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Whey proteins: targets of oxidation, or mediators of redox protection

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ABSTRACT

Bovine whey proteins are highly valued dairy ingredients. This is primarily due to their amino acid content, digestibility, bioactivities and their processing characteristics. One of the reported bioactivities of whey proteins is antioxidant activity. Numerous dietary intervention trials with humans and animals indicate that consumption of whey products can modulate redox biomarkers to reduce oxidative stress. This bioactivity has in part been assigned to whey peptides using a range of biochemical or cellular assays *in vitro*. Superimposing whey peptide sequences from gastrointestinal samples, with whey peptides proven to be antioxidant *in vitro*, allows us to propose peptides from whey likely to exhibit antioxidant activity in the diet. However, whey proteins themselves are targets of oxidation during processing particularly when exposed to high thermal loads and/or extensive processing (e.g. infant formula manufacture). Oxidative damage of whey proteins can be selective with regard to the residues that are modified and are associated with the degree of protein unfolding, with α -Lactalbumin more susceptible than β -Lactoglobulin. Such oxidative damage may have adverse effects on human health. This review summarises how whey proteins can modulate cellular redox pathways and conversely how whey proteins can be oxidised during processing. Given the extensive processing steps that whey proteins are often subjected to, we conclude that oxidation during processing is likely to compromise the positive health attributes associated with whey proteins.

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Introduction

Milk proteins (whey proteins (~20%) and caseins (80%)) are high-quality sources of amino acids (AA) in the human diet. Bovine milk proteins and peptides play important roles in human health not just in terms of nutrition but also in terms of their notable bioactivities. The major bovine whey proteins are α -Lactalbumin (α -Lac) and β -Lactoglobulin (β -Lg) with immunoglobulins, bovine serum albumin (BSA) and lactoferrin as minor proteins (Table 1). Whey proteins provide a complete protein source and are rich in both sulphur-containing and branched-chain AAs. Whey proteins do not coagulate under the acidic conditions present in the stomach, and are considered to be “fast proteins” since they reach the jejunum shortly after entering the gastrointestinal tract and have a digestible indispensable AA score of 1.09 [1]. Whey proteins exhibit a wide range of bioactivities including antioxidant, antibacterial, antifungal,

antiviral, antihypertensive, antithrombotic, opioid and immunomodulatory properties [2]. Consequently, whey products are recognised as value-added ingredients and are commonly used in the sports nutrition market, nutritional beverages for the elderly and infant formula (IF) [3]. These products are also prized in food formulation as they improve product quality by water-binding, stabilising aerated food products and acting as emulsifying agents [4]. Whey products can be supplied as ingredients for food formulations as whey protein concentrate (WPC), whey protein isolate (WPI) or as whey hydrolysates (WH) all of which differ in the degree of processing and protein content (Table 2). However, numerous processing steps from milk to whey powder to food formulation inherently expose whey proteins themselves to redox modifications. This review summarises how whey proteins can modulate cellular redox pathways and conversely how whey proteins can be oxidised during processing.

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Table 1. Bovine whey proteins.

Bovine whey protein	Concentration (g/L) in bovine milk	Molecular mass (kDa)	AAs
Total	7		
β -Lactoglobulin	3.5	18.3	162
α -Lactalbumin	1.2	14.2	123
Immunoglobulins	0.7	150–900	Variable
Bovine serum albumin	0.4	66.4	583
Lactoferrin	0.02–0.35	80	700

Table 2. Composition of different bovine whey products.

Whey powder type	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
Hydrolysed whey protein (WH)	80–90	0.5–8	0.5–10

Whey proteins and their ability to modify redox pathways – studies in animal models and humans

The antioxidant and detoxifying bioactivities of whey proteins are most likely linked to their contribution to glutathione (GSH) synthesis, recently reviewed in Corrochano et al. [5]. Whey proteins are rich in Cys with β -Lg containing 5 Cys residues, α -Lac has 8 Cys, BSA has 35 Cys and lactoferrin contains 34 Cys, although in each case the majority of Cys is present as disulphide bonds. The thiol (R-SH) group of Cys reacts rapidly with many oxidants. This AA when present with Gly and Gln in the tripeptide, GSH, is an important cofactor and antioxidant in mammalian cells and tissues. Reduced GSH, is readily oxidised to the disulphide species, oxidised glutathione (GSSG), with the latter then readily recycled by the enzyme GSH reductase, at the expense of nicotinamide adenine dinucleotide phosphate (NADPH), back to its reduced form. Other oxidised species can however also be formed from GSH including GSH sulphonamide and oxy acids, with the latter species being irreversible products. GSH detoxifies a number of endogenous and exogenous toxins including toxic metals, petroleum distillates, lipid peroxides, quinones, bilirubin and prostaglandins through direct conjugation. Cell lines (e.g. C2C12, MRC-5, PC12, Caco₂, HUVEC, 9HTEo, HepG2, and REPE-1) exposed to various whey products (WPC and WPI) have documented increases in GSH levels with some exceptions [5]. Whey products have also been reported to increase activities of the protective enzymes superoxide dismutase (which removes superoxide radicals) and catalase (which reduces H₂O₂ to water) and decrease levels of reactive oxygen species, lipid peroxidation and DNA damage in cellular assays [5]. However using cell lines with whole proteins has its limitations, not least of which is the altered redox homeostasis of immortalised cell lines [6]

and the nonphysiological exposure of cells to intact dietary proteins.

Human or animal intervention trials with diets that include whey products are the best assessment of impact on cellular redox pathways although reliable and consistent data with well-defined biomarkers is rather limited. In a significant number of cases generic and non-specific assays of antioxidant activity have been used, which is not ideal. Table 3 lists rat and mouse studies with a focus on redox parameters, where animals consumed different whey products (WPC and WPI) at a dosage of 0.02–1 g/kg body mass over a period of 7–84 d. Redox biomarkers were measured in liver, brain, erythrocyte, muscle, serum, kidney, colon, salivary gland or parathyroid gland in the presence or absence of various stressors (exercise, diabetes, heat, brain injury, hepatotoxicity, dyslipidaemia, schizophrenia or aflatoxin). Although conflicting data have been reported [7], the majority of trials have reported increases in GSH and other antioxidant markers. However exactly which species, and what concentrations, are responsible for these changes is unclear in most cases. Human dietary whey intervention trials with a focus on redox readouts have been reviewed previously by Corrochano et al. [5], with additional trials detailed in Table 4. These intervention trials generally recruit participants for, on average, a 3-week study with or without an exercise routine, measuring plasma GSH as an indicator of whole body redox status. Many studies report increases in plasma GSH from time zero with whey intervention but others observe no effect on plasma GSH levels (Table 4) [8,9]. Whether or not a single redox analyte detected at very low levels in the plasma is a good biomarker of redox state at a global or local tissue level is debatable [10,11].

There is also some evidence from intervention trials that whey proteins may decrease plasma GSH levels, cause heart damage and liver injury [12,13]. Oral

Table 3. Animal studies with different whey products and the antioxidant responses reported.

Bovine whey product, source ^a	Dose and time	Species/tissue/organ/disease	Results ^b	Reference
WPC, Dairy Cooperative Poland	0.3 g/kg; 7–14 d	Rat; salivary gland, parotid gland	↑GSH; ↑GSH-Px; ↑SOD; WPC improved redox homeostasis in salivary glands	[131]
WPC, Dairy Cooperative Poland	0.3–0.5 g/kg; 7–21 d	Rat; liver	↑ hepatic GSH; ↑ hepatic MDA	[132]
WPC, Davisco	0.02–0.04–0.06–0.08–0.1 g/kg bw (30 d)	Rat; CCl ₄ hepatotoxicity; liver	↓MDA; WPC prevented liver damage induced by CCl ₄	[133]
Un-denatured WP, Prother [®] , Italy	18%; 3 weeks	Rat; CCl ₄ hepatotoxicity; liver	↑GSH	[134]
WP, Green Land for Food Industries, Egypt	10%; 28 ds	Rat; CCl ₄ hepatotoxicity; liver,	↓MDA; ↑GSH	[135]
WPC, Davisco	0.1 g/mL; 30 d	Rat; CCl ₄ hepatotoxicity; liver,	↓MDA; ↑CAT; ↑GST	[136]
WPI, Davisco	0.1 g–0.2 g/kg;	Rat; lipopolysaccharides/liver, serum	↑SOD; ↓DNA damage; ↓Caspase-3 (apoptosis)	[137]
WPC, Davisco	0.3 g/kg; 30 d	Rat; sflatoxin-contaminated diet; liver, testes	↑GSH, ↓LP, ↓DNA damage; WPC prevents genotoxicity of aflatoxins	[138]
WPC, Davisco	0.3 g/kg; 30 d	Rat; aflatoxin-contaminated diet; liver, testes	↑GSH, ↓LP, ↓DNA damage; WPC reduced oxidative stress induced by aflatoxins	[139]
WPC, Probiotica	0.15 g/kg; 8 weeks	Rat; exercise model; muscle, liver	WP increased hepatic GSH and protected against exercise induced muscle protein oxidation	[140]
WPI, Diamond Whey, Italy	20%	Mouse; exercise model; muscle, liver	↓LP; ↓GSH/GSSG	[141]
WPC, Camillotek, India	0.3 g/kg; 28 d	Rat; erythrocyte from young and aged animals	↓T-SH; ↓sialic acid; ↑ lipid hydroperoxides; ↑ protein carbonyls; WPC restores redox status in erythrocytes	[142]
WPI, Davisco	0.15 g/kg; 28 d	Rat; non-alcoholic fatty liver	↑ hepatic GSH, ↓hepatic MDA	[143]
WP, Immunocal	3.3% twice daily; 28 d	Mouse; traumatic brain injury	↑GSH/GSSG; prevented reduction of GSH/GSSG in brain	[144]
WPI, Bioplex Nutrition, USA	100 g; 12 weeks	Mouse; brain mitochondria	↓MDA; ↓4-hydroxyalkenals; increased brain mitochondria activity	[145]
WP, Immunocal	0.66 g/kg; 60 d	Mouse; ALS model	Treatment prevented disease-associated reductions in whole blood and spinal cord tissue GSH	[146]
WPC	0.3 g/kg; 28 d	Rat; neurodegeneration; brain	↑Beclin-1; ↑Atg-3 (Autophagy); ↑FRAP; ↓PC; ↑T-SH; ↓ROS; ↓NO; ↑acetylcholinesterase	[142]
WPI, Immunocal	0.33 g/mL; 6 weeks	Mouse; schizophrenia model; brain	↑GSH/GSSG ratios; ↑GSH	[147]
WP, Turkey	WP supplemented diet; 21 d	Rat; burn injury model; liver, kidney	Whey protein suppressed burn-induced changes in hepatic and renal tissue	[148]
WP, Turkey	WP supplemented diet; 21 d	Rat; experimental laparotomy and colonic anastomosis; liver, abdominal wall, colon	Whey protein supplementation increased GSH and suppressed MDA in different tissues	[149]
WPI, Balance Muscle Technologies, New Zealand	150–250 g/kg; 4 weeks	Rat; HLAP; colon	No change DNA damage	[150]
WP, mozzarella cheese	10 g/100 g; 6 weeks	Rat; iron overload; toxicity; plasma, erythrocyte	↑SOD, ↑GSH, no change GSH-Px in erythrocytes; no change plasma VitE; WP had antioxidative and antigenotoxic effects	[7]
WP, Immunocal	0.1 g; 4 weeks	Murine; iron overload/toxicity	↓MDA; ↓Hexanal; ↑GSH-Px; ↑GSH	[151]

^aWPI: whey protein isolate; WP: whey protein, type not specified; WPC: whey protein concentrate.

^bGSH: glutathione; GSH – Px: glutathione peroxidase; CAT: catalase; GPT: glutamic-pyruvic acid transaminase; ALP: alkaline phosphatase; SOD: superoxide dismutase; GST: glutathione S-transferase; GSSG: oxidised glutathione; LP: plasma lactate; T-SH: total thiol; PC: protein carbonyl; ROS: reactive oxygen species; NO: nitric oxide; MDA: malondialdehyde, CCl₄: carbon tetrachloride.
FRAP: ferric-reducing antioxidant power.

Table 4. Human intervention trials with whey products and physiological response.

Bovine whey product ^a	Description of the study	Results ^b	Reference
WPI undenatured	Open-labeled clinical trial, 38 patients with non-alcoholic steatohepatitis were given 20 g whey protein isolate for 12 weeks.	Improvements in liver biochemistries, increased plasma GSH, total antioxidant capacity, and reduced hepatic macrovesicular steatosis in patients compared to time zero (study start).	[152]
WPI	23 cancer patients received 40 g WPI plus 2.64 mg zinc and 0.76 mg selenium oral snack for 12 weeks compared to a control group ($n = 19$) who received 40 g maltodextrin.	Baseline assessments at 6 and 12 weeks. Controls showed a significantly lower % change in plasma GSH levels whereas there was a significant time-dependent increase in the intervention group from time zero to 12 weeks and between whey and maltodextrin intervention at both 6 weeks and 12 weeks.	[153]
WP	A pilot open-label study of dietary supplementation with pressurised whey in 27 cystic fibrosis patients. Whey dosage: 20 g/d in patients <18 years and 40 g/d in older patients for 1 month.	Whole blood glutathione levels did not change from time zero to 1 month. Oral supplementation with pressurised whey improves nutritional status and can have additional beneficial effects on inflammation in patients with cystic fibrosis.	[8]
WPC immunocal	10 cystic fibrosis patients received whey protein isolate (20 g/d) for 3 months compared to a cystic fibrosis control group ($n = 11$) who received a casein placebo.	46.6% increase from time zero was observed in the lymphocyte GSH levels with whey.	[154]
WPI	5 d of energy balance, energy deficit, and resistance exercise after energy deficit. 8 males and 7 females healthy resistance-trained subjects completed resistance exercise and consumed either placebo or 30 g whey protein immediately post-exercise.	Muscle biopsies were obtained at 1 and 4 h into recovery in each trial. Resting protein levels of autophagy-related gene protein 5 decreased after energy deficit compared with placebo.	[155]
WPI	Acute study 4 h: Healthy, 9 sedentary male subjects fed two doses (0.8 or 1.6 g/kg body mass). Chronic study: 18 males subjected to 6-week aerobic (bike) training period and whey supplementation	No effect of the supplementation at either dose over the 4-h sampling period on blood glutathione concentration. The aerobic training period resulted in significantly lower glutathione concentrations in whole blood, an effect that was mitigated by WPI supplementation.	[9]
WPI	WPI (40 g/d) were supplemented to 31 overweight people with impaired fasting glucose/DM2 for 12 weeks.	An increase in glutathione peroxidase, a decrease in uric acid and no change in glutathione reductase, total antioxidant status, oxidative damage, inflammation and glucose markers were observed at 12 weeks compared to time zero. Significant improvements in anthropometric parameters and fat mass were also detected.	[156]
WPI	Whey protein supplementation (30 g, three times per day) and resistance training in 10 overweight young men compared to a control group and resistance training only group.	Increased levels of total antioxidant capacity and GSH was observed after whey supplementation compared to other treatment groups and compared to pre-test. Plasma vitamin C levels were significantly increased with whey supplementation compared to pre-test. Although exercise can lead to antioxidant system improvement and reduce some cardiovascular risk factors among overweight subjects, the combination of resistance training and whey consumption was more effective.	[157]
WPI immunocal	The effects of WPI supplementation (20 g/d) for 6 months on 15 Parkinson's disease patient was compared to 16 patients on soy protein supplementation.	Significant increases in plasma concentration of reduced GSH and the ratio of reduced to oxidised glutathione were found in the 15 WPI-supplemented patients compared to baseline. This was associated with a significant decrease of plasma levels of homocysteine.	[158]
WPI immunocal or iroTECTAMIN	Oral supplementation with two different Cys-rich whey protein formulas for 30 HIV-infected patients. Patients were randomised to a supplemental diet with a daily dose of 45 g of whey proteins.	Oral supplementation with Protectamin significantly increased plasma GSH levels in patients with advanced HIV-infection compared to baseline.	[159]
WPC	A double-blind clinical trial for 4 months with 9 HIV-infected children (6 years) who received whey protein (month 1 = 20% RDA protein, month 2 = 30% RDA, month 3 = 40% RDA,	WPC supplementation significantly increased erythrocyte GSH levels and significantly decreased CD8+ cells compared to baseline.	[160]

(continued)

Table 4. Continued.

Bovine whey product ^a	Description of the study	Results ^b	Reference
WPI, pressurised	month 4: 50% RDA) compared to control group. 2-week period, 18 healthy males and 18 healthy females were randomised into three different groups. Each group ingested 15, 30, or 45 g/d pressurised whey protein in the morning in bar format for 14 d.	Increases in lymphocyte GSH levels from pre to post supplementation was affected by the amount of whey protein ingested, with 45 g/d of whey supplementation over 2 weeks increasing lymphocyte GSH by 24% from baseline.	[161]

^aWPI: whey protein isolate; WP: whey protein, type not specified; WPC: whey protein concentrate.

^bGSH: glutathione.

gavage of adult male Sprague Dawley rats ($n = 6$) with the environmental pollutant acrolein (0.005 g/kg body weight/d) for 30 d resulted in a significant decrease in GSH levels (8.38 ± 1.17 nmol/mg protein) in red blood cells compared to the control group (11.31 ± 1.63 nmol/mg protein) ($p < .05$) [12]. Addition of whey protein at a dosage of 0.2 g/kg body weight/d did not halt the loss of GSH. In addition, this co-treatment with whey protein exacerbated an observed increase in plasma homocysteine levels and creatine kinase levels induced by acrolein, such that the levels of these markers in the co-treatment group were significantly higher than in the controls. This led the authors to hypothesise that intake of acrolein together with whey proteins may cause heart damage in rats [12]. Liver may also be adversely affected by whey treatment. Gürgen et al. [13] investigated liver health in Wistar albino male rats ($n = 10$) after a whey protein diet for 5 d (short-term) or 4 weeks (long-term). Hepatic injury was observed by abnormal hepatocyte histology and significantly increased levels of serum aspartate amino transferase and hepatic interleukin-1 β in rats that consumed whey compared to the control group, with the markers of liver injury worsening with increasing time of exposure to whey protein diet ($p < .05$).

Bioavailable antioxidant whey peptides

In addition to providing essential AAs and reduced thiols (Cys residues), whey proteins also contribute peptides with potential antioxidant activity [5,14,15]. However to be bioactive beyond the gut, whey peptides must survive gut transit and be bioavailable to their target [16]. The harsh conditions of the upper gastrointestinal tract function to hydrolyse proteins into individual AAs for transport across the intestinal barrier. Several recent studies have tracked the fate of whey proteins during upper gastrointestinal digestion using *in vitro* digestion models [14,17–20] or gastric [18,21] and jejunal effluents [22,23] from pigs [18,21,24] and humans [19,22,23] post consumption of various dairy foods (IF, WPI, skim milk powder, unpasteurised milk,

whey powder, and lactoferrin). Based on these studies, Table 5 lists locations within individual whey proteins from which peptides have been identified in the intestinal phase. β -Lg has four gut-resistant “hotspots;” consisting of fragments (f) with the following amino acid residues from the primary sequence f(41–58), f(92–100), f(126–138) and f(149–154). α -Lac also has 4 hotspots; f(17–27), f(63–68), f(80–90) and f(97–102). BSA has 5; f(11–18), f(107–114), f(219–224), f(489–495) and f(514–518). Lactoferrin has 7; f(67–77), f(140–145), f(216–228), f(289–295), f(309–318), f(332–337) and f(592–594). This suggests that these regions of the primary sequence are somewhat resistant to gastrointestinal digestion and peptides from these hotspots may survive the gut long enough to be transported across the intestinal barrier. Certainly, the presence of proline and/or aspartic acid, or glutamic acid residues within a peptide appears to confer a resistance to gastrointestinal digestion [23]. It should also be noted that peptide profiles differ according to degree of processing [24]. Table 5 also details whey peptides that have been reported to have potential redox activity encrypted within these hotspots [5,25–35]. These whey peptides have been reported to show redox activity in the ferric-reducing antioxidant power assay (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, oxygen radical absorbance capacity assay (ORAC) or the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. It should however be noted that all of these assays are generic *in vitro* assays conducted in the absence of alternative targets, and hence the data cannot be readily translated to more complex systems. In some cases, however, they have also been shown to boost cellular antioxidant status, albeit *in vitro* [5,15]. Cross matching gastrointestinal resistant hotspots to antioxidant bioactivity allows us to propose a list of potential antioxidant whey peptides likely to arrive in the bloodstream, post whey consumption. Little is known about peptides derived from whey circulating in the bloodstream. Jakobsson et al. [36] quantified α -Lac (140–250 μ g/L serum/L human milk/kg body weight) by radioimmunoassay in blood plasma from 1 month old breastfed

Table 5. Whey peptide sequences resistant to upper gastrointestinal digestion and antioxidant peptides encrypted within these sequences.

Whey protein ^{a,b}	Hotspots: location of peptide sequences resistant to gastrointestinal digestion ^c	Antioxidant whey peptides within these hotspots ^{b,c,d}
β -Lg [14,20,22,23,162]	f(41–58)	f(40–48), f(42–46), f(42–47), f(43–49), f(43–51), f(45–57), f(50–56), f(52–61), f(55–61), f(56–62), f(58–61) [26–28,32,33]
	f(92–100)	f(89–96), f(92–100), f(94–100), f(95–101), f(95–110), f(96–105), f(96–100) [25,27–29]
	f(126–138)	f(122–131), f(122–134), f(123–131), f(123–134), f(123–135), f(124–131), f(125–135) [25,30]
	f(149–154)	f(145–149), f(149–156), f(150–160), f(151–162) [26,27,31]
α -Lac [14,20,22,23]	f(17–27)	f(13–19), f(15–22), f(16–23), f(19–20), f(25–26) [27,33]
	f(63–68)	
	f(80–90)	f(82–88) [27]
BSA [14,22]	f(97–102)	f(99–102), f(101–104) [15,34]
	f(11–18)	
	f(107–114)	
	f(219–224)	
	f(489–495)	
Lactoferrin [14,19]	f(514–518)	
	f(67–77)	
	f(140–145)	
	f(216–228)	f(228–229) [35]
	f(289–295)	
	f(309–318)	
	f(332–337)	
	f(592–594)	

^aWhey proteins: β -Lactoglobulin (β -Lg), α -Lactalbumin (α -Lac), and bovine serum albumin (BSA).

^bReferences are in square brackets.

^cPeptide location (fragment) on primary sequence of whey protein is stated as name of whey protein, fragment (position from amino acid to amino acid) for example β -Lg f(41–58).

^dAmino acid in italic indicates the start or end of the antioxidant peptide sequence falls outside the gut resistant hotspots.

infants ($n = 3$), 30–60 min after feeding. Kuitunen et al. [37] also reported that plasma from 20 full term infants was positive for α -Lac and β -Lg, levels of which declined over time (almost 60% reduction by 8 months of age) as the infant gut barrier matured. No information on the sequences of the bioavailable peptides was provided. However, we have recently identified a number of antioxidant whey peptides post *in vitro* gastrointestinal digestion capable of traversing the tight junctions of Caco₂-HT29 monolayers (a widely accepted model for the intestinal barrier) and arriving in the basolateral compartment [15].

Redox modification of whey during processing

As whey products typically undergo several processing steps from milk to final food matrix, there is substantial evidence that processing can induce or exacerbate redox reactions that modify whey proteins. At the outset, milk produced for consumer consumption is heat-treated to kill bacteria and increase shelf life. Multiple methods are used including low (e.g. 15 s at 74 °C), high (e.g. 15 s at 90 °C), and ultrahigh (e.g. 145 °C for a

few seconds) temperature treatments. Most commercial milk is also homogenised by high pressure treatment to reduce fat globule size. Bovine liquid whey is then produced by either the enzymatic treatment of milk (sweet whey) or by the addition of acids or minerals (acid whey) both of which result in the precipitation, and therefore removal, of caseins. Significant modifications on whey proteins occur with processing, due to heat treatment (pasteurisation and spray drying), exposures to high pressures (e.g. during homogenisation), light exposure, the use of sterilisation/disinfection agents such as H₂O₂ (permitted at concentrations ≤ 16 mM in the USA [38]), high pH values (employed to give hydrolysed protein samples e.g. for IF) and long-term exposure to reducing sugars (e.g. lactose, glucose, galactose. and products from these).

Oxidation, glycation, and racemisation of whey proteins

Thermal treatment of milk and milk products can result in a significant increase in the level of oxidative modification and formation of protein carbonyls (with the

sites of some of these characterised [39]) and crosslinks. The levels of these materials have been proposed as a marker of milk powder quality [40]. The highest levels of protein oxidation products have been reported to be present in powdered IF [39,41–44]. α -Lac has been reported to show enhanced oxidation compared to β -Lg [45]. A recent study has reported that thermal treatment can induce reducible (disulphide) crosslinks in isolated β -Lg (100 μ M), but not isolated α -Lac (150 μ M) in both the absence and presence of H_2O_2 (500 μ M), which appears to be associated with the protein unfolding and the accessibility of the free Cys-121 residue on β -Lg [46,47]. Blocking this thiol prevented cross-link formation, underlining the importance of this residue. Mixed cross-links between β -Lg and α -Lac were detected when both proteins were co-treated [47]. Disulphide cross-links can also be generated *via* thiol-disulphide exchange (i.e. nonoxidative) reactions, with these occurring *via* attack of a free thiol (and usually the more reactive anion form) on a disulphide bond, with this resulting in an exchange of partners, and hence cross-link formation [48,49]. This process occurs more rapidly at higher temperatures and on protein unfolding, as this increases the accessibility of reaction sites [48]. These reactions can be enhanced by the addition of free thiols [50] and higher Ca^{2+} concentrations [51].

Exposure to both heat and H_2O_2 appears to have a greater effect than either agent alone, with this ascribed to the occurrence of a mechanism involving the formation of a sulfenic acid (RSOH species) at Cys121 in β -Lg mediated by H_2O_2 , once this residue is made accessible by thermal unfolding [46,47]. Limited loss of Met and Trp (at very high H_2O_2 concentration) has also been detected with both β -Lg and α -Lac on treatment with heat and H_2O_2 [46,47]. Increased exposure to heat (longer times and higher temperatures) and higher concentrations of H_2O_2 have been shown to enhance the extent of modification, as do combinations of these two factors [46,47]. No protection against damage was detected when radical trapping agents were included, indicating that these are molecular (two electron) and not radical (one electron) reactions [46]. These data indicate that oxidative damage can be selective with regard to the residues that are modified, and that unfolding of the proteins is a critical factor with regard to the extent of damage. Decreasing heat loads and oxidant exposure would therefore be expected to minimise whey protein modification.

Photo-oxidation arising from light exposure of milk preparations containing riboflavin (vitamin B_2 , an endogenous component of milk) can induce changes in milk protein structure (e.g. polymerisation, secondary

and tertiary structure of specific proteins), as well as inducing the formation of protein carbonyls, the Tyr oxidation products, di-tyrosine (di-Tyr) and 3,4-dihydroxyphenylalanine (DOPA), and the Trp-derived species, *N*-formylkynurenine (NFK) and kynurenine (Kyn) [52,53]. Different oxidant systems can therefore generate different patterns and extents of damage, and involve alternative mechanisms, though the overall effects (e.g. aggregation) may be similar. Light exposure has been used as a nonthermal technology to control pathogens and extend product shelf-life but this may result in increased formation of carbonyls and hence protein damage [54].

Presence of oxidation, glycation, and racemised whey proteins in IF

Whey protein modification is a potential health risk for infants fed with IF, and there is convincing data that indicates that breastmilk has considerable health benefits (reviewed [55]). Table 6 summarises the large number of different types and levels of protein modifications detected in IF samples. At a macromolecular level, these include both reducible (presumed to be disulphide-linked species, due to their loss on dithiothreitol treatment) and (multiple types of) nonreducible protein aggregates [56]. Evidence has been presented for the cross-linked species di-Tyr (a species arising from radical reactions), as well as lysinoalanine (LAL), lanthionine (LAN), and the precursor species for the latter two products, dehydroalanine (DHA) (Table 6) [56,57]. Whether the DHA arises from base-catalysed or radical-mediated reactions are unknown. Significant levels of protein carbonyls are also present in the samples, as detected by assaying total carbonyl content and also by using immunoblotting on proteins separated by SDS-PAGE, with the latter experiments indicating that a significant quantity of the carbonyls is present on high-molecular-mass aggregates [56]. The exact nature of the proteins involved and the sites within these proteins remain to be determined, though it is clear that there are significant structural changes to the IF proteins induced by processing [58].

Modifications have also been detected on IF proteins at the AA level, with evidence reported for the formation of Trp oxidation products (*N*-formylkynurenine, NFK; kynurenine, Kyn; and 3-hydroxykynurenine, 3OHKyn), di-Tyr, Phe-derived materials (3-hydroxyPhe, *m*-Tyr) and the Met oxidation product, methionine sulfide [56,59]. Some of these species are consistent with the species detected on isolated whey proteins

Table 6. Selected values of concentrations (nmol/mg protein except where otherwise indicated) of protein oxidation products, advanced glycation endproducts (AGEs) and racemised AAs present in powdered infant formula samples.

Products	Concentrations of oxidation products in infant formula samples	
	Intact protein IF	Hydrolysed protein IF
Protein carbonyls	2.88 ^a 8.6–60.9 [41] 19.4 [42] 6.5 [40]	0.85 ^b 18.3 [42]
Dehydroalanine (DHA)	0.47 ^a	0.58 ^b
Lanthionine (LAN)	0.01 ^a	0.09 ^b
Lysinoalanine (LAL)	0.03 ^a Below limit of detection [57] 18.2 ng mg ⁻¹ protein [42] 150–920 ng mg ⁻¹ protein [163] Higher values detected in liquid IF samples [164,165]	0.29 ^b 63.3 ng mg ⁻¹ protein [42]
Meta-tyrosine	0.97 ^a	1.83 ^b
Kynurenine	1.58 ^a	4.54 ^b
3-hydroxykynurenine	2.82 ^a	7.40 ^b
Methionine sulfoxide	64.97 ^a	24.82 ^b
Dityrosine	3.12 ^a 2.95 ^a 1.3 [42]	6.46 ^b 1 ^b 6.8 [42]
Carboxymethyllysine (CML)	36.3 ^a ng mg ⁻¹ protein 60.1 ng mg ⁻¹ protein [42] ~12 and ~145 ng mg ⁻¹ for low-temperature treated, and high-temperature heated IF respectively [125] 1.2 ng mg ⁻¹ protein [66]	81.0 ^b ng mg ⁻¹ protein 212.4 ng mg ⁻¹ protein [42]
Furosine	2.3 ^a µg mg ⁻¹ protein 5.6 µg mg ⁻¹ protein [42] ~2.7 µg mg ⁻¹ protein [66] 8.0–19.4 µg mg ⁻¹ protein [74] 13.2–15.5 µg mg ⁻¹ protein [166] 0.21–0.81 µg mg ⁻¹ protein [167] ~0.25–1.05 µg mg ⁻¹ protein [168] 7.0 µg mg ⁻¹ protein [169]	3.5 ^b (µg mg ⁻¹ protein) 2.4 µg mg ⁻¹ protein [42] 2.6 µg mg ⁻¹ protein [74] 0.52–0.81 µg mg ⁻¹ protein [167] ~0.6–0.9 µg mg ⁻¹ protein [168]
D-His	2.7 ^{a,c}	2.5 ^{b,c}
D-Lys	1.6 ^{a,c}	4.3 ^{b,c}
D-Phe	1.4 ^{a,c}	3.4 ^{b,c}
D-Tyr	1.1 ^{a,c}	d
D-Arg	5.5 ^{a,c}	32.0 ^{b,c}
D-Ala	1.3 ^{a,c}	d
D-Ile	0.3 ^{a,c}	d
D-Leu	0.7 ^{a,c}	1.4 ^{b,c}
D-Met	7.2 ^{a,c}	d
D-Ser	0.5 ^{a,c}	d
D-Val	4.3 ^{a,c}	0.3 ^{b,c}

Data from [113] except where otherwise indicated.

^aMean data from three commercial brands.

^bData from three replicate measurements from a single commercial brand.

^cData expressed as % of D-isomer relative to total (D+L) isomers.

^dNot detected.

exposed to specific oxidant systems (e.g. heat/H₂O₂ and also light in the presence of riboflavin) [46,47,52,60,61]. A number of the species detected are consistent with the occurrence of radical reactions (e.g. di-Tyr, which appears to be only formed by radical reactions), but others (e.g. methionine sulfoxide) can be formed by both radical and molecular (two electron) processes (e.g. direct oxidation by H₂O₂) [62]. A number of these materials may undergo additional reactions (e.g. redox cycling) that may exacerbate damage [63]. Furthermore, some of these products (e.g. those from Trp) may have multiple biological activities and contribute to disease, if taken up [64].

IF also contain significant levels of advanced glycation endproducts (AGE) materials including the early stage product furosine, and well-characterised AGEs including N^ε-(carboxymethyl)lysine (CML), N^ε-(carboxyethyl)lysine (CEL), pyrroline, and protein crosslinks, such as pentosidine (from Lys and Arg residue linked by a pentose), glyoxal lysine dimer (GOLD), methylglyoxal lysine dimer (MOULD), and Arg-derived products such as argpyrimidine and hydroimidazolone isomers [56,65–72]. The levels of these materials have been examined using a wide variety of both direct and indirect methodologies [66,73,74]. AGE is a chemically heterogeneous group of compounds. AGE formation in dairy products involve Maillard reactions, but

the reaction conditions differ greatly depending on thermal load during processing [75,76]. Multiple AGEs have been characterised in foods (for a review of experimental methods, see Aalaei et al. [77]) and in human tissues, possibly as a result of dietary exposure, including the Lys-derived products: *N*^ε-(carboxymethyl)lysine (CML), *N*^ε-(carboxyethyl)lysine (CEL), pyrroline, and protein crosslinks such as pentosidine (from Lys and Arg residue linked by a pentose), GOLD, MOULD and Arg-derived products including argpyrimidine and hydroimidazolone isomers. Changes in AGE levels, most commonly measured by mass spectrometry [65], have been used to assess glycation/glycooxidation status in foods [78,79].

Analyses for the presence of D-AAAs showed that significant levels of these un-natural AA isomers are also present, though whether these arise *via* oxidation or high temperature/high pH reactions is unclear [56]. In some cases the levels of these species are very high, but it is impossible to eliminate the possibility that some of these arise during processing for analysis, rather than these being present endogenously. Further work needs to be carried out to clarify this point.

Comparison of IF samples containing native bovine proteins with those that contain hydrolysed proteins (hypoallergenic brands, which contain predominantly small peptides and free AAAs), shows that most markers of modification are consistently present at higher levels in IF with hydrolysed proteins [56]. This has been ascribed to the additional processing required to generate these samples (i.e. longer and more complex production times) as well as the decreased steric and electronic hindrance for reactions at free AAAs/small peptides when compared to intact proteins. Thus many modification reactions occur more rapidly with small peptides/free AAAs, than with intact proteins, though this is not always the case. For glycation reactions, a further factor that may enhance the extent of modification in the hydrolysed protein brands, is the elevated level of N-terminal amines generated on proteolysis of the original protein peptide bonds [80]. This may be a significant contributor to the higher levels of AGEs present in these samples. However, the levels of *side-chain* derived species, such as furosine and CML (formed from reaction at the ϵ -amine group of Lys side-chains) have also been reported to be approximately double those present in the intact protein brands [56].

Biological effects of oxidation, glycation, and racemised AAAs

The impact of modified proteins on human health has been investigated to only a limited extent [81], but

there is increasing interest in the effects of oxidised proteins on human health [82]. Consumption of oxidised food components can increase the level of oxidative stress in living tissues and this appears to contribute to the development of some diseases [83–85]. This area has been recently reviewed by Delgado-Andrade and Fogliano [86]. This is considered to be associated with the exposure of the gastrointestinal tract and internal organs to potentially cytotoxic and mutagenic materials [87–89]. Oxidised AAAs/peptides may impair homeostasis and cell toxicity *via* multiple different mechanisms. For example, L-Phe can be oxidised by HO \cdot to give *m*-Tyr, which is cytotoxic by a pathway that appears to involve incorporation of the oxidised AA into proteins [90]. Dietary intake of oxidised Tyr (e.g. di-Tyr) in rats has been reported to induce oxidative damage and hepatic fibrosis *via* MAPK/TGF- β 1 pathway [91]. Exposure to various Kyn species (Trp oxidation products) appears to be injurious as these have been associated with both neurotoxicity and pathogenesis of intestinal diseases [44,92–94]. The high levels of methionine sulfoxide (up to 74% of parent Met) in some milk products appears to be responsible for inducing changes in redox homeostasis, thus showing a toxic potential [59,95]. This may arise from the reduction of the methionine sulfoxide by the family of methionine sulfoxide reductase enzymes present in most organisms (including humans). These enzymes require reducing equivalents from the thioredoxin/thioredoxin reductase/NADPH system, with the consumption of NADPH resulting in a depletion of reducing equivalents, an oxidative stress, and a change in the redox homeostasis of the cell. Oral intake of some oxidised AAAs has been shown to induce hepatic and renal fibrosis in mice, possibly *via* impairment of antioxidant defence systems and modification of the Nrf2-ARE gene pathway [96]. In addition, increased levels of protein carbonylation, di-Tyr and advanced protein oxidation products have been found in the liver, kidney and blood of mice, in response to the intake of modified materials, consistent with these causing oxidative injury *in vivo* [96].

The safety of Maillard reaction products (MRPs) is a concern, as some of these (e.g. acrylamide) are potential, or known, mutagens or carcinogens [97,98]. This area has been recently reviewed [99,100]. However, some MRPs have also been proposed to exert antioxidative, antibiotic, and antiallergic activity [101,102]. Thus reductones have been shown to possess strong antioxidative capacity through electron transfer, hydrogen atom donation and metal ion chelation (e.g. of Cu and Fe ions which might otherwise act as pro-oxidants)

[101–104]. Food-derived AGEs have, however, also been reported to promote oxidative stress and inflammation, and contribute to chronic disease [100,105]. A recent study has demonstrated that oral intake of CML promotes its accumulation in the gastrointestinal tract of rats, stimulates inflammatory responses by downregulating enzymatic antioxidative pathways and increases the levels of inflammatory cytokines [106]. Long-term exposure of rats to dietary AGEs has been reported to increase colon permeability, modulate gut microbial ecology in a detrimental manner and induce host metabolic disturbances which can adversely impact host health [107,108]. In contrast, other studies have reported an absence of deleterious effects [109], and even beneficial effects of dietary AGEs [99], with a significant increase in the total antioxidant capacity of plasma from rats observed after oral administration of a diet supplemented with pronyllysine (a Lys product) [110]. Whether dietary AGEs have a detrimental or beneficial impact on human health is controversial, due to difficulty in carrying out controlled trials [109,111].

As non-native AAs may be absorbed across the gastrointestinal tract, the presence of modified whey protein species in foods or IF may pose a health risk to infants, and consequently a number of studies have examined both the uptake of these modified AAs and peptides across cell monolayers, and the effects of these materials on cells in culture and animal growth [112].

Exposure (for 24–48 h) of nondifferentiated Caco₂ cells (a human intestinal epithelial cell line) to a range of modified AAs species (oxidised, glycated, D-isomers; either singly or as mixtures), at concentrations similar to those reported to be present in IFs (e.g. 20 µM), does not appear to have deleterious effects on cell growth [113]. In some cases, increased metabolic activity (as measured by MTT assay) was detected compared to control cells [113]. However, exposure to combined oxidation products, AGEs or D-AAAs for 2 h at concentrations of 20 or 200 µM, increased protein carbonyl levels in the cells in a dose-dependent manner, consistent with the induction of oxidative stress [113]. Studies with individual protein modification products suggest that *m*-Tyr may be a driver of this change, possibly *via* mis-incorporation (in place of Phe or Tyr) into new proteins. Such incorporation has been shown in previous studies to result in the formation and/or accumulation of truncated and dysfunctional proteins [90,114,115]. Other studies have reported increased extracellular matrix production induced by oxidised Tyr species in rat kidneys [116].

Long term (e.g. 21 d) incubation of Caco₂ cells on filter inserts in transwells results in the formation of intact

monolayers and expression of transport proteins and brush border hydrolases [117]. At the equivalent concentrations to those detected in IF, and a 2-h exposure time (to mimic gut transit times [117]) the modified AAs did not induce changes in the transepithelial electrical resistance (TEER), or permeability as assessed using lucifer yellow [113]. These data indicate that these concentrations of modified materials do not induce gross changes to the monolayers, or cell toxicity, under the conditions employed. Quantification of modified AAs added to the apical side of the transwells, at 20 or 200 µM, showed that there was no significant decrease in the concentration of these species over the 2-h period, indicating that these species do not rapidly equilibrate across the monolayer, and that these materials are stable over this incubation period [113]. However, some material was detected (by LC-MS) in the basolateral compartment, consistent with limited monolayer penetration. The permeability was between 0.003 and 0.095% for the 20 µM concentration group, with 3OHKyn being the least abundant, and *m*-Tyr the highest. This high permeability of *m*-Tyr is consistent with the induction of protein carbonyls by this compound (see above). The permeability increased as the apical concentration was increased, with di-Tyr giving the highest basolateral concentrations [113]. The levels of the modified AAs present in lysates from the Caco₂ cells, after 2-h exposure, were too low to be detected [113]. AGEs appear to be only slowly transported across Caco₂ cell monolayers [113,118]. Whether transepithelial transport occurs *via* diffusion, AA transporters (eg B^o +, b^o +, and y⁺ [119]), endocytosis, or other mechanisms remains to be established. Previous studies have shown that AGEs can penetrate across cell monolayers [118,120–122], but this appears to occur, in at least some cases, by uptake of di-peptides rather than free AAs. Thus, evidence has been presented for uptake of di-peptides containing CML, CEL, ArgP, and pyralline [118,122], *via* specific peptide transporters (e.g. the proton-coupled peptide transporter 1, PEPT1). These di-peptides are then subject to intracellular hydrolysis to the free modified AAs, which subsequently arrive in the basolateral compartment *via* passive diffusion [122]. Whether this is also true for oxidised AAs remains to be determined. Data have been reported on the plasma and urinary levels of CML in breast milk- and formula-fed infants consistent with uptake across the gastrointestinal tract in infants, and subsequent excretion in urine [123]. Furthermore, infants fed on formula have been reported to have significantly increased levels of antibodies against oxidised low-density-lipoproteins,

consistent with significant dietary exposure to oxidising species [124].

Overall, these data suggest that modified AAs from IFs may induce mild oxidative stress to cells, as evidenced by an increase in cellular protein carbonyls [113]. However, these products appear to penetrate across Caco₂ cell monolayers to only a moderate extent under the conditions examined. The effects of long-term exposure, and potential accumulation [106], have not been examined in great detail, and this may be significant given that infants are typically fed every few hours for many months. Whether this results in long-term adverse health effects is controversial, though some epidemiological studies have suggested that long-term feeding with IF can induce inflammatory responses [125] and predispose to an increased risk of diabetes later in life [82,112,126–128]. Whether this is related to exposure to the modified AAs present in IFs, or from other effects (e.g. immunological responses to bovine proteins [129,130]) remains to be determined.

Conclusions

Human intervention studies have provided evidence that consumption of whey proteins can boost antioxidant markers in blood and various organs. This health benefit is likely to be as a result of the contribution of reactive free thiol groups (Cys residues) for GSH synthesis and the presence of bioavailable bioactive peptides. However, whey proteins undergo considerable processing steps from raw milk to consumed products which makes them vulnerable to oxidation, glycation and racemisation. These modifications may have adverse effects on human health particularly when consumed over a prolonged period of time, though there is a lack of definitive data with regard to the specific effects of known concentrations of specific products. Further work is clearly warranted in this area. Alternative processing maps and storage conditions may be needed to protect whey proteins from oxidation during processing and capitalise on whey as a dietary antioxidant.

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