Covalent bonding of 4-methylcatechol to -lactoglobulin results in the release of cysteine-4-methylcatechol adducts after in vitro digestion

Waqar, Khadija; Engholm-Keller, Kasper; Joehnke, Marcel S.; Chatterton, Dereck E.W.; Poojary, Mahesha M.; Lund, Marianne N.

Published in:
Food Chemistry

DOI:
10.1016/j.foodchem.2022.133775

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Covalent bonding of 4-methylcatechol to β-lactoglobulin results in the release of cysteine-4-methylcatechol adducts after in vitro digestion

Khadija Waqar a, b, Kasper Engholm-Keller a, Marcel S. Joehnke a, Dereck E.W. Chatterton a, Mahesha M. Poojary a, Marianne N. Lund a, b, *

a Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark
b Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark

ARTICLE INFO

Keywords:
Protein-polyphenol bonding
Michael addition reaction
Amino acid-polyphenol adducts
4-methylbenzoquinone
Whey protein
Polyphenols

ABSTRACT

Protein-polyphenol adducts are formed upon covalent bonding between oxidized polyphenols and proteins. 4-Methylcatechol (4MC) is a polyphenol with origin in coffee and is oxidized to 4-methylbenzoquinone (4MBQ) under conditions used during food processing. The present study characterizes 4MBQ-induced covalent modifications on β-lactoglobulin (β-LG) from bovine milk, (henceforth β-LG) and the effect on protein digestibility. Significant thiol and amine loss was found in β-LQ compared to β-LG. Site-specific 4MBQ-induced modifications were identified on Cys, Lys, Arg, His and Trp in β-LQ. No significant differences between β-LG and β-LQ on in vitro digestibility were observed by assessment with SDS-PAGE, degree of hydrolysis and LC-MS/MS unmodified peptide intensities. Cys-4MC adduct (1.7 ± 0.1 μmol/g) was released from β-LQ after in vitro digestion. Thus, it is relevant to investigate how released Cys-4MC adducts are absorbed in vivo in future studies.

1. Introduction

Polyphenols have significant biological functions important for human health and disease prevention, which promotes their consumption in the human diet (Williamson, Kay, & Crozier, 2018). Polyphenols can oxidize to reactive quinones in foods by naturally occurring polyphenol oxidases, by transition metal ions (Fe²⁺ or Cu⁺), or via their antioxidative action (Kepper, Schwarz, & van der Goot, 2020). Quinones may react with nucleophilic groups on proteins in foods by Michael addition resulting in the formation of a covalent bond between the quinone and protein (Bittner, 2006). Previous studies have demonstrated covalent bonding between oxidized polyphenols in model systems containing amino acids (Li, Jongberg, Davies, & Lund, 2016), tryptic peptides (Wang, Koivumäki, Kylli, Heinonen, & Poutanen, 2014) and proteins (Rawel, Rohn, Kruse, & Kroll, 2002; Ali, Homann, Khalil, Kruse, & Rawel, 2013). Similarly, in food systems, addition of green tea polyphenols into UHT-treated milk (Poojary, Zhang, Olesen, Rauh, & Lund, 2020) and addition of 4-methylcatechol (4MC) to meat (Arsad et al., 2020) has been shown to result in extensive bonding to proteins. β-Lactoglobulin (β-LG)-catechin/epicatechin covalent bonding has been detected in commercial chocolate milk (Gallo, Vinci, Graziani, De Simone, & Ferranti, 2013). Caffeoquinone-amino acid covalent conjugates in tea and coffee were associated with flavor formation and color formation (Bittner, 2006). Quercetin-protein complexes were detected in peanut proteins with added cranberry (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017). In beer, proteins and polyphenols undergo covalent bonding that contribute to haze formation (Jongberg, Andersen, & Lund, 2020). Stored honey is another example where protein–polyphenol covalent bonding as well as polyphenol mediated protein crosslinking have been reported (Brudzynski, Sjaarda, & Maldonado-Alvarez, 2013). The bonding of polyphenols to food proteins can change protein functional properties; e.g. the antioxidative capacity and thermal stability were increased in whey proteins modified with coffee polyphenols (Ali et al., 2013), textural properties were changed in meat sausages added green tea extract (Jongberg, Terkelsen, Miklos, & Lund, 2014) and rheological properties were changed in meat proteins modified with rosomarinic acid (Tang et al., 2017).

Abbreviations: β-LG, β-lactoglobulin; β-LQ, β-lactoglobulin modified with 4MBQ; Cys-4MC, cysteine-4-methylcatechol adduct; DH, degree of hydrolysis; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); GI, gastro-intestinal; Lys-4MBQ, lysine-4-methylbenzoquinone adduct; 4MC, 4-methylcatechol; 4MBQ, 4-methylbenzoquinone; TMBSA, 2,4,6-trinitrobenzene sulfonic acid; UV–vis, ultraviolet-visible.

* Corresponding author at: Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark.

E-mail address: mnl@food.ku.dk (M.N. Lund).

https://doi.org/10.1016/j.foodchem.2022.133775
Received 8 February 2022; Received in revised form 29 June 2022; Accepted 21 July 2022
Available online 25 July 2022
0308-8146/© 2022 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
Polyphenols may also bind to proteins through non-covalent interactions (usually by hydrophobic interactions and hydrogen bonds). Previous studies have reported various effects of polyphenol-binding on protein digestibility [reviewed by (Kroll, Rawl, & Rohn, 2003)]; non-covalent polyphenol-protein interactions may both decrease and increase protein digestibility, while covalent polyphenol bonding is usually seen to decrease proteolytic digestion of food proteins. However, a detailed chemical characterization of the quinone-modified protein used for digestion studies as well as the resulting digest is lacking. Covalent bonding between polyphenols and proteins may affect proteolytic digestion, which may in turn affect the nutritional quality of proteins. Therefore, it is essential to understand how polyphenol-bonding affects protein digestion on a molecular level. In vitro digestion is a commonly employed method for determining the bioaccessibility of food components in a given matrix, such as the total amount of amino acids and peptides released after digestion of a food matrix (Carbonell-Capella, Buniewska, Barba, Esteve, & Frigola, 2014). Secondly, it may provide information about bioavailability of the proteins and polyphenols during gastro-intestinal (GI) digestion (Carbonell-Capella et al., 2014). Polyphenols can influence protein digestibility by formation of a covalent bond either with a food protein (substrate) or digestive proteases (Kroll et al., 2003). Non-covalent binding of polyphenols to digestive enzymes may also lead to inhibition of their activity (Velickovic & Stanic-Vucinic, 2018). In order to study the digestibility of food protein-polyphenol adducts, free polyphenols in the sample should be removed as they might interact with digestive proteases and hereby influence the digestibility of the substrate protein. Thus, a covalent protein-polyphenol adduct consisting of β-LG covalently modified with 4MC (β-LQ) was synthesized in the present study and free 4MC was removed prior to in vitro digestion. 4-Methylcatechol (4MC) is a polyphenol found in roasted coffee (Lang, Mueller, & Hofmann, 2006), and can also be considered as a model polyphenol for flavonoids found in fruits and vegetables owing to the structural similarity with the antioxidant B-ring of flavonoids. The use of 4MC offers an analytical advantage for characterization of protein-polyphenol bonding without the involvement of highly polymerized products, which are typically formed with complex polyphenols.

Quinones of various polyphenols (e.g. rosmarinic acid, chlorogenic acid, epicatechin, 4MC) under different reaction conditions have been demonstrated to react with multiple amino acid residues on proteins; Cys, Lys, Arg, and His residues (Ali et al., 2013; Arsad et al., 2020; Tang et al., 2017), and some studies have also shown quinone-induced modifications on Trp and Tyr (Prigent et al., 2008). Cys and Lys are believed to be the major targets for quinone-induced modifications, and Cys is kinetically favored (Li et al., 2016). Upon Michael addition re-actions of quinones, the phenolic moiety is regenerated (Fig. 1, reaction 2). The phenolic moiety may undergo a second oxidation leading to a quinone-modified protein or peptide (Fig. 1, reaction 1’), which has been demonstrated for Lys in a model system (Li et al., 2016), but may also take place in the MS source during analysis (Sojo, Chahal, & Keller, 2014). In addition, Lys may also form an imine/Schiff base upon reaction with 4MBQ (Fig. 1, reaction 3). The aim of the present study was to first characterize the covalent modifications induced by 4-methylbenzoquinone (4MBQ), the quinone of 4MC, on the whey protein β-lactoglobulin (β-LG) and thereafter determine the effect of those covalent modifications on in vitro protein digestibility. 4MBQ-induced covalent modifications of Trp, Arg and His residues, which were previously not found in model systems with β-LG, were detected in the present study. In addition, release of Cys-4MC adducts after in vitro digestion of β-LG-4MC covalent adduct was reported here for the first time.

2. Materials and methods

2.1. Chemicals

β-LG from bovine milk (≥90%, with variants A and B), 4MC (≥95%), Amberlyst A-26 (OH) ion exchange resin, periodic acid (99%), acetonitrile (HPLC gradient grade), pepsin from porcine gastric mucosa (≥250 units/mg solid), pancreatic from porcine pancreas (8 × United States pharmacopeia (USP) specification, protease activity of 209 USP units/mg), 5,5-dithiobis(2-nitrobenzoic acid) (>98%, DTNB), 2,4,6-trinitrobenzene sulfonic acid (98%, TNBSA), nitro-blue tetrazolium chloride (98%, NBT), L-alanine (>98.5%), N-α-acetyl L-lysine (>98%), L-cysteine (97% were purchased from Sigma Aldrich, Darmstadt, Germany. Urea Merck (Darmstadt, Germany), ammonium bicarbonate, tris (2-carboxyethyl)phosphine, TrypZean trypsin (>3,350 units/mg solid USP) were from Merck, Saborg, Denmark and formic acid (VWR, Saborg, Denmark), all were of analytical grade. Deionized water obtained from a Milli-Q purification unit (Millipore, Bedford, MA, USA) was used for all experiments.

2.2. Preparation of β-lactoglobulin-4MC adduct (β-LQ)

4MBQ solution was prepared according to a previously described
method (Jongberg, Lund, & Otte, 2015). Briefly, 2.5 mL 4MC solution (0.1 M) was prepared in acetonitrile in 5 mL sample tubes followed by addition of 395 mg per-iodate resin and mixed (under magnetic rotation) for 3 min in the dark. The resin was then filtered (Whatman filter paper, 11 cm diameter, Whatman Ltd, USA) from the 4MBQ solution. The concentration of 4MBQ was determined to be 94 ± 3 mM by ultraviolet–visible (UV–vis) spectrophotometry using an extinction coefficient of 1350 M⁻¹ cm⁻¹ at 395 nm (Li et al., 2016). Freshly prepared 4MBQ in acetonitrile (used within 20 min) and β-LG stock (10 mg/mL in 0.25 M tris buffer, pH 8) were mixed in 1:1 M ratio in a test tube at 22°C for 10 min with stirring in the dark in order to obtain β-LQ. A control sample of β-LG without 4MBQ was prepared and treated similarly.

The concentration of free (unreacted) 4MC and 4MBQ in β-LQ was determined as follows: 0.1 % formic acid in acetonitrile was added to the β-LQ solution (1:3 v/v) in order to precipitate protein. Samples were placed at 4°C for 1 h followed by centrifugation at 15,000 g for 20 min. The resulting supernatant was analyzed by UHPLC as previously described (Poojary et al., 2020), and the content of 4MC and 4MBQ was found to be below limit of detection (1 µM) of the method.

2.3. Characterisation of β-LG and β-LQ prior to in vitro digestion

2.3.1. Quantification of thiol and amine groups in β-LG and β-LQ

Thiol groups in β-LG and β-LQ were quantified by a DTNB assay based on an external calibration curve prepared from l-Cys and results were expressed as µmol/L of protein as previously described (Jongberg et al., 2015).

Total available amine groups were quantified according to previously described method (Habeeb, 1966) with minor modifications. β-LG or β-LQ (125 µL, 0.1 mg/mL in 0.2 M phosphate buffer, pH 8) were mixed with 63 µL TNBSA (0.01 % in the same phosphate buffer). Samples were incubated at 37°C for 2 h. The reaction was stopped by addition of 63 µL of 10 % SDS in milli-Q water and 31 µL 1 M HCl. The absorbance at 355 nm was determined with a UV/Vis spectrophotometer (Varian, Herlev, Denmark). Amine groups were quantified based on an external standard curve prepared from N-α-acetyl-l-lysine by a similar protocol as for the samples. Results are expressed as N-α-acetyl-l-lysine equivalents (µmol/L of protein).

2.3.2. Detection of Cys-4MC adducts in β-LQ

β-LQ (1.5 mL, 20 mg/mL) was mixed with 1.5 mL of 12 M HCl in a microwave hydrolysis vial (Biotage, Uppsala, Sweden). The vials were flushed with nitrogen for 60 s, immediately sealed, and subjected to microwave hydrolysis (Biotage initiator, Uppsala, Sweden) by pre-stirring for 20 s followed by heating at 150°C for 60 s followed by a second heating at 165°C for 10 min. After hydrolysis, samples were subjected to vacuum centrifugation in a SpeedVac (SPD131DDA, Thermo Scientific, Waltham, MA, USA). The resulting pellets were dissolved in 1.5 mL Milli-Q water. Ten microliters of each sample were analyzed by LC/MS/MS after filtering the samples with 0.2 µm syringe filters (Phenomenex, Torrance, CA, USA). The LC/MS/MS system was an Ultimate 3000 UHPLC coupled with a Q Exactive hybrid quadrupole–Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The column used was a Phenomenex Aeries 1.7 µm Particle Size C18 (150 × 2.1 mm). The mobile phase included solvent A (0.1 % formic acid in Milli-Q water) and solvent B (80 % acetonitrile in 0.1 % formic acid).

The flow rate was 0.25 mL and the gradient was as follows: 0.0–4.0 min: 3 % B; 4.0–10.4 min: 3 % to 15 % B; 10.4–13.5 min: 15 % to 100 % B; 13.5–13.6 min: 100 % B, 13.6–17.0 min: 3 % B. The MS was operated in positive ionization mode. Quantification of the Cys-4MC adduct in β-LQ was based on an external calibration curve obtained with an in-house synthesized Cys-4MC standard diluted in Milli-Q water (Arsad et al., 2020).

2.3.3. Amino acid site-specific identification of 4MBQ-derived modification in β-LQ

β-LG and β-LQ (5 µL, 9.9 µg/mL in 0.25 M tris buffer pH 8) was mixed with 5 µL of 8 M urea, 1 µL 1 M ammonium bicarbonate and 1.2 µL of 0.1 M tris(2-carboxyethyl)phosphine and incubated for 30 min at room temperature. Ammonium bicarbonate (36 µL of 50 mM, pH 8.5) was added, followed by 2 µL of 1 µg/µL TrypZea trypsin. After 5 h of hydrolysis at 21°C, another 2 µL of TrypZea solution was added and the reaction was allowed to proceed for 5 h prior to addition of 25 mL 10 % formic acid.

Each sample (50 µL) was injected onto a Phenomenex Aeries C18 (150 × 2.1 mm 2.6 µm particle size) column (at 4°C) using the same LC/MS/ MS system as described above with a 40 min run at a flow rate of 250 µL/min, with the peptides being eluted off the column using an increasing gradient from buffer A (0.1 % formic acid) to B (90 % acetonitrile, 0.1 % formic acid) (0–1.5 min: 2 % B; 1.5–5 min: 2–8 % B; 5–25 min: 8–27 % B; 25–32 min: 27–45 % B; 32–33 min: 45–90 % B; 33–35 min: 90 % B; 35–36 min: 90–2 % B; 36–40 min: 2 % B). Settings were: 3.5 kV spray voltage, sheath gas setting 50, aux gas setting 16, probe heater temperature 320°C. A full MS scan of the m/z 300–1500 range was acquired in the Orbitrap (full width at half maximum (FWHM) resolution of 70,000) with a target value of 1 × 10⁶ ions. For each full scan, the seven most intense ions (charge state 2 + to 8 + ) were selected for higher-energy collision dissociation (HCD) and the fragments detected at a resolution of 35,000 FWHM. Settings for the HCD event were as follows: target value of ions for HCD was 1 × 10⁶, while the threshold for ion selection was 5 × 10⁴, and the maximum injection time was 240 ms at the MS level and 110 at the MS/MS level. The isolation window was 2.0 Da, first fixed mass was 140, normalized collision energy was 28, peptide match was off, exclude isotopes was on, and dynamic exclusion was 8.0 s. To identify and perform relative quantification of 4MC- and 4MBQ-modified peptides from the LC/MS/MS raw data, peak list generation and database searching of the peak lists against a concatenated forward/reverse database comprised of the processed form of bovine β-LG and common contaminants in biological LC-MS/MS experiments (294 proteins in total) was performed using MaxQuant 1.5.8.3 (Cox & Mann, 2008). Default parameters were used except for the following: enzyme was trypsin/P, minimum peptide length was 6, the “match between runs” function was enabled with a matching time window of 0.7 min and an alignment time window of 20 min. The following variable modifications were allowed: 4MC modification of Cys, Lys, Arg, His, Trp and the protein N-terminal (+122 Da), 4MBQ modification (+120 Da) of Cys, Lys, Arg, His, Trp and the protein N-terminal. Schiff base-like 4MBQ modification of Lys, Arg, His, Trp and the protein N-terminal was also included as it was of interest to examine if a carbonyl group on 4MBQ would react directly with amine groups (Lund, 2021). In addition, oxidation (+16 Da) of Met was included as this modification is often observed during MS analysis. Only modified peptides with a site localization probability > 0.75 (which was also manually validated) were accepted. The peptide identifications, intensities and the estimated site modification level, in MaxQuant called “ratio mod/base” (the intensity of the modified form of the peptide in the MS extracted ion chromatogram divided by the sum of intensities of all peptides with the same amino acid sequence that did this contain this modified form), were exported to Microsoft Excel for further calculations. It has to be noted that this estimate relies on the assumption of equal ionization efficiency for all forms of the peptides (both unmodified and various modified forms), an assumption that is not always met, the average of ratio mod/base (n = 2) divided by the highest ratio mod/base obtained was used for determining a relative ratio mod/base for each modification, which gives an estimated relative modification level of all modified β-LQ residues.

2.4. In vitro pepsin and pancreatin digestion of β-LG and β-LQ

To allow for pepsin digestion, 5 mL of β-LG and β-LQ (10 mg/mL) in
0.25 M tris buffer (pH 8.0) were dialyzed separately against 10 mM HCl to achieve acidic pH (pH 2.0). Dialysis was performed using a dialysis tubing cellulose membrane (Sigma Aldrich, Germany) (10 kDa molecular weight cut-off) in the dark at 4 °C. The dialysis buffer was changed twice after every 4 h and later overnight with the third buffer change. After dialysis, the protein concentration (β-LG samples) was determined spectrophotometrically by measuring the absorbance at 280 nm and using an extinction coefficient of 17,600 M⁻¹·cm⁻¹ (Al-Shabib et al., 2018). For in vitro pepsin digestion, samples (5 mL of β-LG or β-LQ) were first incubated at 37 °C for 30 min in a water bath followed by addition of pepsin (0.005 % w/w of protein). Aliquots (10 µL) of digested samples were removed at various time durations (0, 10, 30, 60, 120 min), placed on ice and diluted to 0.1 mg/mL with ice-cold Milli-Q water and stored at –20 °C until further analysis. Following 2 h of digestion, the pH of the remaining samples (4950 µL) was adjusted to 7.8–8.0 by addition of 1.43 M sodium bicarbonate. Pancreatin (1.5 % w/w of protein) was added and samples were removed after 0, 10, 30, 60, 120, 180, and 240 min, placed immediately on ice and diluted in ice-cold Milli-Q water to 0.1 mg/mL and stored at –20 °C. The digested samples were analyzed as described below.

### 2.4.1. Degree of hydrolysis (DH)

Concentration of free amine groups in undigested and in vitro digested samples was determined as described previously (Joehnke et al., 2018). Concentration of total free amines was determined after a complete acidic hydrolysis performed with HCl as described in section 2.4. DH was calculated according to equation 1 and free amines were expressed as γ-alanine equivalents in µmol/g from an external calibration curve.

\[
DH\;[\%] = \frac{\text{free amines (after in vitro digestion) – free amines (undigested sample)}}{\text{total free amines (after total hydrolysis) – free amines (undigested sample)}} \times 100
\]

#### 2.4.2. SDS-PAGE and blotting assay with NBT staining for polyphenol-protein binding

SDS-PAGE of protein samples (undigested and digested at various time points) was performed under non-reducing conditions with Nu-PAGE 12 % Bis-Tris gels (1.0 mm × 15 wells, Thermo Fisher Scientific, Carlsbad, CA, USA). Samples were mixed with LDS sample buffer (Nu-PAGE, Thermo Fisher Scientific, Carlsbad, CA, USA) in a 4:1 ratio, heated at 70 °C for 10 min, and 5 µg of protein was loaded to each well. Gels were run with 1 % Nu-PAGE MES SDS running buffer for 40 min at 200 V (Power Pac 300, BioRad, CA, USA) and subsequently stained overnight with Coomassie Brilliant Blue G-250. Gels were destained with Milli-Q water at room temperature for 2 h and scanned (Epson Perfection V750 Pro, Seiko Epson Corporation, Japan).

Polyphenol-protein binding was evaluated by blotting assay with NBT staining. Protein digestion time course samples in unstained gels were first transferred to a PVDF membrane (IB24002, ThermoFisher Scientific, Waltham, MA, USA) using an iBlot 2 Dry Blotting system (ThermoFisher Scientific) followed by NBT staining, performed in the absence of light as previously described (Pazz, Flückigers, Boakl, Kaganil, & Gallop, 1991).

#### 2.4.3. Peptide identification and relative quantification of in vitro-digested β-LG/β-LQ

Each sample (25 µL, 0.9 µg/µL in 1 % formic acid) was injected onto a Thermo Accucore C18 50 × 2.1 mm column using the same LC-MS/MS system as described above. Peptides were analyzed using a 40 min LC-MS/MS run at a flow rate of 250 µL/min, being eluted off the column using an increasing gradient from buffer A (0.1 % formic acid) to B (90 % acetonitrile, 0.1 % formic acid) (0–1.5 min: 2 % B; 1.5–5 min: 2–8 % B; 5–25 min: 8–27 % B; 25–32 min: 27–45 % B; 32–33 min: 45–90 % B; 33–35 min: 90 % B; 35–36 min: 90–2 % B; 36–40 min: 2 % B). Eluted peptides were introduced into a Q Exactive high-resolution mass spectrometer (3.8 kV spray voltage, sheath gas setting 30, aux gas setting 15, probe heater temperature of 350 °C) (Thermo Scientific, Bremen, Germany) via a heated ESI source (Thermo Scientific, Bremen, Germany). A full MS scan of the m/z range was acquired in the Orbitrap (full width at half maximum (FWHM) resolution of 35,000) with a target value of 1 × 10⁶ ions, with lock mass of m/z 445.120025 being enabled. For each full scan, the ten most intense ions (charge state 1 + to 8 +) were selected for higher-energy collision dissociation (HCD) and detected at a resolution of 17,500 FWHM. Settings for the HCD event were as follows: target value of ions for HCD was 1 × 10⁵, while the threshold for ion selection was 2 × 10⁵, and the maximum injection time was 300 ms at the MS level and 55 at the MS/MS level. The isolation window was 2.0 Da, first fixed mass was 140, normalized collision energy was 28, peptide match was off, exclude isotopes was on, and dynamic exclusion was 5.0 s.

To identify and quantify peptides released from in vitro digested β-LG/β-LQ from the LC-MS/MS raw data, peak list generation and database searching of the peak lists against a concatenated forward/reverse database comprised of the processed form of bovine β-LG and common contaminants in biological LC-MS/MS experiments (294 proteins in total) was performed using MaxQuant 1.5.8.3. Default parameters were used except for the following: enzyme was unspecific and the following variable modifications were allowed: 4MC modification of Cys, Lys, Arg, His, Trp and the protein N-terminal (+122 Da) and 4MBQ modification (+120 Da) of the same positions, and oxidation (+16 Da) of Met. The peptide identifications and intensities from the MaxQuant output were exported to Microsoft Excel and sample replicate areas were averaged. The unmodified peptide sequences with similar intensities from β-LG and β-LQ were searched against a milk bioactive peptide database (Nielsen, Beverly, Qu, & Dallas, 2017) to determine their bioactivity.

LC-MS/MS raw data files along with peak list files and annotated MS/MS spectra of all 4-MC/4-MBQ-modified peptides are available via MassIVE (https://massive.ucsd.edu) with identifier MSV000088002.

#### 2.4.4. Determination of release of Cys-4MC adduct derived from β-LQ after in vitro digestion

In vitro pepsin and pancreatin digested samples (0.5 mg/mL) were syringe filtered (0.2 µm, Phenomenex, Torrance, CA, USA) and transferred to LC-MS vials. Filtered samples (10 µL) were analysed with the same LC-MS/MS method as described in section 2.3.2.

#### 2.5. Statistical analysis

All values are expressed as mean ± SD. Independent Student’s t-test (SPSS version 26) was performed for comparing results from β-LG and β-LQ with p-values < 0.05 considered significant.
residues. These losses indicated covalent protein-quinone bonding, or that the reaction between 4MBQ and 4MBQ during the formation of induced reactions (Li et al., 2016). Free thiol and amine groups were β-lyzed

Table 2

<table>
<thead>
<tr>
<th>Reaction</th>
<th>β-LQ modification level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-4MC</td>
<td>100 ± 2%</td>
</tr>
<tr>
<td>Lys-75</td>
<td>23 ± 1%</td>
</tr>
</tbody>
</table>

a, b Values calculated from independent sample t-test bearing different letters within the same row are statistically significant (p < 0.05).

3. Results and discussion

3.1. Characterization of β-LG and β-LQ prior to in vitro digestion

In the present study, non-covalent binding between 4MC/4MBQ and β-LG was negligible since unreacted 4MC/4MBQ was below LOD (1 μM) after protein precipitation as described in section 2.2.

3.1.1. Quatification of thiol and amine groups in β-LG and β-LQ

Thiol and amine groups were quantified in β-LG and β-LQ since these nucleophilic groups are expected to be the major sites of quinone-induced reactions (Li et al., 2016). Free thiol and amine groups were significantly reduced in β-LQ as compared to β-LG (Table 1), indicating that the reaction between 4MBQ and β-LG modified both Cys and Lys residues. These losses indicated covalent protein-quinone bonding, or could, at least partly, be caused by pro-oxidative effects induced by 4MBQ during the formation of β-LQ. These effects are in accordance with previous studies using the β-LQ model system (Jongberg et al., 2015).

3.1.2. 4MBQ-induced site-specific covalent modifications in β-LQ

Tryptic hydrolysis of β-LQ followed by LC-MS/MS analysis of the resulting peptides showed that a large number of amino acid residues were modified in β-LQ. The LC-MS/MS data including modified peptide intensities and relative modification level for β-LQ modifications are shown in Table 2. The initial Michael addition adduct with 4MC (Fig. 1, reaction 2) was identified on four Cys, 11 Lys, one Arg, two His and one Trp residue (Table 2). The formation of Cys-4MC adducts are in agreement with previous studies where myofibrillar proteins from meat were incubated with 4MBQ (Jongberg et al., 2011) and in meat added 4MC (Arsad et al., 2020), while adduct formation on Lys, Arg, and His residues in addition to Cys on myofibrillar proteins has been observed with other oxidized polyphenols (Tang et al., 2016). In addition, the oxidized form of the Michael addition adduct (Fig. 1, reaction 1') was detected on Cys-119 and Schiff base-like modification of Lys was identified on residues 91 and 14 (Fig. 1, reaction 3).

Relative modification levels of 4MC adducts were found to be highest for Lys-8, and the levels of the rest of the observed modifications were therefore calculated relative to the modification level of this residue. Lys-14 and Trp-19 were also extensively modified with modification levels of ~9 % relative to the Lys-8 residue that was most modified. The modification level of Lys-75 was ~5 % while those of Cys-160, Cys-121, Cys-119 and Arg-124 ranged between 1 and 2 %. The rest of the 4MC adducts on Lys, Cys and His residues were below 0.5 %. A previous study investigated the reaction of different amino acids, peptides and proteins with 4MBQ and showed that Cys residues are kinetically preferred targets for 4MBQ (Li et al., 2016). In the present study, Lys residues were found to be modified to a greater extent than Cys residues in β-LQ, which is likely to be explained by a combination of protein structure and pH. At pH 7.6 close to pH 8 used in the current study, the free thiol (Cys-121) in β-LQ is buried near the beta sheet and alpha helix interface (Papiz et al., 1986). In addition, the high abundance of Lys (9 % of all amino acid residues) in β-LG as compared to the single free buried thiol may be another reason for Lys residues being more modified than Cys. The identification of the oxidized adduct on Cys-119 may be due to oxidation occurring in the MS source during analysis as observed previously (Arsad et al., 2020; Sojo et al., 2014). Modifications of Lys (residues 47, 77 and 138) and Cys (residue 121) were in agreement with a previous study (Ali et al., 2013) where β-LG was modified by oxidized coffee-specific phenols. In the present study, additional amino acid residues were found to be modified in β-LQ (Table 2). The additional modification sites included Cys residues involved in disulfides (residues 66, 119, and 160).

Previous studies (Rawel, Kroll, & Hohl, 2001; Rawel et al., 2002) stated significant loss of Trp in food proteins covalently modified with various polyphenols and reported the Trp loss based on loss of intrinsic Trp fluorescence. Prigent et al., (2008) studied the reaction of oxidized chlorogenic acid with several amino acid residues in a model system and provided MS-based evidence for modification of the Trp side chain by chlorogenic acid quinone, but did not study the extent of modification. Interestingly, our results show that Trp-19 was in fact highly modified by 4MBQ (~9% relative to the Lys-8 residue that was most modified). Overall, 4MBQ induced covalent modifications in β-LQ under the present reaction conditions, with Michael addition adducts being the major modifications observed, while Schiff base modifications were only detected to a minor extent.

3.1.3. Detection of Cys-4MC adduct in β-LQ

Detection of Cys-4MC adducts after total acidic hydrolysis of β-LQ with HCl was performed by LC-MS/MS and an in-house synthesized Cys-4MC standard (Arsad et al., 2020). However, due to lack of a reference protein containing a known concentration of 4MC adducts, it is not possible to estimate the loss of Cys-4MC during acidic hydrolysis. The reported values may therefore not be considered absolute values, but estimated values. After total acidic hydrolysis of β-LQ, 2.3 ± 0.1 μmol/g of Cys-4MC adduct was found. The Cys-4MC adduct was at the same retention time, precursor ion (m/z 244.06) and product ions (m/z 227.03, m/z 155.02) as the standard Cys-4MC standard. The product ion of m/z 227.03 resulted from the loss of a NH2 group, while the product ion of m/z 155.015 corresponded to a C=S bond breakage within the Cys residue. Both fragments were detected in their quinone form and this conversion of OH group in the phenol ring which involves 2H+ losses from the quinone ring may be due to oxidation induced during ESI or HCD fragmentation in LC-MS/MS (Fig. 2).
3.2. In vitro pepsin and pancreatin digestion of β-LG and β-LQ

3.2.1. Degree of hydrolysis (DH) of digested β-LG and β-LQ

DH after 2 h of pepsin digestion followed by 4 h of pancreatic digestion in β-LG and β-LQ are shown in Table 3. β-LQ had a significant increase in DH compared to β-LG after pepsin hydrolysis whereas DH of β-LQ and β-LQ was not significantly different after pepsin followed by pancreatic hydrolysis. The DH value (2.9 ± 0.2 %) for β-LQ after 2 h pepsin hydrolysis is comparable to a previous study (Joehnke et al., 2018) where 2.1 % DH of β-LG was found after 1 h pepsin digestion. The low DH observed after pepsin hydrolysis of β-LQ and β-LQ indicated a resistance to pepsin digestion, which can be explained by the stable globular structure and strong hydrophobic interactions of β-LG under acidic pH, and is in accordance with previous studies on β-LG digestibility (Bossios et al., 2011; Rahaman, Vasiljevic, & Ramchandran, 2017). The observed increase in DH of β-LQ compared to β-LQ after pepsin digestion could possibly be related to cleavage of disulfide bonds by thiol-disulfide exchange reactions and subsequent formation of Cys-4MC adducts at positions 66 and 119. Increase in pepsin-derived digestibility of β-LQ with cleaved disulfide bonds has previously been demonstrated (Reddy, Kella, & Kinsella, 1988).

An increase in DH after pepsin and pancreatin digestion was observed in both β-LG and β-LQ (DH 32 % and 34 %, respectively). This is in accordance with the susceptibility of β-LG towards intestinal enzymes (Rahaman et al., 2017), and is comparable to a previous study where 25 % DH of β-LG was observed after pepsin followed by pancreatin digestion for 2 h (Joehnke et al., 2018). Overall, DH of β-LG and β-LQ after pepsin and pancreatin digestion showed that the combined effect of gastric and intestinal digestion in β-LQ and β-LQ is similar, and thus, that covalent bonding of 4MBQ to β-LG did not affect protein digestibility to any major extent even though multiple amino acid residues were found to be modified by 4MBQ.

3.2.2. SDS-PAGE and NBT staining of digested β-LG and β-LQ

SDS-PAGE was used to evaluate the extent of pepsin and pancreatic digestion of β-LG and β-LQ at various time points (Fig. 3). Mainly monomeric (18 kDa) and dimeric (36 kDa) forms of undigested β-LG and β-LQ were apparent on the gels. In agreement with the DH results, β-LG (in both monomer and dimer form) was resistant during 2 h of pepsin digestion. β-LQ showed a similar digestion pattern as β-LG, apart from the presence of a small band just below the monomer band. This band of lower molecular weight than β-LG was slightly hydrolyzed during pepsin digestion, which can be seen by increased fragmentation and formation of an additional band of even lower molecular weight after 60–120 min of pepsin digestion. This additional band observed in β-LQ is likely due to a slightly lower migration pattern on SDS-PAGE caused by the 4MBQ-induced modification. The increase in fragmented pattern during pepsin digestion can be suggestive of β-LQ being less resistant during pepsin digestion as compared to β-LG, which is consistent with the increase in DH of β-LQ after pepsin hydrolysis.

Already after 5 min of pancreatic digestion a clear loss of β-LG and β-LQ monomers and dimers and formation of smaller peptide fragments below 10 kDa was observed. Following 2 h of pancreatin digestion, all major bands disappeared, indicating that peptides of molecular weight <2.5 kDa were released. The digestion pattern of β-LG is consistent with previous studies (Mandalari et al., 2009). Overall, SDS-PAGE analysis results were in agreement with DH results indicating that β-LG and β-LQ were digested to a similar extent.

Protein bands of β-LQ samples from pepsin digestion from an unstained SDS-PAGE gel were transferred to a membrane followed by NBT staining, which was used to detect protein-phenol covalent bonding (Fig. 3C). NBT staining confirmed polyphenol-bonding in β-LQ samples in agreement with the LC-MS/MS analysis presented above and previous studies (Arsad et al., 2020; Poojary et al., 2020). A vague band was apparent below the monomeric band showing that the additional fragmented bands observed on the SDS-PAGE gels was in fact also modified

---

Table 3

<table>
<thead>
<tr>
<th>Degree of hydrolysis (%) determined by the TNBSA assay for free amines after in vitro pepsin and pancreatin digestion of β-LG and β-LQ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of hydrolysis</td>
</tr>
<tr>
<td>After in vitro pepsin digestion</td>
</tr>
<tr>
<td>After in vitro pancreatin digestion</td>
</tr>
</tbody>
</table>

*Values calculated from independent sample t-test bearing different letters within the same row are statistically significant (p < 0.05).
with 4MBQ. No staining could be visualized on NBT blots during pancreatin digestion due to low levels of undigested protein being present on the SDS-PAGE gels (data not shown).

3.2.3. LC-MS peptide areas of digested β-LG and β-LQ

The intensities of signals of unmodified peptides of digested β-LQ samples were detected by LC-MS and compared to those of digested β-LG. All detected peptides showed similar areas for β-LG and β-LQ (Supporting Information Table S1), which is in agreement with the DH and SDS-PAGE results discussed above showing no obvious differences in in vitro digestion. Bioactive peptide sequences were identified by a database of bioactive peptides from milk (Nielsen et al., 2017). The present study identified eight bioactive peptides of various physiological functions (Nielsen et al., 2017) with similar intensities in β-LG and β-LQ. These results clearly show that the digestibility of identified bioactive peptides released after in vitro digestion was not affected by the covalent modifications induced by 4MBQ.

3.2.4. Release of Cys-4MC adduct after in vitro pepsin and pancreatin digestion

The release of non-protein-bound Cys-4MC adduct in β-LQ after in vitro digestion was assessed by determining the concentration of this adduct in the β-LQ digests. In the present study, out of 2.3 μmol/g of Cys-4MC, which corresponds to the minimum estimated concentration in

Fig. 3. Representative non-reduced SDS-PAGE gel of in vitro pepsin and pancreatin digests of β-LG (A) and β-LQ (B), and NBT staining of 4MBQ-bonding in β-LQ during in vitro pepsin digestion, β-LG is loaded as negative control next to the molecular marker (C).
β-LQ, 1.7 μmol/g of Cys-4MC was released after in vitro digestion, while the remaining Cys adduct was bound to peptides. To rule out the presence of any non-protein-bound Cys in the undigested β-LQ sample, which could have led to an overestimation of Cys-4MC adduct, Schiff base-like adduct was formed on a Lys residue of β-LQ. Previous studies suggest that in the case of non-covalent protein–polyphenol interactions, β-LG showed binding affinity with various polyphenols and can therefore be regarded as a carrier for polyphenols during digestion (Phillips, Whitehead, & Kinella, 1994). However, in the case of covalent bonding of polyphenols to β-LQ, the release of both amino acid- and peptide-polyphenol adducts may have an effect on absorption of either amino acids/peptides or polyphenols, which requires further investigation.

4. Conclusions

A β-LG-4MC covalent adduct (β-LQ) was synthesized and unreacted 4MC removed. A significant loss of free thiol and amine groups was found in β-LQ as compared to β-LG. Site-specific 4MC Michael adducts were identified on His, Arg, Lys, Cys and Trp residues, while a Schiff base-like adduct was formed on a Lys residue of β-LQ. Pepsin and pancreatic digested samples showed no obvious differences in in vitro digestion of β-LQ in comparison with unmodified β-LG. The Cys-4MC adduct (1.7 μmol/g) was released as a free amino acid-polyphenol adduct after in vitro digestion of β-LQ. Intestinal transport and immunological studies of the released Cys-4MC adduct are thus relevant to consider for future investigations.

CRediT authorship contribution statement

Khadija Waqar: Formal analysis, Investigation, Writing – original draft. Kasper Engholm-Keller: Formal analysis, Investigation, Writing – review & editing. Marcel S. Joehnke: Validation, Writing – review & editing. Dereck E.W. Chatterton: Methodology, Validation, Writing – review & editing. Mahesha M. Poojary: Methodology, Supervision, Validation, Writing – review & editing. Mariannene N. Lund: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mariannene Niss Lund reports financial support was provided by Independent Research Fund Denmark. Khadija Waqar reports financial support was provided by Punjab Educational Endowment Fund. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

LC-MS/MS raw data files along with peak list files and annotated MS/MS spectra of all 4-MC/4-MBQ-modified peptides are available via MassIVE (https://massive.ucsd.edu) with identifier MSV000088002

Acknowledgements

Punjab Educational Endowment Fund (PEEF) is acknowledged for financial support of PhD scholarship to KW, and Independent Research Fund Denmark for funding of grant no. 7017-00133B to MNL.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133775.

References
