.-K. Pentzien, G. A. Wright, J.-M. Wal, A. D. Struthers, H. Meisel and R. J. FitzGerald, *J. Diary Sci.*, 2004, **87**, 3845–3857.
48 L. Brennan, A. Shine, C. Hewage, J. P. G. Malthouse, K. M. Brindle, N. McClenaghan, P. R. Flatt and P. Newsholme, *Diabetes*, 2002, **51**, 1714–1721.
49 P. E. Morris and R. J. FitzGerald, in *Whey Processing, Functionality and Health Benefits*, ed. C. L. Onwulata and P. J. Huth, Blackwell Publishing Iowa, 2008, ch. 12, pp. 285–343.
50 A. H. Frid, M. Nilsson, J. J. Holst and I. M. Björck, *Am. J. Clin. Nutr.*, 2005, **82**, 69–75.
51 L. van Loon, W. Saris, H. Verhagen and A. Wagenmakers, *Am. J. Clin. Nutr.*, 2000, **72**, 96–105.
52 D. J. Drucker, *Endocrinology*, 2006, **147**, 3171–3172.
53 G. Tolhurst, F. Reimann and F. M. Gribble, *J. Physiol.*, 2009, **587**, 27–32.
54 W. L. Hall, D. J. Millward, S. J. Long and L. M. Morgan, *Br. J. Nutr.*, 2003, **89**, 239–248.
55 W. A. Blom, A. Lluch, A. Stafleu, S. Vinoy, J. J. Holst, G. Schaafsma and H. F. Hendriks, *Am. J. Clin. Nutr.*, 2006, **83**, 211–220.
56 K. Diepvens, D. Haberer and M. Westerterp-Plantenga, *Int. J. Obes.*, 2007, **32**, 510–518.
57 J. R. Greenfield, I. S. Farooqi, J. M. Keogh, E. Henning, A. M. Habib, A. Blackwood, F. Reimann, J. J. Holst and F. M. Gribble, *Am. J. Clin. Nutr.*, 2009, **89**, 106–113.
58 C. M. Bruen, F. O'Halloran, K. D. Cashman and L. Giblin, *Food Funct.*, 2012, **3**, 1131–1143.
59 G. Tulipano, V. Sibilia, A. M. Caroli and D. Cocchi, *Peptides*, 2011, **32**, 835–838.
60 J. Rahfeld, M. Schierborn, B. Hartrodt, K. Neubert and J. Heins, *BBA-Protein Struct Mol. Enzymol.*, 1991, **1076**, 314–316.
61 A. B. Nongonierma and R. J. FitzGerald, *Food Chem.*, 2014, **145**, 845–852.
62 A. B. Nongonierma, C. Mooney, D. C. Shields and R. J. FitzGerald, *Food Chem.*, 2013, **141**, 644–653.
63 I. M. E. Lacroix and E. C. Y. Li-Chan, *Int. Dairy J.*, 2012, **63**, 97–102.
64 T. Hatanaka, Y. Inoue, J. Arima, Y. Kumagai, H. Usuki, K. Kawakami, M. Kimura and T. Mukaihara, *Food Chem.*, 2012, **134**, 797–802.
65 A. B. Nongonierma, C. Mooney, D. C. Shields and R. J. FitzGerald, *Peptides*, 2014, **57**, 43–51.
66 O. Power, A. B. Nongonierma, P. Jakeman and R. J. FitzGerald, *Proc. Nutr. Soc.*, 2013, **73**, 34–46.
67 J. Floyd, S. Fajans, J. Conn and J. Rull, *J. Clin. Invest.*, 1966, **45**, 1487–1502.
68 A. C. Maritim, R. A. Sanders and J. B. Watkins, *J. Biochem. Mol. Toxicol.*, 2003, **17**, 24–38.
69 H. M. Chen, K. Muramoto, F. Yamauchi, K. Fujimoto and K. Nokihara, *J. Agric. Food Chem.*, 1998, **46**, 49–53.
70 Y. Li, B. Li, J. He and P. Qian, *J. Pept. Sci.*, 2011, **17**, 454–462.
71 Y.-W. Li and B. Li, *J. Theor. Biol.*, 2013, **318**, 29–43.
72 R. J. Elias, D. J. McClements and E. A. Decker, *J. Agric. Food Chem.*, 2005, **53**, 10248–10253.
73 Z. Qian, W. Jung and S. Kim, *Bioresour. Technol.*, 2008, **99**, 1690–1698.

