Journal of Functional Foods 49 (2018) 526–535

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Intestinal health benefits of bovine whey proteins after simulated gastrointestinal digestion

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ARTICLE INFO

Keywords:
Whey protein
In vitro digestion
Bioactive peptide
Glucagon-like peptide-1
Dipeptidyl peptidase IV inhibitor
Antioxidant activity

ABSTRACT

Bovine whey proteins are widely incorporated in foods for their nutritional, health promoting and functional value. However, whey proteins are readily digested in the upper gut. The objective of this study was to determine the fate and bioactivity of bovine whey proteins post simulated gastrointestinal digestion (GID). Our results demonstrated that β-lactoglobulin and α-lactalbumin post GID protect human intestinal cells from free radical formation. Post GID, lactoferrin significantly increased the amount of the intracellular antioxidant enzymes superoxide dismutase 1, 2 and thioredoxin. In addition, all whey samples post GID inhibited the activity of the dipeptidyl peptidase IV. However, the conditions of the gut destroyed the ability of whey proteins to act as glucagon-like peptide-1 secretagogues. The peptide profiles of GID whey protein isolate, β-lactoglobulin, α-lactalbumin, bovine serum albumin and lactoferrin revealed several peptides with bioactive potential.

1. Introduction

Bovine whey proteins are rich in essential amino acids, have a high nutritional value and contain bioactive peptides encrypted in their sequences (Corrochano, Buckin, Kelly, & Giblin, 2018). It has been suggested that these bioactive peptides have several health benefits including antidiabetic (Nongonierma & FitzGerald, 2013), weight management (Chaudhari et al., 2017) and reduction of cellular oxidative stress (Corrochano et al., 2018). Whey peptides can also give rise to food intolerances particularly notable in the infant gut (Brill, 2008). The protein component of bovine whey is composed of β-lactoglobulin (β-LG, 50–60%), α-lactalbumin (α-LA, 15–25%), bovine serum albumin (BSA, 6%), immunoglobulins (10%) and lactoferrin (LF, < 3%) (Corrochano et al., 2018). Commercial whey products differ in their protein content from 95% (whey protein isolate, WPI) to 34% (whey protein concentrate) (Corrochano et al., 2018) and are commonly used as food ingredients especially in the sports nutrition sector. Once ingested, intestinal cells are the first point of contact and where whey proteins are most likely to exert their greatest effect.

The mechanism by which whey proteins may have a positive effect on ameliorating type 2 diabetes or aiding weight management is in their ability to increase the enteroendocrine incretin hormone glucagon-like peptide-1 (GLP-1) (Geraedts, Troost, Fischer, Edens, & Saris, 2011) and inhibit the ubiquitous dipeptidyl peptidase IV (DPP-IV) (Lacroix & Li-Chan, 2014). GLP-1 is produced by L-cells in the gut and functions to stimulate insulin production, increase satiety, influence appetite and regulate gastric emptying and ileal brake. There is conflicting data on whether consumption of whey can promote postprandial GLP-1 levels. Whey protein supplementation (25% energy intake) significantly increased postprandial plasma GLP-1 (425 ± 135 pmol/L h) compared to casein supplementation (161 ± 90 pmol/L h) in 25 healthy individuals (Veldhorst et al., 2009).

Abbreviations: α-LA, α-lactalbumin; β-LG, β-lactoglobulin; ABAP, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; AMC, H-Gly-Pro-7-amino-4-methylcoumarin; BSA, bovine serum albumin; CAT, catalase; DCFH-DA, 2′,7′-dichlorofluorescin di-acetate; DMEM, Dulbecco's modified Eagle medium; DPP-IV, dipeptidyl peptidase IV; FBS, foetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GID, gastrointestinal digestion; GLP-1, glucagon-like peptide-1; GPx, glutathione peroxidase; HBSS, Hank's Balanced Salt Solution; IC_{50}, half maximal inhibitory concentration; LF, lactoferrin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffered saline; PRX2, peroxiredoxin; RFU, relative fluorescence units; SOD, superoxide dismutase; TRX1, thioredoxin; USP, United States pharmacopeia; WPI, whey protein isolate

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https://doi.org/10.1016/j.jff.2018.08.043
Received 15 May 2018; Received in revised form 28 August 2018; Accepted 28 August 2018

In contrast, 14 type 2 diabetic subjects who consumed 36.4 g whey protein per day did not show increases in postprandial serum GLP-1 levels compared to those who consumed a reference diet without whey (Frid, Nilsson, Holst, & Björck, 2005). Certainly, 10 mg/mL of intact whey protein concentrate can stimulate GLP-1 secretion 1.3 fold from the enteroendocrine cells line STC-1 (Power-Grant et al., 2015). Whether whey proteins are pre hydrolysed or intact also appears to influence GLP-1 levels in vitro and in vivo (Gillespie, Calderwood, Hobson, & Green, 2015).

The enzyme DPP-IV is a ubiquitous protease, which is produced and secreted by intestinal cells (Gu et al., 2008) and inactivates GLP-1 by cleavage of N-terminal proline and alanine (Gu et al., 2008). To prolong the antidiabetogenic effect of GLP-1 and reduce diabetes progression, the treatment with DPP-IV inhibitors is being used as antidiabetic therapy (Ahren et al., 2004). Power-Grant et al. (2015) showed that 50% inhibition of the DPP-IV enzyme can be achieved with commercial whey hydrolysates at concentrations 1.5 and 1.1 mg/mL.

Intestinal cells are routinely exposed to exogenous molecules, which can trigger the formation of free radicals and damage the intestinal epithelium and mucus (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Bovine whey products and their hydrolysates have demonstrated antioxidant activity in vitro by chelating metals (Gad et al., 2011; Peng, Kong, Xia, & Liu, 2010), decreasing lipid peroxidation (de Castro & Sato, 2014), reducing ferric ion (Lin, Tian, Li, Cao, & Jiang, 2012), scavenging radicals (peroxyls (Adjonu, Doran, Torley, & Agboola, 2013), hydroxyls (Kerasioti et al., 2014) and superoxides (Zhang et al., 2012)) and also neutralizing synthetic radicals (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Torkova et al., 2016) and 1,1-diphenyl-2-picrylhydrazyl (Mohammadian & Madadlou, 2016)). Whey products (0.02–2.00 mg/mL) can protect against cellular oxidation and boost intracellular antioxidant markers such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in lung fibroblasts, hepatocytes and endothelial cells (Kong, Peng, Xiong, & Zhao, 2012; O'Keeffe & FitzGerald, 2014; Pyo, Yang, Chun, Oh, & Lee, 2016). In the human epithelial colorectal adenocarcinoma cell line, Caco-2, Piccolomini, Iskandar, Lands, & Kubow (2012) reported that a 23 h treatment with digested WPI (0–2 mg/mL) protected H_2O_2-stressed cells against free radical formation and increased the ferric reducing antioxidant power of cellular supernatants.

However, when considering the physiological benefits of whey, it is important to be cognizant that whey proteins do not reach the intestine in their intact form. As they transit the conditions of the upper gut, they will be extensively hydrolysed with a digestibility score of 1.09 (Rutherfurd, Fanning, Miller, & Moughan, 2015).

We therefore pose the question of what happens to the bioactivities of commercial WPI and individual whey proteins after simulated upper gastrointestinal digestion (GID).

2. Materials and methods

2.1. Materials

Bovine WPI from pasteurized milk (≥72 °C, 26 s) used in cheese manufacturing (Isolac, 91.4% protein content) was purchased from Carbery Food Ingredients (Cork, Ireland). The proteins β-LG (92.1% β-LG content) and α-LA (93% α-LA content) were obtained from Davisco Foods International, Inc. (Minnesota, USA). The BSA (98% protein content) was purchased from Sigma-Aldrich (Dublin, Ireland) and LF (Bioferrin 2000, which contains 95% of LF and 0.02% of iron) was donated by Glanbia Nutritionals, Inc. (Wisconsin, USA). Porcine pepsin (4400 U/mg), porcine pancreatin (8 × USP, United States Pharmacopeia) and bovine bile extract were purchased from Sigma-Aldrich (Dublin, Ireland). DPP-IV Drug discovery kit was purchased from Enzo Life Sciences Ltd. (Exeter, UK). Vivaspin Turbo 4 (10 kDa) was purchased from Sartorius Stedim Ltd. (Dublin, Ireland). The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202). The murine enteroendocrine STC-1 cell line (ATCC SD5482) and the human colon adenocarcinoma HT-29 cell line (ATCC HTB-38) were obtained from the American Type Culture Collection. Tissue culture plastic material was from Sarstedt Ltd. (Wexford, Ireland). CellTiter 96 AQueus One Solution reagent was from Promega (MyBio, Kilkenny, Ireland). The mouse metabolic magnetic bead panel for active GLP-1 (#MMHMAG-44K) and the human oxidative stress kit (#H0XSTMAG-18K) were obtained from Merck Millipore (Dublin, Ireland). Halt Protease and Phosphatase inhibitor (100×) and Pierce BCA Protein Assay Kit were purchased from Thermo Fisher Scientific (Dublin, Ireland). RNeasy RNA extraction kit and on-column DNase digestion kit were obtained from Qiagen (Manchester, UK). Tetro cDNA synthesis kit was from Bioline supplied by Medical Supply Company Ltd. (Dublin, Ireland). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). LightCycler 480 SYBR Green I Master was from Roche Diagnostics Ltd. (West Sussex, UK). All other reagents were purchased from Sigma Aldrich (Dublin, Ireland).

2.2. Static simulated upper gastrointestinal digestion

Protein powders (WPI, β-LG, α-LA, BSA and LF) were reconstituted in water, exhibited high solubility, and were subjected to a simulated in vitro GID following Minekus et al. (2014) method. Oral phase was not performed on these liquid formulations. Briefly, protein powder was reconstituted following supplier recommendations to the consumer i.e. 1 g in 5 mL Milli-Q H_2O. This solution was then mixed with 2 mL of simulated gastric fluid, which contained KCl (6.9 mM), KH_2PO_4 (0.9 mM), $NaHCO_3$ (25 mM), NaCl (47.2 mM), $MgCl_2$ (0.1 mM), $(NH_4)_2CO_3$ (0.5 mM) and $CaCl_2$ (0.15 mM). A stock solution (8000 U/mL) of porcine pepsin was prepared in simulated gastric fluid and 2.5 mL was then added to the test samples to reach 2000 U/mL. The gastric phase at pH 3 was performed for 2 h at 37 °C with continuous shaking. Pepsin was inactivated by increasing the pH to 6.5 with NaOH (1 M). For the intestinal phase, bile extract and pancreatin were dissolved in simulated intestinal fluid and added to the gastric chyme (concentrations 10 mM and 100 U/mL, respectively), pH was increased to 7.0 and the volume adjusted to 20 mL with Milli-Q H_2O. The mixture was incubated with continuous shaking at 37 °C for 2 h. The protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (final concentration: 1 mM) was then added to stop the digestion. Samples were aliquoted, snap frozen in liquid nitrogen and stored at −80 °C prior cell exposure. Simulated GID of powder proteins was performed at least in duplicate. Prior experiments, GID samples were diluted to assay concentrations. Before cell treatment, digested samples were ultrafiltered in Vivaspin Turbo 4 tubes (10 kDa cut-off, 5000*g*, 20 min, 4 °C) to remove trypsin (23.3 kDa) and therefore avoid cell detachment during exposure.

A GID control with gut enzymes, electrolytes and bile salts without whey proteins was also performed.

2.3. Ultra-performance liquid chromatography/electrospray ionisation-high resolution tandem mass spectrometry (UPLC/ESI-HR-MS/MS)

Samples were separated by UPLC, and the peptides were identified by HR-MS(/MS). The UPLC/ESI-HR-MS(/MS) analyses were carried by coupling an Acquity UPLC module (Waters, Milford, MA, USA) to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The samples were separated on an Aeris PEPTIDE XB–C18 column (150 × 2.1 mm, 1.7 µm, 100 Å) equipped with a SecurityGuard ULTRA cartridge (Phenomenex, Torrance, CA, USA) kept at 50 °C, using 0.1% formic acid (FA) in MilliQ-treated water (solvent A) and 0.1% FA in acetonitrile (solvent B). The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 2%; 0–5 min, 2%; 5–30 min, 2–35%; 30–35 min, 35–95%; 35–36 min, 95%; 36–37 min, 95–2% (run-to-run time, 47 min). Peptides were

eluted at a flow rate of 0.3 mL/min. The LC eluate was analysed by MS using Full MS and data dependent tandem MS analysis of 10 of the most intense ions [ddMS^2(Top 10)]. Mass spectra were acquired over the *m/z* range from 100 to 1500; 10 of the most intense ions detected in each spectrum underwent HCD fragmentation (data dependent scan acquisition mode). The resolution was set at 70,000 and 17,500, the Automatic Gain Control (AGC) targets were 5×10^5 and 1×10^5, and maximum ion injection times were 50 ms and 110 ms for Full MS and ddMS^2 scan types, respectively. The MS data were processed and the peptides were identified using the Proteome Discoverer 1.4 software (Thermo Scientific, USA). The MS and MS/MS spectra were matched against the database of *Bos taurus* (UniProt taxon ID 9913) whey proteins and caseins inclusive of genetic variations (Farrell et al., 2004) with the additional feature of PeptideRanker, which performed screening for the potential bioactive sequences (Mooney, Haslam, Pollastri, & Shields, 2012). Settings were as follows: enzyme, no-enzyme; mass accuracy window for precursor ions-5 ppm; mass accuracy window for fragment ions, 0.02 Da; no fixed modifications; variable modifications: phosphorylation of serine and threonine, deamidation of asparagine, glutamine and arginine, oxidation of methionine and cyclisation of an N-terminal glutamine to pyro-glutamic acid. A strict false discovery rate of peptide identification was set (FDR = 0.01).

2.4. DPP-IV inhibition activity

The inhibition of the DPP-IV activity was measured using the DPP-IV Drug discovery kit following manufacturer's guidelines. Assay buffer (50 mM Tris, pH 7.5) was added to each well so that the final volume was 100 µL. Then, 15 µL of the DPP-IV stock solution (17.3 µU/mL) was added to each well (except blanks). Intact or GID protein samples (20 µL, final concentration 10 mg/mL) were added. Isoleucyl thiazolidide (10 µL) was the positive control for DPP-IV inhibition. Negative control wells contained only DPP-IV enzyme and substrate in absence of inhibitor or sample. The plate was incubated at 37 °C for 10 min and then, for fluorometric determination, H-Gly-Pro-7-amino-4-methylcoumarin (AMC) substrate (50 µL) was added to each well. Relative Fluorescence Units (RFU) were recorded every 1 min for 1 h at emission at 535 nm and excitation 485 nm using a Synergy HT micro plate reader (BioTek, Vermont, USA). A standard curve was prepared using AMC (30–0.37 µM) and the slope of the curve determined by µM/RFU conversion factor. Results were expressed in pmol/min/mg using the formula:

$$Activity\,(pmol/min) = sample\ slope\,(RFU/min) \times conversion\ factor\,(\mu M/RFU) \times assay\ volume\,(\mu L)$$

2.5. Cell culture

STC-1 and Caco-2 cell lines were grown in Dulbecco's modified Eagle medium (DMEM), containing 4.5 g/L of glucose and 0.581 g/L of L-glutamine. This medium was supplemented with 10% foetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin. HT-29 cells were maintained in McCoy's 5A modified medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were routinely grown in 75 cm^2 tissue culture plastic flasks and maintained in a humidified incubator at 37 °C with a 5% CO_2 air atmosphere. Cells at 70–80% confluence were trypsinised with 0.25% trypsin-EDTA and reseeded. The cell passage number used in this study was between 20 and 40 for the three cell lines.

2.6. Cell viability

The three cell lines were seeded at 10^5 cells/well in a 96 well plate (well growth area: 0.29 cm^2) and incubated in culture conditions. After 24 h, Caco-2 and HT-29 cells were washed twice with phosphate buffered saline (PBS) and then treated for 24 h with 100 µL GID proteins at a range of concentrations (25–0.05 mg/mL) prepared in culture medium. This achieved 0.85 mg test sample/cm^2 well area when 2.5 mg/mL test samples was used. After 24 h, 20 µL CellTiter 96 Aqueous One Solution reagent was added to each well, and cells were incubated for additional 2 h at 37 °C. Absorbance was measured at 490 nm and 630 nm (background) in a Synergy HT microplate reader (BioTek, Vermont, USA). Cell viability was calculated as a percentage of control (cells with culture medium). STC-1 cells were treated for 2 h with 100 µL of intact and GID proteins in Hank's Balanced Salt Solution (HBSS) (4 mg/mL) to achieve assay concentration of 1.37 mg sample/cm^2 well before addition of the CellTitre One Solution reagent and subsequent absorbance measurements.

2.7. Glucagon-like-peptide-1 quantification

The production of GLP-1 in STC-1 cells was determined according to Kondrashina, Papkovsky, and Giblin (2018) with the following modifications. Cells were seeded at 6.25×10^5 cells/well in a 12 well plate (growth area: 3.65 cm^2) and treated with 0.5 mL of pre or post GID samples (10 mg/mL).

2.8. Cellular antioxidant activity

Cellular antioxidant activity of GID proteins was determined in Caco-2 and HT-29 cells as described by Wolfe and Liu (2007) and adapted by Torres-Fuentes, Contreras, Recio, Alaiz, and Vioque (2015). Cells were seeded at 6×10^4 cells/well in a 96 well plate in the correspondent culture medium for 48 h. Cells were washed twice with PBS and treated with 50 µL of GID proteins diluted in HBSS (0.5 mg/mL) together with 50 µL of 2′,7′-dichlorofluorescin di-acetate (DCFH-DA, 50 µM) for 1 h. Once again, cells were washed twice with PBS and treated with 600 µM of the peroxyl radical generator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP) prepared in HBSS. Fluorescence, emission at 535 nm and excitation 485 nm, was recorded at 37 °C every minute for 1 h using Synergy HT microplate reader. Cells treated with HBSS and DCFH-DA were considered the negative control. The oxidative status of cells was calculated in relation to the positive control for oxidation (cells treated only with ABAP and DCFH-DA). N-acetylcysteine (final concentration 5 mM) was used as a positive antioxidant control.

2.9. Quantification of mRNA transcript using real time-PCR (RT-PCR)

Intestinal cells, Caco-2 and HT-29, were seeded in 6 well plates (growth area: 8.87 cm^2) at 5×10^5 cells/well and maintained for 72 h in culture conditions. On the day of the experiment, cells were washed twice with PBS and treated for 4 h with 3 mL of GID proteins at 2.5 mg/mL (0.85 mg sample/cm^2) in HBSS. After treatment, cells were washed twice with PBS and detached with 0.5 mL of trypsin-EDTA solution at room temperature for 5 min. Trypsin activity was stopped by adding complete medium. Cells were pelleted and stored at −80 °C. Total RNA extraction was performed using RNeasy Mini Kit including the step for on-column DNase digestion. Cell disruption was achieved by adding 350 µL lysis buffer directly to the cell pellet. Total RNA was quantified spectrophotometrically with the Nanodrop 1000 (Thermo Fisher Scientific, USA). Quality control checks of 1.8–2.0 for 260/280 and > 1.5 for 260/230 ratios were imposed before proceeding to cDNA synthesis. Reverse transcription to cDNA was performed from 1 µg of total RNA using Tetro cDNA synthesis kit according to manufacturer's instructions. A LightCycler 96 instrument (Roche Diagnostics Ltd., UK) and LightCycler 480 SYBR Green I Master kit were used to quantify the mRNA transcripts of the antioxidant genes SOD1, CAT and Glutathione Peroxidase 2 (GPx2). Primers for human SOD1 (NM_000454): forward 5′-GTGTGGCCGATGTGTCTATT-3′; reverse 5′-CTCAGACTACATCCAAG GGAATG-3′. Primers for human CAT (NM_001752): forward 5′-GGTA

ACCCAGTAGGAGACAAAC-3′; reverse 5′-CGAGATCCCAGTTACCATC TTC-3′. Primers for human GPx2 (NM_002083): forward 5′-CAAGTCC TTCTATGACCTCAGTG-3′; reverse 5′-GACTGTTCAGGATCTCCTCA TTC-3′. Primers for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_002046, housekeeping gene): forward 5′-GTCAACGGAT TTGGTCGTATTG-3′; reverse 5′-TGGAAGATGGTGATGGGATTT-3′. All primers were designed across intron/exon boundaries. The annealing temperature of the primers was 54 °C, optimised by running RT-PCR from 50 to 62 °C. Primer amplification efficiency was in the range of 90–105% determined by running PCR with 4 dilutions (1:1, 1:10, 1:100, 1:1000) of cDNA from both HT-29 and Caco-2. For each LightCycler reaction, 8 μL of Master Mix (LightCycler 480 SYBR Green I Master kit) were mixed with 0.5 μL of each forward and reverse primer at 10 μM and 1 μL of test cDNA. Each cDNA sample was tested in duplicate. The relative amount of target mRNA was calculated using LightCycler 96 Software (Roche Diagnostic Ltd.) in relation to mRNA levels of the house keeping gene, GAPDH. The $\Delta\Delta C_T$ method, using the experimental efficiency of each primer, was followed to determine the relative quantification of each target. Data was represented as fold change compared to non-treated cells.

2.10. Levels of antioxidant enzymes in intestinal epithelial cells

Caco-2 cells were seeded at 5×10^5 cells/well in 6 well plates for 72 h. After this time, cells were washed twice with PBS and treated for 4 h with 3 mL of 5 mg GID sample/mL in HBSS. Following manufacturer's instructions, cells were washed with ice cold PBS; then, cell lysis buffer containing protease and phosphatase inhibitor was added. Cells were scraped, transferred to a tube and centrifuged at 14,000*g* for 20 min at 4 °C. The supernatant was transferred to a chilled tube and stored at −80 °C before quantification of the antioxidant enzymes levels. This determination was performed using the Human Oxidative Stress Milliplex Map Kit. Data were collected using Luminex MagPix xMAP technology and Xponent 4.2 software (Merck Millipore, Ireland). The protein content of the cell supernatants was determined by using Pierce BCA assay kit. Results were expressed as Median Fluorescence Intensity (MFI) per μg of cellular protein.

2.11. Statistical analysis

Results were compared using one-way ANOVA followed by Bonferroni's Multiple Comparison post-hoc test using the PASW Statistics 18 software; a $p < 0.05$ was considered to indicate a statistically significant difference. Results were expressed as mean ± standard deviation. Each cellular treatment was repeated at least in duplicate with two experimental repeats on different days. Cellular antioxidant activity and cellular viability assays were performed in triplicate on two different days. Results of the relative quantification of mRNA after cell treatments were compared with control one-way ANOVA followed by Least Significance Difference (LSD) post-hoc test.

3. Results

3.1. Identification of peptides released after simulated gastrointestinal digestion of whey proteins

Protein powders (WPI, β-LG, α-LA, BSA and LF) were subjected to a simulated in vitro GID by the protocol described by Minekus et al. (2014). Samples were separated by UPLC, and the peptides were identified by HR-MS(/MS). The sequences of the peptides identified in each of the samples are listed in Table 1 and Supplementary Table S1. GID WPI revealed 47 peptides with 14 from β-LG (18.4 kDa), 3 from α-LA (14.4 kDa), 2 from BSA (66.5 kDa) and 2 from LF (80 kDa) (Table 1). Surprisingly, 26 peptides were casein-derived. Length of sequences ranged from 4 to 32 amino acids. PeptideRanker (a software programme that predicts the probability of peptides to be bioactive based on a novel N-to-1 neural network (Mooney et al., 2012), selected 26 peptides as potential bioactives as they exceeded an arbitrary threshold score of > 0.50. Merging data of Table 1 and S1, this selected group consisted of 6 β-LG peptides, 7 from BSA, 3 from LF, 1 from α_{s1}-casein and 9 from β-casein. The peptide PFPGPI located at position 61–66 AA of β-casein had the highest PeptideRanker score, 0.94. For β-LG derived peptides ALPM f(142–145) had the highest score (0.84). For BSA peptides NLPPL f(324–328) had the highest score (0.82) and for LF the peptide GSPPG f(309–313) had the highest score (0.81).

3.2. Inhibition of dipeptidyl peptidase IV by whey test samples

The ability of whey samples to inhibit the ubiquitous protease DPP-IV was evaluated before and after simulated gut transit (Fig. 1). DPP-IV exhibited an activity of 19.99 ± 1.19 pmol/min. Once the known inhibitor, isoleucyl thiazolidide, was added DPP-IV activity was decreased to 1.08 ± 0.49 pmol/min. Intact WPI, β-LG, α-LA and BSA had no inhibitory effect on DPP-IV activity ($p > 0.05$). In contrast intact LF was capable of inhibiting DPP-IV to an activity of 10.13 ± 1.23 pmol/min. Moreover all whey samples exposed to upper GID conditions significantly ($p < 0.05$) reduced DPP-IV activity (86.5%, α-LA – 97.2%, β-LG) to levels comparable to isoleucyl thiazolidide (94.6%) ($p > 0.05$). GID control also exhibited DPP-IV inhibition (9.82 ± 0.95 pmol/min), although this was significantly less than GID test samples ($p < 0.05$).

3.3. Ability of whey samples to induce secretion of GLP-1

Enteroendocrine cells appear throughout the gut with a higher density towards the distal gut; therefore these cells are exposed to both intact and GID proteins (Kondrashina et al., 2018). Intact WPI (10,788 ± 2013 pg/mL), β-LG (5237 ± 318 pg/mL) and α-LA (3721 ± 208 pg/mL) were capable of increasing active GLP-1 secretion from STC-1 cells above levels of Krebs buffer control which contained 10 mM glucose (1972 ± 131 pg/mL, $p < 0.05$, Fig. 2). Simulated GID of whey test samples significantly reduced their ability to stimulate GLP-1 secretion compared to their intact counterparts ($p < 0.05$). For instance, STC-1 cells exposed to GID α-LA secreted 338 ± 74 pg/mL GLP-1 compared to 3721 ± 208 pg/mL GLP-1 as a result of intact α-LA. GLP-1 levels in response to GID WPI, GID β-LG and GID BSA were similar to levels in cells with Krebs alone; whereas GLP-1 levels in response to GID α-LA and GID LF were significantly ($p < 0.05$) below Krebs control. Of note is that the GID control was capable of increasing GLP-1 above Krebs but this was dampened by the addition of whey suggesting an inhibitory effect of GID whey on GLP-1 secretion from enteroendocrine cells. None of the test samples at 10 mg/mL were toxic to the cells (data not shown).

3.4. Ability of whey samples to protect intestinal cells from free radicals

Food digesta is known to be cytotoxic to HT-29 and Caco-2, (Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2013), so initially cells were exposed for 24 h to a range of concentrations of test samples (25–0.05 mg/mL) post simulated upper GID. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay indicated that concentrations below 5 mg/mL were non-cytotoxic (data not shown).

The activity of peroxyl, hydroxyl and other reactive oxygen species within the cell were measured by the fluorogenic dye DCFH-DA (Fig. 3). N-acetylcysteine, which can scavenge free radicals directly and also boosts intracellular levels of the antioxidant tripeptide glutathione was used as the positive control. It reduced the radical formation in ABAP-treated HT-29 cells by 88.5%. Cell treatment with 0.25 mg/mL of GID β-LG and α-LA significantly reduced radical formation in ABAP-treated HT-29 cells compared to radical control ($p < 0.05$) (22.5% and 21.4%, respectively) and compared to GID control.

Table 1
Peptides released after upper gastrointestinal digestion of bovine whey protein isolate.

Protein[1]	Fragment location	Peptide sequence	Accurate mass $[M + H^+]^+$	PeptideRanker score[2]
β-LG	15–18	VAGT	347.1942	0.10
β-LG	45–51	ELKPTPE	813.4384	0.10
β-LG	47–51	KPTPE	571.3114	0.16
β-LG	76–80	TKIPA	529.3370	0.13
β-LG	78–81	IPAV	399.2620	0.24
β-LG	107–114	MENSAEPE	906.3552	0.08
β-LG	108–114	ENSAEPE	775.3140	0.07
β-LG	109–114	NSAEPE	646.2706	0.10
β-LG	110–115	SAEPEQ	660.2864	0.10
β-LG	125–130	TPEVDD	675.2861	0.05
β-LG	125–132	TPEVDDEA	875.3667	0.05
β-LG	126–131	PEVDDE	703.2816	0.06
β-LG	149–154	LSFNPT	678.3488	0.53
β-LG	150–154	SFNPT	565.2645	0.64
α-LA	63–68	DDQNPH	725.2883	0.28
α-LA	82–88	DDDLTDD	808.2883	0.11
α-LA	97–102	DKVGIN	645.3594	0.11
BSA	117–125	PDPN	442.1938	0.58
BSA	568–573	AVEGPK	600.3379	0.15
LF	308–331	LRIPSKVDSALYLGSRYLTTLKNLRETAEEVK	3826.9279	0.03
LF	354–372	EQKKCQQWSQQSGQNVTCA	2168.8805	0.26
α_{s1}-CAS	180–186	SDIPNPI	755.3967	0.57
α_{s2}-CAS	71–76	ITVDDK	690.3702	0.05
α_{s2}-CAS	79–90	QKALNEINQFYQ	1497.7247	0.16
α_{s2}-CAS	115–122	NAVPITPT	812.4553	0.21
β-CAS	33–38	FQSEEQ	847.2902	0.09
β-CAS	58–66	LVYPFPGPI	1002.5697	0.75
β-CAS	59–66	VYPFPGPI	889.4858	0.85
β-CAS	61–66	PFPGPI	627.3531	0.94
β-CAS	61–68	PFPGPIPN	838.4487	0.89
β-CAS	63–66	PGPI	383.2307	0.8
β-CAS	71–77	PQNIPPL	778.4494	0.81
β-CAS	71–82	PQNIPPLTQTPV	1304.7266	0.37
β-CAS	81–86	PVVVPP	607.3843	0.26
β-CAS	81–87	PVVVPPF	754.4535	0.67
β-CAS	100–105	EAMAPK	646.3259	0.25
β-CAS	108–113	EMPFPK	748.3732	0.77
β-CAS	114–119	YPVEPF	751.3693	0.63
β-CAS	174–197	PQKAVPYPQRDMPIQAFLLYQEPV	2844.4847	0.15
κ-CAS	106–111	MAIPPK	656.3831	0.59
κ-CAS	108–111	IPPK	454.3041	0.56
κ-CAS	120–123	PTIE	459.2470	0.09
κ-CAS	125–131	IASGEPT	674.3387	0.16
κ-CAS	132–137	STPTTE	635.2903	0.08
κ-CAS	147–152	EDSPEV	675.2862	0.09
κ-CAS	154–161	ESPPEINT	886.4191	0.23
κ-CAS	166–169	STAV	377.2049	0.06

[1] β-LG = β-lactoglobulin, α-LA = α-lactalbumin, BSA = bovine serum albumin; LF = lactoferrin; CAS = casein.
[2] Data obtained from PeptideRanker, available at http://bioware.ucd.ie/ accessed on January 2018.

In Caco-2 cells, GID whey proteins did not reduce radical formation (data not shown). N-acetylcysteine reduced radical formation in Caco-2 cells exposed to ABAP by 41.7%. Interestingly the level of radical formation in unstressed Caco-2 cells was 18.9% higher than the levels observed in HT-29 cells. Given the rapid digestion of whey in the upper gut (Purpura et al., 2014), intact whey protein was not assayed as it is not physiologically relevant to intestinal epithelial cells.

3.5. Effect of GID whey samples on antioxidant biomarkers

To determine the ability of GID whey proteins to boost antioxidant markers in intestinal cells, mRNA transcripts of SOD1, CAT, and GPx2 were measured in both Caco-2 and HT-29 cells in response to a 4 h incubation with GID whey test samples. GID control significantly increased mRNA levels of SOD1 in Caco-2 cells compared to untreated cells in HBSS (Fig. 4A). Caco-2 treatment with GID α-LA, GID BSA and GID LF were similar to GID control ($p < 0.05$ compared to untreated cells). GID WPI and GID β-LG did not significantly increase SOD1 above untreated cells (Fig. 4A).

All GID samples significantly enhanced mRNA transcripts of CAT in Caco-2 cells compared to untreated cells, with significantly highest levels in GID BSA and GID control (Fig. 4B).

The mRNA levels of GPx2 were also significantly greater after Caco-2 exposure to GID control compared to untreated cells ($p < 0.05$) (Fig. 4C). Levels of GPx2 mRNA were similar with GID whey samples to GID control ($p > 0.05$). Exposure of HT-29 cells to GID whey tests samples did not alter mRNA levels of SOD, CAT or GPx2 compared to untreated cells with one exception, CAT levels for GID α-LA were significantly increased (Supplementary Fig. S1B). GID control significantly increased mRNA levels of SOD and GPx2 in HT-29 cells compared to untreated cells ($p < 0.05$).

Levels of SOD1, SOD2, CAT, thioredoxin (TRX1) and peroxiredoxin 2 (PRX2) enzymes were also quantified in Caco-2 cells in response to 4 h incubation with GID whey test samples (Table 2). The GID LF sample showed significantly higher amount of SOD1 (22.89 ± 3.89 MFI/μg cellular protein), SOD2 (20.75 ± 2.98 MFI/μg cellular protein) and TRX1 (11.62 ± 1.41 MFI/μg cellular protein) compared to untreated cells and compared to GID control cells (Table 2,

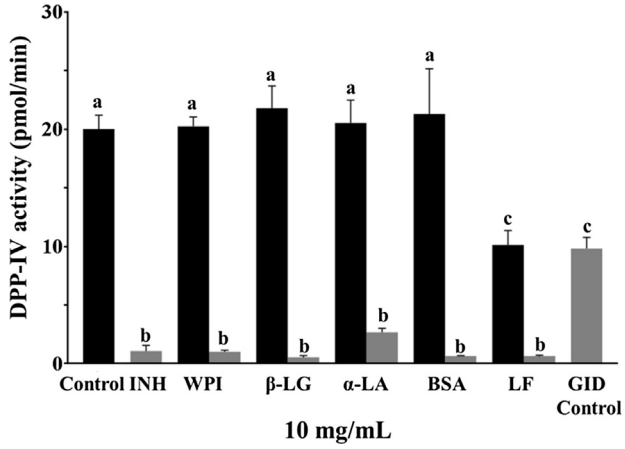

Fig. 1. DPP-IV enzyme activity (pmol/min) of intact (black bars) and gastrointestinal digested (grey bars) whey products (10 mg/mL). Control = DPP-IV enzyme and substrate (H-Gly-Pro-7-amino-4-methylcoumarin); INH = isoleucyl thiazolidide (DPP-IV inhibitor, 1 mM), WPI = whey protein isolate; β-LG = β-lactoglobulin, α-LA = α-lactalbumin, BSA = bovine serum albumin; LF = lactoferrin; GID Control = gut enzymes, bile salts and electrolytes without whey proteins. Experiments were performed in duplicate on two different days. Different letters indicate significant difference ($p < 0.05$).

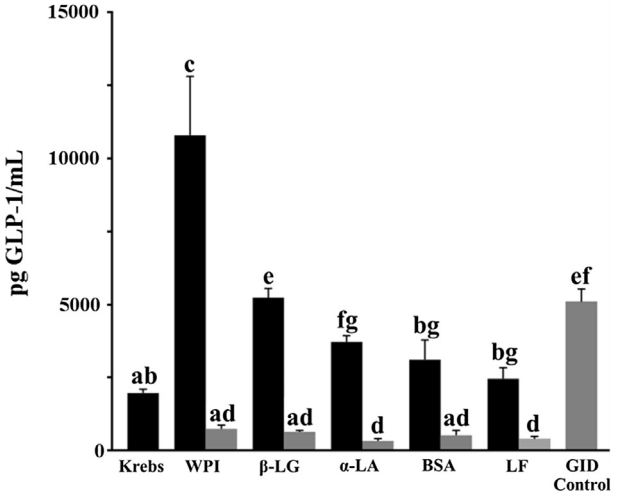

Fig. 2. GLP-1 secretion (pg/mL) in 6.25×10^5 STC-1 cells/well after 4 h treatment with 10 mg/mL of intact (black bars) and gastrointestinal digested (grey bars) whey products. Krebs = cells treated with Krebs buffer, which contained 10 mM glucose, WPI = whey protein isolate; β-LG = β-lactoglobulin, α-LA = α-lactalbumin, BSA = bovine serum albumin; LF = lactoferrin; GID Control = gut enzymes, bile salts and electrolytes without whey proteins. Cell exposure was performed in duplicate on two different days. Different letters indicate significant difference ($p < 0.05$).

$p < 0.05$). Treatments with GID WPI, GID β-LG, GID α-LA, GID BSA or GID enzymes did not significantly alter intracellular levels of these antioxidant enzymes.

4. Discussion

The whey fractions β-LG and α-LA exposed to the conditions of the upper gut were able to protect HT-29 cells from free radical formation. GID LF also increased the amount of the antioxidant proteins SOD1, SOD2 and TRX1 present in Caco-2 cells. All GID whey samples inhibited the protease DPP-IV activity. However, the hydrolytic conditions of the upper gut destroyed the ability of whey proteins to increase secretion of GLP-1. The peptide profiles revealed several sequences common to those previously identified in human jejunum effluents, some of which

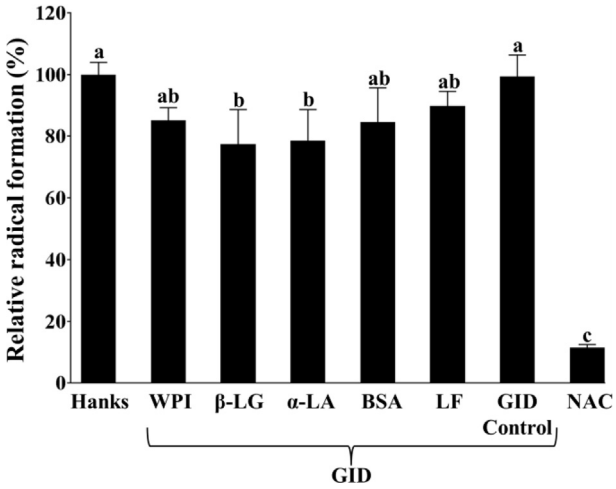

Fig. 3. Relative % of radical formation in HT-29 cells (seeded at 6×10^4 cells/well) after 1 h exposure to gastrointestinal digested (GID) whey samples (0.5 mg/mL). Hanks = cells treated with Hank's Balanced Salt Solution, WPI = GID whey protein isolate; β-LG = GID β-lactoglobulin; α-LA = GID α-lactalbumin; BSA = GID bovine serum albumin; LF = GID lactoferrin; GID Control = gut enzymes, bile salts and electrolytes without whey proteins; NAC: N-acetylcysteine (antioxidant positive control). Different letters indicate significant difference ($p < 0.05$). Cell treatment was performed in triplicate on two different days.

are undoubtedly responsible for these bioactivities. In addition, the gastrointestinal environment itself increased mRNA levels of the antioxidant markers SOD1, CAT and GPx2, in Caco-2 cells and SOD1 and GPx2 in HT-29 cells.

Previously, several whey peptides have been identified in the proximal jejunum after ingestion of 30 g of ^{15}N labelled whey protein by 4–6 healthy subjects (Boutrou et al., 2013; Sanchón et al., 2018). After a 4 h simulated static GID of WPI, we identified 14 peptides from β-LG derived from 6 regions which agree with previous in vivo GID results (β-LG regions: 15–18, 45–51, 76–81, 107–115, 125–132 and 149–154). Furthermore, α-LA peptides from the regions 63–68, 80–89 and 97–102 were common to both our in vitro study and in vivo data (Boutrou et al., 2013; Sanchón et al., 2018). The BSA peptide, KDDS-PDLK f(130–138) and derivatives of KFPK f(245–248), KVPQ f (437–440) and LTPDET f(514–519) (Table S1) were also identified in vivo (Boutrou et al., 2013). Derivatives of the LF peptides EAGRDPY f (66–72), FENLPE f(215–220), ADRDQYE f(222–228), FGSPPGQ f (289–295), RIPS f(309–312) and KPVTEA f(579–584) were found in the duodenum of 2 subjects who received 2 g of bovine LF (95% purity) (Furlund et al., 2013). Interestingly, the β-LG sequences DAQSAPL f (33–39), TPEVDDEA f(125–132) and LSFNPT f(149–154) (Table S1) were not found in vivo but have been recently discovered in post dynamic GID skim milk powder (10% w/v) (Egger et al., in press). The detection of casein peptides in WPI suggests that casein leakage into whey fraction during processing. There are limitations with the detection range of UPLC HR-MS(/MS) methodology with no doubt a considerable amount of peptides < 4 amino acids and free amino acids also present in the samples. UPLC-HR-MS(/MS) analysis was also not performed on non-digested samples.

Whey protein samples after simulated GID inhibited DPP-IV similar to the known inhibitor isoleucyl thiazolidide (94.6%). Recently GID WPI (62.5 μg/mL) also inhibited DPP-IV activity by 15%, although this inhibition was lower compared to the positive control diprotin A, where 12.5 μg/mL inhibited 49% enzymatic activity (Lacroix, Chen, Kitts, & Li-Chan, 2017). Power-Grant et al. (2015) observed that commercial whey hydrolysates, with a degree of hydrolysis of 32% and 45%, maintained DPP-IV inhibition after simulated GID. Whey protein hydrolysis with the enzyme papain at pH 6.3 also increased the ability of

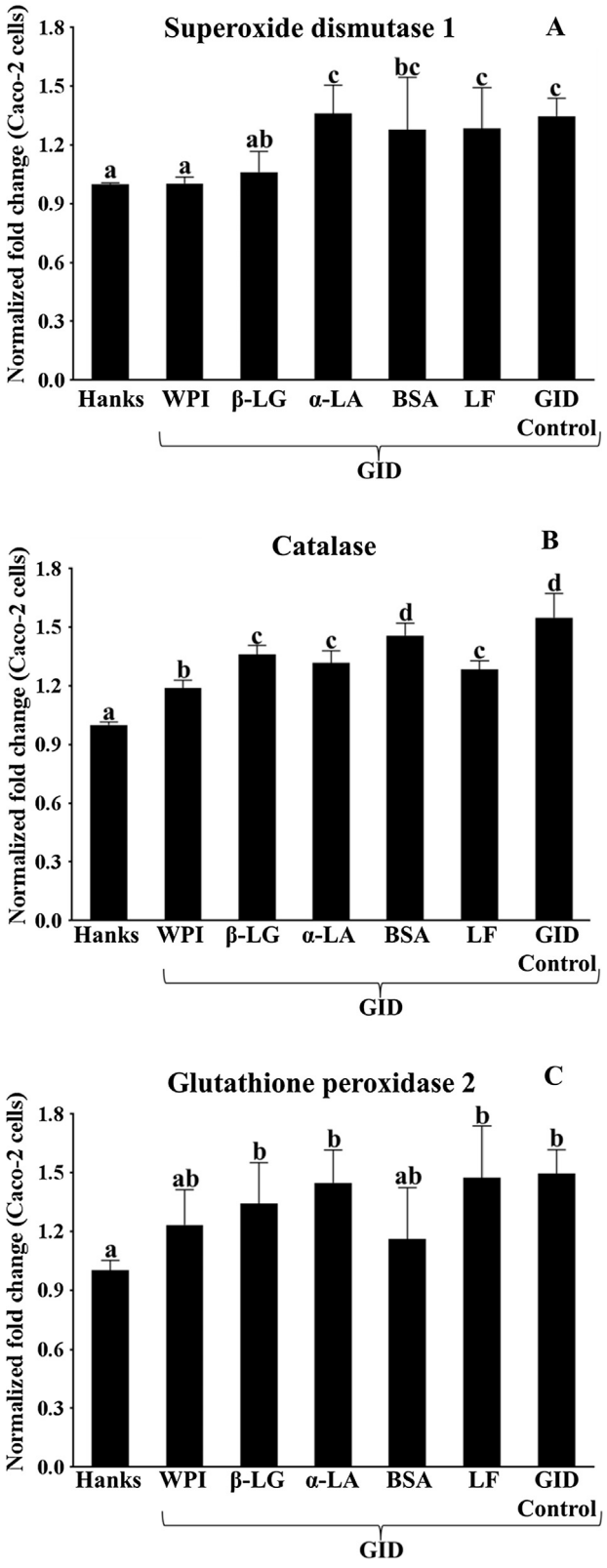

Fig. 4. Relative quantification of mRNA of superoxide dismutase 1 (A), catalase (B) and glutathione peroxidase 2 (C) in Caco-2 cells after 4 h exposure to gastrointestinal digested (GID) samples (0.85 mg sample/cm^2, seeded at 5×10^5 cells/well). The relative quantification was calculated using the housekeeping gene GAPDH following the $\Delta\Delta C_T$ method; data were normalized to untreated cells (Hanks alone). WPI = GID whey protein isolate; β-LG = GID β-lactoglobulin; α-LA = GID α-lactalbumin; BSA = GID bovine serum albumin; LF = GID lactoferrin; GID Control = gut enzymes, bile salts and electrolytes without whey proteins. Cell exposure and mRNA quantification were performed in duplicate on two different days. Different letters indicate significant difference ($p < 0.05$).

whey protein concentrate to inhibit DPP-IV activity (IC_{50} = 1.40 mg/mL) compared to unhydrolysed whey protein (IC_{50} > 2.5 mg/mL). The fate of this bioactivity after gut transit was not tested (Le Maux, Nongonierma, Barre, & FitzGerald, 2016). In the present study, GID WPI exerted the same DPP-IV inhibition than the individual whey proteins. In contrast, hydrolysates from whey protein concentrate produced by a serine protease enzyme found in Asian pumpkin was better at inhibiting DPP-IV activity (IC_{50} < 0.55 mg/mL) than equivalent β-LG hydrolysates (Konrad et al., 2014). The discrepancies between studies are most likely as a result of different enzyme specificity resulting in distinctive peptide profiles. Previously, > 50 dairy peptides have been proven to inhibit DPP-IV activity in vitro (Lacroix & Li-Chan, 2016). Tulipano, Sibilia, Caroli and Cocchi (2011) observed that 49 μM of synthetic IPA, found at position 78–80 AA of β-LG, can inhibit DPP-IV by 50%. In our study, simulated GID of WPI and β-LG resulted in the production of the IPA encrypted peptides IPAV f(78–81) and TKIPA f(76–80). Others have observed β-LG peptides, IPAVF f (78–82), VAGTWY f(15–20) and TPEVDDEALEK f(125–135), with a DPP-IV IC_{50} of 47.7 μM, 174.0 and 319.5 μM, respectively (Silveira, Martinez-Maqueda, Recio, & Hernandez-Ledesma, 2013; Uchida, Ohshiba, & Mogami, 2011). In our study, GID of both WPI and β-LG released similar peptides, i.e. VAGT and TPEVDDEA. In fact, the peptide VAGT possesses an alanine at the penultimate location at the N-terminal side of the peptide, which is known as a substrate for DPP-IV (Caron et al., 2016). The sequences identified DKVGIN, α-LA f(97–102), and LKPTPEG, β-LG f(46–52), (Table S1) were also present in peptides previously described as DPP-IV inhibitors, ILDKVGINY (IC_{50} = 263 μM) and LKPTPEGDL (IC_{50} = 45 μM) (Lacroix & Li-Chan, 2014). Interestingly, the peptide LKPTPEGDL f(46–54) was also proven to survive brush border membrane peptidases and be transported across in vitro Caco-2 monolayers (Lacroix, et al., 2017). Other whey peptides with DPP-IV inhibitory activity including 12 dipeptides (Nongonierma & FitzGerald, 2013) and peptides produced by pepsin hydrolysis of WPI or α-LA (Lacroix & Li-Chan, 2014) were not found during our simulated GID nor in the human proximal jejunum (Sanchón et al., 2018).

Intact whey proteins promoted the secretion of GLP-1 in enteroendocrine cells although this effect was not maintained after simulated GID. In agreement, Geraedts et al. (2011) demonstrated that whey and casein lost their GLP-1 secretagogue effect after hydrolysis with trypsin. Interestingly, dietary supplementation with intact whey protein maintained plasma GLP-1 levels longer than commercial whey hydrolysates (Calbet & Holst, 2004). Degree of food digestion and indeed levels of oxygen in the gut influence GLP-1 response to food (Kondrashina et al., 2018).

There is some evidence that oxidative stress may aggravate diabetes by triggering associated complications (Matough, Budin, Hamid, Alwahaibi, & Mohamed, 2012). Therefore, the discovery of ingredients with a dual function, antioxidant and antidiabetic, may be highly promising in the treatment of type 2 diabetes mellitus (Milani et al., 2017; Power, Nongonierma, Jakeman, & FitzGerald, 2014). Interestingly, 60% of the peptides released from β-LG after simulated gut transit are contained in one of three antioxidant hotspots previously identified in the β-LG structure, 42–61 AA, 77–110 AA and 123–135AA (Corrochano et al., 2018). In addition, outside these locations, two β-LG peptides, VAGT f(15–18) and IIAE f(71–74), have known peroxyl radical scavenger ability (O'Keeffe, Conesa, & FitzGerald, 2017). It is interesting to note that only VAGT survived simulated GID (Table 1). This may explain the ability of GID β-LG to reduce free radical formation in HT-29 cells by 22.5%. However, β-LG post GID did not increase the

Table 2
Levels of antioxidant enzymes in Caco-2 cells after gastrointestinal digested bovine whey protein exposure (MFI/μg cellular protein[1]).

	CAT[2]	SOD1	SOD2	TRX1	PRX2
Control	10.84 ± 1.13	15.69 ± 0.49	15.92 ± 1.47	8.38 ± 1.03	1.68 ± 0.07
GID WPI	11.25 ± 0.71	17.60 ± 0.09	16.29 ± 0.68	8.59 ± 0.52	2.07 ± 0.33
GID β-LG	9.29 ± 0.82	16.66 ± 0.56	15.30 ± 0.95	8.57 ± 0.34	2.10 ± 0.13
GID α-LA	8.87 ± 0.36	15.04 ± 1.69	15.00 ± 0.74	7.69 ± 0.57	2.03 ± 0.16
GID BSA	10.09 ± 1.36	17.75 ± 2.38	16.45 ± 1.37	8.72 ± 0.73	2.06 ± 0.22
GID LF	11.80 ± 0.07	22.89 ± 3.89* a	20.75 ± 2.98* a	11.62 ± 1.41* a	2.08 ± 0.10
GID Control	9.63 ± 0.68	17.15 ± 0.95	15.68 ± 0.88	8.44 ± 0.79	2.06 ± 0.54

Levels of antioxidant proteins in Caco-2 cells (seeded at 5×10^5 cells/well in a 6 well plate) after a 4 h exposure to gastrointestinal digested (GID) whey protein test samples (1.69 mg/cm[2]) were quantified by the Human Panel Oxidative stress Magnetic Bead Panel. Results were expressed as median fluorescence intensity (MFI) ± SD per μg cellular protein.

* Statistically significant results ($p < 0.05$) compared with untreated cells in basal conditions (Control).

[a] Statistically different results to GID Control (gut enzymes, bile salts and electrolytes without whey proteins).

[1] GID WPI = GID whey protein isolate; GID β-LG = GID β-lactoglobulin, GID α-LA = GID α-lactalbumin, GID BSA = GID bovine serum albumin; GID LF = GID Lactoferrin.

[2] CAT = catalase; SOD1 = superoxide dismutase 1, SOD2 = superoxide dismutase 2, TRX1 = thioredoxin; PRX2 = peroxiredoxin.

amount of antioxidant enzymes nor SOD1 mRNA levels in Caco-2 cells.

Caco-2 cells exposed to GID LF significantly increased the amount of SOD1, SOD2 and TRX1 enzymes compared to untreated and GID control cells ($p < 0.05$) and also produced noteworthy increases in mRNA levels of SOD1, CAT and GPx2 albeit not more than GID control. A 24 h pretreatment with iron-unsaturated human LF also protected H_2O_2-stressed Caco-2 cells against oxidation (Shoji, Oguchi, Shinohara, Shimizu, & Yamashiro, 2007). In agreement, LF decreased the production of free radicals in H_2O_2-stressed mesenchymal stem cells after 24 h pretreatment (Park et al., 2017). Free radical production also decreased in H_2O_2-stressed Caco-2 cells after a 23 h treatment with 2 mg/mL of GID WPI, which contains < 3% LF (Piccolomini et al., 2012). However, to mimic physiological conditions, a concentration of 0.25 mg/mL and shorter exposure times (1–4 h) were selected in our study. A 1 h pretreatment with GID α-LA protected ABAP-stressed HT-29 cells from free radicals by 21.4%. Previously, α-LA hydrolysates produced by gastric enzymes also inhibited peroxyl radicals in chemical antioxidant assays with an activity of 1.065 ± 0.056 μmol of Trolox/mg protein (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005). Preliminary work in our laboratory demonstrated that α-LA has the greatest antioxidant potential of all whey proteins (FRAP, ABTS and ORAC methodologies) once exposed to the conditions of the gastrointestinal tract. It is noteworthy that the antioxidant status of Caco-2 cells differs from HT-29 by higher raw Ct values for CAT and GPx2 mRNA levels and 18.9% more radical formation. Therefore, the same test compound may induce distinct effects depending of the target cells and their redox status before treatment (Amorati & Valgimigli, 2015). Previously, SOD, CAT and GPx activity in muscle and lung cells has been influenced by whey protein products (Kong et al., 2012; Xu, Liu, Xu & Kong, 2011). CAT activity was also greater in umbilical vein endothelial cells after 48 h exposure to low molecular weight fractions (< 5 kDa) of whey hydrolysates produced by different commercial enzymes (O'Keeffe & FitzGerald, 2014). Interestingly, high molecular weight fractions (2 μm–5 kDa) and intact whey protein concentrate did not produce such antioxidant response.

However, these studies did not consider the effect of the gut conditions or the bioavailability of whey across the intestinal barrier to reach muscle, lung fibroblasts or endothelial cells. It is of note that a 30 day supplementation with 1250 or 2500 mg bovine LF/kg body weight did increase the expression of the antioxidant enzymes SOD1, CAT and GPx in serum of healthy piglets (Wang, Xu, An, Liu, & Feng, 2008).

Other interesting observations included the ability of GID control to modulate SOD1, CAT and GPx2 mRNA levels. This effect could be explained by the release of bioactive peptides from the endogenous gut enzymes that are also able to regulate the gut function (Dave et al., 2016). In addition, the antioxidant up regulation might be produced by the bile extract, which contains bile salts and conjugates of hyodeoxycholic acid with the antioxidant amino acids glycine and taurine. Similarly to our study, a mixture of bile salts (0.2–0.8 mM) also regulated antioxidant enzymes in unstressed esophageal squamous carcinoma cells by increasing the enzyme SOD2 after 4 h exposure (Schiffman et al., 2011).

5. Conclusions

Conditions of the gut modulated the bioactivity of WPI and individual whey proteins. In particular in vitro evidences demonstrated that WPI lost its ability to stimulate GLP-1 but gained DPP-IV inhibition suggesting digestion facilitates or impedes bioactivity. Some antioxidant benefits were noted but these observations were intestinal cell model dependant. Whether some or all of the peptides identified were capable of crossing the intestinal barrier to subsequently have a health benefit to downstream target organs has yet to be determined.

Acknowledgement

This work was supported by the Department of Agriculture, Food and the Marine (Dublin, Ireland; FIRM project 13 F 354-WheyGSH). A. R. Corrochano is in receipt of a Teagasc Walsh Fellowship.

Conflict of interest

None.

Ethics statement

I have read and adhere to the Publishing Ethics.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.08.043.

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