High hydrostatic pressure enhances whey protein digestibility to generate whey peptides that improve glutathione status in CFTR-deficient lung epithelial cells

Regina Maria Vilela[1], Larry C. Lands[2], Hing M. Chan[1,3], Behnam Azadi[1] and Stan Kubow[1]

[1] School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada
[2] Montreal Children's Hospital, Division of Respiratory Medicine, Montreal, Quebec, Canada
[3] Centre for Indigenous Peoples' Nutrition and Environment (CINE), Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada

Whey protein isolates (WPI) may provide anti-inflammatory benefits to cystic fibrosis (CF), which could be mediated via peptides, as proteolytic digests of WPI enhance intracellular glutathione (GSH) concentrations. The objectives of this study were to investigate whether high hydrostatic pressure can (i) improve the *in vitro* digestibility of WPI; and (ii) generate low molecular weight (< 1 kDa) peptides from WPI hydrolysates that exert GSH-enhancing and anti-inflammatory properties in wild type and mutant CF transmembrane conductance regulator (CFTR) tracheal epithelial cells. Hydrostatic pressure processing enhanced the *in vitro* digestibility of WPI to proteolytic enzymes resulting in altered peptide profiles as assessed by CZE and GC-MS. The exposure of mutant CFTR cells to low molecular weight (< 1 kDa) peptides isolated from WPI hydrolysates exposed to pressure processing (pressurized WPI hydrolysates, pWPH), showed increased intracellular levels of reduced GSH and total GSH relative to treatment with peptides obtained from native WPI hydrolysates (nWPH). A tendency for decreased interleukin-8 secretion was associated with the pWPH and nWPH treatments in mutant CFTR cells, which was not observed in wild type cells. Hydrostatic pressure processing of whey proteins appears to enhance their impact on cellular GSH status in cells with the mutant CFTR condition.

Keywords: Cystic fibrosis / Digestibility / Glutathione / Hydrostatic pressure / Peptides

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1 Introduction

There is increasing use of bovine whey proteins in either native or predigested forms in infant formulas and medical foods for nutritional and therapeutic applications [1]. In this regard, whey proteins contain bioactive peptides in their primary sequence that can be released during gastrointestinal protein hydrolysis to exert physiological effects. Bioactive peptides in whey protein hydrolysates have demonstrated cytoprotective effects towards oxidative stress [2], and have been shown to induce levels of the major intracellular antioxidant, glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) in cultured prostate cells [3]. Human trials have demonstrated that intake of whey proteins, which are cysteine-rich, can increase GSH levels in lymphocytes [4]. Whey proteins such as BSA, β-lactoglobulin (β-LG) and immunoglobulins contain glutamic acid-cysteine segments in their primary sequences that form disulfide bridges. It has been postulated that during digestion of undenatured whey protein isolates (WPI), low-molecular-weight peptides such as γ-glutamylcysteine or cystine are released and absorbed intact to stimulate GSH biosynthesis [5]. Cystine, the disulfide form of cysteine, can be used towards intracellular synthesis of GSH [6], as cystine is reduced to two moles of cysteine, which is the rate-limiting amino acid in GSH biosynthesis. The GSH molecule is

Correspondence: Dr. Stan Kubow, School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, 21,111 Lakeshore, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9
E-mail: stan.kubow@mcgill.ca
Fax: +1-514-398-7739

Abbreviations: β-LG, β-lactoglobulin; **CF**, cystic fibrosis; **CFTR**, cystic fibrosis transmembrane conductance regulator; **GSH**, glutathione; **GSSG**, glutathione disulfide; **IL**, interleukin; **LSD**, least square difference; **MEM**, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **MWCO**, molecular weight cut-off; **nWPH**, native whey protein hydrolysates; **OPA**, *o*-phthaldialdehyde; **pWPH**, pressurized whey protein hydrolysates; **ROS**, reactive oxygen species; **TNF-α**, tumor necrosis factor-α; **WPI**, whey protein isolate

a ubiquitous cellular non-protein sulfhydryl that functions as a potent cellular antioxidant because the reduced form of GSH can readily donate its sulfhydryl proton to quench reactive oxygen species (ROS). The oxidized form of GSH forms a disulfide linkage with a second oxidized GSH molecule to yield glutathione disulfide (GSSG). A high intracellular GSH/GSSG ratio provides strong protection against cell damage induced by oxidative stress [7]. Major developments in the isolation and processing of whey proteins have improved their physicochemical and sensory properties as ingredients within foods [8]. There is relatively little knowledge, however, regarding the impact of food processing on the digestibility and antioxidant activity of whey proteins. The sensitivity of proteins to digestion as well as the type of peptides formed from the proteolysis is dependent on the amino acid sequence and three-dimensional structure of proteins. In this regard, whey proteins have a globular conformation that makes them more resistant to digestive enzyme proteolysis [9]. High hydrostatic pressure processing is a food processing technique used as an alternative to heat treatment for food sterilization whereby the proteins are subjected to pressures of several hundred MPa [10]. Although the primary structure of proteins remains intact during pressure treatment [11], high-pressure treatment above 200 MPa can cause changes in the secondary and tertiary structure that can lead to irreversible denaturation of the protein [12]. Several studies have demonstrated that *in vitro* proteolytic digestion of individual whey proteins such as β-LG and α-lactalbumin is enhanced when carried out under elevated hydrostatic pressures [13]. It is unclear, however, whether the prior exposure of whey proteins to high hydrostatic pressure can enhance their subsequent *in vitro* digestibility under ambient conditions or whether hydrostatic pressure processing could alter the profile of bioactive peptides released upon hydrolysis. The impact of peptides generated from whey protein hydrolysis has not been studied previously in lung epithelial cells, particularly in relation to their impact on inflammation and GSH status. High concentrations of GSH are present in lung epithelial lining fluid and GSH is important in maintenance of the integrity of the lung airspace epithelia *in vitro* and *in vivo* [14]. There is abundant evidence that GSH also plays a major role in regulating inflammatory mediators such as cytokines increased by oxidative stress [15]. The ROS signaling regulating the transcription of interleukin (IL)4 [16], IL6, IL8 [17] and tumor necrosis factor (TNF)-α [17] involves thiol-dependent mechanisms. Depletion of lung epithelial lining fluid GSH has been described in lung inflammatory disorders such as cystic fibrosis (CF), adult respiratory distress syndrome [18], and idiopathic pulmonary fibrosis [19]. Cellular GSH depleting agents such as L-buthionine-(S,R)-sulfoximine (BSO) can enhance cytokine secretion by up-regulating ROS [17]. Conversely, the GSH precursor N-acetyl-L-cysteine (NAC) inhibits TNFα-induced activation of nuclear factor (NF)-κB activity and IL8 promoter-mediated reporter gene expression in airway epithelial cells [20]. The CF condition is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene that encodes a cAMP-dependent chloride channel located in the apical membrane of epithelial cells. Lung damage due to chronic inflammation, mucus accumulation and chronic infection is the major cause of death in CF. Chronic inflammation is also associated with increased oxidative stress in CF patients [21]. Antioxidant therapy such as supplementation of WPI to induce tissue GSH has been proposed to combat chronic inflammation in the CF lung [4]. CFTR deficiency is known to result in decreased GSH export leading to a reduction of GSH in the CF apical fluid [22]. However, relatively little is known regarding GSH status of airway epithelial cells or whether intervention with peptides derived from whey proteins can stimulate GSH status and diminish IL8 secretion in mutant CFTR lung epithelial cells.

The first part of this study utilizes an *in vitro* enzymatic digestion protocol to study the effect of high hydrostatic pressure on the digestibility and peptide profile of whey protein subjected to different forms of high hydrostatic pressure. A combination of ultrafiltration techniques and enzymic hydrolyses were used to produce a hydrolysate in which peptide fragments >1 kDa were removed, as these latter peptides are considerably less bioavailable and are not observed in the ileal juices in the pig model of human digestion following protein feeding [23]. Treatment of wild-type tracheal epithelial cells (9HTEo-) and mutant CFTR cells (CFTE29o-) with the low molecular weight peptides obtained from hydrolysates of native and pressurized whey proteins was undertaken to examine whether such peptides could lead to differential effects on GSH homeostasis. The present studies also tested whether whey peptide treatment could induce intracellular GSH or affect the release of the cytokine and chemokine, IL8, under pro-inflammatory conditions, as raised secretory levels of this cytokine are recognized as a major contributory factor in lung pathophysiology [24].

2 Materials and methods

2.1 Materials

InPro™ WPI was obtained from Volac Nutrition (UK) and contains a 92% protein content obtained from cross-flow microfiltration. To perform *in vitro* protein digestion, pepsin (catalog # P-7012) from porcine stomach mucosa and pancreatin (catalog # P-1625) from porcine pancreas were obtained from Sigma-Aldrich (Oakville, Ontario) and prepared in 0.01 M HCl and phosphate buffer pH 7.0, respec-

tively. Dulbecco's PBS was obtained from Gibco BRL (Burlington, Ontario). To determine protein content the Bio-Rad protein assay dye reagent was obtained from ICN Biomedicals (Aurora, OH). Determination of the α-amino group content required preparation of an OPA (*o*-phthaldialdehyde) solution, which used the reagents sodium tetraborate, SDS and 2–mercaptoetanol obtained from Sigma-Aldrich. Amicon ultrafiltration regenerated cellulose membranes (NMWL: 1 kDa) and ultrafiltration stirred units for isolation of low molecular weight peptides from enzymic hydrolysates were obtained from Millipore (Nepean, Ontario). Wild-type (non-CF; 9HTEo-) and mutant ΔF508 CFTR (CF; CFTE29o-) human tracheal epithelial cells were gifts from Dr. D. Gruenert (University of California). The supplies for the maintenance of cell culture such as minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, L- glutamine, and Dulbecco's PBS were obtained from Gibco BRL (Burlington, Ontario). Trypsin-EDTA solution (0.25%) was obtained from Sigma-Aldrich. The solution used to coat the T-75 flasks and 24-well plates was prepared with collagen type I bovine, and human fibronectin was obtained from BD Biosciences (Oakville, Ontario). BSA and LHC basal medium were obtained from Biosource-Biofluids Division (Camarillo, California). Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma-Aldrich. To determine IL8 release ELISA kits (catalog # 550999, OptEIA Human IL8 Set, PharMingen) were obtained from BD Bioscience.

2.2 Hydrostatic pressure treatment and sample preparation for enzymatic hydrolyses

Pressure treatment was applied to WPI solutions at single cycle of 550 MPa or triple cycle of 400 MPa. Briefly, a 15% solution w/v of WPI was prepared using doubly distilled water and inserted into sealed plastic bags. Pressurization of the WPI solutions was undertaken using a high-pressure machine from GEC Alstom ACB (Nantes, France) with a maximum capacity of 690 MPa. The pressure chamber was filled with water as the hydrostatic fluid and sealed plastic bags containing the WPI solution were submerged to receive the pressure treatment. During the pressure cycles, the temperature was kept below 26 – 27°C and after reaching 550 MPa (within 3 – 4 min) the pressure was released within 1 min (single cycle pressure treatment). For the triple cycle pressure treatment, the process of pressurization at 400 MPa was repeated three times, *i. e.* increasing pressure to 400 MPa and then releasing it to ambient pressure for three consecutive times. The native 15% w/v WPI solution and the pressurized 15% w/v WPI solutions were lyophilized by placement into vacuum chambers of Flexi-Dry MP Lyophilizer (FTS Systems, Stone Ridge, NY) and freeze-dried under −80°C and 90 MT vacuum in preparation for future *in vitro* digestibility studies. The time needed to freeze-dry the samples depended on the volume used and varied from 48 to 72 h.

2.3 *In vitro* enzymatic digestion

The enzymatic digestion method was a modification of the methods of Multilagui *et al.* [9] and Kitabataki and Kinekawa [25] that were developed to simulate *in vivo* gastrointestinal digestion of milk proteins in terms of biochemical, physical and mechanical conditions including the enzyme concentration, pH adjustment (*i. e.* pH 1.5 and then 7.8), temperatures for optimal enzyme activity (37 and 40°C), shaking and incubation time. The 30-min incubation period with pepsin corresponded to the *in vivo* gastric half-emptying time described by Mah *et al.* [26]. After the lyophilization treatment described above, native and pressurized whey proteins were diluted in double distilled water at a concentration of 3 mg protein/mL and the pH of the solution was adjusted to 1.5 with HCl. Digestion experiments were performed using triplicate solutions at 37°C in 50-mL Erlenmeyer flasks placed in a shaking water bath. Digestion was initiated by the addition of freshly prepared enzyme stock solution (5 mg pepsin/mL in 0.01 M HCl) to the protein solutions to reach an enzyme to substrate ratio of 1 : 100. The peptidic digestion was interrupted after 30 min by adding 1.0 M NaOH solution, which raised the pH to approximately 6, thereby inactivating pepsin irreversibly. After pepsin inactivation, the digests were put on ice and the pH was adjusted to 7.8 with 1.0 M NaOH and kept at –80°C until the next day for further digestion with pancreatin or for future analytical analyses. The peptidic digests were thawed at room temperature and placed in a water bath at 40°C. Freshly prepared pancreatin stock solution (5 mg/mL in sodium phosphate buffer pH 7.0) was added to the pepsin pre-digested mixture to reach an enzyme to substrate ratio of 1 : 30. After 60-min incubation, 150 mM Na_2CO_3 solution was added to stop the reaction. The pancreatic digests were stored at −20°C until subsequent isolation of low molecular weight peptides.

2.4 Peptide isolation

After the pepsin-pancreatin digestion, the resulting hydrolysates from native, single-cycle and triple-cycle pressurized WPI were subject to ultrafiltration to remove the low molecular weight peptides via cellulose membranes with a molecular weight cut-off (MWCO) of 3 or 1 kDa (Millipore) in a stirred ultrafiltration membrane reactor (Model 8050, Millipore, Nepean, Ontario) under nitrogen gas pressure of 40 psi. The process was performed under a refrigerated environment at 4°C. The peptides in the permeate were freeze-dried in a vacuum concentrator (Flexi-Dry MP Lyophilizer, FTS Systems, Stone Ridge, NY) at −80°C and

90 MT vacuum for further analyses and cell culture experiments. The freeze-dried peptides were stored in sealed tubes flushed with N_2 under $-20^{\circ}C$ if not used immediately.

2.5 Protein content determination

For the pepsin digestion experiments, the protein content of the whey protein solutions was determined at 5-min intervals for 30 min after starting the digestion with pepsin. Protein content was determined via the Bradford method [27] according to the instructions of the kit manufacturer (catalog #500-0006, Bio-Rad, Mississauga, Ontario). Briefly, aliquots taken from the samples during the experiment were mixed with the dye reagent according to the manufacturer's recommendations and, after incubation for 5 min at room temperature, the OD at 540 nm was measured. The results were expressed as% of control (time 0), which corresponds to the total of whey protein in the solution before digestion. Considering that the protein content decreases with the digestion time, the volume of the aliquot to be taken was determined at time 0 and was based on the maximum linear Ae obtained from the standard curve using BSA with the concentration ranging from 0.2 to 0.9 mg/mL.

2.6 α-Amino group analysis

The amount of released α-amino groups can be measured using reagents that react specifically with amino groups, yielding derivatives that can be detected spectrophotometrically via OPA. The α-amino group content was determined according to a spectrophotometric assay, which is based on the OPA reaction with α-amino groups released by hydrolysis as described by Church *et al.* [28]. Briefly, 50 mL of OPA solution was freshly prepared as follows: 25 mL of 100 mM sodium tetraborate solution in water; 2.5 mL of 20% w/w SDS; 40 mg of OPA (dissolved in 1 mL of ethanol); 100 μL of β-mercaptoethanol, and water to complete the volume. Aliquots of each sample were collected before and after digestion with pepsin and pancreatin and added to 1 mL of OPA solution and incubated for precisely 2 min. Considering that the α-amino group content increases as the digestion time progresses, the volume of the aliquot to be taken was determined at time 0 and was based on the minimum linear A obtained from the standard curve using Phe-Gly with the concentration ranging from 25 to 150 μM. The OD was measured 2 min later at 340 nm wavelength because OPA absorption is stable only after 20 min [29]. Because A was sensitive to the pH, the efficiency of the digestion was determined by measuring the OD at 0 and 30 min at pH 1.5 for pepsin digestion and at 0 and 60 min at pH 7.8 for pancreatin digestion. The OD was also determined after the ultrafiltration to detect the peptides with molecular weight less than 1 kDa. The efficiency of the digestion was determined taking into consideration the net α-amino groups detected (OD before filtration less OD after filtration) after digestion with pepsin and after digestion with pancreatin. The results were expressed as μM of Phe-Gly.

2.7 CZE analyses

Following pancreatin treatment, the lyophilized permeate containing <1 kDa peptides obtained from hydrolysates of native WPI and single-cycle and triple-cycle pressure-treated WPI were analyzed by CZE. Prior to CZE analysis, the peptide solutions were filtered through a 0.2-μm low binding cellulose acetate membrane (Nalgene-nalge®, Nunc International Corporation, Rochester, NY). The running sodium phosphate buffer (0.1 M, pH 2.5) was obtained from Sigma-Aldrich. The sample stock peptide solutions were prepared by dissolving 50 mg of the freeze-dried filtered hydrolysates in 1.0 mL of the running buffer that was diluted (1:10) with nanopure water. The solutions were either kept on ice for immediate CZE analysis or stored at $-80^{\circ}C$ for later CZE analyses. The separation buffer solution was degassed before use. The CZE analysis was carried out using a Beckman P/ACE™ 2200 HPAC instrument (Beckman Instruments, Fullerton, CA) coupled to an IBM PC 486 computer (IBM Corp., Portsmouth, UK) for data acquisition and analysis. A neutral uncoated (57×50 μm, the length from intake to detector was 50 cm) fused silica capillary column was assembled in a P/ACE cartridge (Polymicro Technologies, Phoenix, Arizona) for the capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest run time. The CZE analyses were performed for a total duration of 30 min at a constant voltage (27 kV) and temperature ($3^{\circ}C$) using a UV-detection at 214 nm. Before the first sample application, the capillary column was pre-rinsed with nanopure water for 2 min and separation buffer for additional 2 min or until the baseline was stable. Between sample runs, the capillary was flushed with NaOH 1 M for 2 min, and HCL 1 N for additional 2 min followed by the pre-rinse. A mixture of peptide standards (peptide standard for CZE, catalog # P2693; Sigma-Aldrich) was used to verify the precision and accuracy of the method. The best peak resolution was achieved by injecting 40 μL of samples containing 2 mg/mL of lyophilized peptide extracts obtained following the dual enzyme digestion of pepsin and pancreatin digestion described above. The migration time of the individual peptides were compared relative to the migration time of BSA and results were expressed as% of the internal standard (BSA; 400 μg/mL).

2.8 Free amino acid content analysis

The free amino acid content of the lyophilized MWCO fractions of less than 1 kDa from the hydrolysates of native

WPI and single-cycle pressure-treated WPI was determined using lithium ion exchange HPLC (Biochrom 30, UK) with postcolumn ninhydrin detection. A 50-μL peptide solution (20 mg/mL; w/v in distilled water) was injected onto the HPLC and the separation of the amino acids was accomplished through an optimal combination of pH and cation strength (*i. e*. lithium citrate concentrations ranged 0.2–1.65 M and pH ranged 2.8–3.5, depending on the amino acids eluted) with a temperature gradient to enhance separation. A constant flow rate of 25 mL/h was used during 135 min run and the postcolumn reaction between ninhydrin and eluting amino acids from the column was monitored at 440 and 570 nm. These wavelengths detect the characteristic purple color resulting from the reaction between the free amino and carboxyl groups of the amino acids with ninhydrin. The identification and quantitation of the amino acids in the samples was obtained using a standard curve based on an external standard, which contained of all the known amino acids. To assure precision between runs, an internal standard composed of D-glucosaminic acid was used. To ensure the correct identification of the amino acids in the chromatograms, samples were spiked with the external standard.

2.9 MALDI-TOF spectrometry

MALDI-MS was carried out on a TOF-mass spectrometer (Voyager DE-STR; Applied Biosystems, Palo Alto, CA) with a laser at 337 nm and an acceleration voltage of 20 000 V. The lyophilized MWCO fractions of less than 1 kDa from native WPI and single-cycle pressure-treated WPI were dissolved in 0.5 mM ammonium acetate in methanol. In order to break disulfide bridges 10 μL DTT (Sigma, St Louis, MO) was added to each tube of the lyophilized peptidic mixtures for reduction.

2.10 Cell culture

2.10.1 General procedures

Cells were grown in pre-coated T-75 flasks in Eagle's MEM containing 10% FBS, re-fed every 2–3 days until confluent. The confluent, adherent monolayers were then released from the plastic surface after treatment with PVP-trypsin-EDTA and were seeded to 24-well plates or 60-mm dishes for 24 h before receiving the treatments.

2.10.2 Cell culture treatment with whey peptides and TNF-α

Wild-type and mutant ΔF508 CFTR cells seeded at 0.4 and 0.6×10^6 cells/mL respectively, were grown in MEM containing 10% FBS for 24 h until nearly confluence. Cells were treated with MWCO fractions of less than 1 kDa from the hydrolysates of native WPI (nWPH) and single-cycle pressure-treated WPI (pWPH) at doses of 12.5 and 500 μg/mL for the GSH experiments, and 12.5 μg/mL for the IL8 release experiments. These peptide doses have been demonstrated previously to exert bioactive effects on immune function and GSH status in cell culture. For example, the 12.5-μg/mL dose of whey peptides with molecular weights < 1 kDa was shown to stimulate lymphocyte proliferation [30]. Thymic peptides at a dose of 12.5 μg/mL were effective in stimulating intracellular GSH concentrations in vascular endothelial cells [31]. Kent *et al.* [3] demonstrated that a dose of hydrolyzed WPI of 500 μg/mL increased intracellular GSH in prostatic epithelial cells. Following the first 24-h incubation, the MEM 10% FBS was replaced with fresh medium containing 2% FBS and filtered sterilized nWPH and pWPH solutions. The cells were allowed to grow for 24 h at 37°C in 5% CO_2 and the medium was replaced with freshly prepared peptide solution in MEM 2%. After an additional 24 h of incubation, either the cells or the supernatant were collected to assess the impact of nWPH and pWPH on intracellular GSH production, and IL8 release, respectively. Intracellular GSH and IL8 release were performed in independent experiments using 60-mm dishes and 24-well plates, respectively. To assess the effect of whey protein hydrolysates on IL8 production in a stimulated state, after the initial 24-h incubation with nWPH and pWPH, cells were treated with MEM 2% FBS containing 12.5 μg/mL of nWPH or pWPH and concurrently stimulated with human recombinant TNF-α (10 ng/mL) for an additional 24 h. All experiments included unstimulated negative control wells. The viability of the peptide-treated and untreated cells was determined by the MTT assay.

2.10.3 IL8-release and cell viability assays

After the peptide treatments described above, the supernatant was collected to determine IL8 released using commercially available ELISA kits. Briefly, 96–well plates were coated with capture antibody (anti-IL8) overnight, washed with 0.05% Tween-20 in PBS and coated with 10% FBS in order to block nonspecific binding. Known concentrations of IL8 (standard) and the samples containing the IL8 released by the cells after treatment (supernatant) were added as aliquots into appropriate wells, incubated for 2 h and decanted from the wells. Anti-IL8 plus enzyme reagent (biotinylated detection antibody conjugated to streptavidin-horseradish) were added and incubated for 1 h. After washing the plate, a solution was added, which contained a substrate for the enzyme (TMB-peroxide chromogen) present in the anti-IL8 + enzyme reagent mixture and the plate was incubated for 30 min. The reaction was stopped using a 2N H_2SO_4 solution and A was read at 450 nm using a Titertek II Multiscan MCCB40 (Labsystems, Finland). The OD were then used to calculate the IL8 concentration from the standard curve and adjusted by their dilution factor.

Cell viability was assessed using the MTT assay as previously described [32]. Briefly, after collecting the supernatant to determine IL8 release, the cells were gently washed with PBS. Cells were incubated with MTT at 0.5 mg/mL in culture medium free of phenol red for 3 h at 37°C. After incubation, the supernatant was aspirated and HCl-isopropanol solution (0.04 N HCl in isopropanol) was added to dissolve the formazan crystals formed in viable metabolic active cells. Solubilization of the formazan crystals results in a colored solution allowing the quantification of viable cells. After 5 min of treatment with isopropanol, the OD were measured at 540 nm using an automated series 750 microplate spectrophotometer (Cambridge Technology, Cambridge, MA). The OD values were converted into cell numbers by using a cell proliferation standard curve with a cell seeding concentration ranging from 0.4 to 1×10^6/mL cells.

2.10.4 Analysis of cellular GSH

Quantitative determination of the total intracellular GSH and glutathione disulfide (GSSG) was performed spectrophotometrically by the glutathione reductase-recycling assay according to a modification of the method of Anderson [6]. Adherent epithelial cell lines, grown on coated 60-mm dishes with MEM 10% FBS during the first 24 h after seeding and for additional 48 h with MEM 2% FBS were washed twice with 5 mL PBS containing 25 mg% BSA. The cells were treated with 1.5 mL volume of 25 mg% BSA in PBS solution, scraped, and then subjected to centrifugation at $500 \times g$ for 10 min (Microlite Microcentrifuge OM 3580, Thermo IEC, Needham Heights, MA) in order to remove any extracellular GSH and GSSG. The supernatant was discarded and the cells were resuspended in 1 mL of PBS followed by another centrifugation at $400 \times g$ for 6 min. The supernatant was discarded and the cells were washed once with 0.45 mL PBS and re-suspended with 0.45 mL PBS. Samples were diluted with 0.1 mL 10 mM HCl and sonicated for two cycles of 20 s to disrupt cell membranes. The cell suspension was filtered using a centrifugal Millipore filter (catalog #42407, Millipore) at $14\,000 \times g$ for 60 min at 4°C. The protein concentration of the unfiltered portion of the samples was determined by the Bradford protein assay, according to the manufacturer`s instructions (catalog #500-0006, Bio-Rad). From the filtrate, two aliquots of each sample were taken to determine GSH and GSSG, respectively. In one aliquot, GSH was derivatized with 1 M solution of 2-vinylpyridine in ethanol for 60 min to quantify GSSG exclusive of GSH, while the second aliquot was ready to assay for GSH. In 96-well microplates GSH and GSSG concentrations were measured by using the GSH reductase recycling method of Anderson [6] following treatment of the samples with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The microplate was placed on the series 750 microplate reader from Cambridge Scientific (Cambridge, MA) using a 420-nm filter and A was read for 25 min.

The filtered samples were compared with the GSH and GSSG calibration curves to determine the GSH and GSSG concentrations in each well. The linearity of the GSH and GSSG calibration curves ranged from 0.1 to 16 μM and from 0.25 to 8 μM, respectively. The concentration of GSH and GSSG for each sample was determined from a standard curve and expressed as nmoles of GSH. The amount of the reduced form of GSH was calculated as the difference between the total GSH and oxidized GSH (GSSG), (*i.e.* total GSH − oxidized GSH = reduced GSH). The GSH content was expressed as nmol/mg protein or as the GSH redox ratio (reduced GSH/GSSG).

2.11 Statistical analysis

Statistical analysis was performed using an ANOVA with Tukey's *post hoc* test to determine significant statistical differences between groups in the digestibility studies and least square difference (LSD) *post hoc* comparison was applied for values of cytokines, cell numbers, and intracellular GSH concentrations. Two-tailed Student's *t*-test was used to determine the significance of the comparison between intracellular GSH and GSSG content in the comparison of CF *vs.* non-CF cells and in peptide content between native *vs.* triple-cycle pressure-treated WPI. All digestions and experiments were carried out in triplicate and data were expressed as mean ±SEM. SPSS 11.0 for Windows (SPSS, Chicago, Illinois) was used in all statistical analyses. $p < 0.05$ was considered as statistically significant.

3 Results and discussion

3.1 Hydrostatic pressure treatment increases WPI digestibility and alters peptide profiles generated from enzymatic hydrolyses

Figure 1 shows the *in vitro* protein digestibility with pepsin of native, single-cycle and triple-cycle pressure-treated WPI. Analysis of variance showed significant ($p < 0.05$) main effects of both length of time of enzyme exposure and type of protein treatment. Native whey proteins were more resistant to pepsin hydrolysis, as a 20-min digestion was needed to show a significant ($p < 0.05$) decrease in protein content and only a 30.9% decrease in protein content was observed after the 30-min pepsin digestion (Fig. 1). In contrast, WPI that had undergone single-cycle or triple-cycle pressure treatment showed significant ($p < 0.05$) decreases in protein content by 5 min of pepsin treatment and demonstrated decrease in protein content of 51 and 68%, respec-

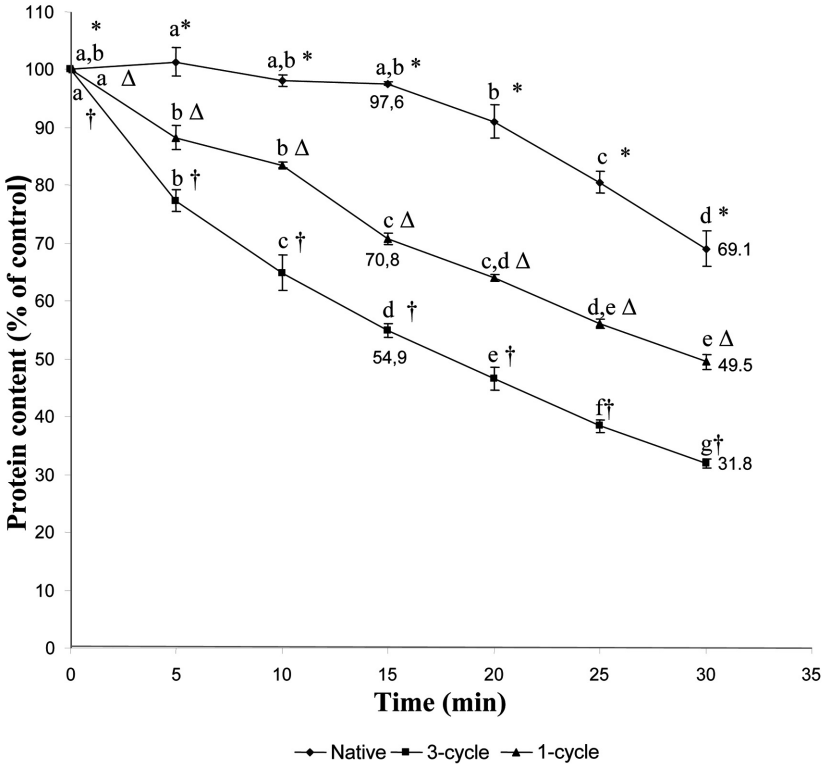

Figure 1. Effect of hydrostatic pressure treatment on *in vitro* digestion of WPI. WPI were exposed to either triple-cycle treatment at 400 MPa or single-cycle treatment at 550 MPa and the solutions were lyophilized. Using lyophilized native WPI and pressure-treated WPI samples, 3% solutions w/v were prepared and digested with pepsin for 30 min at 37°C. Aliquots were taken every 5 min and the protein content was determined at 590 nm ($n = 3$). Error bars show 95% confidence interval of mean. The numbers along the curves represent the percentage of proteins detected at 15 and 30 min. Time points within the same treatment not sharing common letters differed significantly ($p < 0.05$; ANOVA) by Tukey's *post hoc* comparison. Treatments not sharing common symbols (*, △, ⋄) indicate significant differences ($p < 0.05$, ANOVA) between groups at each time point by Tukey's *post hoc* comparison.

tively, following 30-min pepsin digestion. The Bradford protein assay used for protein determination does not detect peptides smaller than 3 kDa as these low-molecular weight peptides do not form the dye-protein complex needed for colorimetic detection [33]. It thus appears that single- and triple-cycle hydrostatic pressure treatment increases the digestibility of whey proteins to an extent that there is greater release of low-molecular weight peptides of <3 kDa from pepsin digestion. At all incubation times with pepsin, single- and triple-cycle pressure-treated WPI showed a significantly ($p < 0.05$) greater degree of proteolysis than native WPI. Likewise, triple-cycle pressure-treated WPI demonstrated a significantly ($p < 0.05$) greater degree of digestibility with pepsin at all incubation times as compared to single-cycle pressure-treated WPI. In preliminary tests, lyophilized pressurized proteins could be stored at –20°C for periods up to 12 months, without significant changes in their digestibility profiles (data not shown).

Since β-LG is the major protein in WPI, the high resistance of β-LG to pepsin-mediated hydrolysis is likely a major reason for the low digestibility of native WPI. Numerous studies have demonstrated that there is a low accessibility to peptide bonds localized to the interior of the globular structure of β-LG to hydrolytic action of pepsin [9, 25] and large oligopeptides are produced as pepsin hydrolysis is limited to peptide bonds involving only phenylalanine or tyrosine [34]. Enzyme-substrate binding is required for protein hydrolysis to occur. However, most peptide bonds are located in the interior of globular proteins such as β-LG and thus are not accessible to proteolytic enzymes. For globular proteins, denaturation of the protein is required for protein breakdown because a greater number of peptide bonds are exposed after denaturation. Recent ESI-MS studies have indicated that both single-cycle pressure treatment at 550 MPa as well as triple-cycle pressure treatment at 400 MPa caused a partial unfolding of genetic variants of β-LG as exhibited by a higher proportion of charges in the charge-state-distribution of pressurized β-LG relative to the native forms [35]. Hence, it is likely that the partial unfolding of β-LG induced by pressure treatment exposed the hydrophobic amino acids buried in the interior of the mole-

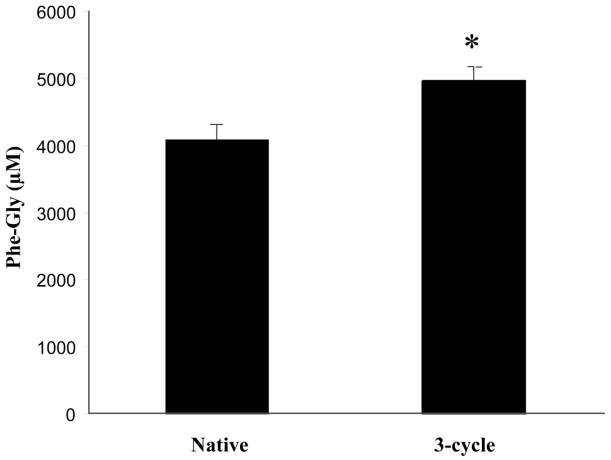

Figure 2. Effect of hydrostatic pressure treatment on *in vitro* digestion of WPI. WPI was untreated (native) or exposed to either triple-cycle treatment at 400 MPa and the solutions were lyophilized. Using lyophilized native WPI and pressure-treated WPI samples, 3% solutions w/v were prepared and digested with pepsin for 30 min followed by pancreatin digestion for an additional 60 min. Ultrafiltration was used to separate peptides with molecular weight lower than 3 kDa. The amino acid content of the filtrate was determined at 340 nm ($n = 3$). Error bars show 95% confidence interval of mean. Asterisks (*) indicate significant differences ($p < 0.05$) between the treatments by two-tailed Student's *t*-test.

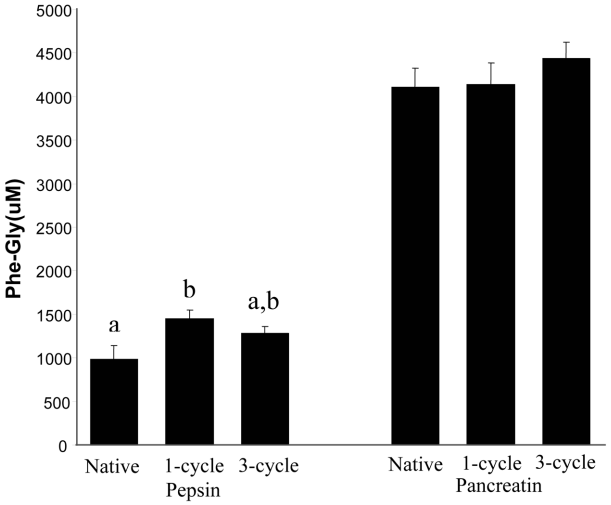

Figure 3. The content of peptides with molecular weight lower than 1 kDa in native WPI, WPI treated at single-cycle pressure at 550 MPa and WPI treated at triple-cycle pressure at 400 MPa after *in vitro* enzymatic digestion by pepsin alone, and by pepsin followed by pancreatin. The enzyme/substrate ratio used was 1/100 for pepsin and 1/30 for pancreatin. In both experiments, peptides were separated by ultrafiltration and the amount of peptides/amino acids released at the end of the digestion with pepsin (left) and pancreatin (right) was determined at 340 nm. Experiments were performed six times. Error bars show 95% confidence interval of mean. Statistical analysis was performed by ANOVA, followed by Tukey's *post hoc* comparison test. Columns not sharing common letters represent means that differed significantly ($p < 0.05$).

cule to the proteolytic enzymic action thus increasing the rate of hydrolysis as only the unfolded molecules are susceptible to degradation by proteolytic enzymes [36].

Further confirmation of the increased digestibility of pressure-treated whey proteins is shown by the significantly ($p < 0.05$) greater release of peptides with molecular weights < 3 kDa from pressure-treated WPI hydrolysates relative to native WPI hydrolysates following pepsin and pancreatin digestion and ultrafiltration (Fig. 2). As peptides with molecular weights larger than 1 kDa have not been observed in digestive juices following protein feeding in animal models [23], peptides under molecular weight of 1 kDa were isolated by ultrafiltration following pepsin as well as pepsin followed by pancreatin digestion. After 30-min pepsin digestion, enzymic hydrolysates from single-cycle pressurized WPI showed a significantly ($p < 0.05$) higher content of peptides smaller than 1 kDa as compared to native WPI hydrolysates (Fig. 3). Interestingly, triple-cycle pressure-treated whey proteins did not show an enhanced release of peptides smaller than 1 kDa following pepsin digestion relative to single-cycle treated or native WPI despite the significantly greater drop in protein content in the triple-cycle pressure-treated WPI during pepsin digestion (Fig. 3). As the Bradford protein assessment method does not detect polypeptides smaller than 3 kDa [33], the present results thus indicate that single-cycle pressurization of WPI was relatively more effective in generating peptides < 1 kDa following pepsin digestion as compared to triple-cycle pressurization that appeared to generate more peptides with molecular weights < 3 kDa. In contrast to the finding of a greater content of < 3 kDa peptides in digests of triple-cycle pressure-treated *vs.* native WPI following digestion with pepsin-pancreatin (Fig. 2), no differences in total content of < 1 kDa peptides were observed among all the treatment groups (Fig. 3). Pancreatin enzymes including the endopeptidase enzymes, trypsin, chymotrypsin and elastase and the exopeptidase enzymes, aminopeptidase and carboxypeptidases, are highly efficient in cleaving proteins and peptides into low molecular peptides, which could account for the similar < 1 kDa peptide content among the treatment groups [34]. Hydrolysates showing a similar extent of hydrolysis or release of small molecular weight peptides, however, may differ in their peptide composition as measured by MS, CZE and RP-HPLC [9, 33].

Characterization of the wide variety of low molecular weight peptides released from hydrolysis of native and pressure-treated WPI following pepsin and pancreatin digestion was carried out to further define whether these fractionated hydrolysates could differ in terms of their peptide profiles. Peptides released from the pepsin digests of native and tri-

ple-cycle pressurized whey proteins were analyzed by MALDI-TOF. Although it is difficult to distinguish between the different peptide mixtures due to the large number of peptide molecular ions present, the MS analysis of the products after 30 min of pepsin digestion demonstrated that different peptide profiles were observed when pressure treatment was applied. In particular, the peaks at mass 2429 and 2430 were absent in the pressure-treated

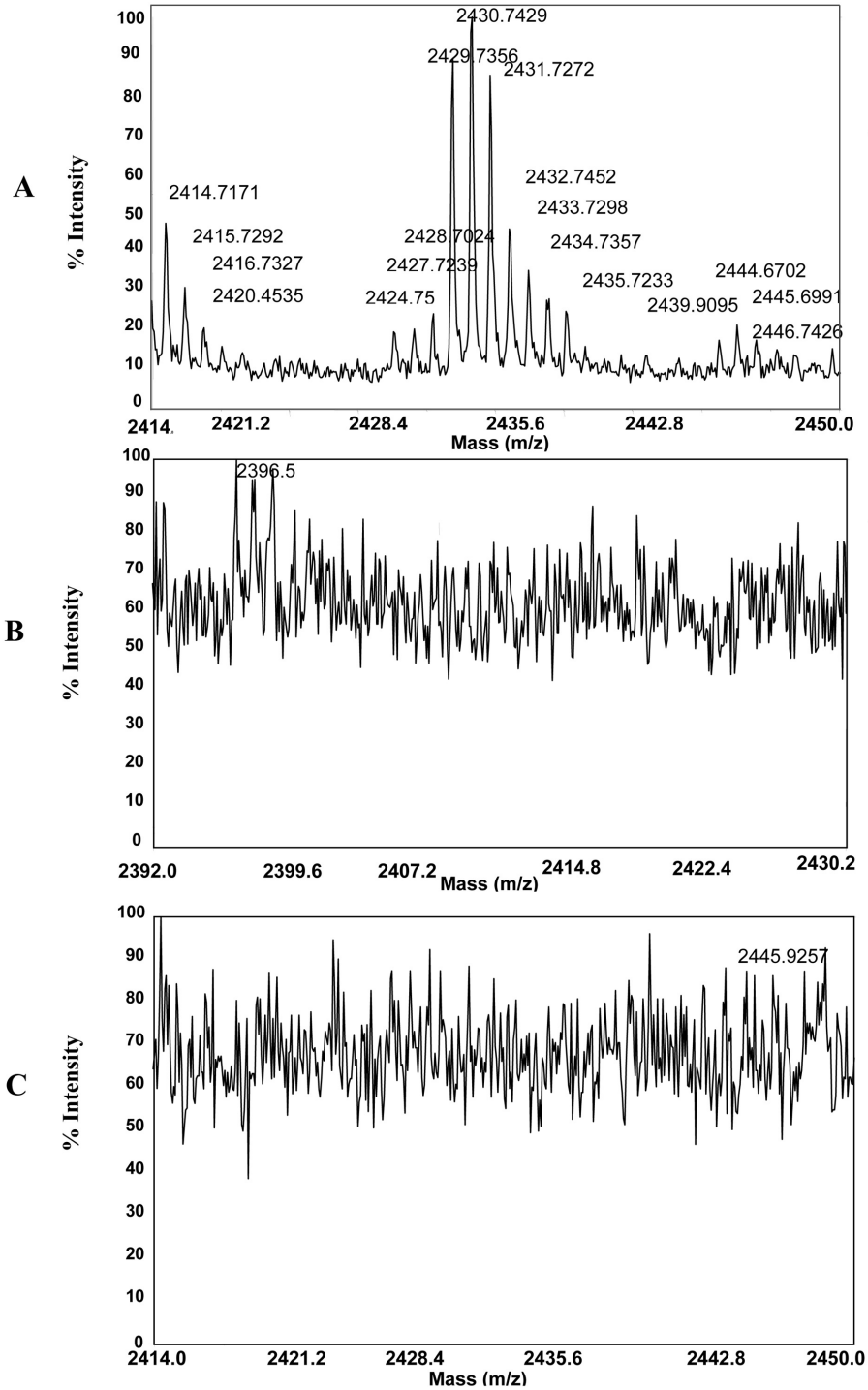

Figure 4. MALDI-MS profiles of peptide extracts with molecular weight < 1 kDa. The peptides were obtained from *in vitro* pepsin digestion and ultrafiltration of (A) native WPI; (B) WPI treated at triple-cycle pressure at 400 MPa; and (C) reducing DTT treatment of < 1 kDa peptides obtained from *in vitro* pepsin digestion of native WPI.

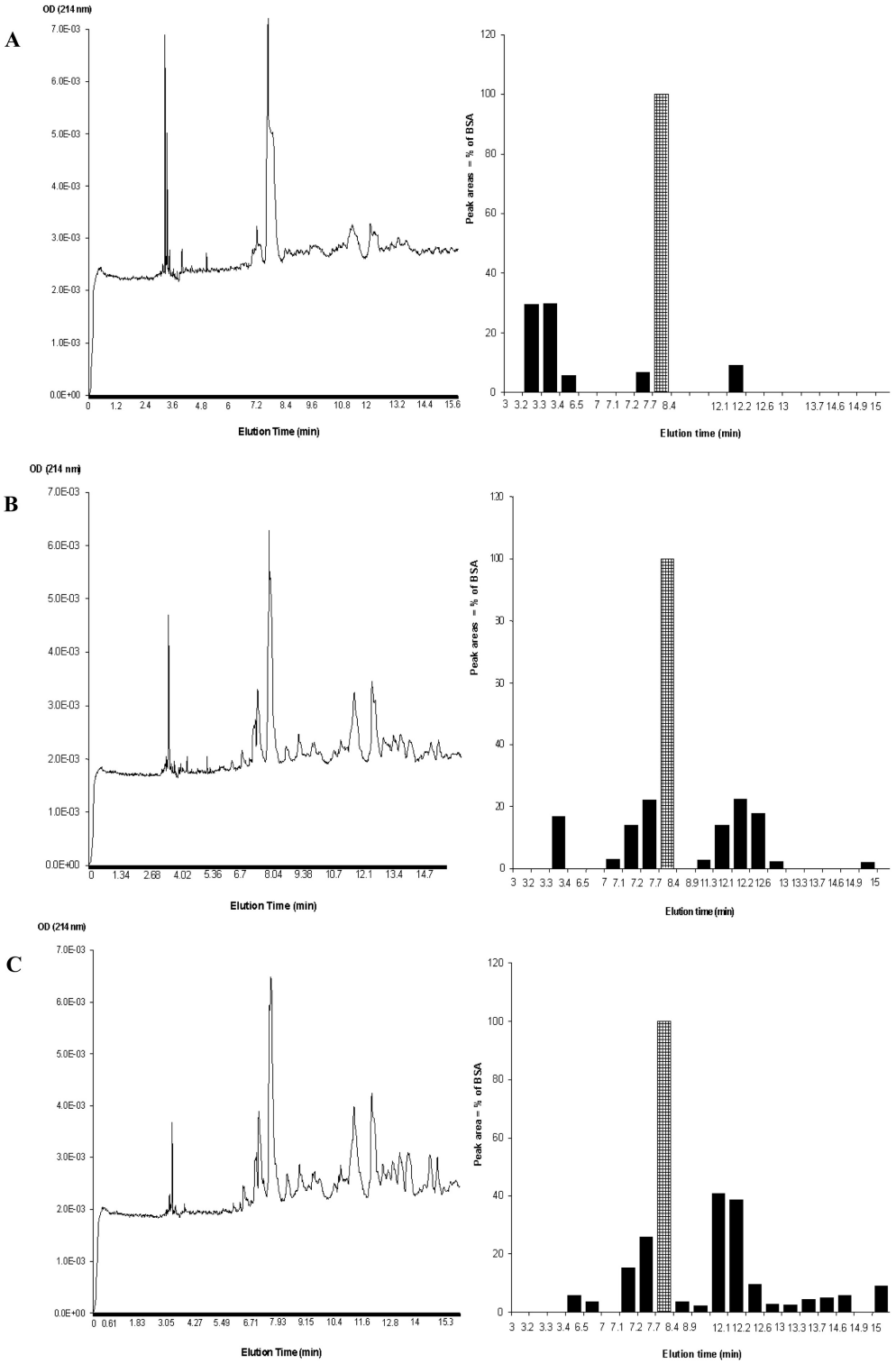

Figure 5. Profiles of peptide extracts with molecular weight <1 kDa. The peptides were obtained following *in vitro* digestion and ultrafiltration of (A) native WPI, (B) WPI treated at single-cycle pressure at 550 MPa, and (C) WPI treated at triple-cycle pressure at 400 MPa. CZE was used to generate the electropherograms of the separated peptides, which were detected at 214 nm. CZE conditions: 40 μL of the sample solution at a concentration of 40 mg/mL was injected and the peptides migrated from the positive to the negative pole at 27 kV and 30°C using 0.1 M phosphate buffer (pH 2.5). BSA (400 μg/mL) was used as internal standard. Graphs on the right represent the relative amounts of peptides as compared to the internal standard BSA (small grid background) as calculated from the peak areas (100% corresponds to the albumin peak area).

WPI (Fig. 4). These peptides were also removed by DTT treatment of the native WPI, suggesting that the peptides were likely to be dimers with disulfide bonds. Extensively disulfide-linked proteins such as whey proteins may be resistant to specific proteolysis such as trypsin [37]. However, more aggressive proteolysis with nonspecific proteases such as pepsin can generate cysteinyl peptides whose mass is affected by reducing agents such as DTT [37, 38]. The present findings thereby indicate that pressure treatment can facilitate the breakage of disulfide bonds in association with pepsin digestion, which could enhance pepsin-mediated whey protein digestion. In support of this latter concept, previous studies have shown a dramatic enhancement of *in vitro* pepsin-mediated digestion of β-LG after treatment with thioredoxin to break the intramolecular disulfide bonds [39].

Protein hydrolysates of native and pressure-treated whey proteins were characterized by CZE, which provides peptide separation based on size and charge [40]. Figure 5 represents typical electropherograms of the profiles of peptides smaller than 1 kDa that were isolated from pepsin-pancreatin hydrolysates of native, single- and triple-cycle pressure-treated WPI. The number of peaks and the peak areas relative to the internal BSA standard differed among the hydrolysates as single- and triple-cycle pressure-treated whey proteins generated a greater number of peptide fragments than native whey proteins as well as a different migration pattern and peak areas relative to BSA. These latter findings suggest that despite similar total content of small molecular weight peptides among the three hydrolysates, different profiles of peptides were generated following pancreatin digestion of pressurized whey proteins as compared to native whey proteins. Similar peptide profiles were observed in the electropherograms of peptides obtained from single- and triple-cycle pressure-treated WPI although there were a decreased number of peptide fragments in single-cycle as compared to triple-cycle pressure-treated whey protein hydrolysates. The larger number of small peptides associated with pressure treatment also implicates a greater degree of hydrolysis as an increased variety of small peptides is produced with a greater extent of hydrolysis as reported for enzymic hydrolysates generated under various processing conditions [41].

The electropherograms of peptides resulting from pancreatin digestion of pressurized whey protein showed a larger number of fragments eluting later than BSA (Fig. 6), which was not observed in the chromatograms obtained from the peptides obtained from native protein hydrolysates. The additional peaks observed with single- and triple-cycle pressure treatments could possibly have been the result of cleavage of original peptides found in hydrolysates of native WPI, as some of the peptide fragments associated with native whey samples were not observed with pressure

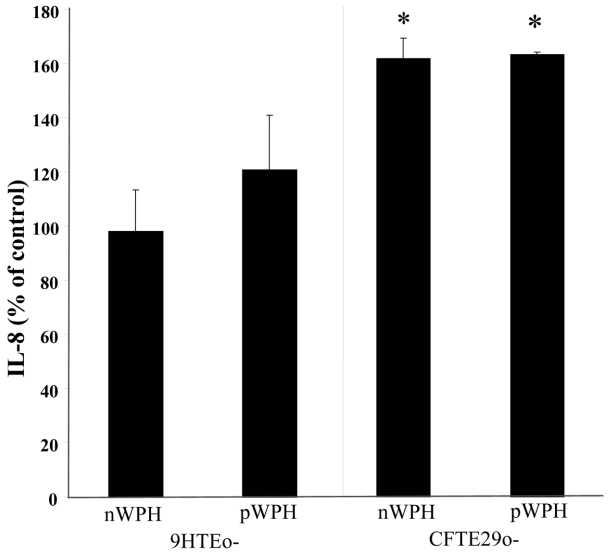

Figure 6. IL8 release after treatment of non-CF and CF cells with pWPH and nWPH under unstimulated conditions. As described in Section 2, wild-type tracheal epithelial cells (non-CF 9HTEo-) and CFTR-deficient cells (CF cells CFTE29o-), were treated with 12.5 μg/mL of pWPH and nWPH solution w/v for 48 h in order to characterize the impact of nWPH and pWPH on IL-8 release in an unstimulated basal condition. Results are means ± SE of three independent experiments. Asterisks (*) indicate significant differences ($p < 0.05$) as compared to untreated controls by ANOVA and LSD *post hoc* analysis. The IL8 release values for the controls were 287 ± 43 pg/mL for non-CF cells and 166 ± 21 pg/mL for CF cells.

treatment. As the migration time in CZE depends on the *m/z*, it is expected that large molecules with p*I* higher than 2.5 would have a slow migration speed considering that the running buffer used was adjusted to pH 2. 5. Since the peptides and amino acids isolated from the hydrolysates had molecular weights lower than BSA, it is reasonable to assume that peptides that eluted earlier than BSA are more positively charged than the peptides that eluted later than BSA, which supposedly have negative or neutral net charges. Hence, it appears that pressure treatment of whey proteins allows digestive enzymes to produce peptides with different molecular weights and charges as compared to peptides derived from native whey proteins.

The amino acid analysis demonstrated that the permeates from the filtered native and single-cycle pressure-treated whey protein hydrolysates obtained following pancreatin digestion differed in terms of the profiles and content of free amino acids and their derivatives (Table 1). The profile of free amino acids also differed as a higher content of all amino acids was observed in the pressure-treated sample apart from cystine and proline. The most evident differences between the native and pressure-treated samples were observed in terms of the amino acid derivatives. Markedly

Table 1. Amino acid analysis of whey protein hydrolysates (total free amino acid content < 1 kDa)

Amino acid compounds (570 nm)	nWPH (μmol/L)	pWPH (μmol/L)
Amino acids		
Alanine	135	169
Arginine	688	694
Asparagine	28	36
Aspartic acid	24	33
Cystine	5	5
Glutamic acid	36	37
Glutamine	530	559
Glycine	93	98
Histidine	200	232
Isoleucine	368	380
Leucine	2391	2538
Lysine	1934	2086
Methionine	268	296
Phenylalanine	1045	1056
Proline[a)]	41	23
Serine	34	36
Threonine	269	290
Tryptophan	369	488
Tyrosine	660	691
Valine	624	646
Amino acid derivatives		
Argininosuccinic acid	187	61
β-Alanine	213	211
α-Aminoisobutyric acid	97	121
α-Aminoadipic acid	14	10
γ-Aminobutyric acid (GABA)	N.D.[b)]	40
α-Amino-n-butyric acid	6	21
Citrulline	45	63
Ethanolamine	43	59
Hydroxyproline[a)]	25	33
Ornithine	5	10
o-Phosphoethanolamine	N.D.	2
o-Phosphoserine	11	23
Sarcosine	127	107

a) Measured at 440 nm.
b) N.D. = not detectable.

higher concentrations of argininosuccinic acid and sarcosine were observed in the filtered native protein hydrolysates whereas γ-aminobutyric acid, a known neuropeptide involved in the modulation of corticotropin-releasing hormone from the hypothalamus [42], and *o*-phosphoethanolamine, a molecule that is part of the structure of glycerolipids and sphingolipids such as phosphatidylethanolamine and phosphorylceramide [43] were detected only in the pressure-treated whey samples. These results are in concert with the CZE analyses showing differing peptide and amino acid profiles following *in vitro* enzymatic hydrolysis of pressure treated *vs.* native whey proteins.

Although hyperbaric pressure processing has been demonstrated to increase whey protein digestibility *in vitro* in previous studies, such pressure treatment was carried out in the presence of proteolytic enzymes as pressurization was shown to increase the catalytic effect of proteolytic digestive enzymes such as pepsin [13]. The present work extends such previous studies to indicate that after exposure to higher hydrostatic pressures, whey proteins subsequently show enhancement of *in vitro* digestibility, without the need of pressure treatment of the proteolytic enzymes. In this regard, it is noteworthy that the lyophilized pressurized proteins could be stored at −20°C for periods up to 12 months, without significant changes in their digestibility profiles (data not shown), suggesting that the pressure-induced conformational changes were relatively stable, at least under cold storage.

3.2 Effect of peptides isolated from whey protein hydrolysates on cell viability and IL8 secretion

In view of the above findings that pressure treatment increased digestion efficiency generating unique peptide fragments and that bioactive peptides present in whey protein hydrolysates have been shown to play a role in immune modulation and GSH homeostasis [44, 45], peptides isolated from native and pressurized whey proteins hydrolysates were examined for their capability to down-regulate the excessive inflammatory response in CF cells. To test this possibility, cultured tracheal epithelial CF and non-CF cells were treated with peptides of MWCO < 1 kDa isolated from native (nWPH) and single-cycle pressure-treated (pWPH) whey protein hydrolysates and IL8 was analyzed release in both non-stimulated and TNF-α-stimulated conditions. TNF-α is recognized to play an important role in disease pathogenesis during acute inflammation and is an important mediator of multiple inflammatory events in lungs [46].

Unstimulated CF and non-CF control cultures produced similar low levels of IL8 release (166 ± 21 and 287 ± 43 pg/mL, respectively) under basal conditions. Sham treatment (ethanol) did not increase IL8 secretion in either type of culture (data not shown). The present results are similar to previous cell culture studies that indicate small or no differences in basal levels of the pro-inflammatory response in CF epithelial cells [47, 48].

Exposure of cells to 10 ng/mL TNF-α resulted in more than a 114-fold increase (19 009 ± 943 pg/mL) in secreted IL8 levels in CF cells whereas non-CF cells exhibited greater than 230-fold increase (78 202 ± 9940 pg/mL) in IL8 concentrations relative to the unstimulated MEM controls. Similarly, Massengale *et al.* [49] demonstrated that non-CF cells are more sensitive to lipopolysaccharide-mediated IL8 stimulation than CF cells. However, a consistent pattern of differences in the secretion of IL8 between CF and non-CF epithelial cells under TNF-α is not always present. CF cells

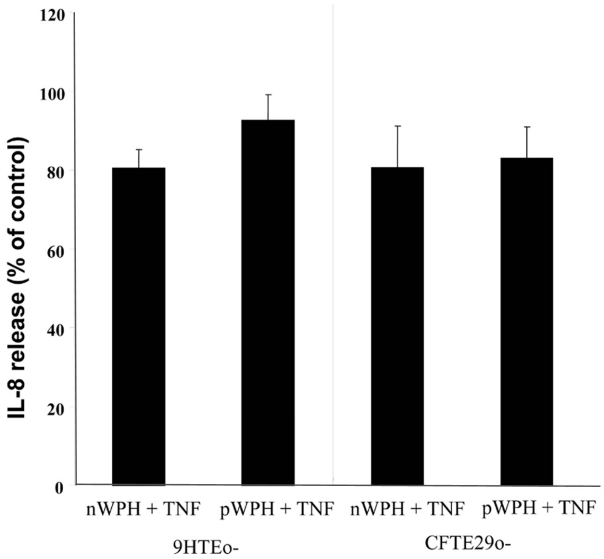

Figure 7. IL8 release after treatment of non-CF and CF cells with pWPH and nWPH under TNF-α-stimulated conditions. As described in Section 2, wild-type tracheal epithelial cells (non-CF 9HTEo-), and CFTR-deficient cells (CF CFTE29o-), were treated with 12.5 μg/mL of nWPH and pWPH in MEM 2% FBS for 24 h. After 24 h, the medium was replaced by fresh MEM 2% FBS containing 12.5 μg/mL of WPH concurrently with human recombinant TNF-α (10 ng/mL) for an additional 24 h. All experiments included stimulated negative control wells. Results are means ± SE of three independent experiments and ANOVA and LSD *post hoc* statistical analysis was used. The IL8 release values for the stimulated controls were 78 202 ± 9 940 pg/mL for non-CF cells and 19 009 ± 943 pg/mL for CF cells and were used to express the results as% of the controls.

have also been shown to be equally sensitive to TNF-α when compared to non-CF cells [47].

The viability of the peptide treated cells as assessed by the MTT reduction assay was unaffected by peptide treatment both at the 12.5 and 500 μg/mL peptide concentrations (data not shown). This result concurs with the findings of Kent *et al.* [3] who noted no significant decrease in cell viability following treatment of whey protein hydrolysates in cultured human prostate epithelial cells at a concentration of 500 μg/mL. Although the MTT assay detects mitochondrial activity rather than cell viability, these phenomena are intimately related and MTT has been widely used to assess cell injury [50].

Non-CF cells in either basal or stimulated conditions were unaffected by either the nWPH or pWPH treatments in terms of IL8 release as compared with control cells (Figs. 6 and 7) although a strong tendency for decrease IL8 secretion in response to nWPH treatment was observed (p = 0.09) under stimulated conditions, which was not seen with pWPH. On the other hand, CF cells were more sensitive to hydrolysate treatment as both nWPH and pWPH significantly ($p < 0.05$) stimulated IL8 production in CF cells under basal conditions. Conversely, there was also a strong tendency for both the nWPH and pWPH treatments to decrease inflammatory response in CF cell lines under TNF-α-stimulated conditions (p = 0.08 and 0.11, respectively; Fig. 7). The low dose of whey peptides (12.5 μg/mL) used was based on previous cell culture studies demonstrating immunomodulatory effects in response to such low peptide doses [30, 31]. The present results thereby indicate the possibility that low-dose pWPH treatment could have the potential as a co-adjuvant treatment to attenuate the pro-inflammatory condition of CF cells without affecting non-CF cells in this regard.

3.3 Effect of peptides isolated from whey protein hydrolysates on GSH status

The intracellular concentrations of total GSH and reduced GSH of CFTR-deficient cells seen in the present study were approximately eight- to tenfold higher than in wild-type cells ($p < 0.05$) (Figs. 8 and 9). Similar findings were also observed by Jungas *et al.* [51] with mutant CFTR HeLa cells. It is possible that the relatively higher GSH levels in CFTR-deficient cells will indicate a compensating mechanism to reduce excessive oxidative stress as cells utilize intracellular GSH to protect themselves against oxidants. Moreover, more than threefold lower GSH/GSSG ratios were observed in mutant CFTR epithelial cells as compared to wild-type cells under basal conditions (Figs. 8 and 9). As GSH and GSSG are the major redox pair involved in cellular redox homeostasis, a lower cellular GSH/GSSG ratio is regarded as a representative marker for oxidative stress, which can perturb cellular function leading to an induction of pro-inflammatory responses [7]. The lower basal GSH/GSSG ratios in the CF as compared with mutant CFTR epithelial cells ($p < 0.05$) may thus explain the increased propensity towards oxidative stress in the CFTR-deficient epithelial cells. Oxidative stress leading to redox activation of NF-κB has been proposed as an explanation of the persistent inflammation and lung damage in CF [21]. CF patients generally have poor antioxidant status [52] and antioxidant therapy has been proposed [53]. As whey protein hydrolysates were shown to induce cellular GSH concentrations [3], the impact of nWPH and pWPH to improve the redox status in CF and non-CF cells as measured by intracellular levels of GSH and GSSG was assessed.

The GSH modulatory activity in mutant CFTR cells from exposure of 12.5 and 500 μg/mL doses of peptides (MWCO <1 kDa) isolated from native and pressurized whey protein hydrolysates following pepsin and pancreatin treatment is shown in Figs. 8 and 9. Treatment of CF cells with pWPH at the dose of 12.5 μg/mL showed marginally significant

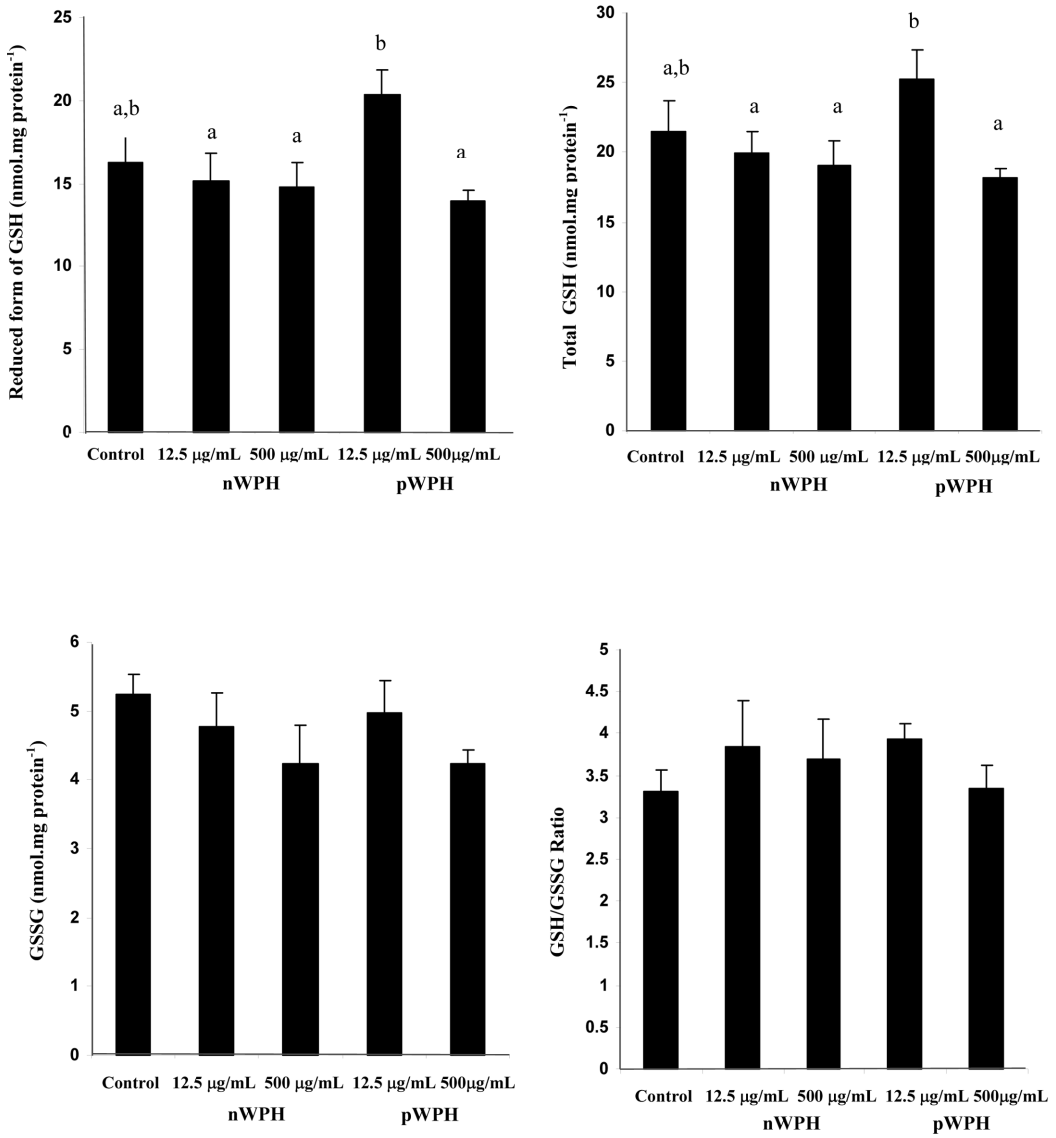

Figure 8. Intracellular levels of total GSH, the reduced form of GSH, GSSG and the GSH/GSSG ratio in CFTE29o- cells in response to WPH. Nearly confluent CFTR-deficient human epithelial cells (CFTE29o-) were incubated for 48 h in MEM containing 2% FBS with two different concentrations (12.5 and 500 μg/mL) of peptides smaller than 1 kDa obtained from either nWPH or pWPH (single-cycle, 550 MPa). Wells containing MEM with 2% FBS were used as controls. After 48 h, the cells were collected and the intracellular GSH and GSSG were determined as described in Section 2. Intracellular GSH was divided by intracellular GSSG to establish the GSH/GSSG ratio. Columns not sharing common letters represent means that differed significantly ($p < 0.05$, ANOVA) by LSD *post hoc* comparison.

increases in the reduced form of GSH ($p = 0.09$), total GSH ($p = 0.15$) and the GSH/GSSG ratio ($p = 0.09$) relative to untreated controls (Fig. 8). Previous studies have shown that thymic peptides at doses of 12.5 μg/mL increase GSH concentrations in cultured bovine pulmonary artery endothelial cells [31]. In contrast to CF cells, non-CF cells treated with pWPH at 500 μg/mL had a significant ($p < 0.05$) increase in GSSG relative to untreated controls and the nWPH and pWPH treatments at the 500 and 12.5 μg/mL dose, respectively. In addition, a decrease in the GSH/GSSG ratio was promoted at the 12.5 μg/mL of nWPH (Fig. 9). Taken together, the above findings indicate that the observed differences in peptide profiles in pWPH *vs.* nWPH resulted in differences in their biochemical properties in terms of GSH modulation. The reason for the contrasting finding for differences in the response of CF *vs.* non-CF cells to the WPH treatment in terms of modulation of GSH status is not explainable from this experimental set-up but could be due to intrinsic metabolic factors that affect the relative amounts of GSH and GSSG in the two cell lines.

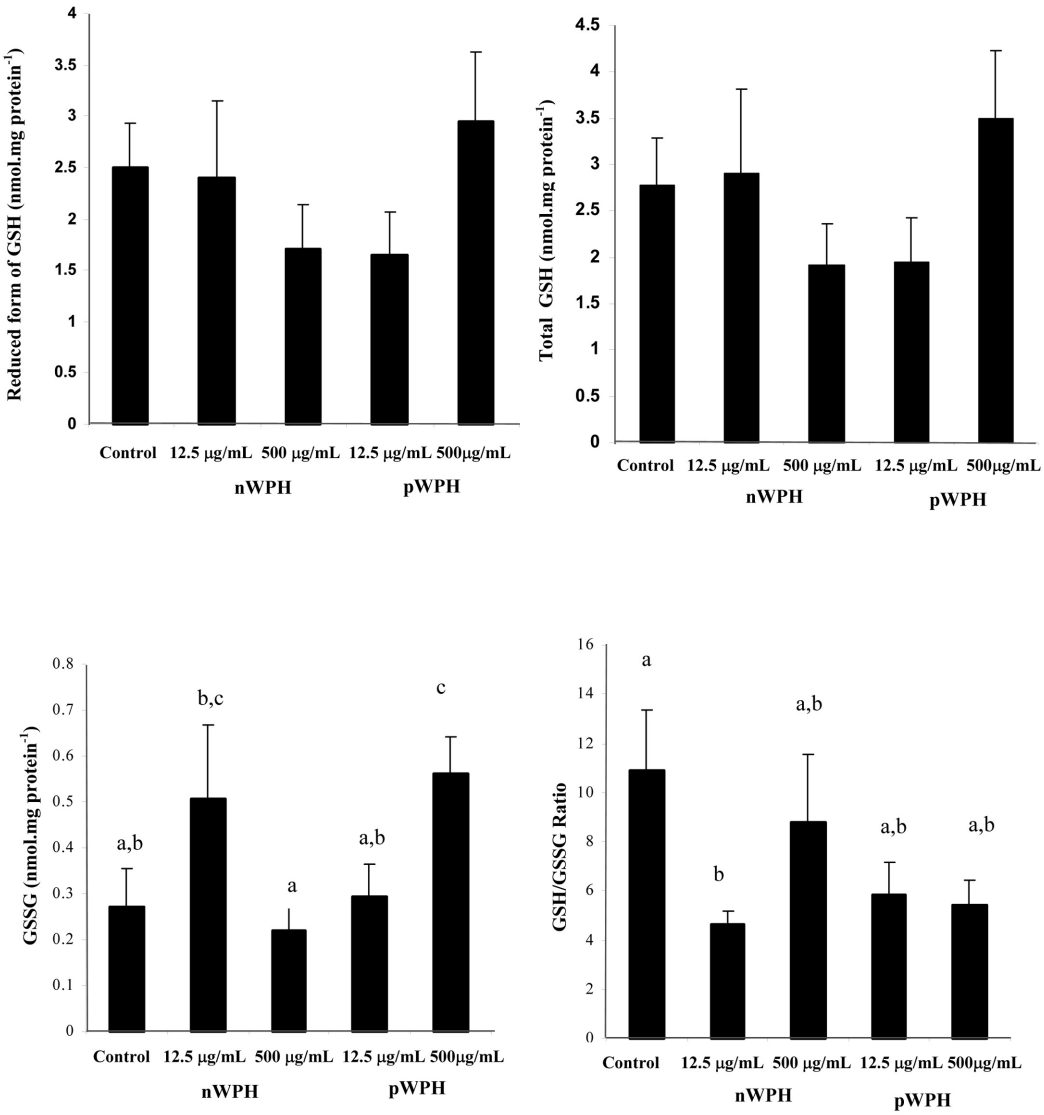

Figure 9. Intracellular levels of total GSH, the reduced form of GSH, GSSG and the GSH/GSSG ratio in 9HTEo- cells in response to WPH. Nearly confluent CFTR-deficient human epithelial cells (CFTE29o-) were incubated for 48 h in MEM containing 2% FBS with two different concentrations (12.5 and 500 μg/mL) of peptides smaller than 1 kDa obtained from either nWPH or pWPH (single-cycle, 550 MPa). Wells containing MEM with 2% FBS were used as controls. After 48 h, the cells were collected and the intracellular GSH and GSSG were determined as described in Section 2. Intracellular GSH was divided by intracellular GSSG to establish the GSH/GSSG ratio. Columns not sharing common letters represent means that differed significantly ($p < 0.05$, ANOVA) by LSD *post hoc* comparison.

The factors that may differ between the two cell lines might include the rates of biosynthesis and utilization of GSH in oxidation/reduction reactions as well as involvement of GSH in a variety of reactions requiring reducing equivalents such as direct interaction with free radical species, enzymatic reduction of lipid hydroperoxides and the regeneration of reduced forms of redox pairs such as cysteine/cystine [54]. In that regard, it is noteworthy that abnormal GSH transport is associated with a defective CFTR with a lower GSH efflux in CFTR-deficient cells versus CFTR-repleted cells [22].

The uptrend in intracellular GSH levels in CFTR-deficient cells associated with pWPH as compared to the other whey hydrolysate treatments could be an outcome of an increase in GSH synthesis and/or a reduction in GSH efflux out of the cell [55]. There is a possibility that pWPH supplies intracellular GSH stores via provision of cell-permeant GSH precursors in the form of peptides as demonstrated previously with thymic peptides at the dose of 12.5 μg/mL [31]. Although marked differences were observed in the free amino acid profiles between nWPH and pWPH, the amino acid content of the whey protein hydrolysates are

unlikely to exert GSH-modulating effects. GSH-stimulating effects of amino acid supplements such as cystine and glutamine in cell cultures have been noted at the millimolar level [56] as opposed to the micromolar concentrations present in the whey protein hydrolysates. The nature and mechanism of action of the GSH-modulating peptides cannot be discerned with the present experimental context. A rate-limiting factor in the synthesis of GSH in hepatic cells is cystine uptake through the Xc-system, which supplies cystine and glutamate required for GSH synthesis [57]. Kent *et al.* [3] proposed that the GSH-stimulating activity of the WPH in human prostate epithelial cells could be attributed to an increased availability of cysteine. Supplementation of non-cysteine containing peptides could also play a role, as the di-peptide, alanylglutamine, was shown to increase GSH concentrations in CaCo-2 cells that were depleted after H_2O_2 treatment [58]. Moreover, oxidant stress has been reported to increase both the uptake of cystine-glutamate and the synthesis of GSH in endothelial cells [57]. Hence, it is possible that the isolated peptides from whey protein hydrolysates could have exerted more potent effects on improving intracellular GSH status under conditions of oxidant stress.

The results of the present work differ from Kent *et al.* [3] who noted that treatment of human prostate epithelial cells with hydrolyzed WPI (500 μg/mL) for 24 or 48 h was associated with significant increases in intracellular GSH as compared with control cells receiving no hydrolyzed WPI. The impact of hydrolyzed WPI on GSSG content, however, was not reported by Kent *et al.* [3]. Apart from possible metabolic differences between the cell lines used, it is possible that the divergent digestion protocols could account for the discrepancy in GSH stimulating activity of the whey protein hydrolysates tested. Kent *et al.* [3] used trypsin, chymotrypsin and peptidase, whereas in the present study WPI was hydrolyzed initially via pepsin, followed by treatment with pancreatin that contains a mixture of trypsin, chymotrypsin and elastase. Previous literature has consistently demonstrated that the profile and bioactivity of peptides isolated from whey proteins can vary considerably depending on the digestive enzymes used [59]. Another possible explanation is that Kent *et al.* [3] did not fractionate their whey protein hydrolysates to isolate the low molecular weight peptides with MWCO < 1 kDa that are more bioavailable [23]. Hence, it is conceivable that the *in vitro* GSH-stimulating activity in human normal epithelial cells observed by Kent *et al.* [3] were due to relatively larger molecular weight peptides that were excluded in the present study.

4 Concluding remarks

In summary, the data from the present study indicate that high hydrostatic pressure processing of whey protein can enhance *in vitro* proteolysis. The altered profile of low molecular weight peptides isolated from hydrolysates of pressurized whey proteins was associated with a strong uptrend in the total and reduced GSH content in cultured mutant CFTR cells in comparison to peptides released from native whey hydrolysates. The present study results also suggest that bioactive peptides produced from native and pressurized whey protein hydrolysates may have utility acting as anti-inflammatory agents via inhibition of IL8 release in cells with the mutant CFTR condition.

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