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Proteomic tracking of hydrothermal Maillard and redox modification in lactoferrin and β-lactoglobulin: Location of lactosylation, carboxymethylation, and oxidation sites

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ABSTRACT

Lactoferrin and β-lactoglobulin are important protein components of mammalian milk. Maillard reactions, as well as redox chemistry, are of particular interest for dairy products because they are known to occur during common processing steps, notably heating procedures such as pasteurization. Using a redox proteomics approach, we characterized AA residue side-chain modification across a range of heating times and with or without the specific addition of lactose, to both map the key modification sites within these proteins and evaluate their sensitivity to process-induced modification. Heating in the presence of lactose resulted in significant Maillard modification (both lactosylation and carboxymethylation) to both bovine lactoferrin and β-lactoglobulin. Notably, Lys47, a key residue in the bioactive peptide lactoferricin, was particularly susceptible to modification. Lactoferrin appeared to be fairly robust to hydrothermal treatment, with relatively low levels of oxidative modification observed. In contrast, β-lactoglobulin was susceptible to significant oxidative modification under hydrothermal treatment, with the range and type of modifications observed suggesting compromised nutritional value. These results have important implications for processing applications in dairy foods where retention of biological function and optimal protein quality is desired.
Key words: lactoferrin, β-lactoglobulin, Maillard reaction, redox chemistry, proteomics

INTRODUCTION

Lactoferrin and β-LG are important protein components of mammalian milk (Farrell et al., 2004). Lactoferrin, a globular glycoprotein of approximate molecular weight of 80 kDa also known as lactotransferrin, is a multi-functional component of the immune system, with reported antimicrobial, antifungal, and antiviral properties (Adlerova et al., 2008). It has an important immunomodulatory role and can be found in a range of secretory fluids in addition to milk, including tears and saliva (Adlerova et al., 2008). Digestion with pepsin and chymosin, such as occurs during consumption of dairy foods, is believed to result in the release and uptake of bioactive peptides from lactoferrin (Tomita et al., 2009). β-Lactoglobulin is the major protein present in whey from cow, goat, and sheep milk, but is notably absent in human milk. It is also a globular protein with a molecular weight of approximately 18 kDa (precursor 20 kDa, mature protein 18 kDa), but is yet to be robustly assigned a specific cellular or systemic function (Kontopidis et al., 2004).

Maillard chemistries can profoundly affect the color, flavor, digestibility, and nutritional value of food. They encompass a complex array of reactions, starting with the glycation of protein AA residues, with lysine being particularly susceptible, and progressing to form sugar-derived protein adducts and cross-links also known as advanced glycation end products. The consequences of such chemistry can be positive, such as flavor development, or negative, such as unwanted coloration changes, and thus understanding and controlling Maillard chemistry is important in food processing. For instance, impaired nutritional value can occur due to changes in protein integrity and function through protein cross-linking mediated by glycation products, which could limit the bioavailability of the AA lysine.

Maillard reactions are of particular interest for dairy products, as they are known to occur during common processing steps, notably heating procedures such as pasteurization (Meyer et al., 2012). Milk has a high lactose content (Cerbulis and Farrell, 1975), making it particularly susceptible to lactosylation during storage and

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processing (Guyomarc'h et al., 2000). The implication for dairy foods is significant, with potential changes in structure, physicochemical properties, susceptibility to proteases, nutritional value, and bioactivity. Whereas Maillard reaction products of caseins and major whey proteins have been relatively well characterized (Jing and Kitts, 2004; Aminlari et al., 2005; Meltretter et al., 2007), only very limited previous work has been done with lactoferrin (Joubran et al., 2013), and the location of modifications in these proteins is not well understood.

The effects of the Maillard reaction in the case of lactoferrin have important potential implications with respect to its use as a functional ingredient. Specific regions of lactoferrin responsible for bioactive function, for example, iron-binding and antimicrobial peptides, contain lysine. Lactosylation of these lysine residues is likely to have a profound effect on bioactivity, either altering it or eliminating activity completely. Lactosylation is an early stage Maillard product. Lactosylated residues can subsequently undergo oxidative cleavage to form protein-bound carboxymethyllysine. We here postulate that hydrothermal treatment, such as is commonly applied in milk processing and production of dairy products, leads to both lactosylation and subsequent formation of carboxymethyllysine from susceptible lysine residues. The occurrence of lactosylation in some milk proteins has been reported previously (Fogliano et al., 1998), but detailed mapping of susceptibility has not been performed.

Redox proteomics is a sub-discipline of proteomics that focuses on the characterization of often low abundance oxidative modifications to proteins. Application of redox proteomics to a range of systems has led to the development of a powerful set of mass spectrometric and bioinformatic tools, and these have been used effectively to map modifications in proteins exposed to oxidative and other insults (Dyer et al., 2006a, 2010; Grosvenor et al., 2010, 2011). In this study, we applied a redox proteomic approach to specifically characterize sites of lactosylation in lactoferrin, and performed a parallel analysis of another key whey protein, β-LG. We characterized AA residue side-chain modification across a range of heating times with or without the specific addition of lactose, to both map the key modification sites within these proteins and evaluate their sensitivity to process-induced modification.

MATERIALS AND METHODS

Sample Preparation

Disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were from BDH (Poole, UK). Bovine lactoferrin, β-LG, and lactose and all other general chemicals were obtained from Sigma (St. Louis, MO). Lactoferrin and β-LG were each dissolved in PBS (10 m*M*, pH 6.8) at a concentration of 0.15 m*M* (11.1 mg/mL of lactoferrin, 2.67 mg/mL of LG). The solutions were heated at 55°C in the presence or absence of lactose (146 m*M*) with agitation using a Mini LabRoller rotator (Labnet, Woodbridge, NJ) for 24, 48, 72, 96, 120, 144, and 168 h. This resulted in a 973-fold molar ratio excess of lactose:protein, reflecting a biologically relevant lactose concentration and the lactose:substrate ratios used in Dalsgaard et al. (2007) and Meltretter et al. (2007).

Proteomic Evaluation

Enzymatic Digestion. Samples were prepared for mass spectrometric analysis by dialysis in Slide-A-Lyzer Mini dialysis units fitted with 2,000 molecular weight cutoff membranes (Thermo, Rockford, IL) against 100 m*M* ammonium bicarbonate, which was the solvent for subsequent steps. Reduction was performed with 50 m*M* Tris(2-carboxyethyl)phosphine at 56°C for 45 min, followed by alkylation with 360 m*M* acrylamide in the dark at ambient temperature for 30 min and digestion using 1 μg of sequencing grade porcine trypsin (Promega, Madison, WI) in 10% acetonitrile, 50 m*M* ammonium bicarbonate for 18 h at 37°C.

Liquid Chromatography-Tandem Mass Spectrometry. The resulting peptides were diluted 1:500 with loading solvent (2% acetonitrile, 0.2% formic acid). Liquid chromatography-tandem mass spectrometry (**LC-MS/MS**) was carried out on an Ultimate nanoflow HPLC equipped with a Famos autosampler and Switchos column switching module (LC-Packings, Amsterdam, the Netherlands). A 10-μL sample was loaded on a C18 trap column (300 μm internal diameter, 5 μm particles, 300 Å pore size) at a flow rate of 8 μL/min. The trap column was then switched in line with the analytical column (C18, 20 cm, 75 μm internal diameter, 5 μm particles, 300 Å pore size), and eluted at a flow rate of 150 nL/min, with a gradient from 2 to 55% B in 50 min. Solvent A was HPLC-grade water with 0.2% formic acid, solvent B was LCMS-grade acetonitrile with 0.2% formic acid. Using a stainless-steel nanospray needle (Proxeon, Odense, Denmark), the column outlet was directly connected to a QSTAR Pulsar i mass spectrometer (Applied Biosystems, Foster City, CA), which was programmed to acquire 3 MS/MS traces of 1+, 2+, 3+, 4+, and 5+ peptides per MS survey scan.

Data Analysis. Mascot Daemon (v2.2.2) was used to convert data files to peak lists (Mascot Script for Analyst v1.6b25), which were imported in ProteinScape