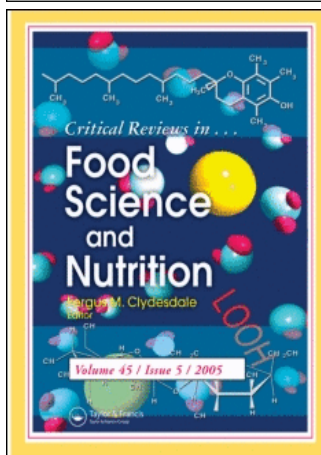


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Antioxidant Activity of Proteins and Peptides

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Proteins can inhibit lipid oxidation by biologically designed mechanisms (e.g. antioxidant enzymes and iron-binding proteins) or by nonspecific mechanisms. Both of these types of antioxidative proteins contribute to the endogenous antioxidant capacity of foods. Proteins also have excellent potential as antioxidant additives in foods because they can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems. A protein's overall antioxidant activity can be increased by disruption of its tertiary structure to increase the solvent accessibility of amino acid residues that can scavenge free radicals and chelate prooxidative metals. The production of peptides through hydrolytic reactions seems to be the most promising technique to form proteinaceous antioxidants since peptides have substantially higher antioxidant activity than intact proteins. While proteins and peptides have excellent potential as food antioxidants, issues such as allergenicity and bitter off-flavors as well as their ability to alter food texture and color need to be addressed.

Keywords protein oxidations, food emulsions, antioxidants, lipid oxidation

INTRODUCTION

Oxidative reactions in foods lead to the deterioration of quality attributes such as flavor, aroma, texture, and color. The main targets of oxidative reactions that affect food quality are lipids and proteins. In lipids, these reactions typically involve free radical pathways that result in the formation of lipid hydroperoxides, which are eventually decomposed to low molecular weight carbonyls in the presence of prooxidants. These carbonyls are associated with rancid aroma and can interact with compounds such as proteins to alter functionality. However, free radicals mechanisms are also involved in protein oxidation. To date, there is a significant body of evidence in the literature that demonstrates the oxidation of individual amino acid residues within proteins, with particular emphasis given to those residues containing sulfur or aromatic R-groups (Davies, 2005; Hernandez-Ledesma et al., 2005; Levine and Stadtman, 2001; Stadtman and Levine, 2001; Stadtman and Levine, 2003; Stadtman et al., 2003). In recent years, a number of studies have linked protein oxidation with a variety of human diseases, including diabetes (Dean et al., 1997; Hicks et al., 1988; Wolff and Dean, 1987), atherosclerosis

(Dean et al., 1997; Parhami et al., 1993), and neurodegenerative disorders (Dyrks et al., 1993; Smith et al., 1991; Smith et al., 1992). For example, it has been suggested that the symptoms of Alzheimer's disease is attributable to the accumulation of aggregated amyloid β -protein as neurofibrillary tangles in the brain resulting from oxidation reactions (Butterfield, 2002; Nunomura et al., 2001; Shringarpure et al., 2000; Varadarajan et al., 2000). Furthermore, the content of protein carbonyls in Alzheimer's brain samples is greater than in age-matched controls (Dean et al., 1997; Dyrks et al., 1993), which indicates that protein oxidation is more pervasive in those with the disease. The mounting evidence that proteins are susceptible to oxidation suggests that free radicals could negatively affect both proteins and lipids in foods. However, the ability of proteins to interact with free radicals in foods could also lead to the development of novel antioxidant technologies if proteins could be used to inhibit oxidative reactions thus protecting oxidatively labile fatty acids. While many proteins have antioxidant potential, it should be recognized that some proteins such as heme proteins and lipoxygenases are prooxidative. The remainder of this paper will focus on proteins that could be used to inhibit oxidative reactions.

The oxidative stability of foods is related to the balance between antioxidative and prooxidative factors. The biological tissues from which foods are obtained contain numerous antioxidant systems to maintain the antioxidant/prooxidant

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balance in favor of antioxidative protection, thus protecting those tissues from oxidative damage. These antioxidant systems include free radical scavengers (e.g. ascorbate and tocopherols), metal chelators (e.g. organic acids), and enzymes (e.g. catalase and superoxide dismutase) that inactivate reactive oxygen species. However, non-enzyme proteins also contribute to the antioxidant capacity of biological tissues. For example, it is estimated that blood proteins provide 10–50% of the peroxyl radical trapping activity of plasma (Frei et al., 1998; Wayner et al., 1987).

While the antioxidant-prooxidant balance in healthy biological tissues is normally controlled to provide an antioxidative environment, this is not always true in foods. Food processing operations increase oxidative stress by introducing oxygen (e.g. grinding, mixing, and homogenizing operations), removal of natural antioxidants (e.g. physical and chemical separation steps of oil refining), destruction of endogenous antioxidants (e.g. heat inactivation of antioxidant enzymes), and increasing prooxidative factors (e.g. light exposure that generates singlet oxygen, and thermal treatments that release protein-bound transition metals). Another factor that increases oxidative stress is the current trend of incorporating oxidatively labile unsaturated fatty acids, such as omega-3 fatty acids, into foods for the benefit of consumer health.

The number of antioxidant intervention strategies available for foods is limited and will likely decline as the consumer demand for “all natural” products increases. New antioxidant technologies are clearly needed. The ability of proteins to inhibit lipid oxidation makes them an important component of the antioxidant defense of biological tissues from which foods are produced. Therefore, it may be possible to increase the oxidative stability of a food by protecting endogenous antioxidant enzymes, enhancing the activity of proteins found naturally in foods by altering protein structure, introducing antioxidant proteins by genetic engineering, or through the use of proteins or peptides with antioxidant activity as food additives. By taking advantage of the antioxidant properties of proteins, manufacturers could have an added tool to improve the oxidative stability of foods. However, the use of proteins to inhibit lipid oxidation could be limited by the ability of proteins to impact the texture (through gelation and viscosity enhancement), color (through light scattering and formation of Maillard browning products) and flavor (as flavor reactants and bitter compounds, e.g. peptides).

PROTEIN OXIDATION REACTIONS

As is the case with most macromolecules of biological origin (e.g. lipids, carbohydrates, DNA), proteins are susceptible to oxidative modification (Davies and Dean, 1997). There is a wide range of reactive oxygen species (ROS) and free radicals capable of promoting protein oxidation *in vivo* and in foods. These reactive species are generated from a number of pathways which may include radiative processes (e.g. γ -rays, x-rays, ultraviolet radi-

ation), air pollutants, metal-catalyzed oxidation reactions, lipid oxidation, and the production of ROS from the electron transport chain of mitochondria, neutrophils and macrophages (Davies et al., 1997; Dean et al., 1997; Levine et al., 2001; Stadtman and Berlett, 1997). The oxidation of proteins typically results in the modification of amino acid R-groups, although polymerization and/or fragmentation reactions of proteins are also possible under certain oxygen concentrations. For example, when bovine serum albumin (BSA) is oxidized with hydroxyl radicals in the absence of oxygen, extensive protein cross-linking is observed while in the presence of oxygen, alteration of protein size occurs only to a limited extent (Dean et al., 1997). Abstraction of hydrogen atoms alpha to backbone carbons can result in fragmentation of peptide bonds (Davies et al., 1997; Viljanen et al., 2005).

The oxidative susceptibility of a given amino acid residue to free radical attack is dictated in large part by its functional R-group (side chain); however, solvent accessibility and the chemical properties of vicinal residues are also important (Davies et al., 1997; Levine et al., 1996). Despite the fact that all 20 biologically-derived amino acids are potentially oxidizable, the most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine, and phenylalanine) from which hydrogen is easily abstracted. Histidine's imidazole-containing side chain is also oxidatively labile. The oxidative modification of a given amino acid can yield a variety of products. The most common oxidation products formed from the free amino acids cysteine, methionine, tryptophan, tyrosine, phenylalanine, and histidine, as well as their respective mechanisms of formations, are summarized in Table 1.

The oxidation chemistry of free amino acids is believed to be very similar to the products detected on amino acids in peptides or proteins (Davies et al., 1997). However, once an amino acid residue is oxidized, radical transfer reactions (e.g. transfer of damage from one side chain to another) can take place. The intramolecular transfer of radicals from one amino acid residue to another is largely dependent on the protein's physical structure. In the case of erabutoxin *b*, a neurotoxin with one tryptophan (position 25) and one tyrosine (position 29), very little radical transfer is observed between the two side chains, despite the fact that the residues are only 1.3 nm apart (Butler et al., 1982; Davies et al., 1997; Prutzel et al., 1982). This has been attributed to the especially rigid nature of the erabutoxin *b*, which has a relatively inflexible tertiary structure due to the presence of four disulfide bonds (Davies et al., 1997). Chemical reduction of the disulfide bridges allowed the protein to adopt a more flexible conformation, and the rate at which Trp25 and Tyr29 exchanged radical damage was found to concomitantly increase (Prutzel et al., 1982).

The selectivity of radical attack on amino acid side chains is inversely related to the energy of the oxidative insult. For example, the highly reactive hydroxyl radical is capable of attacking the side chain of any amino acid residue, and is therefore considered non-selective. In cases where high-energy radicals are present, side chain damage is typically a function of the

Table 1 Stable, or semi-stable, products generated as a result of exposure to selected amino acids to radical species and their postulated mechanisms of formation; adapted from (Davies and Dean, 1997)

Amino acid	Product	Mechanisms of formation 1017
Cysteine (Cys)	(1) Cystine (2) Oxy acids	(1) Hydrogen abstraction from SH group and subsequent radical dimerization (2) Hydrogen abstraction from SH group, subsequent reaction with oxygen, and isomerization
Methionine (Met)	Methionine sulfoxide	Variety of routes including both radical and non-radical reactions
Tryptophan (Trp)	<i>N</i> -formylkynurenine, kynurenine, 5-hydroxytryptophan, 7-hydroxytryptophan	HO· attack or one-electron oxidation of ring
Tyrosine (Tyr)	(1) 3,4-dihydroxy- phenylalanine (2) Di-tyrosine (3) 3-chlorotyrosine (4) 3-nitrotyrosine (5) Tyrosine hydroperoxide and subsequent cyclicized materials	(1) HO· attack or one-electron oxidation of aromatic ring (2) HO· attack or one-electron oxidation of tyrosine and subsequent radical-radical dimerization; HOCl (3) Chlorination of tyrosine as a result of exposure to HOCl or other chlorinating agents (4) Reaction of reactive nitrogen species with tyrosine (5) Formation of tyrosine phenoxyl radical in presence of O ₂ · ⁻
Phenylalanine (Phe)	(1) <i>o</i> -, <i>m</i> -tyrosine (2) Dimers of hydroxylated aromatic amino acids	(1) HO· attack or one-electron oxidation of aromatic ring. Possibly via reaction nitrogen species (2) HO· attack or one electron oxidation before or after dimerization
Histidine (His)	2-oxo-histidine	HO· attack or one-electron oxidation

physical location of where the radical is produced (i.e. the amino acids closest to the site of radical production is oxidized). Conversely, in systems containing lower-energy radical species, the most labile amino acid side chains (cysteine, methionine, tryptophan, tyrosine, histidine, and phenylalanine) are oxidized more frequently than non-reactive, aliphatic residues (Davies et al., 1997). Hydroperoxyl radicals are commonly observed in oxidized food lipids. These radical species have low to intermediate reduction potential values ($E^{0'} \approx 1000$, pH 7) relative to those of hydroxyl radical ($E^{0'} = 2310$ at pH 7) (Buettner, 1993); therefore, hydroperoxyl radicals are considerably more selective in attacking reactive side chains than hydroxyl radicals. The fact that proteins can interact with free radicals and ROS suggests that they could protect lipids from oxidation if they are oxidized preferentially to unsaturated fatty acids. Preferential oxidation of proteins could occur if available amino acids are more oxidatively labile than unsaturated fatty acids, or if the physical location of a protein places it near the site of free radical or ROS generation where the protein would be able to rapidly scavenge the free radical prior to the migration of the radical to lipids.

The oxidation of proteins can be detected in biological tissues and foods by a variety of methods. These include electron paramagnetic resonance (EPR), protein fragmentation and/or polymerization rates, changes in proteolytic susceptibility, and the detection of specific amino acid oxidation products (Stadtman and Levine, 2003). Many of the amino acid oxidation products contain carbonyl derivatives that can be measured easily by spectrophotometric methods (Requena et al., 2003). In living tissues, proteins carbonyls increase with oxidative stress and age (Stadtman, 2006). Protein carbonyls have also been detected in meat and dairy products, indicating that protein oxidation occurs during food processing and storage (Srinivasan et al., 1996; Fedele and Bergamo, 2001; Stagsted et al., 2004; Salminen et al., 2006). Unfortunately, many of these studies do not address the relative kinetics of lipid and protein oxidation; therefore, it is

unclear if the protein protects the lipid or if the lipid causes the oxidation of the protein.

ANTIOXIDANT ACTIVITY OF PROTEINS AND PEPTIDES ADDED TO FOODS

There are several studies that demonstrate the ability of proteins to inhibit lipid oxidation in foods. Proteins originating from milk, blood plasma, and soy protein all have been shown to exhibit antioxidant activity in muscle foods (Table 2). Porcine blood plasma (2.5%) contains antioxidant proteins such as serum albumin and transferrin, and can retard the formation of thiobarbituric acid reactive substances (TBARS) in both salted ground pork (Faraji et al., 1991) and cooked ground beef (Shantha and Decker, 1995). Whey protein concentrate is antioxidative in cooked beef (Shantha and Decker, 1995), and whey and soy proteins inhibit lipid oxidation in cooked pork patties containing 2% protein (Pena-Ramos and Xiong, 2003), with soy protein isolate being more effective than whey proteins. Whey proteins have also been found to inhibit lipid oxidation in oil-in-water emulsions (Taylor and Richardson, 1980; Allen and Wrieden, 1981a and b; Donnelly et al., 1998; Tong et al., 2000; Elias et al., 2005). Park et al. (2005) found that soy protein inhibited the oxidation of ethyl esters of eicosapentaenoic acid dried in a maltodextrin-stabilized, freeze-dried emulsion powder system.

Peptides can also inhibit lipid oxidation in foods. Whey, casein, soy, and egg yolk hydrolysates have been shown to inhibit lipid oxidation in various muscle foods, such as beef, pork, and tuna (Sakanaka and Tachibana, 2006; Diaz et al., 2005; Sakanaka et al., 2005; Pena-Ramos and Xiong, 2003). Endogenous peptides found in foods can also act as antioxidants. Carnosine and anserine are histidine-containing dipeptides found in skeletal muscle that exhibit antioxidant properties (Chan and Decker 1994). Addition of carnosine to muscle foods can inhibit lipid

Table 2 Examples of protein sources, specific proteins and protein hydrolysates or peptides that exhibit antioxidant activity in food systems

Type	Reference
<i>Protein source</i>	
Dairy	Taylor and Richardson, 1980
Soybean	Pena-Ramos and Xiong, 2003
	Park et al., 2005
	Faraji et al., 2004
Egg yolk	Sakanaka and Tachibana, 2006
Blood Plasma	Faraji et al., 1991
Potato	Shantha and Decker, 1995
Gelatin	Wang and Xiong, 2005
	Park et al., 2005
<i>Specific protein</i>	
Casein	Diaz et al., 2003
	Diaz et al., 2005
β -Lactoglobulin	Elias et al., 2005
Transferrin	Mancuso et al., 1999
Lactoferrin	Satue-Gracia et al., 2000
	Nielsen et al., 2004
Zein	Kong and Xiong, 2006
<i>Peptides</i>	
Hydrolyzed Egg Proteins	Sakanaka and Tachibana, 2006
Hydrolyzed Gelatin	Park et al., 2005
Hydrolyzed Soy Proteins	Pena-Ramos and Xiong, 2003
	Park et al., 2005
Hydrolyzed Whey Proteins	Elias et al., 2005
	Pena-Ramos and Xiong, 2003
	Hernandez-Ledesma et al., 2005
	Sakanaka et al., 2005
Hydrolyzed Casein	Rival et al., 2001
	Diaz et al., 2003
	Diaz et al., 2005
Hydrolyzed Potato	Wang and Xiong, 2005
Carnosine	Chan and Decker, 1994
Anserine	Chan and Decker, 1994

oxidation and inhibit myoglobin discoloration (Calvert and Decker, 1992; Decker and Crum 1991). Park et al. (2005) found that soy peptide, gelatin peptide, and carnosine all inhibited the oxidation of freeze-dried ethyl esters of eicosapentaenoic acid encapsulated in maltodextrin.

ANTIOXIDANT MECHANISMS OF PROTEINS

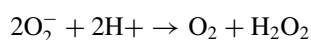
Proteins can inhibit lipid oxidation by biologically designed mechanisms (e.g. antioxidant enzymes and iron-binding pro-

teins) or by nonspecific mechanisms. Both of these types of antioxidative proteins contribute to the endogenous antioxidant capacity of foods and could also be used as potential antioxidant additives. Overall, the antioxidant activity of proteins is due to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, enzymatically eliminate specific oxidants, and alter the physical properties of food systems in a way that separates reactive species (Table 3). Proteins are somewhat unique in this way compared to other food antioxidants, in that they can potentially act as multifunctional antioxidants that can inhibit several different lipid oxidation pathways.

Antioxidant Enzymes

Superoxide Dismutase

The addition of an electron to molecular oxygen produces the relatively reactive superoxide anion. Superoxide anion is prooxidative due to its ability to reduce transition metals, release protein-bound metals, and form perhydroxyl radical which can directly catalyze lipid oxidation under acidic conditions (pH < 4.8) (Decker, 2002). Because superoxide anion can promote oxidative reactions, many biological organisms keep this reactive oxygen species in check with the enzyme superoxide dismutase (SOD). Isoforms of SOD contain either copper plus zinc in the active site or manganese, and both catalyze the conversion of superoxide anion to hydrogen peroxide by the following reaction:



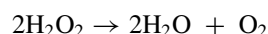
Peroxidases

Hydrogen and lipid peroxides are important in oxidative reactions because they are commonly found in foods where they can decompose to form free radicals. For example, hydrogen peroxide is decomposed by the reduced state of transition metals (e.g., Fe and Cu) to the hydroxyl radical, an extremely reactive radical that can oxidize lipids, proteins, and most organic matter at diffusion-limited rates. Catalase (CAT) is a heme-containing enzyme found in many biological systems that catalyzes the

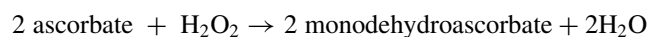
Table 3 Ways in which proteins and peptides inhibit or reduce the net production of volatiles associated with oxidative rancidity

Precursors to rancidity	Potential strategies for inhibition	Example
Location of lipid and prooxidant	Partitioning and/or physical hindering substrate prooxidant interactions	Engineering of emulsion droplets to produce thick and/or cationic interfaces
Prooxidants	Inactivation or inhibition of prooxidative metals	Metal chelation
Peroxy radicals	Free radical scavenging	Sacrificial amino acid oxidation
Lipid hydroperoxides	Decomposition to non-reactive species	Reduction of lipid hydroperoxides to lipid hydroxides by methionine
Secondary Products	Aldehyde binding	Formation of Schiff base adducts between nucleophilic amino acid R-groups and β -scission products

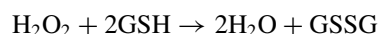
conversion of hydrogen peroxide to water by the following pathway:



In plants, hydrogen peroxide can also be removed by ascorbate peroxidase via the following mechanism:



Most biological tissues also contain glutathione peroxidase (GSH-Px). GSH-Px is capable of inactivating both lipid and hydrogen peroxides. GSH-Px uses selenium in its active site and reduced glutathione (GSH) to convert hydrogen or lipid (LOOH) hydroperoxide to water:



or



where GSSG is oxidized glutathione and LOH is a fatty acid alcohol.

Inactivation and Sequestration of Prooxidative Metals

Redox active transition metals such as iron and copper are capable of catalyzing the reduction of hydroperoxides to reactive radical species, and are important prooxidants in food lipids.



Protein chelators can inhibit oxidative reactions by changing the physical location of transition metals (e.g. partitioning metals away from oxidatively labile lipids or hydroperoxides), forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and/or sterically hindering the interaction of metals and dispersed lipids (Diaz et al., 2003). Foods contain proteins whose specific function it is to bind metals, whereas other food proteins nonspecifically bind prooxidant metals. Proteins whose biological function is to chelate and store or transport catalytically inactive metals are found in most biological systems (Table 4). Examples include transferrin, ovotransferrin (conalbumin), lactoferrin, and ferritin. Transferrin (blood) and lactoferrin (milk) bind two ferric ions apiece, while ovotransferrin binds three. Ferritin is an iron storage protein that can bind up to 4,500 ferric ions (Fennema, 1996; Weinberg, 1990). Transferrin, ovotransferrin, lactoferrin, and ferritin control iron reactivity by binding iron in its less active ferric state and by sterically hindering metal-peroxide interactions. Iron is released from these proteins by reducing agents (ascorbate, cysteine, superoxide anion), low pH, and processes that denature

Table 4 Metal-binding protein antioxidants, adapted from (Symons and Gutteridge 1998)

Protein	Function
Ferritin	Binds and stores ferric ions within helical fold
Transferrin	Binds ferric ions (2 per mole of protein)
Lactoferrin	Binds ferric ions at low pH (2 per mole protein)
Haptoglobins	Binds hemoglobin
Hemopexin	Binds heme
Albumin	Binds copper and heme
Ceruloplasmin	Non-specific copper binding

proteins. Ferritin is a good example of a protein that reduces iron's reactivity by interfering with its redox cycling capacity. In the case of this protein, ferrous (Fe^{+2}) ions are oxidized to the ferric (Fe^{+3}) oxidation state after binding to a specific site located inside the subunit helical fold (i.e. the ferroxidase center) (Arosio and Levi, 2002). Once oxidized, iron migrates to the protein's cavity where it nucleates and aggregates to form a ferric hydroxide core (Arosio et al., 2002; Carrondo, 2003). Thus, iron is sequestered and maintained in its ferric state, and is unavailable to participate in redox reactions (e.g. lipid hydroperoxide decomposition). Lactoferrin has been reported to be an effective antioxidant in food, where the protein inhibited the lipid oxidation in infant formula (Satue-Gracia et al., 2000), milk, and mayonnaise (Nielsen et al., 2004). Furthermore, apotransferrin (31 μM) was shown to inhibit lipid oxidation in a Tween 20-stabilized salmon oil-in-water emulsion at pH 7 (Mancuso et al., 1999). Not only does this data suggest that transferrins reduce the reactivity of iron in foods, but they offer strong evidence that iron is the most important prooxidant in oil-in-water emulsions, since transferrin has a very high binding specificity for iron. Additionally, haptoglobins and hemopexins are known extracellular antioxidants, capable of binding hemoglobin and heme, respectively (Symons and Gutteridge, 1998).

Copper, like iron, is a prooxidative transition metal in lipid systems and is typically found in food ingredients (albeit at significantly lower concentrations than iron). Copper is a more effective catalyst than iron in hydroperoxide decomposition reactions (Halliwell and Gutteridge, 1990). As is the case with iron, proteins are capable of forming strong complexes with copper and thus can affect lipid oxidation. In biological tissues, copper is bound by proteins such as serum albumin (one cupric ion per protein) and ceruloplasmin (six cupric ions per protein). These proteins can inhibit lipid oxidation as can be seen in the copper-catalyzed oxidation of low-density lipoprotein (LDL), where oxidation rates are inversely related to the concentration of BSA (Bourdon et al., 1999; Schnitzer et al., 1997).

Many proteins whose specific biological function is not to store or transport metals are still capable of chelating metals. This is because amino acid residues such as histidine, glutamic acid, aspartic acid, and phosphorylated serine and threonine are known to bind metals. If these amino acids are exposed on the surface of the proteins they are able to chelate metals. The ability of a protein to chelate metals is dependent on pH. For example,

a net anionic charge will be established on a protein at pH values above its pI. This results in electrostatic attraction between the protein and cationic transition metals, which ultimately inhibits lipid oxidation reactions.

Numerous proteins whose major function are not to transport or store transition metals have been reported to bind prooxidative transition metals. These include casein (Diaz et al., 2005; Diaz et al., 2003), whey proteins (Faraji et al., 2004; Tong et al., 2000), soy proteins (Faraji et al., 2004), bovine serum albumin (Villiere et al., 2005), zein (Kong and Xiong, 2006), and potato protein (Wang and Xiong, 2005). For example, whey protein isolate, soy protein isolate, and sodium caseinate (10 mg/mL) were capable of binding 185, 405, and 980 μ moles of iron, respectively (Faraji et al., 2004). These non-specialized protein chelators have been observed to inhibit lipid oxidation in oil-in-water emulsions (Villiere et al., 2005; Faraji et al., 2004; Diaz et al., 2003; Tong et al., 2000) and meats (Wang and Xiong, 2005).

Non-specialized protein chelators can inhibit lipid oxidation in foods by several mechanisms including altering the physical or redox state of the metal, or by increasing the proteins ability to scavenge free radicals. Proteins can alter the redox state of transition metals by preferentially binding one state of the metal. For example, the polar domains of caseins (α_{s1} , α_{s2} , and β) contain phosphorylated serine residues which form complex ferric ions, thus shifting the equilibrium and promoting a conversion from iron's ferrous to ferric state (Diaz et al., 2003; Vegarud et al., 2000). A net decline in the pool of ferrous ions has the effect of decreasing oxidative stress, since the ferrous state is the most important species in hydroperoxide decomposition (Decker and McClements, 2001).

The metal-catalyzed decomposition of lipid hydroperoxides is thought to be the dominant oxidative pathway in processed foods, and in particular oil-in-water emulsions (McClements and Decker, 2000). This reaction can occur at the surface of the emulsion droplet because lipid hydroperoxides are surface active and migrate to the water-oil interface (Nuchi, et al.). One way in which proteins can inhibit lipid oxidation reaction in oil-in-water emulsions is by changing the physical location of aqueous prooxidants. In this scenario, metals are moved away from the emulsion droplet surface, reducing its ability to decompose lipid hydroperoxides. Tong and coworkers (2000) found that whey proteins were able to both inhibit lipid oxidation and remove iron from the surface of a bovine serum albumin-stabilized salmon oil-in-water emulsion, suggesting that at least part of its antioxidant activity is due to its ability to change the physical location of iron.

Metals bound to non-specialized protein chelators can remain prooxidative if they retain their ability to redox cycle (Davies et al., 1997). In such a system, a hydroperoxide is reduced to a reactive radical species when it comes into contact with the protein-bound metal. However, if the resulting radical is sufficiently potent (e.g. hydroxyl radical), it will react with neighboring amino acid side chains on the protein instead of diffusing outward and promoting further radical reactions

Ability of Proteins to Inhibit Lipid Oxidation by Altering the Physical State of Foods

In many foods, lipid oxidation reactions do not occur randomly but instead occur at specific locations based on the physicochemical properties of the food matrix. This is exemplified in the case of metal-catalyzed oxidation in lipid dispersions, where surface active lipid hydroperoxides concentrate at oil-water interfaces. Subsequently, aqueous phase transition metals can catalyze the decomposition of these hydroperoxides, yielding radical species. Proteins can inhibit lipid oxidation in such lipid dispersions by hindering access of metals to the water-oil interface through electrostatic repulsion. This effect has been demonstrated in protein-stabilized oil-in-water emulsions wherein lipid oxidation rates are substantially slower at pH values below the isoelectric point of the proteins. A net cationic charge is established at the water-oil interface, repelling iron and physically hindering the ability of cations from binding to the droplet interface (Donnelly et al., 1998; Hu et al., 2003a; Hu et al., 2003b; Kellerby et al., 2006).

Another factor that can impact lipid oxidation kinetics in oil-in-water emulsions is the thickness or existence of a thick emulsion droplet interface that physically inhibits the ability of iron to access lipid hydroperoxides at the droplet surface. For example, the ability of iron to promote cumene hydroperoxide decomposition as well as the oxidation of salmon oil is lower in emulsion droplets stabilized by Brij 700 than Brij 76. These two surfactants have the same hydrophobic tail group length ($\text{CH}_3(\text{CH}_2)_{17}-$), but vary in the length of their polar head groups (Brij 700 is comprised of 100 oxyethylene head groups whereas Brij 76 has 10). A decrease in lipid hydroperoxide decomposition and lipid oxidation rates in Brij 700-stabilized oil-in-water emulsions suggests that a thicker interfacial layer provided was able to act as a physical barrier to decrease lipid-prooxidant interactions (Silvestre et al., 2000). This could also occur in protein-stabilized oil-in-water emulsions if proteins could form a thick interfacial layer that inhibits metal-lipid interactions. For example, casein forms an interfacial layer around dispersed oil droplets of up to 10 nm compared to 1–2 nm for whey proteins (Dalgleish et al., 1995). The ability of casein to form a thick layer around emulsion droplets could help explain why casein-stabilized emulsions had greater oxidative stability than those prepared with whey protein, despite the fact that casein-stabilized emulsion droplets were less cationic (+29.9 mV) than whey protein-stabilized emulsion droplets (+ 55.9 mV).

Scavenging of Free Radicals and Reactive Oxygen Species

The ability of proteins to interact with free radicals has been documented in many systems. These studies have typically employed the use of free radical generators, and a determination is made if a given protein can quench the radical species produced. For example, the oxygen radical absorbance capacity (ORAC) assay is a common method that measures the ability

of a free radical scavenger to quench peroxy radicals generated by 2,2-azo-bis (2-methylpropionamide) dihydrochloride (AAPH) and protect the fluorescent marker, fluorescein. Using this technique, β -lactoglobulin (Elias et al., 2006) and casein (Diaz et al., 2005) were shown to scavenge peroxy radicals. Radical scavenging activity has also been measured using free radicals generated by 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to show that zein (Kong and Xiong, 2006) and potato (Wang and Xiong, 2005) proteins quench radicals. A third radical scavenging assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH) has shown that egg yolk proteins can scavenge free radicals (Sakanaka and Tachibana, 2006). Interactions between free radicals and proteins can also be measured by electron paramagnetic resonance (EPR, for review see Davies and Hawkins, 2004), or electron spin resonance (ESR), as it is sometimes referred. Using EPR, bovine serum albumin, β -lactoglobulin, and lactoferrin were shown to scavenge free radicals generated from cumene hydroperoxide and iron (Pazos et al., 2006). EPR is also useful for directly observing amino acid radicals in proteins, such as tyrosinyl radicals, which have been measured directly in bovine serum albumin (Ostval et al., 1999).

The ability of proteins to merely scavenge free radicals is not conclusive evidence that they are antioxidants. For a free radical scavenger to be an effective antioxidant in foods, it must be more oxidatively labile than unsaturated fatty acids and the resulting protein radical must not be powerful enough to promote lipid oxidation. In a study on the role of β -lactoglobulin as an antioxidant in a model oil-in-water emulsion system, continuous phase protein was found to inhibit lipid oxidation (Elias et al., 2005). Evaluation of the oxidative stability of cysteine, tryptophan, and methionine residues in continuous phase β -lactoglobulin found that tryptophan and cysteine, but not methionine, oxidized prior to lipid oxidation, thus indicating that these amino acids were oxidized preferentially to unsaturated fatty acids. Methionine was not found to oxidize in this system, despite its reported high susceptibility to oxidation. The fact that β -lactoglobulin's methionine residues are buried within its hydrophobic core, and therefore possibly inaccessible to aqueous phase oxidants, could explain this observation (Elias et al., 2005).

All 20 amino acids found in proteins have the potential to interact with free radicals if the energy of the radical insult is high (e.g. hydroxyl radical). However, the ability of a protein to behave as antioxidants by scavenging free radicals is incumbent upon the condition that the resulting protein radical has insufficient energy to substantially initiate or propagate further oxidation reactions. Thus, a protein is of little or no value as an antioxidant in a food if it simply serves, and transports high energy free radicals to lipid thus promoting oxidation. The antioxidant activity of proteins in radical-mediated oxidation reactions may be due to their ability to act as radical trapping devices (Neuzil et al., 1993; Ostval et al., 2002). For example, tyrosine radicals formed on bovine serum albumin are significantly longer lived and are therefore less reactive than free tyrosine radicals (Ostval, et al., 1999). The long half-lives of tyrosinyl

radicals in bovine serum albumin is likely due to the ability of the protein to transfer radicals on surface exposed amino acid residues to tyrosine residues buried in the protein's hydrophobic core. It is conceivable that a protein's antioxidant activity is at least partially attributable to the lower reactivity of protein radicals if those radicals are transferred to the interior of the protein where they are unable to physically interact with lipids. However, it should be noted that it is possible that not all protein radicals are transferred into the protein's interior since bovine serum albumin radicals have been found to promote the oxidation of linoleic acid emulsions (Ostval et al., 2002).

Methionine has been proposed to be an important free radical scavenger in proteins in biological systems (Levine et al., 1999; Levine et al., 2000; Levine et al., 1996; Stadtman et al., 2003). This is because methionine residues are very labile to oxidation and can potentially scavenge radicals before they are able to attack other amino acid residues that are critical to protein structure or function (Levine et al., 1999; Levine et al., 1996). For example, the high concentration of methionine residues at the active site of bacterial glutamine synthetase may serve as a "last chance" antioxidant defense system by oxidizing preferentially to other amino acids that are critical to enzyme function (Levine et al., 1996). The role of methionine as an important free radical scavenger in biological systems can also be argued based on the ubiquity of methionine sulfoxide reductases (enzymes capable of repairing oxidized methionine residues) in virtually all forms of life. Thus methionine could act as a free radical scavenger that can be regenerated in biological tissues similar to α -tocopherol, which is thought to be regenerated by ascorbic acid (ascorbic acid is subsequently regenerated by NADPH). Regeneration of a free radical scavenger would obviously increase its role as an antioxidant since it would not be lost in the early stages of oxidation as are many other free radical scavengers. Whether oxidized methionine residues can be regenerated in foods has not been determined.

Non-Radical Reduction of Lipid Hydroperoxides

Another antioxidant mechanism of proteins could result from their ability to reduce lipid hydroperoxides to relatively non-reactive lipid hydroxides by non-radical reactions (Garner et al., 1998a; Garner et al., 1998b; Pryor et al., 1994; Pryor and Squadrito, 1995). Methionine residues are thought to be central to this process, as it has been observed that canine high density lipoprotein (HDL), which lacks methionine residues Met112 and Met148, showed weaker lipid hydroperoxide reducing activity than human HDL (Garner et al., 1998a). The proposed mechanism for the reduction of lipid hydroperoxides to lipid hydroxides (Fig. 1) involves a direct two-electron transfer from the sulfide of methionine's thioether group, resulting in the oxidation of methionine to methionine sulfoxide (MetO) (Garner et al., 1998a; Garner et al., 1998b; Panzenbock and Stocker, 2005). In effect, methionine residues reduce lipid hydroperoxide to non-reactive species, which in turn interferes

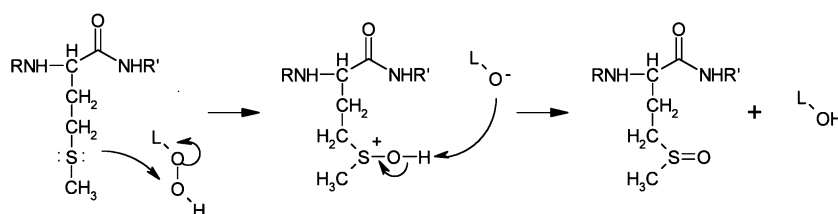


Figure 1 Proposed two-electron reduction of lipid hydroperoxide (LOOH) by thioether-containing side chain of methionine (Garner, Waldeck et al., 1998; Garner, Witting et al., 1998; Panzenbock and Stocker, 2005).

with lipid oxidation propagation. In processed foods, this reaction would not be an important antioxidant mechanism once all available methionine residues have been oxidized. However, in some foods that are physiologically active under postharvest or postslaughter conditions (e.g. fruits, vegetables, minimally processed muscle foods), methionine residues could be vastly more important antioxidants if methionine sulfoxide reductases are active.

Interactions with Aldehydes Arising from Lipid Oxidation

Oxidation of lipids do not result in the development of rancidity until fatty acid hydroperoxides decompose via β -scission reactions into low molecular weight, volatile compounds that are perceived as off-flavors and aroma. These compounds, known as secondary lipid oxidation products, are deleterious to the sensory properties of foods. In addition, these products adversely affect the physiochemical properties of food proteins because lipid-derived aldehydes can react with the side chains of certain amino acid residues by either Schiff base reactions or Michael additions, yielding aldehydic adducts (Fig. 2). Schiff bases are imines that form in complex food systems when the carbonyl groups of aldehydic lipids react with the side chains of certain nucleophilic amino acid residues (e.g. cysteine's thiol moiety). Depending on the characteristics of the lipid oxidation products, Schiff base formation can be accompanied or eclipsed by competing reactions, such as 1,4 (Michael) addition reactions. In general, polyunsaturated aldehydes react faster with proteins than saturated aldehydes (Chan et al., 1997; Zhou and Decker, 1999a and b). This is likely due to the fact that Michael addition reactions, that only occur with α,β -polyunsaturated aldehydes, are faster than Schiff base reactions that occur between proteins and either saturated or unsaturated aldehydes. For example, greater than 99% of β -lactoglobulin B and human hemoglobin is modified via Michael addition reactions (vs. Schiff base forma-

tion) in the presence of the lipid oxidation product 4-hydroxy-2-nonenal (Bruenner et al., 1995).

Since volatile aldehydic lipid oxidation products impact the sensory quality of foods, pathways that reduce the volatility of aldehydes can improve food quality. Although this pathway is not truly an antioxidant mechanism since it does not inhibit lipid oxidation pathways, it will inhibit perceived oxidative rancidity by transforming lipid oxidation products into non-volatile, high molecular weight covalent adducts. In this way, proteins are capable of altering the development of rancidity in unsaturated fats and oils by adducting volatile aldehydes. While proteins can react with aldehydes to decrease rancidity, these reactions can also be deleterious to other food quality parameters, such as color. Several studies have shown that aldehydic lipid oxidation products react with myoglobin, which adversely affects the color of myoglobin-containing meat products (Alderton et al., 2003; Faustman et al., 1999; Lynch and Faustman, 2000). Myoglobin (Mb) is the major heme-containing sarcoplasmic protein in skeletal muscle that can exist in three forms dictated by the redox state of its heme iron: ferrous deoxymyoglobin (deoxyMb) and ferrous oxymyoglobin (oxyMb), or oxidized ferric metmyoglobin (metMb) (Alderton et al., 2003). In Mb-containing foods, oxyMb is important because it is responsible for the characteristic cherry red appearance of fresh meats; however, the oxidation of oxyMb to MetMb results in meat discoloration from red to brown (Alderton et al., 2003), which is generally unacceptable to consumers. Chan and coworkers (1997) demonstrated that the oxidation of oxyMb to metMb was hastened in the presence of reactive aldehydic oxidation products. Furthermore, Lynch and Faustman (2000) reported that 4-hydroxy-2-nonenal adducts render metMb a less suitable substrate for metmyoglobin reductase, thus significantly decreasing its ability to be enzymatically reduced back to oxyMb. Mb modification by 4-hydroxy-2-nonenal also resulted in increased rates of lipid oxidation, suggesting that these adducts confer a structural change upon metMb that enables its heme group to more readily catalyze oxidation reactions (Lynch et al., 2000). Therefore, not all interactions between aldehydic lipid oxidation products are beneficial to food quality.

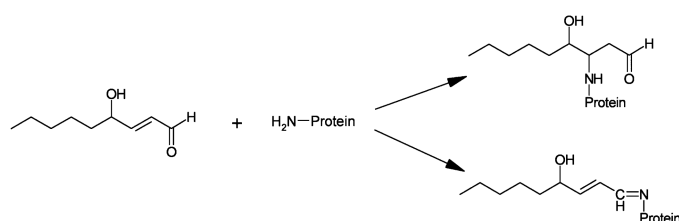


Figure 2 Protein modification by Michael addition (top pathway) or Schiff Base formation (bottom pathway).

INCREASING THE ANTIOXIDANT ACTIVITY OF PROTEINS

The antioxidant activity of proteins can be increased by changes in concentrations, reactivity, and physical structure. The

level of antioxidant enzyme activity in foods is typically a function of genetics and tissue source (i.e. tissues that are involved in oxidative metabolism generally have greater antioxidant activity) (Decker and Xu, 1998; Lee et al., 1997). In addition, environmental conditions can also influence gene expression and, thus, the level of antioxidant enzymes in foods (Chanjirakul et al., 2006; Cao et al., 2006).

Many protein antioxidant mechanisms are dependent on amino acids composition (e.g. metal chelation, free radical scavenging, hydroperoxide reduction, aldehyde adduction). However, the antioxidant activity of these amino acids residues is limited by the tertiary structure of the polypeptide, since many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants. For example, in native β -lactoglobulin (β -Lg), the majority of free radical scavenging amino acids are located in the protein interior and therefore may not be able to contribute to the protein's overall antioxidant activity. Table 5 details the solvent accessibility of the major free radical scavenging amino acids in native β -Lg. These values were calculated using an algorithm for determining solvent accessible surface area based on the protein's crystal structure. As shown in Table 5, 17 of native β -Lg's most oxidatively labile amino acids are not solvent accessible, while tyrosine 20, tryptophan 61, and histidine 146 are partially exposed. Therefore, these amino acids are unlikely to possess strong free radical scavenging activity when the protein is in its native state.

One approach to increasing a protein's overall antioxidant activity is through tertiary structure disruption (i.e. partial denaturation), which may potentially increase the solvent accessibility of oxidatively labile amino acid residues. Taylor and Richardson (1980) studied the antioxidant activity of heated skim milk in a methyl linoleate emulsion with hemoglobin used as a lipid oxidation catalyst. The authors found that heat treatment (70 to 130°C for up to 30 min) increased the antioxidant activity of skim milk, an observation that was partially attributed to exposure of cysteine's sulfhydryl groups. Heat treatment resulted in an increase in reactive sulfhydryls (starting reactive sulfhydryl concentration of 48 μ M was increased to 71 μ M after a 130°C for 30 min). It was also shown that the optimum heat treatment for whey proteins to increase reactive sulfhydryl concentration without decreasing total sulfhydryl was 80°C. In a separate study, β -Lg heated at 95°C for 30 min was demonstrated to increase the protein's antioxidant activity in a menhaden oil-in-water emulsion (Elias et al., 2007). The observed increase in antioxidant activity was likely due to an increase, solvent exposure of free radical scavenging amino acid residues, since heating increased sulfhydryl exposure and peroxy radical scavenging capacity but lowered iron chelation capacity. It should also be noted that heating proteins in the presence of sugars (such as the experiments with skim milk) can produce Maillard reaction products that are antioxidative. This possibility is discussed later in this review.

Increasing the exposure of antioxidant amino acids in proteins can also be accomplished by enzymatic hydrolysis. Increased

Table 5 The solvent accessible surface area (SASA) of β -Lg's oxidatively labile amino acid residues calculated using the program GETAREA (Fraczkiewicz and Braun 1998). The ratio of side-chain surface area to random coil value of each amino acid residue is listed in the 4th column. The "random coil" value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations. Residues are considered to be solvent exposed if the side-chain surface area to random coil value ratio value exceeds 50% and to be buried if the ratio is less than 20% (Fraczkiewicz and Braun, 1998). Buried residues are denoted with an "i". Cys residues in bold typeface represent residues involved in disulfide bonds (i.e. cystine). A probe radius of 1.4 Å was used

Residue	Sidechain	Random coil	Ratio (%)	In/out
Met 7	25.58	158.3	16.2	i
Trp 19	0.00	224.6	0.0	i
Tyr 20	53.55	193.1	27.7	
Met 24	0.00	158.3	0.0	i
Tyr 42	16.58	193.1	8.6	i
Trp 61	93.04	224.6	41.4	
Cys 66	10.51	102.3	10.3	i
Phe 82	1.58	180.1	0.9	i
Tyr 99	35.28	193.1	18.3	i
Tyr 102	13.81	193.1	7.2	i
Phe 105	16.40	180.1	9.1	i
Cys 106	2.45	102.3	2.4	i
Met 107	7.34	158.3	4.6	i
Cys 119	0.15	102.3	0.1	i
Cys 121	0.00	102.3	0.0	i
Phe 136	0.10	180.1	0.1	i
Met 145	15.17	158.3	9.6	i
His 146	43.60	154.6	28.2	
Phe 151	12.67	180.1	7.0	i
Cys 160	12.38	102.3	12.1	i

antioxidant activity in hydrolyzed proteins has been reported for dairy (Ostdal et al., 1999; Rival et al. 2001; Pena-Ramos and Xiong, 2003; Hernandez-Ledesma et al., 2005; Sakanka et al., 2005; Diaz et al., 2005; Elias et al., 2005) soy (Pena-Ramos and Xiong, 2003; Park et al., 2005), zein (Kong and Xiong, 2006), potato (Wang and Xiong, 2005), gelatin (Park et al., 2005), and egg yolk (Sakanaka and Tachibana, 2006) proteins. The observed increase in antioxidant activity due to hydrolysis may result directly from increased solvent exposure of amino acids. This exposure leads to increased metal chelation capacity (Wang and Xiong, 2005; Kong and Xiong, 2006; Elias et al., 2006) and free radical scavenging activity (Ostdal et al., 1999; Rival et al. 2001; Wang and Xiong, 2005; Park et al., 2005; Hernandez-Ledesma et al., 2005; Sakanka et al., 2005; Diaz and Decker, 2005; Elias et al., 2006; Kong and Xiong, 2006; and Sakanaka and Tachibana, 2006).

In general, free amino acids are not effective antioxidants and extensive proteolysis results in decreased antioxidant activity (Ostdal et al., 1999; Rival et al. 2001; Sakanka et al., 2005; Hernandez-Ledesma et al., 2005; Zhou and Decker, 1999a and b and Chan et al., 1994). The increased antioxidant activity of peptides is related to unique properties contributed by their chemical composition and physical properties. Peptides are potentially better food antioxidants than amino acids due to their increased free radical scavenging activity, metal chelation,

and aldehyde adduction activity (Chan et al., 1994; Zhou and Decker, 1999a and b). For example, proteolytic degradation of bovine serum albumin results in protein radicals with longer half-lives relative to native bovine serum albumin while extensive proteolysis yields short-lived BSA radicals (Ostdal et al., 1999). Pietraforte and Minetti (1997) corroborate this conclusion by demonstrating that tyrosyl radicals can be detected in tyrosine-containing peptides after treatment with peroxyntirite only if the tyrosine residue is located in a central position of a relatively long peptide sequence. Thus the increased ability of protein hydrolysates to decrease the reactivity of a free radical is related to an increase in the exposure of the amino acids, which leads to increased peptide-free radical reactions as well as the ability of the peptide to decrease the energy of the scavenged free radical which decreases its ability to oxidized lipids.

The antioxidant activity of proteins can also be increased by interactions with reducing sugars to produce Maillard reaction products (for review see Bailey and Um, 1992 and Zamora and Hidalgo, 2005). Protein-reducing sugar Maillard products include a broad array of structures that contain furans, reductones, Schiff bases, and aldehydes. The Maillard reaction products can inhibit lipid oxidation by free radical scavenging and metal chelation. Antioxidative Maillard reaction products can be water soluble or produced on the surface of protein-stabilized emulsion droplet. Augustin et al. (2006) found that heating casein-stabilized fish oil-in-water emulsions in the presence of glucose resulted in the formation of Maillard reaction products that were capable of inhibiting lipid oxidation.

Potential Problems with Protein Antioxidants in Foods

While proteins and peptides have excellent antioxidant activity and great potential as food additives, they may not be suitable in all food applications. A major concern associated with the use of protein and peptides as antioxidants is the potential issue of allergenicity, especially with proteins derived from dairy, soy, nuts, and eggs. Also of concern is the potential problem of bitter off-flavors from peptides. Proteins and peptides could also cause problems in food if they alter the texture (e.g. increased viscosity or gelation) or color (light scattering or Maillard reactions) of the food product. Finally, certain Maillard reaction products have been implicated as carcinogenic, making their addition to foods problematic.

CONCLUSIONS

Proteins are unique antioxidants in that they can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems. Proteins are important in the endogenous antioxidant capacity of foods

and have potential as food antioxidant additives. The antioxidant activity of proteins can be increased in a number of ways (e.g. Maillard reaction chemistry, increasing the accessibility of antioxidative amino acids by thermal processing, or hydrolysis, etc.). Peptides show the most promise as proteinaceous antioxidants since a number of studies have shown that they have substantially higher activity than intact proteins. While hydrolyzed proteins have good antioxidant activity, it is still not well-understood how the composition of peptides influences their ability to inhibit lipid oxidation. Understanding the relationship between peptide composition and antioxidant activity could lead to the development of new class of extremely effective, multifunctional, generally recognized as safe (GRAS) antioxidants that could be used in many food applications, including the development of functional foods fortified with oxidatively unstable, yet healthy, unsaturated fatty acids.

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