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Influence of cysteine and methionine availability on protein peroxide scavenging activity and phenolic stability in emulsions

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ABSTRACT

Plant phenolics are secondary metabolites that have been shown to confer beneficial health effects in humans. However, many of these compounds undergo metal-catalysed oxidation reactions, leading to the generation of hydrogen peroxide (H_2O_2) and other reactive oxygen species that may negatively impact product stability. In proteins, methionine (Met) and cysteine (Cys) are capable of reacting directly with peroxides. Thus, the dairy proteins, casein (CAS) and β-lactoglobulin (BLG), were examined for their ability to scavenge H_2O_2 (400 μM) and influence (−)-epigallocatechin-3-gallate (EGCG) oxidation (400 μM) in Tween- or sodium dodecyl sulphate (SDS)-stabilised hexadecane emulsions. To examine the effect that the accessibility of these amino acids have on their peroxide scavenging activities, proteins were pre-treated with tert-butyl hydroperoxide (TBHP), a bulky peroxide, to oxidise only solvent accessible Met residues or H_2O_2, the smallest peroxide, to oxidise buried Met residues. In CAS treatments, higher Met content yielded greater peroxide scavenging activity and EGCG stability. CAS treatments also showed significantly higher peroxide scavenging activity compared to the corresponding BLG treatment. However, BLG peroxide scavenging activity was greatly enhanced in SDS-stabilised emulsions due to protein denaturation and subsequent exposure of previously buried Cys residues.

1. Introduction

Polyphenols are attractive functional ingredients in foods; they serve as non-synthetic (i.e., "natural") antioxidants in foods (Ganhão, Estévez, & Morcuende, 2011; Sørensen, Nielsen, & Jacobsen, 2010; Zhong & Shahidi, 2012) and their consumption is associated with numerous health benefits (Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012). Polyphenols are known to act as antioxidants under many conditions by scavenging free radicals (Iacopini, Baldi, Storchi, & Sebastiani, 2008; Rice-Evans, Miller, & Paganga, 1996) and by chelating transition metal catalysts (Elhabiri, Carrër, Marmolle, & Traboulsi, 2007; Hider, Liu, Khodr, & Lester, 2001). Phenolics containing di- or trihydroxy substituted phenol rings, such as (−)-epigallocatechin-3-gallate (EGCG) are especially effective radical scavengers due to resonance stabilisation of their one electron oxidation product (the semiquinone radical) (Kumamoto, Sonda, Nagayama, & Tabata, 2001). However, di- and trihydroxy substituted phenolics are also capable of reducing transition metals (Fe^{2+}, Cu^{1+}) (Chvátalová, Slaninová, Brezinová, & Slanina, 2008; Mira, Fernandez, Santos, Rocha, Florêncio, & Jennings, 2002), resulting in the generation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot -}$) and hydroperoxyl radicals ($HO_2^{\cdot}$), which are subsequently reduced to H_2O_2 and, eventually, hydroxyl radicals (**.**OH) (Scheme 1a). The metal-catalysed oxidation of phenolics not only results in the undesirable loss of a potentially bioactive compound, it also has the potential to decrease product shelf-life due to sensory defects, such as undesired colour changes from phenolic polymerisation (Li, Guo, & Wang, 2008),or metallic and fishy notes from increased lipid oxidation. Pro-oxidant activity in oil-in-water emulsions resulting from the incorporation of phenolics has previously been observed (Huang & Frankel, 1997).

In a previous study, we examined the use of metal chelators as a strategy for controlling phenolic oxidation in food emulsions (Zhou & Elias, 2012). The ferric chelator, ethylenediaminetetraacetic acid (EDTA), was shown to increase EGCG stability in emulsions under acidic conditions, but induced rapid EGCG oxidation at neutral pH, suggesting that different approaches to preventing phenolic oxidation will be necessary depending on food matrix conditions. Proteins may be an alternate solution due to their ability to scavenge H_2O_2, thereby preventing **.**OH formation. H_2O_2 is a known precursor for the **.**OH, a potent and non-selective oxidant (Makrigiorgos, Bump, Huang, Baranowska-Kortylewicz, & Kassis, 1995) for which chain-breaking antioxidants are largely ineffective. It therefore stands to reason that the most effective way to prevent **.**OH mediated oxidation is to quench its parent compound, H_2O_2, before it is reduced by the Fenton reaction to these radicals. In proteins, Cys (Luo, Smith, & Anderson, 2005) and Met (Garner, Waldeck,

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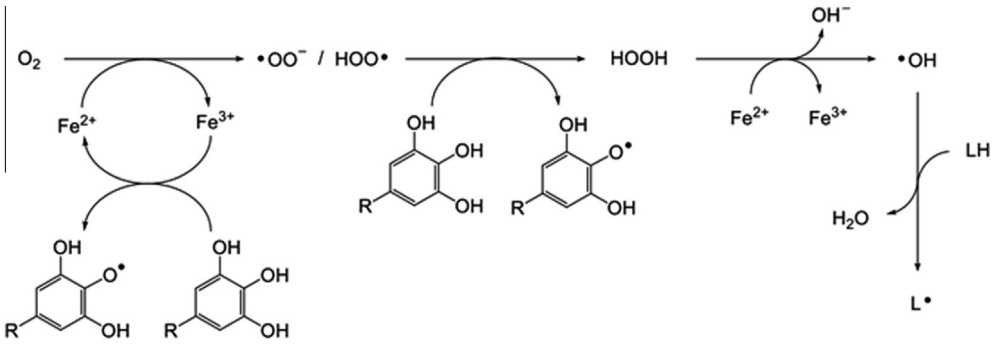

Scheme 1a. Proposed prooxidant reactions resulting from metal-catalysed phenolic oxidation in lipid dispersions depicted with a galloyl group. Abbreviations are as follows: oxygen (O_2), superoxide ($^{\bullet}OO^-$), hydroperoxyl radical (HOO•), hydrogen peroxide (HOOH), hydroxyl radical (•OH), lipid (LH), lipid alkyl radical (L•).

Witting, Rye, & Stocker, 1998a,b) residues have been shown to react directly with peroxides *via* two electron (i.e., non-radical) reduction to yield non-reactive alkoxides. However, peroxide scavenging capability is dependent on more than total Met and Cys content. Differences in the relative solvent accessibility of Cys and Met on the two proteins may offer an explanation, as both Cys and Met residues are relatively hydrophobic and, thus, often positioned within the hydrophobic domains of proteins. Studies have shown preferential oxidation of solvent accessible Met residues in proteins (Requena et al., 2004; Snijder, Rose, Raijmakers, & Heck, 2010). After oxidation with H_2O_2, LC–MS analysis of prion proteins after digestion demonstrated that Met residues in the unstructured, solvent accessible region were the most readily oxidised, while oxidation of buried Met residues were undetectable (Requena et al., 2004). Similarly, Met oxidation of specific residues in calmodulin were well-correlated with their calculated solvent accessibility (Snijder et al., 2010).

The objective of this experiment was to examine the H_2O_2 scavenging activity of CAS and β-lactoglobulin (BLG). H_2O_2 was either added as an exogenous component, or H_2O_2 was generated directly from EGCG oxidation. The effect of emulsifier type (Tween vs. SDS) on peroxide scavenging capacity of the proteins was also investigated. A specific focus was placed on the role of Met's and Cys's solvent accessibility on peroxide scavenging activity.

2. Materials and methods

2.1. Materials

Phthaldialdehyde (OPA) reagent, methionine, methionine sulfoxide, tryptophan, tyrosine, sodium dodecyl sulphate (SDS), Tween-80, ethylenediaminetetraacetic acid (EDTA), xylenol orange tetrasodium salt, ferrous sulphate heptahydrate and the enzymes leucineaminopeptidase M (L5006), protease (P5147), and prolidase (P6675) were purchased from Sigma (St. Louis, MO, USA). n-Hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ, USA). Sodium caseinate (Alanate 191) was obtained from New Zealand Milk Proteins. β-Lactoglobulin was kindly donated by Davisco Foods International (Eden Prairie, MN, USA). H_2O_2 (30%, w/v) was purchased from EMD Chemicals (Gibbstown, NJ, USA). 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), guanidine hydrochloride, tert-butyl hydroperoxide (TBHP) (70%), phenylalanine and D-sorbitol (98% purity) were purchased from Alfa Aesar (Ward Hill, MA, USA). Ferric chloride 6-hydrate lumps and sodium azide were purchased from Mallinkrodt (Phillipsburg, NJ, USA). Trichloroacetic acid (crystalline) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Leucine was acquired from Amresco (Solon, OH, USA). BD Falcon™ Clear 96-well Microtest™ Plates, 0.45 μm polytetrafluoroethylene (PTFE) syringe filters and Spectra/Por® regenerated cellulose dialysis tubing with a 12–14 kDa molecular weight cut off (MWCO) were purchased from VWR (Radnor, PA, USA). BCA Protein Assay Kit was purchased from Thermo Fisher (Rockford, IL, USA). All electrophoresis materials listed were acquired from Biorad (Hercules, CA):4–20% Mini-PROTEAN® TGX™ Precast Gel, glycine (aminoacetic acid) powder, Tris base (Tris[hydroxymethyl]aminomethane) and Precision Plus Protein™ Kaleidoscope Standards. All other chemicals and solvents were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA, USA) purification train.

2.2. Preparation of methionine-oxidised protein

Protein solutions (1% w/v CAS or BLG) were prepared in 10 mM phosphate buffer pH 7 and oxidised with H_2O_2 (2 mM) or TBHP (7.77 mM) for 5 days at room temperature. NaN_3 (0.02%) was added to inhibit microbiological activity. The concentrations selected were previously shown to oxidise Met residues while minimizing the oxidation of other oxidatively labile amino acids (Keck, 1996). Controls without added peroxides were also incubated under identical conditions. CAS was precipitated by adjusting the pH to its isoelectric point (pH 4.6). BLG was precipitated by adjusting the pH to 2.6 followed by the addition of NaCl (30%, w/v). Protein was collected after centrifugation at 3300*g* for 15 min at 4 °C. Proteins were then resuspended in water and dialysed (MWCO = 10 kDa) against water for 3 days at 4 °C with three water changes per day. The water was analysed for residual peroxides using the FOX method described below to ensure their complete removal. Following dialysis, protein samples were frozen at −80 °C and lyophilised using a FreeZone 2.5 L Benchtop Freeze Dry System (Labconco, Kansas City, MO, USA). Dry protein samples were stored at 4 °C until use. Native-PAGE was run on all protein samples, as well as freshly prepared casein and BLG solutions. No differences in relative mobility were observed (data not shown).

2.3. Emulsion preparation

Oil-in-water emulsions were prepared by dispersing 10 wt% hexadecane in water with 2 wt% emulsifier (Tween-80 or SDS). Hexadecane was selected as the organic dispersed phase for the model system because it is a saturated hydrocarbon that is oxidatively inert. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, USA) on high speed for 0.5 min. Fine emulsions were prepared by passing coarse emulsions twice through a microfluidiser with the interaction chambers, H307-200 μm porosity and F20Y – 75 μm porosity placed in series (Microfluidics M-110Y, Newton, MA, USA) at a pressure of 40 psi. All emulsions had a mean particle size