Interpretive Summary

Whey proteins as antioxidants and promoters of cellular antioxidant pathways. By Corrochano. Bovine whey is a valuable source of proteins. Both whey and individual whey proteins are used as ingredients in a wide range of foods. One of the health promoting benefits of whey proteins is thought to be their antioxidant activity. This review critically assesses the ability of whey products to exhibit antioxidant activity but more importantly their ability to boost antioxidant levels in target cells. It poses the question whether the antioxidant compounds in whey ever actually reach their target organs as whey proteins are rapidly digested to individual amino acids in the gastrointestinal tract. It examines the processing and treatment attempts to increase the antioxidant bioactivity and compares whey from different sources. The antioxidant potency of whey proteins is also compared to that of other known food antioxidants. The review summarizes the antioxidant outputs from animal and human trials where the diet is supplemented with whey products.

Whey proteins as antioxidants and promoters of cellular antioxidant pathways

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ABSTRACT

Oxidative stress contributes to cell injury and aggravates several chronic diseases. Dietary antioxidants help the body to fight against free radicals and therefore, avoid or reduce oxidative stress. Recently proteins from milk whey liquid have been described as antioxidants. This review summarises the evidence that whey products exhibit radical scavenging activity and reducing power. It examines the processing and treatment attempts to increase the antioxidant bioactivity and identifies one enzyme, Alcalase, which consistently produces the most potent whey fractions. The review compares whey from different milk sources and puts whey proteins in the context of other known food antioxidants. However for efficacy, the antioxidant activity of whey products must not only survive processing but also upper gut transit and arrival in the bloodstream, if whey proteins are to promote antioxidant levels in target organs. Studies reveal that direct cell exposures to whey samples increase intracellular antioxidants such as glutathione. The physiological relevance of these in vitro assays however is questionable and there is conflicting evidence from dietary intervention trials, with both rats and humans, that whey can boost cellular antioxidant biomarkers.

Key words: whey products, whey proteins, bioactive peptides, antioxidant activity, oxidative stress

INTRODUCTION

Within each cell of the body, metabolic processes generate free radicals and antioxidant systems are in place to effectively disarm them. However, this homeostatic balance can be altered due to excess free radical production, antioxidant depletion, or both. When the controls fail, cells are exposed to high levels of free radicals (reactive oxygen (**ROS**), reactive nitrogen or reactive sulphur species). Oxidative stress ensues leading to cell injury such as protein and lipid peroxidation, DNA strand breakage, racemization or decarboxylation of amino acids, enzyme dysfunction and oxidative breakdown of carbohydrates (d'Ischia et al., 2006, Li et al., 2015)**.** Sustained oxidative stress is considered a causative agent of neurodegenerative disorders (Gilgun-Sherki et al., 2001, Klein and Ackerman, 2003), cancer (Waris and Ahsan, 2006), liver injury (Li et al., 2015), aging (Lee et al., 2004) and appears to aggravate diabetes (Rochette et al., 2014), cystic fibrosis (Galli et al., 2012), chronic pancreatitis (Zhou et al., 2014) and cardiovascular disease (Sugamura and Keaney, 2011, Lonn et al., 2012). Cells protect themselves from oxidative damage by (1) prevention, (2) repair, (3) antioxidant production or (4) uptake of dietary antioxidants or their precursors (Valko et al., 2007, Niki, 2010). Endogenous antioxidants include the intracellular enzymes superoxide dismutase (**SOD**) and catalase (**CAT**). The metal binding enzyme, SOD, converts superoxide anion to hydrogen peroxide plus oxygen whilst CAT converts hydrogen peroxide to water (Weydert and Cullen, 2009). The cytosolic cysteine tripeptide, γ-Glutamyl-cysteinyl-glycine, reduces hydroperoxides to alcohols and hydrogen peroxide to water by converting from its reduced (**GSH**) to its oxidized form (**GSSG**). Well documented dietary antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), polyphenols and carotenoids (Fiedor and Burda, 2014). Recently dairy proteins obtained from whey have received considerable attention for their antioxidant bioactivity (Bayram et al., 2008, Haraguchi et al., 2011, Zhang et al., 2012). Bovine whey proteins (**WP**) are widely used in various foods for

69 their nutritional, health promoting and functional value (Ramos et al., 2017). Bovine liquid
70 whey is produced by enzymatic treatment of milk (sweet whey) or addition of organic acids
71 or minerals (acid whey) (Yadav et al., 2015). Bovine WP account for 6 - 10% of liquid whey.
72 The other components of bovine liquid whey are lactose (46 – 52%), fat (5%), minerals (5%)
73 and vitamins (A, C, E and B group, especially folate (B12) and pantothenic acid (B5)) (Miller
74 et al., 2006, Yadav et al., 2015). The protein component of whey provides a complete protein
75 source, is rich in sulphur containing amino acids (1.7%) (Fox et al., 2015) and in branched
76 chain amino acids (26%) (Ha and Zemel, 2003, Paul, 2009). It is composed of β-
77 lactoglobulin (50 - 60%), α-lactalbumin (15 - 25%), bovine serum albumin (6%), lactoferrin
78 (< 3%) and immunoglobulins (< 1%) (Madureira et al., 2007). Beta-lactoglobulin is a small
79 globular protein, composed of 162 amino acids with a molecular weight ($\mathbf{M_W}$) of
80 approximately 18,300 g/mol (Rade-Kukic et al., 2011). It contains all 20 essential amino
81 acids and is a rich source of sulphur. From a GSH precursor perspective, it has 5 cysteine
82 residues, 4 of them involved in disulphide bonds with the remaining one having a free
83 reactive thiol group (Le Maux et al., 2014). Alpha-Lactalbumin is a small protein with a M_W
84 of 14,200 g/mol consisting of 123 amino acids arranged in a single peptide chain (Konrad and
85 Kleinschmidt, 2008). It has 8 cysteines as 4 disulphide bonds and therefore unlike β-
86 lactoglobulin, has no free thiol group (Konrad and Kleinschmidt, 2008, Pepe et al., 2013).
87 Bovine serum albumin is composed of 583 amino acids with a M_W of 66,430 g/mol
88 (Hirayama et al., 1990). It contains 35 cysteine groups making 17 disulphide bonds in
89 addition to a free cysteine which can facilitate intra-molecular disulphide interactions
90 (Madureira et al., 2007). Lactoferrin (M_W 80,000 g/mol) is an iron-binding monomeric
91 globular glycoprotein (Wakabayashi et al., 2006) which contains 708 amino acids of which
92 34 are cysteines, all of which participate in disulphide bonds (Marshall, 2004, O'Halloran et
93 al., 2009). In addition, each lactoferrin monomer can bind 2 Fe^{3+} ions, with a binding affinity

of 10 - 20 M (Baker and Baker, 2004). This iron binding capacity is likely to contribute to its antioxidant potential (Baker and Baker, 2004, Kim et al., 2013). The bovine whey fraction also contains dilute concentration of immunoglobulins (Ig_A, Ig_M, Ig_G (Ig_{G1} and Ig_{G2})). These are quaternary structure molecules, either monomers or polymers with 4 chains, consisting of 2 light polypeptide chains (M_W 25,000 g/mol) and 2 heavy chains (M_W between 50,000 and 70,000 g/mol) linked by disulphide bonds (Madureira et al., 2007). Several bovine whey ingredients are produced commercially (Table 1) and differ primarily in protein content and lactose concentration.

DOES WHEY SHOW ANTIOXIDANT ACTIVITY IN VITRO?

The antioxidant potential of WP has been assessed by different in vitro methodologies (1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical assay, (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC)). Tables 2a, 2b and 2c detail the most recent studies of the antioxidant activity of WP ingredients after processing, or enzymatic hydrolysis, or both. Other noteworthy studies have been reviewed previously (Power et al., 2013, Brandelli et al., 2015). The WP antioxidant activity was shown to be dose-dependent (20 - 100 mg/mL) by the DPPH assay which measures the ability of a compound to scavenge the DPPH radical (Gad et al., 2011). Hydrolysis of pre-heated Whey Protein Isolate (**WPI**) with the enzyme Alcalase (EC 3.4.21.62), a non-specific endopeptidase purified from *Bacillus licheniformis* and commercially available with specific activities ranging from 0.6 - 2.5 U/g, increased its DPPH scavenging activity from 11.4% to 62.9% (Peng et al., 2010). Hydrolysates of Whey Protein Concentrate (**WPC**) produced by Alcalase also showed significantly greater inhibition that WP hydrolysates (**WPH**) produced by other microbial enzymes (Dryáková et al., 2010, Lin et al., 2012, O'Keeffe and FitzGerald, 2014). Dryáková et al. (2010) demonstrated 35.5% greater inhibition of the ABTS radical with WPC hydrolyzed by

Alcalase rather than by the enzymes Neutrase (EC 3.4.24.28) or Protamex (EC 3.4.21.14). WPC hydrolyzed by Alcalase also showed more ferric reducing power (0.55 mM $FeSO_4$ equivalents) than trypsin (EC 3.4.21.4), pepsin (EC 3.4.23.1), or Flavourzyme (EC 3.4.11.1) hydrolysates (0.35 mM $FeSO_4$ equivalents, $P < 0.05$). This activity was further increased by heat-treatment (95°C, 5 - 10 min) of WPC before hydrolysis (Lin et al., 2012). However Adjonu et al. (2013) observed that heat-pretreatment (80°C, 15 min) did not improve antioxidant activity of WPI hydrolysates from pepsin (non-heated-WPH = 0.32 ± 0.03 μmol Trolox Equivalents (**TE**)/mg protein, heated WPH = 0.30 ± 0.03 μmol TE/mg protein) or chymotrypsin (EC 3.4.21.1) (non-heated-WPH = 0.27 ± 0.04 μmol TE/mg protein, heated WPH = 0.31 ± 0.02 μmol TE/mg protein). In this study ORAC methodology was employed, which scavenges peroxyl radicals and compares levels to the Vitamin E analogue, Trolox (Adjonu et al., 2013). In an effort to increase antioxidant properties of peptides from WP, Le Maux et al. (2016) altered the hydrolysis conditions (pH, enzyme type, reaction time and temperature). Hydrolysis of WP (81% protein) test samples with papain (EC 3.4.22.2) at constant pH 7.0 gave significantly higher ORAC values (285.32 ± 36.71 μM TE/g powder) than those obtained from hydrolysates generated under non-controlled pH conditions (192.54 ± 42.61 μM TE/g powder, $P < 0.05$). To characterize the functional fraction of whey, WPC was hydrolyzed by several enzymes and the resultant hydrolysates were fractioned by size using gel or membrane filtration (Peng et al. 2009, Onay-Ucar et al. 2014, Tarango-Hernandez et al. 2015). ORAC results showed that fractions containing peptides with smaller molecular weight (Alcalase hydrolysate 1 kDa permeate = 0.91 μM TE/mg powder) exhibit more antioxidant activity than those fractions containing larger peptides (Alcalase hydrolysate 5 kDa permeate = 0.75 μM TE/mg powder) (O'Keeffe and FitzGerald, 2014). Mass spectrometry analysis has revealed the amino acid sequences of peptides in antioxidant fractions (Table 3a). The bioavailability of whey fractions is under investigation (Picariello et

al., 2013). Several peptides from β-lactoglobulin were identified in antioxidant fractions produced by enzymatic hydrolysis of whey products. Many of these peptides occurred within 3 location hotspots (42 - 61AA, 77 - 110AA and 123 - 135AA). Interestingly 123 - 135AA contains the iron-binding peptide TPEVDDEALEK (Cruz-Huerta et al., 2016). Bertucci et al. (2015) also discovered several α-lactalbumin peptides in fractions exhibiting antioxidant activity. In this case, peptides from location 15 - 23AA were frequently identified. To date none of these peptides have been synthesized and tested in DPPH, ABTS, ORAC or FRAP assays for antioxidant activity. The free amino acids present in these fractions, some of which may contribute to the antioxidant activity (tryptophan, phenylalanine, tyrosine, cysteine and histidine), has also not been described. In contrast (Table 3b), Hernandez-Ledesma et al. (2005) identified several peptides and amino acids from a 3 kDa permeate of the β-lactoglobulin hydrolyzed by Corolase PP. Three peptides MHIRL, YVEEL and WYSLAMAASDI were synthesized and exhibited antioxidant activity by ORAC; MHIRL 0.306 μM TE/μM peptide, YVEEL 0.799 μM TE/μM peptide and WYSLAMAASDI 2.621 μM TE/μM peptide. In particular the antioxidant activity of WYSLAMAASDI is comparable to the synthetic antioxidant butylated hydroxyanisole (2.43 μM TE/μM pure compound) but 1.7 - 4 fold lower than the plant polyphenols catechin and quercetin (14.9 and 10.5 μM TE/μM compound, respectively) (Dávalos et al., 2004). Additionally, Hernandez-Ledesma et al. 2007 identified 3 synthetic peptides derived from β-lactoglobulin (19 - 24AA) (WY, WYS and WYSLAM) which exhibited ORAC values (4.45 (WYS) to 7.67 (WY) μM TE/μM peptide) higher than equimolar mixtures of their corresponding free amino acids. Nongonierma and Fitzgerald (2013) identified a synthetic dipeptide WS present in α-lactalbumin (60 - 61AA) and lactoferrin (8 - 9AA, 341 - 348AA) which exhibited 50% DPPH scavenging capacity at 0.26 mM, equivalent to 17.2 nM Trolox. Moreover, purified peptides,

LDQW and INTW, derived from thermolysin (EC 3.4.24.27) hydrolysis of α-lactalbumin were capable of a 100% ABTS radical inhibition at 2.5 μM (Sadat et al., 2011).

In addition to processing, whey origin may also play a role in antioxidant activity. Salami et al. (2010) hydrolyzed camel whey with either chymotrypsin, trypsin, proteinase K (EC 3.4.21.64) or thermolysin. Camel whey is rich in α-lactalbumin and lysozyme but lacks β-lactoglobulin. It exhibited 40% higher antioxidant activity by ABTS than bovine whey (Salami et al., 2010). In addition, sheep whey was found to be more active against the DPPH radical, requiring 3.1 ± 0.09 mg/mL to inhibit 50% of the radical compared to 8.2 ± 0.77 mg bovine whey protein/mL, (Kerasioti et al., 2014). It also exhibited greater iron reducing power than bovine whey although, in this case, ABTS data was similar (Kerasioti et al., 2014). Indeed, conflicting data between different radical scavenging methods (ORAC, DPPH, ABTS) is common to the majority of studies (Adjonu et al., 2013, Kerasioti et al., 2014) indicating the need to perform several antioxidant assays to be confident of results. Interestingly, there are other components in milk which appear to synergistically enhance the antioxidant activity of whey. Zulueta et al. (2009) showed higher ORAC values for pasteurized milk (13,935 μM TE) than from whey obtained after casein precipitation of pasteurized milk (1,078 μM TE). In this regard, Conway et al. (2013) observed that hydrolysates from buttermilk protein (54.6% protein content) were more effective ($P < 0.05$) at scavenging free radicals than those from WPC (74.5% protein content). ORAC values were 1,319.6 ± 46.7 μM TE/g protein for buttermilk compared to 782.5 ± 34.8 μM TE/g protein for WPC. Although buttermilk is unlikely to contain large quantities of whey, analysis revealed 4 β-lactoglobulin peptides which were proposed to contribute to the antioxidant activity (Conway et al., 2013).

Recently, whey ingredients have been added to nutritional beverages to boost their antioxidant capacity. Supplementation of a lemon drink with 1% WP hydrolyzed by Alcalase

increased the antioxidant activity of the beverage from 0.75 mmol TE/L to 7.79 mmol TE/L (Athira et al., 2014). In addition, a flavored milk beverage fortified with 1 or 2% WP hydrolysates from different enzymes (Flavourzyme, Alcalase or Corolase) increased the ABTS radical inhibition of the beverage by 21 and 33%, respectively. Interestingly, adding intact whey (1 - 2%) to the beverage did not alter ABTS values (Mann et al., 2015). A polyphenol rich beverage (chlorogenic (0.01%) or catechin (0.01%)), thermally treated (121°C, 10 min) at pH 3.7, exhibited ABTS values of 0.45 - 1.22 mM TE/L respectively. However, the addition of whey (0.2%) (ABTS = 0.45 mM TE/L) to this model beverage did not result in additive antioxidant activity, although ABTS results were higher than the beverage with polyphenol values alone 0.90 - 1.77 TE/mL (He et al., 2015). Indeed, addition of WP (0.5, 2.0, 4.0 or 6.0%) did not significantly change ($P > 0.05$) the antioxidant activity of a beverage with 0.0032% lutein, a carotenoid antioxidant (Rocha et al., 2017). Interestingly, the combination of whey and the algae *Spirulina platensis*, rich in carotenoids, tocopherol and phycocyanin, showed less antioxidant power (130 TE mg/L of sample) than *Spirulina platensis* (100 mg/mL) alone (170 TE mg/L of sample) which indicates that whey can exert an antagonistic effect on the antioxidant activity of other compounds (Gad et al., 2011).

How WP compares in terms of its antioxidant activity to other proteins and known antioxidant compounds has been investigated (Davalos et al., 2004, Hernandez-Ledesma et al., 2007, Castro and Sato, 2014). Intact WP showed significantly lower DPPH radical inhibition (17.13 ± 2.33%) than soy protein isolate (27.18 ± 0.15%) or egg white protein (33.39 ± 0.26%) (Castro and Sato, 2014), although the purity of each protein was not described. Interestingly, no significant differences in DPPH inhibition were found between whey (29.81 ± 0.48%) and egg (31.50 ± 0.24%) hydrolysates using Flavourzyme (Castro and Sato, 2014). In contrast, ORAC values for WP hydrolysates were lower (160.72 ± 26.26 μM TE/g) than

results obtained for their counterparts from egg (546.45 ± 55.75 μM TE/g) or soy (1157.18 ± 134.66 μM TE/g), which again underlines the inconsistencies across antioxidant assays (Castro and Sato, 2014). It is noteworthy that 100 g of WPC (79.0% protein) results in ORAC values of 13,662 ± 1018 μM TE (Power-Grant et al., 2015) whereas 100 g of concentrated green tea extract results in 758,000 μM TE (de la Luz Cadiz-Gurrea et al., 2014). However as a protein, WP can be added to foods at concentration of 22.2% (Chavan R. S. et al., 2015) whereas green tea extract is usually added to foods at concentrations less than 0.04% (Maruyama et al., 2017).

CAN WHEY BOOST INTRACELLULAR ANTIOXIDANT DEFENCES IN VITRO?

According to the Swedish Agency for Health Technology Assessment and Assessment of Social Services and cited by the World Health Organization, boosting antioxidants capabilities (GSH, CAT and SOD) in cells by the diet will achieve long life and well-being (SBU, 1997). At the cellular levels, GSH 1) directly scavenges free radicals (for example hydroxyl radicals); 2) is a substrate for the antioxidant enzymes glutathione peroxidase (**GPx**) and glutathionetransferase; 3) facilitates transport of amino acids, specially cysteine, across the plasma membrane; 4) regenerates antioxidants (for example Vitamins C and E) to their functional form and 5) forms conjugates with toxic electrophilic compounds, catalyzed by glutathionetransferase, which are excreted from cells (Pastore et al., 2003, Masella et al., 2005, Valko et al., 2007). Tseng et al. (2006) reported that the rat renal cell line PC12, pre-treated with WPC at 10 mg/L for 24 h prior to an ethanol stress, produced 59.4.0 mM GSH/mg protein compared to 29.9 mM GSH/mg protein, ($P < 0.05$) for cells with no WPC pretreatment. This indicates whey may offer a protective benefit to cells when stressed. In agreement, stressing myoblast cells C_2C_{12} with 0.3mM tert-butyl hydroperoxide (**t-BHP**) for 30 mins decreased GSH levels by 31.5% compared with control, as measured by flow cytometry. These t-BHP stressed C_2C_{12} when pre-treated for 24 hours with sheep WP at 1.56,

3.12 and 6.24 mg increased GSH levels 112.9%, 118.0% and 138.0% compared to levels of t-BHP stressed cells. However it is interesting to note that 0.78 mg/mL WP actually reduced GSH levels by 25.7% in the same experiment (Kerasioti et al., 2014). In a recent study, t-BHP was also used to stress human hepatocytes (HepG2) for 2 h after 24 h WPC treatment (100 μg/mL) (Pyo et al., 2016). WPC treatment increased GSH levels (130%) from basal conditions and also recovered GSH levels from stressed cells (80%). In an attempt to identify which whey fraction is responsible for increasing GSH, O'Keeffe and FitzGerald (2014) used enzymatically hydrolyzed WPC to treat human umbilical vein endothelial cells (**HUVEC**) and GSH levels were monitored. Whey was hydrolyzed by Alcalase, Neutrase, Corolase PP and Flavourzyme and resulting peptide fractions separated according to size using 0.2 μm, 10 kDa, 5 kDa and 1 kDa cut off membranes. Whey hydrolysate fractions by Alcalase, Neutrase, Corolase PP and Flavourzyme at 1mg/mL significantly increased intracellular GSH in HUVEC cells ($P < 0.05$) after 48 hours incubation compared to HUVEC cells cultured in media alone. The 1 kDa permeate of hydrolysate from Alcalase treatment increased GSH levels by 153% in HUVEC cells compared with media alone ($P < 0.001$). Kent et al. (2003) incubated prostate epithelial cells (**RWPE-1**) for 48 hours with (a) 0.5 mg/mL hydrolyzed WPI, (b) 0.5 mg/mL hydrolyzed casein, (c) 500 μM buthionine sulfoxime (GSH synthesis inhibitor) or (d) 500 μM N-acetylcysteine (GSH stimulant). N-acetylcysteine increased GSH by 88% in RWPE-1 cells. Whey hydrolyzed with trypsin, chymotrypsin and peptidase increased GSH by 64% compared with hydrolyzed-casein-treated and control cells ($P < 0.05$). Interestingly, the 50% reduction of GSH levels in RWPE-1 cells by buthionine sulfoxime could not be reversed by cotreatment with WPI but could be reversed with N-acetylcysteine (Kent et al., 2003). Vilela et al., (2006) evaluated a combination of high hydrostatic pressure processing and low molecular weight whey peptides fractions but did not observe a boost in GSH levels in human tracheal epithelial cells (9HTEo- cell line).

To investigate if WP increases CAT activity in cells, C_2C_{12} muscle cells were treated with 0.1 - 0.4 mg/mL whey (80.05% protein) for 24 h and then stressed with 0.75 mM H_2O_2 for 1 h (Xu et al., 2011). The CAT activity was significantly enhanced from 15.1 ± 0.7 units to 23.7 ± 1.3 units/mg total cellular protein ($P < 0.05$) by WPC. Similarly, CAT activity was increased 141% in HUVEC cells after a 48 h incubation with 1 mg/mL of 1 kDa permeate of whey hydrolyzed with Corolase PP compared with media alone ($P < 0.01$) (O'Keeffe and FitzGerald, 2014). In addition, CAT activity also increased in H_2O_2-stressed lung fibroblasts (MRC-5 cell line) after 24 h treatment with 100 μg/mL Alcalase whey hydrolysates from 25 CAT U/mg protein to 65 CAT U/mg protein (Kong et al., 2012).

The SOD activity was also determined in stressed C_2C_{12} cells (Xu et al., 2011). Once again, cells were pre-incubated with WP (0.1 to 0.4 mg/mL) for 24 h, and then stressed for 1 h with 0.75 mM H_2O_2. Pre-treatment with WP significantly increased SOD levels (11.7 ± 0.5 U/mg protein) in stressed cells compared to cells that did not receive whey (5.27 ± 0.41 U/mg protein). In addition, WP also increased SOD activity in non-stressed cells from 13.4 ± 0.82 units/mg protein to 19.4 ± 0.6 units/mg protein. Similarly, 24 h pre-treatment with Alcalase hydrolysates of whey (20 μg whey/mL) increased SOD activity in H_2O_2-stressed lung fibroblasts compared with non-whey treated cells by 248% (Kong et al., 2012).

The vast majority of experiments to date expose cells lines to whey test samples. The usual mode of delivery of whey is via food consumption so the physiological relevance of such experiments is questionable. Target cells will only be exposed to whey components arriving in the bloodstream from the gut. Whey protein is easily and rapidly digested to individual amino acids in the gastrointestinal tract, showing maximum concentration of total amino acids in plasma at 69 min post WPI consumption (Purpura et al., 2014). Indeed WPI has a digestible indispensable amino acid score (**DIAAS**) of 1.09 (Rutherfurd et al., 2015). Power-Grant et al. (2015) performed a simulated gastrointestinal digestion of intact WPC and then

293 measured its antioxidant activity by ORAC. Gastric digestion of WPC increased its ORAC
294 values by 2.5 fold compared to intact WPC. However when the WPC was in a hydrolyzed
295 form, gastric digestion resulted in a 22% decrease in ORAC values. This indicates that
296 bioactivity of hydrolyzed whey samples was reduced during gut transit (Power-Grant et al.,
297 2015). To assess the antioxidant benefit to intestinal cells exposed to gastric digested whey,
298 the intestinal epithelial cell line, Caco2, was stressed with 0.25 mM H_2O_2 for 1 h and then
299 treated with gastric digested WPI (0 - 2 mg/mL) for 23 h. ROS activity in Caco2 cells were
300 reduced by 32.5% when cells were treated with 2 mg/mL gastric digested WPI compared to
301 ROS values from stressed cells (Piccolomini et al., 2012). Although physiological biomarkers
302 are limiting, human/animal intervention trials with diets that include whey are the best
303 assessment of antioxidant benefit. Table 5 summarizes the most recent animal trials which
304 tested the antioxidant effect of WP rich diets.

305 DOES WHEY ACT AS ANTIOXIDANT PROTECTOR IN VIVO?

306 Bounous et al. (1989) proposed that a diet rich in GSH amino acid precursors, such as
307 cysteine, would boost cellular GSH production. As WP are cysteine rich, Bounous et al.
308 (1989) fed elderly (17 - 20 months old) C7BL/6NIA male mice a diet rich in WPC (20
309 g/100g diet) for 3 months. Animals were sacrificed and GSH levels in liver and heart were
310 measured. Mice on whey diets had significantly higher, GSH levels in liver (9 μM GSH/g
311 liver) and heart (1.6 μM GSH/g heart) than those animals fed a casein rich diet (20 g/100 g
312 diet) or a control chow diet (8 μM GSH/g liver and 1.3 - 1.5 μM GSH/g heart) ($P < 0.05$) for
313 the same time period. In addition, the WP rich diet appeared to extend the lifetime of the aged
314 mice with a 55% mortality rate reached at 125.0 ± 41.6 days compared to 92.2 ± 55.2 and
315 92.7 ± 31.7 days for mice fed casein rich or chow diets respectively ($P < 0.05$). Liver GSH
316 was also increased in Fisher rats fed with WP (150 g/1000 g diet) (55 nM GSH/mL tissue
317 extract) during 8 weeks compared to those on a casein rich diet (44 nM GSH/mL) (Haraguchi

et al., 2011). Interestingly, a diet supplemented with 10% whey protected Wistar rats against induced CCl_4 hepatotoxicity (Ashoush et al., 2013). Ashoush et al. (2013) proposed that this protection was as a result of an increase in total GSH plasma levels (CCl_4 plus WP = 16.74 ± 1.2 mg/dL *vs.* CCl_4 = 9.94 ± 0.84 mg/dL). As a model of oxidative stress, Sprague-Dawley rats were fed a diet high in iron (2,000 mg/kg) for 6 weeks. Those animals that also received 10% WP had increased GSH in blood erythrocytes (11.43 ± 0.71 μM) compared to controls (GSH = 8.75 ± 0.71 μM) (Kim et al., 2013). However, CAT levels were not significantly increased after WP supplementation (Kim et al., 2013). In agreement, a combination of exercise and whey intake over an 8 week test period had little impact on liver CAT activity in Fisher rats fed a whey rich diet (150 g/1000 g) compared to those on a casein rich diet (30 U/mg protein) (Haraguchi et al., 2011). In contrast, Athira et al. (2013) observed a significant increase in liver CAT levels in Swiss albino mice who received an intraperitoneal injection of WP hydrolyzed by Alcalase (4 mg/kg body weight) (CAT=193.66 ± 18.61 U/mg protein) compared to mice without WP administration (149.67 ± 12.83 U/mg protein). All of the Swiss albino mice in this study had received paracetamol orally (300 mg/kg body weight) for 2 days to induce oxidative stress prior to WP administration (Athira et al., 2013).

In a human intervention study, blood GSH levels were evaluated over 6 weeks in 18 male participants subjected to strenuous aerobic training and a dietary supplement of 1 g of WPI per kg body mass per day. Blood GSH levels were significantly lower in those subjects who performed exercise that those who did not ($P < 0.05$). The addition of WP supplementation to an exercise regime prevented this GSH depletion in blood (Middleton et al., 2004). In agreement, Sheikholeslami Vatani and Ahmadi Kani Golzar (2012) observed increased GSH plasma levels in 30 overweight young men who consumed whey and performed resistance training for 8 weeks (173 ± 22 nM GSH/L versus control group: 144 ± 20 nM GSH/L, $P < 0.05$) (Sheikholeslami Vatani and Ahmadi Kani Golzar, 2012). Levels of GSH in plasma

were also increased by 23% in steatohepatitis patients who received 20 g whey/day for 12 weeks compared to GSH levels before whey supplementation (Chitapanarux et al., 2009). In another human trial, 31 subjects received 15 - 45 g pressurized WPI/day over a 2 week period. The GSH levels in lymphocytes extracted from blood were 24% higher after 45 g WPI consumption than participants who did not consume WPI (Zavorsky et al., 2007). In contrast, blood GSH levels remained unchanged over the 4 h sampling period in male subjects who received an acute dose of WPI (0.8 - 1.6 g WPI/kg body weight) (Middleton et al., 2004). Measuring levels of GSH in plasma is one of the most common techniques to detect whey antioxidant protection in vivo. There is a positive correlation between low plasma GSH levels and disorders in which oxidative stress is a contributing factor, such as cardiovascular disease (Shimizu et al., 2004), polycystic ovary syndrome (Murri et al., 2013) and autism (Frustaci et al., 2012). However, plasma GSH levels are unlikely to reflect intracellular GSH in target organs such as the liver, brain or muscle that are routinely exposed to oxidative stress (Ballatori et al., 2009). In contrast, charting the oxidation levels of particular proteins involved in disease onset and progression, would provide more relevant biomarkers in cellular assays and dietary intervention trials (Frijhoff et al., 2015).

CONCLUSIONS

Bovine whey and individual whey proteins exhibit antioxidant activity. This bioactivity is observed with different commercial whey products (WPI, WPC), is relatively resistant to processing method and is increased by enzymatic hydrolysis. Alcalase appears the enzyme of choice to deliver the most potent whey hydrolysate fractions.

Several synthetic peptides derived from β-lactoglobulin and α-lactalbumin have demonstrated antioxidant activity. It is also likely that free amino acids released during hydrolysis contribute to this bioactivity. It is important to note that there is conflicting readout from

different antioxidant methodologies, different whey products, different dosages and in the translation of cellular assays to plasma biomarkers. Exposing cell lines to whey test samples directly results in an increase in GSH levels. However the biological relevance of these experiments is questionable. Whether the bioactivity survives gut transit, passes through the intestinal barrier and reaches its target cells will ultimately determine its efficacy as a dietary antioxidant ingredient. Certainly there is some evidence that WP supplementation alters plasma biomarkers for antioxidant activity, especially in individuals exposed to high oxidative stress levels, either from illness or intense exercise. However the number of participants in these studies is small and the relevance of these biomarkers to target organs exposed to oxidative stress requires further investigation.

In conclusion, there is ample evidence that bovine whey exhibits antioxidant activity in vitro, in animal trials and in human dietary intervention studies. The antioxidant potency of whey is however lower than well-known plant antioxidants such as green tea, although whey can be added to food at much higher concentrations. Therefore for whey to be an effective antioxidant, future studies should focus on the synergistic or antagonistic effect of novel combinations of whey with other known antioxidants within food matrices.

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769 7):380-385. http://dx.doi.org/10.1016/j.idairyj.2009.02.003.
770

771 Table 1. Composition of commercial whey protein products

	Composition		
Whey products	Protein (%)	Fat (%)	Lactose (%)
Whey Protein Concentrate (WPC)	34 - 80	1 - 7	4 - 52
Whey Protein Isolate (WPI)	90 - 95	0.5 - 1	0.5 - 1
Hydrolyzed Whey Protein (WPH)	80 - 90	0.5 - 8	0.5 - 10

772

773

774 Table 2a. Effect of processing treatments and enzyme hydrolysis on Whey Protein

775 Isolate antioxidant activity

Products	Heat treatment	Hydrolysis/ Filtration/ Others [2]	Antioxidant assay[3]	Results[4]	Reference
WPI[1] (95% protein)	Preheating 95°C, 5 min	Alcalase (pH 8.5, 65°C, 1, 2, 3, 4, 5 and 8 h)	Inhibition liposomes peroxidation TBARS FRAP Metal-chelating activity DPPH	Hydrolysis ↑ AOX (DPPH inhibition: WPI = 11.4%, 5h-WPH = 62.9%)	Peng et al. (2010)
WPI	Preheating 85°C, 15 min	Papain (pH 8.0, 35 - 55°C, 2 - 6 h)	Reducing power assay DPPH	< AOX at 3.6 h, 45.7°C (DPPH = 31.6%)	Zhidong et al. (2013)
WPI	Unheated or Preheated (80°C, 15 min)	Pepsin (pH 2.6, 37°C) Trypsin (pH 7.8, 37°C) Chymotrypsin (pH 7.8, 37°C) 12 or 24 h	ABTS ORAC	Hydrolysis ↑ AOX (WPH trypsin = 0.32, WPI = 0.08 μM TE/mg protein) No differences between enzymes, time or preheating.	Adjonu et al. (2013)
Native and pressurized WPI (1 mg/mL)		Pressure treatment (1 cycle 550 MPa) followed by SGID: - (Pepsin (pH 1.9, 37°C, 0.25 h), trypsin, chymotrypsin and peptidase (pH 7.4, 37°C, 1.0 h) Membrane filtration (10 kDa permeate)	FRAP	SGID pressurized WPI showed 21% more AOX activity than SGID native WPI	Iskandar et al. (2015)
WPI		Papain (pH 7.0, 65°C, 0 - 5h) Pepsin (pH 2.0, 37°C, 0 - 5 h) Alcalase (pH 8.3, 55°C, 0 - 5 h)	Ferric reducing power	Papain AOX results were time-dependent No change in AOX activity of Alcalase or pepsin hydrolysates	Mohan et al. (2015)
WPI (≥90% protein)	Preheated (85°C, 5 h, pH 2.0)	Corolase N (pH 7.7, 55°C, 5 h)	DPPH Reducing power	Hydrolysis and heat ↑ AOX activity (Scavenging activity: WPI = 13%, WPH = 60%)	Mohammadian and Madadlou (2016)
WPI (90% protein)	Preheating 80°C, 7 min	Pepsin (pH 2.0, 37°C, 0 - 12 h)	Ferric reducing power	Maximum AOX activity at 6 h	Nourbakhsh et al. (2017)

[1] WPI = Whey Protein Isolate

[2] MPa = Megapascal; SGID = Simulated Gastrointestinal Digestion; kDa = Kilodalton

[3] TBARS = Thiobarbituric Acid Reactive Substances; FRAP = Ferric Reducing Antioxidant Power; DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical; ABTS = 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ORAC = Oxygen Radical Absorbance Capacity

[4] AOX = Antioxidant; WPH = Whey Protein Hydrolysate, μM TE = μM Trolox Equivalents

785 Table 2b. Effect of processing treatments and enzyme hydrolysis on Whey Protein
786 Concentrate antioxidant activity

Products	Heat treatment	Hydrolysis/ Filtration/ Others	Antioxidant assay[3]	Results[4]	Reference
WPC[1] (80% protein)			DPPH Ferrous-chelating activity	WPC AOX activity dose-dependent (20 - 100 mg/mL)	Gad et al. (2011)
WPC	Unheated or Preheated (95°C, 5 or 10 min)	Pepsin (pH 2.0, 37°C, 2 h) Trypsin (pH 8.0, 37°C, 2 h) Alcalase (pH 9.0, 50°C, 2 h) Flavourzyme (pH 7.0, 50°C, 2 h)	Total AOX activity FRAP DPPH	Hydrolysis ↑ AOX activity (Alcalase = 62% DPPH inhibition > trypsin or pepsin)	Lin et al. (2012)
WPC	Preheating 90°C, 5 min	Pepsin (pH 1.5, 37°C) followed by trypsin (pH 7.6, 50°C) for 1.5 h	DPPH Superoxide anion radical scavenging activity Ferric reducing power	WPH dose dependent AOX activity (0 - 10 mg/mL)	Zhang et al. (2012)
WPC, Commercial WPH (DH = 32%; DH = 45%)		SGID[2]: Pepsin (pH 2.0, 37°C, 1.5 h) followed by Corolase PP (pH 7.5, 37°C, 2.5 h)	ORAC	WPH AOX activity 3 - 6 fold > WPI. SGID ↑ AOX WPC (WPC = 13,662; SGID WPC = 36,605 μM TE/100g powder)	Power-Grant et al. (2015)
WPC (4% protein)	Preheating 50 - 54°C, 10 min	Protamex, Alcalase or both (pH 7.0; 0.5, 1, 1.5 h; 45, 50, 55°C)	ABTS ORAC	WPC = 2.83 mM TE WPH Protamex = 4.27 mM TE WPH both enzymes = 6.33 mM TE	Torkova et al. (2016)

787 [1] WPC = Whey Protein Concentrate; WPH = Whey Protein Hydrolysate; DH = Degree
788 of Hydrolysis

789 [2] SGID = Simulated Gastrointestinal Digestion

790 [3] DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical; AOX = Antioxidant; FRAP = Ferric
791 Reducing Antioxidant Power; ORAC = Oxygen Radical Absorbance Capacity; ABTS =
792 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

793 [4] μM TE = μM Trolox Equivalents

794

795 Table 2c. Effect of processing treatments and enzyme hydrolysis on whey antioxidant
796 activity

Products[1]	Hydrolysis/ Filtration/ Others	Antioxidant assay[2]	Results[3]	Reference
WPX (11% protein)		DPPH	WP 72.15% scavenging activity	Ashoush et al. (2013)
WPX	Flavourzyme Alcalase Protease from *Aspergillus oryzae* Optimal temperature and pH,4 h	ORAC DPPH	Hydrolysis ↑ AOX activity of WP Hydrolysates by protease from *Aspergillus oryzae* > ORAC values (172.11 mM TE/g) and DPPH inhibition (69.53 %)	de Castro and Sato (2014)
Fresh sweet and acid whey	Polymerization by glycation (pH 7.0 and 9.0) Hydrolyzed by biomass of *Bacillus subtilis* (pH 7.0, 50°C, 24 h)	ABTS	Hydrolysis and glycation ↑ AOX activity (acid WPC = 55%, WPH = 85%, WPC glycated = 75%, WPH glycated = 95%)	Ortega et al. (2015)
MPC, Commercial WPH (DH = 32%, 78% protein; DH = 45%, 75% protein)		ORAC	WPH45 = 77691 μM TE/100g powder > WPH32 = 37391 μM TE/100g powder > MPC (15678 μM TE/100g powder	Power-Grant et al. (2016)
WPX (81% protein)	Papain (pH 7.0, 6.3 and without pH control, 50°C, 3 h)	ORAC	Higher AOX at constant pH 7.0 (WP = 71.52 19.29μM TE/g protein, WPH = 285.32 μM TE/g protein)	Le Maux et al. (2016)

797 [1] WPX = Whey Protein type not specified; MPC = Milk Protein Concentrate; WPH =
798 Whey Protein Hydrolysate; DH = Degree of Hydrolysis

799 [2] DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical; ORAC = Oxygen Radical Absorbance
800 Capacity; ABTS = 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

801 [3] AOX = Antioxidant; TE = Trolox Equivalents

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803

804 Table 3a. Peptides identified in antioxidant bovine whey fractions

Products[1]	Hydrolysis/ Filtration/Other pretreatments[2]	Antioxidant assay[3]	Peptides in antioxidant fractions	Reference
WPC	Thermolysin (pH 8, 80°C, 8 h) followed by membrane filtration 3 kDa cut-off	ORAC	β-LG: f(58-61) LQKW, f(95-101) LDTDYKK	Contreras et al. (2011)
WPI (95% protein)	Alcalase (pH 8.5, 65°C, 5 h) followed by gel filtration (> 40, 2.8 - 40, 0.1 - 2.8, < 0.1 kDa	FRAP Cellular assays	Fraction 0.1 - 2.8 kDa VHLKP, protein not described	Kong et al. (2012)
Fresh whey	Alcalase (pH 7 - 9, 50 - 7 C, 2 - 8 h) followed by membrane filtration 3 kDa cut-off	ABTS	β-LG: f(96-100) DTDYK, f(94-100) VLDTDYK, f(123-131) VRTPEVDDE, f(122-131) LVRTPEVDDE, f(124-131) RTPEVDDEALE, f(123-134) VRTPEVDDEALE, f(122-134) LVRTPEVDDEALE	Athira et al. (2014)
WPC	Alcalase (pH 8.5, 65°C, 5 h) Flavourzyme (pH 6.5, 50°C,8 h) Corolase (pH 7.5, 45°C,7 h) followed by membrane filtration: 3 kDa cut-off	ABTS	Corolase fraction: β-LG: f(50-56) PEGDLEI, f(43-49) VEELKPT, f(43-51) VEELKPTPE, f(123-135) TPEVDDEALEK	Mann et al. (2015)
WPX	Serine peptidase from *Maclura pomífera* (pH 6.5, 45°C, 0 - 3 h)	ABTS	α-LA: f (32-38) HTSGYDT, f(15-22) LKGYGGVS, f(16-23) KGYGGVSL, f(13-19) KDLKGYG, f(82-88) DDDLTDD, f(5-12) KCEVFREL, f(6-13) CEVFRELK, f(104-113) WLAHKALCSE, f(40-57) AIVQNNDSTEYGLFQINN β-LG: f(42-47) YVEELK, f(89-96) ENKVLVLD, f(55-61) EILLQKW, f(56-62) ILLQKWE, f(52-61) GDLEILLQKW, f(77-87) KIPAVFKIDAL, f(150-160) SFNPTQLEEQC, f(151-162) FNPTQLEEQCHI, f(95-110) LDTDYKKYLLFCMENS, f(10-28) LDIQKVAGTWYSLAMAASD, f(11-29) DIQKVAGTWYSLAMAASDI	Bertucci et al. (2015)
β-LG	Alcalase (pH 7, 50°C, 2 h) under high-hydrostatic pressure	FRAP, Iron Chelating Activity	β-LG: f(27–38) DIQKVAGTWYSL, f(33-38) GTWYSL, f(39-48) AMAASDISLL, f(40-48) MAASDISLL, f(61-73) ELKPTPEGDLEIL, f(87-98) IIAEKTKIPAVF, f(112-121) DTDYKKYLLF, f(165-172) LSFNPTQL	Bamdad et al. (2017)

805 [1] WPC = Whey Protein Concentrate, WPI = Whey Protein Isolate, WPX = Whey
806 Protein type not specified

807 [2]kDa = Kilodalton

808 [3] FRAP = Ferric Reducing Antioxidant Power; ABTS = 2,2'-Azino-bis(3-
809 ethylbenzothiazoline-6-sulfonic acid), ORAC = Oxygen Radical Absorbance Capacity

Table 3b. Synthetic antioxidant peptides derived from whey proteins

Substrate	Hydrolysis/ Filtration/Other pretreatments[2]	Antioxidant assay[3]	Antioxidant peptides[4]	Reference
β-LG	Corolase PP (pH 8, 37°C, 24 h)	ORAC	β-LG: f(19-29) WYSLAMAASDI, f(42-46) YVEEL, f(145-149) MHIRL	Hernandez-Ledesma et al. (2005)
Synthetic peptides		ORAC	β-LG: f(19-20) WY, f(19-21) WYS, f(19-22) WYSL, f(19-23) WYSLA, f(19-24) WYSLAM	Hernandez-Ledesma et al. (2007)
Purified peptides	Thermolysin (pH 7.2, 70°C, 15 min)	ABTS	α-LA: f(101-104) INTW, f(115-118) LDQW	Sadat et al. (2011)
Synthetic peptides		DPPH	β-LG: f(60-61) KW α-LA: f(19-20) WY, f(25-26) EW, f(60-61) WC, f(103-104) YW, f(104-105) and f(118-119) WL BSA: f(133-134) FW LF: f(16-17) WF, f(22-23) WQ, f(24-25) WR, f(124-125) and f(466-467) GW, f(137-138) SW, f(138-139) WT, f(198-199) LW, f(448-449) and f(467-468) WN	Nongonierma and Fitzgerald (2013)
β-LG	Trypsin (pH 8, 37°C, 24 h)	ORAC	β-LG f(15–20)VAGTWY	Power et al. (2014)
WPC[1]	Corolase PP (pH 7.0, 50°C, 4 h) followed by membrane filtration: 0.65 kDa cut-off	ORAC	β-LG: f(15-18) VAGT, f (24-26) MAA, f(71-74) IIAE	O'Keeffe et al. (2017)

[1] WPC = Whey Protein Concentrate

[2] kDa = Kilodalton

[3] ORAC = Oxygen Radical Absorbance Capacity, ABTS = 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical

[4] LF = Lactoferrin

817 Table 4a. Antioxidant activity of whey products determined by cell culture[1]
818

Whey products	Protein pre-treatment: Hydrolysis/Filtration/Others[2]	Cell line	Incubation time/Stress	Antioxidant assay[4]	Results	Reference
WPC (0.4 mg/mL)		C2C12 (mice myoblast)	24 h followed by H_2O_2 (0.75 mM, 1h)	GSH CAT SOD TBARS GPx DNA oxidative damage AOX gene expression	After H_2O_2 stress, WPC ↑ GSH, CAT, SOD,GSH-Px HO-1 = NRF2 and NQO1 ↓ Lipid peroxidation and DNA damage	Xu et al. (2011)
WPI (4, 20 or 100 µg/mL), 2.8 - 40 kDa fraction	Preheating 95°C, 5 min Alcalase (pH 8.5, 65C, 5h) Followed by gel filtration (>40, 2.8-40, 0.1-2.8, <0.1 kDa	MRC-5 (human lung fibroblast)	24 h followed by H_2O_2 (1mM, 24 h)	AOX enzymes: SOD, CAT, GPx - Cellular lipid oxidation inhibition	WPI (20 and 100 µg/mL), protected against induced-lipid peroxidation by increasing SOD, CAT, GPx	Kong et al. (2012)
WPC (0.05 - 0.4mg/mL)	Preheating 90°C, 5 min Pepsin (pH 1.5, 37°C) followed by trypsin (pH 7.6, 50°C) for 1.5 h	PC12 (rat pheochromocytoma)	2h followed by H_2O_2 (100 µM, 24h)	Cell viability	WPH protected against H_2O_2 cytotoxicity	Zhang et al. (2012)
Native and pressurized WPI (0 - 2 mg/mL)	Pressure treatment (1 cycle 550 MPa) followed by SGID (Pepsin (pH 1.9, 37°C, 0.5 h) ,trypsin and chymotrypsin (pH 7.4, 40°C, 1.5 h); 10 kDa permeate	Caco-2 (human colonic adenocarcinoma)	H_2O_2 (0.25 mM, 1 h) with or without SGID whey (23 h)	Intracellular ROS - FRAP	↓ ROS at 2 mg/mL: SGID native WPI = 32.5%, SGID pressurized WPI = 76.1% compared to stressed but non-WPI treated cells FRAP activity of SGID WPI treated-cell medium > non-treated > stressed-cell medium	Piccolomini et al. (2012)

WPC (1 mg/mL)	Alcalase Neutrase Corolase PP Flavourzyme General conditions: pH 7.0, 50°C, 4 h Followed by membrane filtration (0.2 μm, 5 and 1 kDa)	HUVECs (human umbilical vein endothelial)	48 h	GSH CAT Microarray analysis	WPC did not ↑ GSH nor CAT All 1 kDa hydrolysates ↑ GSH All 5 kDa hydrolysates ↑ CAT unlike 0.2 μm Gene expression of GPX3, NADPH dehydrogenase quinine 1 and 2, Aldehyde dehydrogenase 3 family, member A1 ↑ with 5 kDa permeates of Alcalase and Neutrase compared to baseline	O'Keeffe and FitzGerald (2014)
Native (nWPI) and pressurized WPI (pWPI) (0 - 1 mg/mL)	Pressure treatment (1 cycle 550 MPa) followed by SGID: - Pepsin (pH 1.9, 37°C, 0.25 h) followed by trypsin, chymotrypsin and peptidase (pH 7.4, 37°C, 1.0 h). Then, membrane filtration (10 kDa permeate)	1HAEo- (human tracheobronchial epithelial)	6 h	FRAP	FRAP of SGID pWPI-treated-cell 35% greater than control	Iskandar et al. (2015)
WPC (0.1 mg protein/mL)	Glucose-WPC conjugate	HepG2 (human hepatocytes)	24 h followed by *t*-BHP[3] (1 mM, 2 h)	GSH ROS production AOX gene expression	WPC and Glucose-WPC ↑ GSH (130%, 150%), ↓ROS (140 %, 120%) from control (100%) and stressed cells (GSH: 80%, ROS: 160%)	Pyo et al. (2016)

[1] WPC = Whey Protein Concentrate, WPI = Whey Protein Isolate

[2]kDa = Kilodalton; MPa = Megapascal; SGID = Simulated Gastrointestinal Digestion

[3] t-BHP = tert-butylhydroperoxide

[4]AOX = Antioxidant; GSH = Glutathione, CAT = Catalase, SOD = Superoxide dismutase,TBARS = Thiobarbituric Acid Reactive Substances ; GPx = Glutathione peroxidase ROS = Reactive Oxygen Species; FRAP = Ferric Reducing Antioxidant Power

826 Table 5. Antioxidant effect of whey in vivo

Products[1]	Protein pre-treatment	Food matrix	Subjects	Dosage	Time	AOX[3] parameters	Results[4]	Reference
WPI (InPro 90)	Pressurized	Bars (17 g protein/bar), orally	Men & women (31)	15, 30 or 45 g/d	2 wk	GSH in lymphocyte	30 and 45 g/day ↑ 24% GSH 15 g/day = GSH	Zavorsky et al. (2007)
Whey-derived peptide (NOP-47)		Powder in water, orally	Men & women (20)	5 g peptide/d	2 wk	FRAP and oxidative stress in plasma	FRAP value (375 ± 80 μM TE/L, pre-ingestion: 354 ± 82 μM TE/L)	Ballard et al. (2009)
WPI		Powder in water, orally	Patients (38) with non-alcoholic steatohepatitis	20 g/d	12 wk	ABTS Plasma glutathione level	↑ Plasma total AOX capacity (61%) compared to baseline ↑ Plasma GSH (28%) compared to baseline	Chitapanarux et al. (2009)
WPX (Peptamen 1.5)	Hydrolyzed	Enteral formula	25 elderly men and women with ischemic stroke	1.2g protein/kg/d	5 d	GPx in blood	GPx ↑ in whey diet (39.9 ± 4.8 U/g Hb) compared to casein (26.2 ± 6.7 U/g Hb)	de Aguilar-Nascimento et al. (2011)
WPX		ND	Rats (32) + resistance exercise	150 g protein/ kg	8 wk	GSH, CAT in liver Lipid peroxidation in liver and muscle	After exercise: WP ↑ GSH ↓ Lipid peroxidation compared to casein diet No differences in CAT	(Haraguchi et al., 2011)
WPC		Powder in water, orally	Rats (80, male), CCl_4-induced hepatotoxicity	50 mg/d	30 d	Total AOX activity in liver Lipid peroxidation in liver	↑ AOX activity ↓ lipid peroxidation	Gad et al. (2011)
WPI		Powder, orally	Overweight men (30) + resistance exercise	30 g/d	6 wk	GSH in blood	Whey diet and exercise GSH ↑ compared to control: 173 ± 22 nm/L Control = 144 ± 20 nm/L Placebo = 163 ± 26 nm/L	Sheikholeslami Vatani and Ahmadi Kani Golzar (2012)

Fresh whey	Preheating 70°C, 10 min Hydrolysed by Alcalase (pH 8.0 - 9.0, 55 - 60°C, 8 h)	Orally i.p. injection	Mice (24, male), paracetamol-induced oxidative stress	Oral: 8 mg/kg Injection: 4 mg/kg	4 d	Serum oxidative biomarkers (GPT,ALP, creatinine, BUN) CAT, SOD, GPx Lipid peroxidation inhibition	↓ Oxidative biomarkers and lipid peroxidation ↑ CAT, SOD and GPx	Athira et al. (2013)
WPX		Powder	Rats (40, male), carbon tetrachloride (CCl_4)-induced hepatotoxicity	10% whey	28 d	GSH in plasma Lipid peroxidation inhibition	↑ GSH levels (CCl_4 plus WP = 16.74 ± 1.2 mg/dl, CCl_4 = 9.94 ± 0.84 mg/dl) ↓ Lipid peroxidation	Ashoush et al. (2013)
Fresh WP		Pellet, orally	Rats (30 male), iron overload-induced oxidative stress	10 g protein/100 g	6 wk	Plasma radical trapping potential GPx, CAT, SOD, GST, GSH DNA damage Plasma lipid peroxidation	↑ Plasma AOX activity, SOD and GSH in erythrocytes No differences in GPx ,CAT and GST ↓ Lipid peroxidation and DNA damage	Kim et al. (2013)
Whey peptides	Commercial peptides	Orally with gastric tube	Mice (28, male) ultraviolet B radiation	400 or 800 mg peptide/kg	17 wk	DNA oxidative damage (detection of 8-OHdG[1])	Prevention of DNA damage caused by radiation	Kimura et al. (2014)
WPI	Chymotrypsin (pH 7.0, 37°C, 2 h) followed by filtration (30, 10 and 5 kDa[2] cut-off)	Liquid, orally	Mice (140) + exercised-induced fatigue	1.5 g protein/kg/d	6 wk	SOD, GPx in mitochondria and gastrocnemius	Mitochondria: SOD and GPx ↑with WPH < 5kDa (SOD: 549.20 ± 19.08 U/mg protein, Control = 388.41 ± 34.56 U/mg protein) and WPH5-10 kDa Gastrocnemius: no	Liu et al. (2014)

							differences in SOD GPx ↑ with WPH < 5kDa and WPH 5 - 10 kDa	
Mixture of MPC, Commercial WPHs (DH = 32%, 78%protein; DH = 45%, 75% protein)	Hydrolyzed	Liquid, orally	Women	0.3 g/kg	Acute ingestion	ORAC in plasma	AOX activity ↑ 23% from baseline	Power-Grant et al. (2016)

827 [1] WPI = Whey Protein Isolate; WPX = Whey Protein type not specified; WPC = Whey Protein Concentrate; MPC = Milk Protein
828 Concentrate; WPH = Whey Protein Hydrolysate; DH = Degree of Hydrolysis

829 [2] kDa = Kilodalton

830 [3] AOX = Antioxidant; GSH = Glutathione; GPx = Glutathione peroxidase; CAT = Catalase; GPT = glutamic-pyruvic acid transaminase;
831 ALP = alkaline phosphatase; BUN = blood urea nitrogen; SOD = Superoxide dismutase; GST = glutathione S-transferase; 8-OHdG= 8-
832 hydroxy-2'-deoxyguanosine;

833 [4] μM TE = μM Trolox Equivalents; U/G Hb = Units per gram of hemoglobin

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