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Invited review: 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 summarizes 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 1 enzyme, subtilisin, 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 proteins must not only survive processing, but also upper gut transit and arrival in the bloodstream, if whey products are to promote antioxidant levels in target organs. Studies reveal that direct cell exposure to whey samples increases intracellular antioxidants such as glutathione. However, the physiological relevance of these in vitro assays is questionable, and evidence is conflicting from dietary intervention trials, with both rats and humans, that whey products 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 sulfur species]. Oxidative stress ensues, leading to cell injury such as protein and lipid peroxidation, DNA strand breakage, racemization or decarboxylation of AA, 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., 2015), and cardiovascular disease (Sugamura and Keaney, 2011; Lönn 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, whereas CAT converts hydrogen peroxide to water (Weydert and Cullen, 2010). The cytosolic Cys tripeptide, γ-glutamyl-cysteinyl-glycine, reduces hydroperoxides to alcohols and hydrogen peroxide to water by converting from its reduced (**GSH**) to its oxidized form. 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 their nutritional, health-promoting, and functional values (Ramos et al., 2017). Bovine liquid whey is produced by enzymatic treatment of milk (sweet whey) or addition of organic acids or minerals (acid whey) with the precipitation and removal of casein (Yadav et al., 2015). Bovine WP account for 11 to 14.5% of dry whey; the other components of bovine whey powder are lactose (63–75%), fat (1–1.5%), minerals (8.2–8.8%), and vitamins (A, C, E,

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and B groups; Miller et al., 2006; Yadav et al., 2015). The protein component of whey provides a complete protein source and is rich in sulfur-containing AA (1.7%; Fox et al., 2015) and in branched-chain AA (26%; Ha and Zemel, 2003; Paul, 2009). It is composed of β-LG (50–60%), α-LA (15–25%), BSA (6%), lactoferrin (<3%), and immunoglobulins (<10%; de Wit, 1998; Madureira et al., 2007; Le Maux et al., 2014). Beta-lactoglobulin is a small globular protein, composed of 162 AA with a molecular weight ($\mathbf{M_W}$) of approximately 18,300 g/mol (Rade-Kukic et al., 2011). It contains all 20 EAA and is a rich source of sulfur. From a GSH precursor perspective, it has 5 Cys residues, 4 of them involved in disulfide bonds with the remaining 1 having a free reactive thiol group (Le Maux et al., 2014). Alpha-lactalbumin is a small protein with a M_W of 14,200 g/mol consisting of 123 AA arranged in a single peptide chain (Konrad and Kleinschmidt, 2008). It has 8 Cys as 4 disulfide bonds and, therefore unlike β-LG, has no free thiol group (Konrad and Kleinschmidt, 2008; Pepe et al., 2013). Bovine serum albumin is composed of 583 AA with a M_W of 66,430 g/mol (Hirayama et al., 1990). It contains 35 Cys groups making 17 disulfide bonds in addition to a free Cys which can facilitate intramolecular disulfide interactions (Madureira et al., 2007). Lactoferrin (M_W 80,000 g/mol) is an iron-binding monomeric globular glycoprotein (Wakabayashi et al., 2006) that contains 708 AA, of which 34 are Cys and all of which participate in disulfide bonds (Marshall, 2004). In addition, each lactoferrin monomer can bind 2 Fe^{3+} ions, with a binding affinity of 10 to 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). Bovine WP also contains dilute concentrations of immunoglobulins [Ig_A, Ig_M, and 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 50,000–70,000 g/mol) linked by disulfide bonds (Madureira et al., 2007). Several bovine whey products are produced commercially (Table 1) and differ primarily in protein content and lactose concentration.

Table 1. Composition of commercial whey protein products

	Composition		
Whey products	Protein (%)	Fat (%)	Lactose (%)
Whey protein concentrate	34–80	1–7	4–52
Whey protein isolate	90–95	0.5–1	0.5–1
Hydrolyzed whey protein	80–90	0.5–8	0.5–10

DO WHEY PRODUCTS 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**). Table 2 details the most recent studies of the antioxidant activity of whey products after processing, 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 subtilisin (EC 3.4.21.62), a nonspecific endopeptidase purified from *Bacillus licheniformis* and commercially available as Alcalase (Novozymes A/S, Bagsvaerd, Denmark), with specific activities ranging from 0.6 to 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 subtilisin also showed significantly greater inhibition than 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 subtilisin rather than by the enzymes as bacillolysin [EC 3.4.24.28, commercial source Neutrase (Novozymes A/S, Bagsvaerd, Denmark)] or Protamex (EC 3.4.21.14, Novozymes A/S). The WPC hydrolyzed by subtilisin also showed more ferric-reducing power (0.55 m*M* $FeSO_4$ equivalents) than trypsin (EC 3.4.21.4), pepsin (EC 3.4.23.1), or leucyl aminopeptidase (EC 3.4.11.1, commercial source Flavourzyme, Novozymes A/S) hydrolysates (0.35 m*M* $FeSO_4$ 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 [nonheated WPH = 0.32 ± 0.03 μmol of Trolox equivalents (**TE**)/mg of protein; heated WPH = 0.30 ± 0.03 μmol of TE/mg of protein] or chymotrypsin (EC 3.4.21.1; nonheated WPH = 0.27 ± 0.04 μmol of TE/mg of protein; heated WPH = 0.31 ± 0.02 μmol of TE/mg of protein). In Adjonu et al. (2013), ORAC methodology was employed, which scavenges peroxyl radicals and compares levels to the vitamin E analog, Trolox.