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A kinetic and product investigation

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Reactivity and mechanism of the reactions of 4-methylbenzoquinone with amino acid residues in β-lactoglobulin: A kinetic and product investigation

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ABSTRACT

Quinones, produced by the oxidation of phenolic compounds, covalently bind to nucleophilic groups on amino acids or proteins. In this study, the reactions of 4-methylbenzoquinone (4MBQ) with β-lactoglobulin (β-LG) and amino acids at neutral pH were investigated. LC-MS analysis revealed that Cys121 was likely the most modified residue in β-LG. Identification of reaction products by LC-MS/MS showed that Michael addition occurred in all reactions with amino acids tested. The formation of Schiff base and a di-adduct was found in His and Trp samples. Apparent second-order rate constants (k2) were determined at 25 °C and pH 7.0 by stopped-flow spectrophotometry. The rate of reactions decreased in the order: β-LG > His > Trp > Arg > Nα-acetyl His > Nα-acetyl Arg > Nα-acetyl Trp. The rate constants correlated with the pK values of the amino acids, showing that the amount of unprotonated amine is the major factor determining the reactivity.

1. Introduction

Phenolic compounds are secondary metabolites found in plants, and are abundant in vegetables, fruits, coffee beans, wine, and tea (Bennick, 2002; Ramos, Santos, Daguer, Valese, Cruz, & Granato, 2017; Xu, Hao, Sun, & Tang, 2019). They are applied as ingredients in foods for well-documented biological activities, including antioxidant, anti-inflammatory, antibacterial and anticarcinogenic activity (Li et al., 2021; Coppo & Marchese, 2014). Extracts rich in phenolic compounds, especially those from green tea, have been added to various types of foods, including dairy and meat products, in order to reduce lipid oxidation and Maillard reactions (Jansson et al., 2017; Jongberg, Skov, Torgren, Skibsted, & Lund, 2011; O’Connell & Fox, 2001). After being oxidized to their corresponding quinones or semi-quinones (Bittner, 2006), phenolic compounds can irreversibly react with nucleophilic groups, such as thiol and amine groups of proteins, peptides or free amino acids through Michael addition or Schiff base formation (Chu, Bao, & Wu, 2018; Liu, Poojary, Thygesen, Jensen, Andersen, & Lund, 2023; Lund, 2021; Rohn, 2014). The covalent bonding between phenolic compounds and proteins can be used to improve the thermal stability of whey protein (Ali, Homann, Khalil, Kruse, & Rawel, 2013). However, if the extent of covalent bonding between proteins and quinones is not controlled, it may lead to undesirable changes of food quality, such as haze formation in beer (Jongberg, Andersen, & Lund, 2020), reduction of myofibrillar protein gelation (Tang et al., 2017), and disruption of the protein network of meat emulsions (Jongberg, Terkelsen, Miklos, & Lund, 2015).

Whey protein concentrate or isolate is a side stream from cheese production and widely used as a food ingredient, due to its functional properties and nutritional value (Yildirim-Elikoglu & Erdem, 2017). β-Lactoglobulin (β-LG) is the most abundant protein in bovine whey. It is a globular protein with a molecular mass of 18.2 kDa and 162 amino acid residues, including five cysteine (Cys), two histidine (His), 15 lysine (Lys), three arginine (Arg), and two tryptophan (Trp) residues (Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998). The conjugation of caffeic acid to β-LG increased the antioxidant capacity and thermal stability of the protein (Abd El-Maksoud et al., 2018). β-LG conjugation with epigallocatechin 3-gallate and chlorogenic acid were confirmed by MALDI-TOF-MS and SDS-PAGE, and were found to effectively reduce the IgE binding capacity of β-LG (Wu et al., 2018). However, the relative

Abbreviations: 4MBQ, 4-methylbenzoquinone; 4MC, 4-methylcatechol; kobs, observed rate constant; k2, apparent second-order rate constant; β-LG, β-lactoglobulin; β-LQ, 4MBQ-modified β-lactoglobulin; TCEP, tris(2-carboxyethyl)phosphine; MCR, multivariate curve resolution.

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reactivity of the amino acid residues in β-LG towards quinones has not
been established. A previous study tentatively assigned the ε-amino
groups of Lys47, Lys77, Lys91, Lys138 and the thiol group of Cys121 in
β-LG as modification sites for quinones of a green coffee bean extract
based on MALDI-TOF-MS analysis after a tryptic digestion. However,
because of high peptide mass error, this requires further confirmation
(Ali et al., 2013). In a recent study, β-LG was found to be modified by 4-
methylecatechol (4MC), a model compound for a typical plant phenolic
mixture (Jongberg, Lund, Waterhouse, & Skibsted, 2011), on 11 residues
of Lys (8, 14, 47, 70, 75, 77, 83, 91, 135, 138, and 141), four Cys (66,
119, 121, and 160), two His (146 and 161), one Arg (124) and one Trp
(19) at pH 8.0 by LC-MS/MS analysis after tryptic digestion (Waag,

4MC can be oxidized to its o-quinone form, 4-methylbenzoquinone
(4MBQ), which is convenient for kinetic studies as it is more stable
than quinones derived from plant phenolics. We previously studied the
kinetics of the reactions between 4MBQ and thiol and amino groups of
amino acids, peptides and proteins (Cys, Nε-acetyl-Cys, Lys, Nε-acetyl-
Lys, Nε-acetyl-Lys glutathione, bovine serum albumin, hum serum
albumin, and α-lactalbumin) (Li, Jongberg, Andersen, Davies, & Lund,
2016; Liu et al., 2023). But the reactivity of 4MBQ to amino acid residues in
proteins. To comprehensively investigate the reactivity of 4MBQ to
β-LG, the present study includes a kinetic investigation of the reaction between 4MBQ and β-LG as well as Arg, Nε-
acetyl Arg, His, Nε-acetyl His, Trp, and Nε-acetyl Trp at pH 7.0 at 25 °C.
The modification sites of 4MBQ on β-LG were identified by LC-MS/MS
analysis after enzymatic digestion by either trypsin or Glu-C and rela-
tive quantification was performed to examine the reaction preference of
4MBQ for amino acid residues on β-LG at pH 7. The reaction products of
4MBQ and the free amino acids were also identified by high-resolution LC-MS/MS analysis.

2. Materials and methods

2.1. Chemicals and reagents

4-Methylecatechol (4MC, ≥95%), L-His (≥98%), Nε-acetyl-L-His
(≥98%), L-Trp (≥98%), Nε-acetyl-L-Trp (≥98%), L-Arg (≥98%), Nε-
acetyl-L-Arg (≥98%), β-LG from bovine milk (variant A/8, Lot
SLSB5636, ≥90%), TrypZean™, and acetonitrile (HPLC grade) were
purchased from Merck (Seborg, Denmark). The protease Staphylococcus aureus
endoproteinase-Glu-C (Glu-C) was obtained from Worthington
(NJ, USA). Formic acid was purchased from VWR (Seborg, Denmark).
Ultra-pure water produced by a Milli-Q water purification system
(Millipore, Bedford, MA) was used for all the experiments.

2.2. Generation of 4MBQ

4MBQ was generated by electrolysis according to the method re-
ported by Li et al., (2016) with modifications described in Liu et al.
(2023). 4MBQ was generated from 4MC (2 mM) in phosphate buffer
(pH 9.1)) were mixed in a 50 mL centrifuge tube.

The product of 4MBQ reacted with β-LG was named β-LQ and pre-
pared as described in Waag et al., (2022) with a few modifications.
Aliquots of 5 mL of 4MBQ (0.5 mM) and 5 mL of β-LG (0.5 mM in 0.2 M
of phosphate buffer (pH 9.1)) were mixed in a 50 mL centrifuge tube.

The resulting pH was 7.0 instead of pH 8.0 as previously described
(Waag et al., 2022). The β-LQ sample was dialyzed against milli-Q
water for 24 h in the dark at 4 °C (with 12 kDa molecular weight cut-
off membrane (Merck, Seborg, Denmark) and change of Milli-Q water
every 4 h) in order to remove unreacted 4MBQ, and was then lyoph-
ilized. A control sample of β-LG was prepared and treated in the same
way but without addition of 4MBQ. β-LG and β-LQ were dissolved in
milli-Q water. Aliquots of β-LG or β-LQ (5 µL, 0.5 mM) were 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 (TCEP) and incubated for 30 min at room

temperature. Ammonium bicarbonate (36 µL, 50 mM, pH 8.5) was
added, followed by 2 µL of 1 µg/µL TrypZean trypsin or Glu-C. After 5 h
of digestion at 21 °C, another 2 µL of TrypZean or Glu-C solution was
added, and the reaction was allowed to proceed for 5 h before the
addition of 25 µL 10% formic acid. The non-reduced samples were
prepared according to the same procedure but without TCEP addition.

Each sample (5 µL) was injected onto a Phenomenex Aeras C18 (2.1 ×
150 mm, 2.6 µm particle size) column (at 40 °C) using an Ultimate 3000
UHPLC system (Thermo Scientific, Bremen, Germany).

Peptides were analyzed using an 18 min LC-MS/MS run at a flow rate of
250 µL/min, being eluted off the column using an increasing gradient
from mobile phase A (0.1% formic acid) to B (90% acetonitrile, 0.1% formic acid)
(0.0-2.0 min: 5% B; 2.0-3.0 min: 5-15% B; 3.0-11.0 min: 15-35% B; 11.0-12.0 min: 35-90% B; 12.0-13.0 min: 90% B; 13.0-14.0
min: 90-50% B; 14.0-18.0 min: 5% B). Eluted peptides were introduced
into the Q Exactive mass spectrometer in positive ion mode with the following conditions: 3.5 kV spray voltage, sheath gas setting 50, aux gas
setting 10, probe heater temperature of 325 °C. A full MS scan of the m/z
200–2000 range was acquired in the orbitrap (full width at half maximum (FWHM) resolution of 60,000) with an AGC target of 1 × 106.
For each full scan, the 10 most intense ions with an intensity higher than
2 × 1010 (charge state 1 to 2+) were selected for higher-energy collision
dissociation (HCD) and detected at a resolution of 7,500 FWHM. Settings
for the HCD event were as follows: The AGC target was 1 × 106, and
the maximum injection time was 120 ms at the MS level and 200 ms at the
MS/MS level. The isolation window was 1.4 Da, first fixed mass was 140,
normalized collision energy was 26, peptide match was off, exclude isotopes was on, and dynamic exclusion was in auto mode.

To identify the peptides containing modified amino acids 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 bio-
logical LC-MS/MS experiments (294 proteins in total) was performed using Proteome Discoverer 2.5 (Thermo Fisher Scientific, Roskilde,
Denmark). The search parameters were left as default apart from the
following: Enzyme was Trypsin/P or Glu-C with up to two missed

cleavages, peptide mass was 600 to 8000 Da, peptide length was 4 to 141
amino acids, precursor and fragment mass tolerance was 10 ppm, and false discovery rate (FDR) was 5% at the peptide-spectrum match (PSM)
level. The following variable modifications were allowed: 4MC modific-
ation (+122 Da) of Cys, Lys, Arg, His, Trp and the protein N-terminal,
4MBQ modification (+120 Da) of Cys, Lys, Arg, His, Trp and the protein
N-terminal, and oxidation (+16 Da) of Met. The modified sites were
manually checked according to the fragment spectra. Then, the chro-
matograms and MS/MS spectra of peptides from Proteome Discoverer
were exported to Skyline for relative peptide quantification of the
modification levels as previously described (Li, Paulsen, Akillioglu,

2.4. Kinetic investigation of the reaction between 4MBQ and β-LG or
amino acids

The kinetic measurements were carried out at 25 °C based on
the method of Liu et al. (2023). Aliquots of 25 µL of 4MBQ and 25 µL of
amino acids (or β-LG) were mixed by injection into the reaction chamber
at the same time by the nitrogen drive, and the pH of the mixture was
7.0. The concentration of 4MBQ in the reaction chamber was 0.2 mM
when reacted with amino acids. The concentration of amino acids was
12.5–250-fold higher than 4MBQ to ensure pseudo-first-order conditions, which is a well-established approach to determine rate constants (Van Boekel, 2008). Similarly to what was used in a previous study for proteins (Li et al., 2016), the concentration of 4MBQ in the reaction mixture was 0.04 mM in reactions with β-LG, and the concentration of β-LG was only 5.00–8.75-fold higher than 4MBQ due to the lower solubility of β-LG compared to amino acids. The concentrations of each of the amino acids and β-LG are shown in Supplementary Materials Table S1. The absorbance was recorded from the injection triggered during a period of 50–300 s with 200 points recorded for each reaction. Single or double exponential fitting was applied for the fitting of absorbance changes at 401 nm (R² > 0.9700), and the observed rate constant (kobs) was calculated as described previously (Liu et al., 2023). Linear fitting was applied to the plots of kobs against concentrations of amino acids or β-LG. The ranges of concentrations of β-LG and amino acids, the fitting equations used for each sample, and kobs are listed in Supplementary Materials Table S1. All the reactions were performed in triplicates. The apparent second-order rate constants, k2, were determined as the slope of the linear regressions.

2.5. Multivariate curve resolution (MCR)

For the reaction of 4MBQ with His, a complex absorption spectrum was observed, which hindered the use of exponential fitting on the change in absorbance to find the rate constant kobs. In this case, to deconvolve the chemical components present in the system and confirm the results obtained on the pure absorbance spectra, Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS) (de Juan, Jaumot, & Tauler, 2014) was employed using the MCR-ALS 2.0 Toolbox (Jaumot, Gargallo, de Juan, & Tauler, 2005) available online (https://www.mcrails.info) and operating in a Matlab environment. Data-elaborations and plotting were done using Matlab R2020a (The Mathworks, Inc., Natick, MA, USA).

The MCR model was built on an augmented data set including all the kinetic experiments performed with 4MBQ and His or N⁰-acetyl-His with different concentration of the amino acids. The final model was obtained with non-negativity constraint on both the concentration and spectral profile. A complexity of five components was chosen based on the eigenvalues and the interpretability of the components. This model showed an explained variance of 99.96% and a lack of fit of 2.07%.

2.6. Identification of adducts formed between 4MBQ and amino acids by LC-MS/MS

Aliquots of 0.5 mL of 4MBQ (0.2 mM) and 0.5 mL of amino acids (20 mM, dissolved in 0.2 M of phosphate buffer at a specific pH for individual amino acids) were mixed in 2.0 mL Eppendorf tubes to make sure the resulting pH was 7.0. The mixture was stirred for two minutes at room temperature. The reaction products were analyzed by LC-ESI-MS/MS according to our previous study (Zhu, Poojary, Andersen, & Lund, 2019) with some modifications. Samples were filtered through 0.22 μm syringe filters and injected (10 μL) into an Ultimate 3000 UHPLC system (Thermo Scientific, CA, USA) equipped with a Synchronis Aq C-18 column (Thermo Scientific, CA, USA; 100 mm length, 2.1 mm ID, 1.8 μm particle size). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 100% acetonitrile (mobile phase B) with a flow rate of 0.25 mL/min. The gradient program was as follows, 0–6.0 min: 0% B; 6.0–14.1 min: 40% B; 14.1–14.2: 95% B; 14.2–15.8 min: 95% B; 15.8–15.9: 0% B; 15.9–21.4 min: 0% B. The column was operated at 40 °C. Mass spectra were obtained using an Orbitrap Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). The heated electrospray ionization (HESI) source of the Q Exactive was operated in positive ion mode with the following conditions: spray voltage: 3.8 kV; capillary temperature: 300 °C; sheath gas flow rate: 28 arbitrary units; auxiliary gas flow rate: 15 arbitrary units; sweep gas flow rate: 4 arbitrary units; S-lens RF level: 60%. The full MS scans were acquired from 70 to 1000 m/z at a resolution of 70,000. The MS/MS fragmentation patterns of amines and adducts with 4MBQ were obtained by HCD-based MS/MS mode (resolution: 17,500) with a normalized collision energy of 30.

2.7. Statistical data analysis

Analysis of variance (ANOVA) was carried out in SPSS version 28.0.0.0. Significance was defined as p value < 0.05. The curve fitting and the calculation of rate constants were performed using the OriginPro 2016 software (OriginLab Co., Northampton, USA).

3. Results and discussion

3.1. LC-MS identification and relative quantification of modified sites in β-LQ

The β-LQ sample was prepared by mixing equal molar ratios of 4MBQ and β-LG. Relative quantification of the 4MBQ-modified residues in β-LQ was performed by using the peak area of modified peptides divided by the sum of the peak areas of both modified and unmodified peptides as previously described. This method assumes that the modified and unmodified forms of each peptide are ionized equally in the MS instrument and relies on the assumption of complete detection and quantification of all modified species. β-LG and β-LQ were analyzed with or without reduction by TCEP prior to LC-MS analysis, and trypsin and Glu-C were used separately for protein digestion in the present study in order to identify as many modified sites as possible compared to digestion with a single enzyme. However, for quantitative purposes, trypsin was not as useful compared to Glu-C, as the tryptic cleavage sites at Lys and Arg are both potential modification sites for 4MBQ-based Michael addition or Schiff base formation, which block the cleavage by trypsin. This might lead to an underestimated level of unmodified peptides and thus an overestimated relative modification level when trypsin is used. Therefore, the results obtained from tryptic digestion are only reported as the peak area of the modified peptides in Supplementary Materials Fig. S1A.

Digestion by Glu-C allowed relative quantitation of modified sites of Cys121 or Cys119, seven Lys residues (8, 75, 77, 91, 135, 138, and 141), His146, and Arg148, with Cys121 or Cys119 giving the highest relative modification level (~ 4.7%) followed by Lys141 (detected on several peptides) (Fig. 1). Due to the lack of presence of the key fragment ions b5, b6, y7 or y8 (Supplementary Materials Fig. S2) of the most modified peptide, QSLACQLVRTP, it was not possible to assign the modification to only one of the Cys residues (Cys119 or Cys 121) on this peptide. However, since Cys121 is the only free thiol group in β-LG, Cys121 is the most likely modification site. This is supported by a previous study, which found that Cys121 was the most reactive residue toward 3,4-dihydroxyphenylalanine-quinone (Doblas, Hägglund, Fuentes-Lemus, & Davies, 2023). Apart from the modified peptides shown in Fig. 1, two additional peptides were found to be modified on Cys119 and Cys121 (Supplementary Materials Fig. S1B) but could not be used for the calculation of relative modification levels as the unmodified forms were not detected. Altogether (for digestion with both trypsin and Glu-C), the detected modification sites included ten Lys (Lys8, Lys60, Lys75, Lys77, Lys91, Lys100, Lys101, Lys135, Lys138 and Lys141), one Arg (Arg148), one His (His146) and two Cys (Cys66 and Cys119 or Cys121) residues. The number of identified sites was lower in the present study compared to our previous study (Waqr et al., 2022), which is likely to be caused by a higher pH (pH 8.0) in the latter study, rendering the amino acids more reactive. Therefore, more modification sites on Lys residues, as well as on Trp, were observed in the previous study (Waqr et al., 2022). Interestingly, Cys106-Cys119 and Cys66-Cys160 are disulfides in the native β-LG, and not available for reaction with 4MBQ, but 4MBQ-modified sites were identified on Cys66 and Cys119. This indicated 4MBQ-induced cleavage of the two disulfide bonds, or that prior thiol-disulfide exchange had taken place, thereby making Cys119...
and Cys66 available for reaction with 4MBQ. As seen in Fig. 1, many of the modified peptides were only observed in the samples where disulfides were reduced with TCEP, and not in the samples without TCEP. Reduction of disulfides converts cross-linked disulfide peptides into linear peptides, which in contrast to cross-linked peptides can be identified by the Sequest algorithm in Proteome Discoverer. On the other hand, some modified peptides were only observed in samples without TCEP, which may be because TCEP can dissociate the protein-phenol adducts (Jongberg, Lund, & Otte, 2015), thereby lowering the abundance of modified peptides in TCEP-containing samples (Fig. 1). Apart from Michael addition (+120 or +122 Da, which correspond to the addition of 4MBQ or 4MC, respectively), modification based on Schiff base formation (+104 Da) was also observed to occur on Lys8, Lys77, Lys100, Lys101, Lys135, Lys141 and His146 in β-LQ.

3.2. Reactivity of β-LQ towards 4MBQ

4MC and 4MBQ had characteristic UV–vis absorption bands with maxima around 285 and 401 nm, respectively (Fig. 2A and 2B) (Li et al., 2016). Absorption spectra of mixtures of 4MBQ and β-LQ showed a typical decrease in intensity at 401 nm, indicating the disappearance of the base formation (addition of 4MBQ or 4MC, respectively), modification based on Schiff from Michael addition (compound B in Fig. 5), and (+120) represents the preceding amino acid was modified by 4MBQ through Michael addition and then oxidized (compound C in Fig. 5).

The observed rate constants, $k_{obs}$ and $k'_{obs}$, indicating a fast initial reaction and a slower subsequent reaction. The observed rate constants, $k_{obs}$ and $k'_{obs}$, were $(335.1 \pm 3.3) \times 10^{-3}$ s$^{-1}$ and $(37.1 \pm 0.7) \times 10^{-3}$ s$^{-1}$, respectively. Since $k_{obs}$ was approximately 8-fold higher than $k'_{obs}$ (Fig. 2D), the $k_{obs}$ values were used for the determination of the apparent second order constant, $k_2$. A plot of $k_{obs}$ as a function of the concentration of β-LQ showed linear correlation, which indicated that the reaction between 4MBQ and β-LQ followed pseudo-first-order rate conditions and provided a $k_2$ of $(1.3 \pm 0.1) \times 10^4$ M$^{-1}$ s$^{-1}$. Our previous study found $k_2$ of the reaction of 4MBQ with α-lactalbumin and BSA to be $(4.0 \pm 0.2) \times 10^2$ M$^{-1}$ s$^{-1}$ and $(3.1 \pm 0.2) \times 10^4$ M$^{-1}$ s$^{-1}$, respectively (Li et al., 2016) (Table 1). The differences in reactivity between proteins are likely caused by several factors, including the number of nucleophilic amino acid residues (especially number of Cys residues due to the kinetically preferred reaction of 4MBQ), and folding of the protein (i.e. degree of exposure of nucleophilic residues to the environment). The low value of $k_2$ for α-lactalbumin may be due to the lack of free thiol groups, while the higher reactivity of BSA may be due to the presence of a free thiol group in addition to the higher molecular weight of BSA (ca. 66 kDa) compared to β-LQ (18.2 kDa) and thus potentially more nucleophilic sites available for reaction with 4MBQ.

3.3. Kinetics of reactions between amino acids and 4MBQ

Because previous studies have only determined $k_2$ for Cys and Lys residues (Table 1), the present study therefore extended our kinetic investigation to include His, Arg and Trp residues as well in order to gain better insight into the reactivity of those residues that were identified as being modified by 4MBQ in β-LQ.

The absorption changes observed during reaction of 4MBQ with the amino acids tested in the present study are shown in Fig. 3A for Arg, and in Supplementary Material Fig. S3 for the remaining amino acids. For Arg, absorption increased at 285 nm, 308 nm, and 491 nm and decreased at 401 nm, while the changes observed for N$^4$-acetyl Arg, N$^4$-acetyl His, Trp and N$^4$-acetyl Trp were more simple except for His, which will be discussed separately below. In all cases, except for His, the absorption decrease at 401 nm was used to calculate the rate constants for the initial reaction steps of the reactions of 4MBQ with the different amino acids. In most cases the absorption could be fitted to a single exponential decay giving observed rate constants, $k_{obs}$ for the reaction. However, a sum of two exponential decays were needed in the case of
Arg to give satisfactory fits resulting in two observed rate constants, $k_{obs}$ and $k'_{obs}$, indicating a fast initial reaction and a slower subsequent reaction with absorption changes at 401 nm (Supplementary Material Table S1). The rate constants, $k_{obs}$, for the fastest reactions were chosen for determination of the apparent second-order rate constants ($k_2$). Notably, $k_{obs}$ for the reaction of 4MBQ and the indole group of Nα-acetyl Trp (50 mM) was $(18.3 \pm 0.1) \times 10^{-3}$ s$^{-1}$, which was not significantly different from the value of $k_{obs}$ for the spontaneous decay of 4MBQ in the absence of other reactants, which was $(18.4 \pm 0.1) \times 10^{-3}$ s$^{-1}$ ($p > 0.05$).

As for His (Fig. 4A), more complex changes in absorbance at 401 nm over time were observed, where the absorbance first decreased and then increased after around 10 s (Supplementary Materials Fig. S4A). In this case, only the first decreasing part was used for single exponential fitting, which gave an apparent second-order rate constant of $13.4 \pm 0.7$ M$^{-1}$ s$^{-1}$ (Supplementary Materials Fig. S4B). The absorbance changes suggested a complex reaction pathway with more than one reaction and intermediate products also giving rise to UV–Vis absorption (Fig. 4A). Therefore, multivariate curve resolution-alternating least squares (MCR-ALS) (de Juan et al., 2014) was employed to deconvolve the chemical components of the reaction of 4MBQ with His. A five-MCR components model was applied (Fig. 4B). Components 1 and 5 had very low intensities in the concentration profile and could be attributed to noise or background signals. Both components 2 and 4 had concentration profiles that increased with the reaction time and was therefore attributed to products, however only the increase of component 4 appeared to depend on the initial amino acid concentration. Component 3 showed a concentration profile decreasing over the course of the reaction and a spectral profile dominated by the peak at 401 nm (Fig. 4C). The intensity of component 3 over time was used for determination of $k_{obs}$ at 401 nm (Fig. 4D). The $k_2$ based on component 3 was $17.4 \pm 1.9$ M$^{-1}$ s$^{-1}$ (Fig. 4D insert), which was slightly higher than $k_2$ ($13.4 \pm 0.7$ M$^{-1}$ s$^{-1}$) determined directly from the exponential fitting without the use of MCR modelling (Supplementary Materials Fig. S4B). According to the MCR spectral loadings, an increase was shown from 10 to 20 s in the plot of component 3 (Fig. 4D), indicating that an intermediate was generated and consumed subsequently.

The values of $k_2$ for the amino acids determined in the present study decreased in the order of His $>$ Trp $>$ Arg $>$ Nα-acetyl His $>$ Nε-acetyl...
Apparent second-order rate constants ($k_J$) for reaction of 4MBQ with Arg, $N^\alpha$-acetyl Arg, His, $N^\alpha$-acetyl His, Trp, $N^\alpha$-acetyl Trp, and $\beta$-LG at pH 7.0 and 25 °C at 401 nm. Rate constants determined in previous studies are included for comparison: The rate constants of 4MBQ reacted with $\beta$-LG, $\alpha$-LA, BSA, Cys and $N^\alpha$-acetyl Cys were obtained from Li et al. (2016); the rate constants of 4MBQ reacted with Lys, $N^\alpha$-acetyl Lys and $N^\alpha$-acetyl Lys were obtained from Liu et al. (2022). The pKa value for Cys is the pKa for the thiol group in Cys. The pKa values for Lys, $N^\alpha$-acetyl Lys, Arg, and Trp are of the corresponding $\alpha$-amino group for the $\alpha$-amino groups are the dominant reactive group in these amino acids, and the pKa values for $N^\alpha$-acetyl Lys, $N^\alpha$-acetyl Arg and $N^\alpha$-acetyl His are of the side chains (Lide, 2004). The nucleophilicity values of Arg, His, and $2$-imidazole methylidazolide (equivalent to $N^\alpha$-acetyl His) were obtained from Bentley (2011).

<table>
<thead>
<tr>
<th>Amines</th>
<th>$k_J$ ($M^{-1} s^{-1}$)</th>
<th>pKa</th>
<th>Nucleophilicity ($N^\alpha$, $\varepsilon$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-LG</td>
<td>1.3 ± 0.1 × 10^{-3}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\alpha$-LA</td>
<td>4.0 ± 0.2 × 10^{-3}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BSA</td>
<td>3.1 ± 0.2 × 10^{-5}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cys</td>
<td>7.0 × 10^{-3}</td>
<td>8.18</td>
<td>9.5</td>
</tr>
<tr>
<td>$N^\alpha$-acetyl Cys</td>
<td>5.2 ± 10^{-3} *</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Arg</td>
<td>2.3 ± 0.2</td>
<td>9.04</td>
<td>6.05</td>
</tr>
<tr>
<td>$N^\alpha$-acetyl Arg</td>
<td>0.1 ± 0.0</td>
<td>12.48</td>
<td>–</td>
</tr>
<tr>
<td>His</td>
<td>17.4 ± 1.9</td>
<td>9.17</td>
<td>6.26</td>
</tr>
<tr>
<td>$N^\alpha$-acetyl His</td>
<td>0.0 ± 0.0</td>
<td>6.00</td>
<td>3.97</td>
</tr>
<tr>
<td>Trp</td>
<td>4.5 ± 0.2</td>
<td>9.99</td>
<td>–</td>
</tr>
<tr>
<td>$N^\alpha$-acetyl Trp</td>
<td>Too slow</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lys</td>
<td>4.5 ± 0.1</td>
<td>8.95</td>
<td>–</td>
</tr>
<tr>
<td>$N^\alpha$-acetyl Lys</td>
<td>1.4 ± 0.1</td>
<td>10.53</td>
<td>–</td>
</tr>
<tr>
<td>$N^\alpha$-l-lys</td>
<td>1.8 ± 0.1</td>
<td>8.95</td>
<td>–</td>
</tr>
</tbody>
</table>

* Rate constant for Cys was determined at pH 6.0, and for $N^\alpha$-acetyl Cys at pH 6.5.

3.4. Identification of 4MBQ-amino acid products

Based on our previous work on the reaction of 4MBQ and Lys (Liu et al., 2023), we know that Michael addition and Schiff base formation are competitive reactions in the mixture and that temperature did not affect the adduct distribution. To investigate whether different types of amino acid substrates would impact the adduct distribution, the products formed from the reaction of 4MBQ with Arg, $N^\alpha$-acetyl Arg, His, $N^\alpha$-acetyl His, Trp and $N^\alpha$-acetyl Trp were identified by LC-MS/MS. The retention time of the adduct-related peaks in UHPLC-DAD chromatograms (detection wavelength 285 nm), as well as the fragment mass of the all the identified adducts from the LC-MS/MS analyses are shown in Supplementary Material Table S2, with the corresponding chromatograms and MS/MS spectra shown in Supplementary Materials Fig. S5-S10. Reaction schemes of 4MBQ with individual amino acids are shown in Supplementary Materials Schemes S1-S5. An overall reaction scheme based on the identification of products is shown in Fig. 5. Several positions on 4MBQ can undergo Michael addition and Schiff base formation but for simplicity, only one possibility is shown.

The Michael addition reaction pathway of reactions 1–3 and Schiff base formation pathway of reaction 4–5 as well as the formation of Michael addition/Schiff base di-adduct (compound G) (reaction 7 and 8) are similar to reactions previously observed by Liu et al. (2023). The identified reaction products show that these reactions also occur for Arg, $N^\alpha$-acetyl Arg, $N^\alpha$-acetyl His and Trp reacting with 4MBQ. The reaction mixture containing 4MBQ and Arg gave a single peak with $R_f$ of 10.22 min (Supplementary Material Fig. S5A), and the MS analysis revealed that the peak had a molecular ion $[M + H]^+$ at m/z 297.1556 (Supplementary Material Fig. S5B), which was tentatively identified as an oxidized Michael addition type of product (compound C, Fig. 5). The MS/MS spectrum had a distinct fragmentation pattern with product ions of $m/z$ 280.1293, $m/z$ 253.1662, and $m/z$ 138.0555, corresponding to the loss of hydroxyl or amino groups, carboxyl group, and aliphatic chain of Arg, respectively. According to the masses of the fragments, the product ion at $m/z$ 158.0925 could be derived from the Arg moiety (Supplementary Material Fig. S5B). Similarly, 4MBQ-$N^\alpha$-acetyl Arg adduct was also identified as a Michael addition product (compound C, Fig. 5). The two peaks related to 4MBQ-$N^\alpha$-acetyl His adducts were both identified as Michael addition products (compound C, Fig. 5), indicating different reaction sites on 4MBQ as it is known that either C3, C5, or C6 can be involved in the formation of adducts (Jongberg, Torngren, Gunwig, Skibsted, & Lund, 2013). Adducts of 4MBQ-Trp included a Michael addition adduct ($m/z$ 325.1188), a Schiff base ($m/z$ 309.1234) (compound E, Fig. 5) and the hydrated product from the Schiff base ($m/z$ 311.1390). The products of 4MBQ reacted with $N^\alpha$-acetyl Trp were identified as a Michael addition product ($m/z$ 369.1445) and a re-oxidized product ($m/z$ 367.1288). A di-adduct of Michael addition with $[M + H]^+$ of $m/z$ 611.2116, formed...
from an additional Michael addition, was also observed (compound F, Fig. 5). The adducts of 4MBQ-His were identified as a Michael addition adduct (compound C, Fig. 5) and a di-adduct (compound I, Fig. 5), and the formation is shown in Supplementary Materials Scheme S3. The Michael addition adduct with a molecular ion [M + H]$^+$ of m/z 276.0981 was observed (Compound C, Fig. 5, with MS/MS spectrum shown in Supplementary Materials Fig. S7B). 4MBQ was also found to react with His at the carbonyl group of C1 or C2 to form an amino-hydroquinone (Compound D, Fig. 5), which lost water to form the Schiff base (Compound E). Subsequently, the Schiff base underwent Strecker degradation to form a di-Schiff base adduct (Compound H, Fig. 5), which may then react with an additional His to form a catechol-benzoquinone di-His Michael addition/Schiff base adduct (Compound I, Fig. 5) with [M + H]$^+$ of m/z 381.1557 (MS/MS spectrum is shown in Supplementary Materials Fig. S7C). A previous study reported a similar reaction pathway for Lys and the quinone of epicatechin (Yin, Hedegaard, Skibsted, & Andersen, 2014). However, the elimination of carbon oxide and the di-Schiff base adduct formation were only found in heated samples, and the authors did not report the further reaction between di-Schiff base adduct and additional amino acids (Yin et al., 2014).

Overall, the reaction pathways of 4MBQ and amino acids included Michael addition, Schiff base formation and oxidation and varied with individual amino acids. His-related reactions were the most complicated reactions. Collectively, in addition to the single Michael addition, for formation of Schiff base, and Michael addition/Schiff base di-adducts, other types of di-adducts were identified where the Michael addition product may have reacted with another amino group through Michael addition to form a di-adduct (compound F, Fig. 5). The Schiff base can undergo a carbon dioxide elimination and then react with another 4MBQ to form a di-Schiff base adduct (compound H, Fig. 5) as also shown in Supplementary Materials Scheme S3). The di-Schiff base adduct can further react with a nucleophile to form compound I in Fig. 5, which can also be generated from the reaction between a Michael addition/Schiff base di-adduct (compound G, Fig. 5) and 4MBQ.

3.5. Factors influencing the reaction between 4MBQ and \(\beta\)-LQ

The reactions of 4MBQ with thiol groups are kinetically preferred...
over the amine groups (Table 1), therefore the reactivity of proteins are highly dependent on the accessibility of their thiol groups (Li et al., 2016). β-LG contains five Cys residues, of which four are linked in disulfide bonds (Cys66-Cys160 and Cys106-Cys119) and one is free (Cys121) (Braunitzer, Chen, Schrank, & Stangl, 1973). Cys121 is buried in the native protein structure at pH 7.0 (Burova, Choiset, Tran, & Haertlé, 1998), which makes the thiol group less accessible for reaction. Nevertheless, the peptide containing Cys121 was still the peptide with the highest modification level among all residues in β-LG, even though it was not possible to distinguish between Cys119 and Cys121 modification in the current study (Fig. 1). Another critical factor for the reaction between 4MBQ and β-LG is pH of the environment. Firstly, pH affects the structure of the β-LG, leading to a more unfolded structure at pH 8.0 than at pH 7.0. Secondly, pH strongly affects the reactivity of nucleophiles with 4MBQ, as a higher pH will increase the proportion of deprotonated nucleophiles, increasing the reactivity toward 4MBQ (Li et al., 2016). At pH 8.0, the reactivity of both thiol and amino groups are significantly increased compared to pH 7.0. This may explain why more amino acid residues of β-LG, including Trp, were found to react with 4MBQ at pH 8.0 (Waqar et al., 2022) than at pH 7.0. Additionally, due to the many potential reaction sites (Cys, His, Lys, Arg and Trp) on β-LG (Fig. 1) (Waqar et al., 2022), even if the concentration of β-LG was only 1–1.75 times that of 4MBQ, the reaction still followed the pseudo-first-order rate conditions (Fig. 2). In the case of free amino acids, apart from the reaction involving thiols, the reactivity of free amino acids towards 4MBQ mainly depends on the pKa values of the nucleophilic groups of the amino acids.

4. Conclusion

In β-LG, Lys, Arg, His and Cys residues were identified to be modified at pH 7.0 upon reaction with 4MBQ, with Cys being the most abundantly modified residue. The reactivity of β-LG was lower than that of thiol-containing amino acids but higher than non-thiol amino acids. This observation indicates the reactivity being determined by the distribution and accessibility of nucleophilic groups, especially thiol groups. β-LG and free amino acids react with 4MBQ via Michael addition and Schiff base formation. The adduct distribution of 4MBQ reacted with amino acids varied with the type of amino acid, forming Michael addition product, Schiff base, and di-adducts (Michael addition/Michael addition di-adduct, Michael addition/Schiff base di-adduct, Schiff base/Schiff base di-adduct and di-quinone-Michael addition/Schiff base di-adduct). The findings in the present study provide more knowledge on the molecular reactions between proteins and phenolic compounds, which can be useful in the application of quinone-protein modifications to produce food products with high nutritional values and good functionalities.
5. Associated content

**Supplementary Materials:** Table S1 Observed pseudo-first-order rate constants ($k_{obs}$) and second-order rate constant ($k_2$) for the reactions of 4MBQ with Arg, $N^\alpha$-acetyl Arg, His, $N^\alpha$-acetyl His, Trp, $N^\alpha$-acetyl Trp, or $\beta$-LG at pH 7.0 and 25 °C; Table S2 LC-ESI-MS/MS data for 4MBQ-amino acid adducts; Fig. S1 Quantification of the peak area of modified peptides derived from the tryptic digestion of $\beta$-LQ and Glu-C hydrolysis; Fig. S2 MS/MS spectra of Cys-modified peptide, QSLACQCLVRTPE and the relative unmodified peptide. Fig. S3 Changes of the UV-visible absorption spectra of reaction in mixtures containing 4MBQ and $N^\alpha$-acetyl Arg, His, $N^\alpha$-acetyl His, Trp and $N^\alpha$-acetyl Trp; Fig. S4 Time-dependent absorbance (at 401 nm) changes of the reaction mixture containing 4MBQ and His. Plot of pseudo-first-order rate constant at 401 nm ($k_{obs}$) against His concentration; Fig. S5-S10 Chromatograms and MS/MS spectra of adducts formed from 4MBQ and Arg, $N^\alpha$-acetyl Arg, His, $N^\alpha$-acetyl His, Trp and $N^\alpha$-acetyl Trp, respectively; Scheme S1-S6 Schemes of 4MBQ reacted with the amino acids.

**CRediT authorship contribution statement**

**Jingyuan Liu:** Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **Kasper Engholm-Keller:** Methodology, Formal analysis, Validation, Writing – review & editing. **Mahesha M. Poojary:** Methodology, Validation, Writing – review & editing. **Marta Bevilacqua:** Formal analysis, Visualization, Writing – review & editing. **Mogens L. Andersen:** Conceptualization, Validation, Writing – review & editing, Supervision. **Marianne N. Lund:** Conceptualization, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

**Declaration of Competing Interest**

The 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**

Data will be made available on request.

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Appendix A. Supplementary material
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References