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Original article

Key role of cysteine residues and sulfenic acids in thermal- and H_2O_2-mediated modification of β-lactoglobulin

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ABSTRACT

Oxidation results in protein deterioration in mammals, plants, foodstuffs and pharmaceuticals, via changes in amino acid composition, fragmentation, aggregation, solubility, hydrophobicity, conformation, function and susceptibility to digestion. This study investigated whether and how individual or combined treatment with heat, a commonly encountered factor in industrial processing, and H_2O_2 alters the structure and composition of the major whey protein β-lactoglobulin. Thermal treatment induced reducible cross-links, with this being enhanced by low H_2O_2 concentrations, but decreased by high concentrations, where fragmentation was detected. Cross-linking was prevented when the single free Cys121 residue was blocked with iodoacetamide. Low concentrations of H_2O_2 added before heating depleted thiols, with H_2O_2 alone, or H_2O_2 added after heating, having lesser effects. A similar pattern was detected for methionine loss and methionine sulfoxide formation. Tryptophan loss was only detected with high levels of H_2O_2, and no other amino acid was affected, indicating that sulfur-centered amino acids are critical targets. No protection against aggregation was provided by high concentrations of the radical scavenger 5, 5-dimethyl-1-pyrroline *N*-oxide (DMPO), consistent with molecular oxidation, rather than radical reactions, being the major process. Sulfenic acid formation was detected by Western blotting and LC–MS/MS peptide mass-mapping of dimedone-treated protein, consistent with these species being significant intermediates in heat-induced cross-linking, especially in the presence of H_2O_2. Studies using circular dichroism and intrinsic fluorescence indicate that H_2O_2 increases unfolding during heating. These mechanistic insights provide potential strategies for modulating the extent of modification of proteins exposed to thermal and oxidant treatment.

1. Introduction

Oxidation is a major cause of protein deterioration in mammals, plants, foodstuffs and pharmaceuticals, with this giving rise to changes in amino acid composition, fragmentation, aggregation, solubility, hydrophobicity, conformation, susceptibility to digestion and function [1–7]. Exposure to elevated temperatures can unfold proteins and deplete or inactivate protective systems (both low-molecular-mass antioxidants and also enzymatic defenses), potentially making proteins more susceptible to oxidation, fragmentation and/or aggregation. However the role of heating in exacerbating oxidation-induced changes, and vice-versa, is not fully established and each process might be expected to enhance the effects of the other. In particular, it is unclear whether, and how, oxidation exacerbates protein unfolding and cross-linking induced by thermal treatment, and what role particular amino acid residues play in these processes.

Multiple amino acids can play critical roles in the maintenance of protein structure, including cysteine/cystine (through the formation of disulfides, and potential thiol-disulfide exchange), aromatic residues (though hydrophobic interactions such as pi-stacking and exclusion of water molecules), hydrogen-bond donors and acceptors, and charged residues (via ionic interactions such as salt-bridges). All of these to a greater or lesser extent, can be modified by oxidation, and hence might be expected to modulate protein integrity [1,2,4,5,8]. Conversely, thermal unfolding may expose previously buried residues (such as Cys, Met and

Abbreviations: BSA, bovine serum albumin; dimedone, 5,5-dimethyl-1,3-cyclohexanedione; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO); DTNB;, 5,5′-dithio-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; β-Lg, β-lactoglobulin; MetSO, methionine sulfoxide; MSA, methanesulfonic acid; OPA, *o*-phthaldialdehyde; TBS, Tris-buffered saline, pH 7.4; TBST, Tris-buffered saline containing Tween 20; TFA, trifluoroacetic acid; TNB, 5-thio-2-nitrobenzoic acid; TRIS, tris(hydroxymethyl)aminomethane; UHT, ultra high temperature

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aromatic residues) making these more susceptible to oxidation, and it is known that many of these reactions result in the formation of products that have increased hydrophilicity as a result of the formation of oxygenated products (alcohols, carbonyls, sulfoxides, oxy acids), with this resulting in destabilization of the original (folded) structure [1,4,5,8]. Gaining an understanding of how such oxidation and unfolding processes interact is critical to the development of methods to stop undesirable changes and promote favorable outcomes, with this being of particular importance in the pharmaceutical and food industries where heat treatment and (often adventitious/unintended) oxidation occur frequently (e.g. during sterilization, lyophilisation, cooking and product formulation) (e.g. [6,7,9]). These alterations can result in a loss of function (e.g. enzyme or antibody activity [10,11]), altered activity (e.g. lowered immunoreactivity [12]), changes in structures (unfolding, aggregation, fragmentation [1,8]), decreased transport capacity [13], modified solubility, decreased product quality (e.g. decreased tenderness of meats, generation of off-flavors and aromas, lower digestibility) and a loss of nutritional value as a result of depletion of essential amino acids [6,7].

The current study investigated the individual and mutual effects of H_2O_2 and thermal treatment on β-lactoglobulin (β-Lg), the major (~50%) whey protein of bovine milk that is routinely exposed to elevated temperatures during processing (e.g. 135 °C for 1 s during UHT treatment [14]). The thermal behavior of this protein is well described in the literature (reviewed [15]), and it is known to undergo aggregation at elevated temperatures, starting at 65–75 °C, where the parent dimer monomerizes and adopts a "molten globule" conformation. This change results in the exposure of a previously buried single Cys residue (Cys121), with subsequent formation of new disulfide bonds and aggregation. The mechanism(s) of these changes are incompletely understood, with both free radical and thiol-disulfide shuffling proposed as mechanisms (reviewed [15]). This prior data makes β-Lg a good model system to investigate the individual and combined effects of thermal treatment and oxidation on proteins.

β-Lg accounts for approximately 10–15% of total milk proteins and is a relatively small globular protein consisting of 162 amino acids with a molecular mass of 18.4 kDa. Both the free thiol (Cys121), and one disulfide bond (Cys106-Cys119) are located in the hydrophobic core which is created by two β-strands and covered by a 3-turn α-helix on the outer surface [16]. A second disulfide bond is present between Cys66 and Cys160. Upon heating to 65–75 °C partial unfolding occurs, which results in loss of the α-helical structure [17,18], exposure of Cys121 and dimer formation with another reactive monomer [19–21]. Oxidation of whey protein isolate, of which β-lactoglobulin is a major component, by an iron/ascorbate/H_2O_2 system results in a significant increase in protein carbonyl levels, the formation of dityrosine cross-links (generated via reaction of tyrosine-derived phenoxyl radicals, reaction 1) and loss of thiols, with this resulting in changes to the protein structure and decreased digestibility [9]. In addition to the formation of new disulfides via thiol-disulfide exchange (reaction 2) it is also well established that these species can be formed via both the formation and subsequent dimerization of thiyl radicals ($RS^{\bullet}$; reaction 3), as well as via two-electron reactions involving sulfenic acids (RSOH) (reactions 4,5) (reviewed [22]). Furthermore a number of studies have provided evidence for cross-linking of proteins via (readily oxidized) tryptophan and tyrosine residues (reactions 6–8) (e.g. [23–25]) and via Schiff base formation (and subsequent downstream chemistry) between protein carbonyls and free amine groups (e.g. Lys residues) [26], amongst others. Thus there are multiple mechanisms by which cross-links may be generated by oxidant and thermal treatment of proteins.

$$2 \times \mathrm{TyrO}^{\bullet} \rightarrow \mathrm{Tyr\text{-}Tyr} \quad (1)$$

$$\mathrm{R^1SH} + \mathrm{R^2SSR^3} \rightarrow \mathrm{R^1SSR^2} + \mathrm{R^3SH} \quad (2)$$

$$2\mathrm{RSH} + 2\mathrm{X}^{\bullet} \rightarrow 2\mathrm{XH} + 2\mathrm{RS}^{\bullet} \rightarrow \mathrm{RSSR} \quad (3)$$

$$\mathrm{R^1SH} + \mathrm{H_2O_2} \rightarrow \mathrm{H_2O} + \mathrm{R^1SOH} \quad (4)$$

$$\mathrm{R^1SOH} + \mathrm{R^2SH} \rightarrow \mathrm{R^1SSR^2} + \mathrm{H_2O} \quad (5)$$

$$2 \times \mathrm{TrpN}^{\bullet} \rightarrow \mathrm{Trp\text{-}Trp} \quad (6)$$

$$2 \times \mathrm{TyrO}^{\bullet} \rightarrow \mathrm{Tyr\text{-}Tyr\ (dityrosine\ and\ isodityrosine)} \quad (7)$$

$$\mathrm{TrpN}^{\bullet} + \mathrm{TyrO}^{\bullet} \rightarrow \mathrm{Trp\text{-}Tyr} \quad (8)$$

In the light of these multiple proposed mechanisms for β-Lg aggregation, studies have been carried out where the protein has been exposed to heat alone, H_2O_2 alone, and with H_2O_2 added prior to or post heat treatment. Analyses have been carried out at both the molecular and structural level, together with studies on the mechanism(s) of aggregation.

2. Materials and methods

2.1. Chemicals

β-Lactoglobulin (β-Lg) from bovine milk (L3908, a mixture of isoforms A and B), amino acid standards, trichloroacetic acid (TCA), o-phthaldialdehyde (OPA), methanesulfonic acid (MSA; 4 M containing 0.2% tryptamine), iodoacetamide, methanol, sodium thiosulfate, silver nitrate, formaldehyde, sodium carbonate, ethylenediamine tetraacetic acid (EDTA), acetic acid, bovine serum albumin (BSA), 30% hydrogen peroxide (H_2O_2) solution, sodium phosphate dibasic dodecahydrate, sodium phosphate monobasic dehydrate, tris(hydroxymethyl)aminomethane (TRIS), Tween-20, sodium chloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), urea, 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO), 5,5-dimethyl-1,3-cyclohexanedione (dimedone), DL-dithiothreitol (DTT), trifluoroacetic acid (TFA), and acetonitrile were purchased from Sigma Aldrich Chemie GmBh (Steinheim, Germany). The biotin-tagged reagent DCP-Bio1 was purchased from Kerafast (Boston, Massachusetts).

2.2. Sample preparation

Stock solutions of β-lactoglobulin (molecular mass 18.4 kDa) were dissolved by solvation of the solid protein in 10 mM sodium phosphate buffer at pH 7, at a concentration of 200 μM as determined by absorbance measurement at 280 nm using an extinction coefficient, ϵ of 17210 M^{-1} cm^{-1} [27]. The protein solution (final concentration 100 μM) was either left untreated, exposed to heat alone, or treated with different molar ratios of β-Lg to H_2O_2 (1:0; 1:1, 1:2; 1:10; 1:20; 1:100, 1:200; 1:2000), with the H_2O_2 addition occurring without heating, before heating, or after gradual heating from 20 to 100 °C over 3 h in an oven (Thermo Scientific, Heratherm) with a temperature ramp of 0.45 °C/min.

2.3. Gel electrophoresis

SDS-PAGE of the samples, denatured for 5 min at 90 °C in the presence of LDS sample buffer (4 ×) (Invitrogen), was carried out after loading 1 μg protein per lane on 12% Bis-Tris gels (Invitrogen). For reducing gels, 500 mM dithiothreitol at a 10 × concentration (Invitrogen) was added to the samples before loading.