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Short communication

Kinetic investigation of the trapping of Nε-(carboxymethyl)lysine by 4-methylbenzoquinone: A new mechanism to control Nε-(carboxymethyl) lysine levels in foods

Yuting Li[a,b], Lin Li[a,b,c,*], Marianne N. Lund[d,e], Bing Li[a,c,*], Yi Hu[a], Xia Zhang[a,c]

[a] School of Food Science and Technology, South China University of Technology, Guangzhou 510640, China
[b] School of Chemical Engineering and Energy Technology, Dongguan University of Technology, Dongguan 523808, China
[c] Guangdong Province Key Laboratory for Green Processing of Natural Products and Product Safety, Guangzhou 510640, China
[d] Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg, Denmark
[e] Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen, Denmark

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ABSTRACT

o-Benzoquinones, formed during oxidation of polyphenols, react with amines through a Michael addition. In the present study, the ability of 4-methylbenzoquinone (4MBQ) to trap Nε-(carboxymethyl)lysine (CML) through a Michael addition with the amine groups on CML was investigated at different pH values. Apparent second order rate constants (k_2) for the reaction of 4MBQ with CML were determined by stopped-flow spectrophotometry at 25 °C to be ~0.0 $M^{-1} s^{-1}$ at pH 5, 9.5 $M^{-1} s^{-1}$ at pH 7, and 164.5 $M^{-1} s^{-1}$ at pH 8 based on the loss of 4MBQ at 401 nm. The reaction between 4MBQ and CML generated coloured CML-quinone compounds via colourless CML-phenol species as identified by LC-ESI-MS/MS. These data provide evidence that CML formed during food production can be trapped by *o*-benzoquinones, which is a new mechanism by which polyphenols may be used to control CML levels in foods.

1. Introduction

Advanced glycation end products (AGEs) are a series of complex highly oxidizing compounds, mainly formed in the advanced stage of the Maillard reaction (Ahmed et al., 2005; Lund & Ray, 2017; Uribarri et al., 2010). Nε-(carboxymethyl)lysine (CML), as a well-established AGE marker in foods and *in vivo* (Assar, Moloney, Lima, Magee, & Ames, 2009; Teerlink, Barto, Brink, & Schalkwijk, 2004), is formed by modification of free lysine or lysine residues in peptides and proteins with glucose, glyoxal, or ascorbate autoxidation products (Chuyen, Kurata, & Fujimaki, 1973; Dunn et al., 1990; Han et al., 2013). CML is extensively formed in foodstuffs, with 46.1 mg/kg in wholemeal bread crust (Assar et al., 2009), 46.2 mg/kg in evaporated whole milk (Assar et al., 2009), and 378–989 mg/L in soy sauce (Li et al., 2015). CML concentration in foods is significantly influenced by cooking methods. Frying and boiling beef have resulted in ~15.5 and ~7.0-fold higher levels of CML, respectively, compared to raw beef (Assar et al., 2009). Long term ingestion of excessive dietary CML has been found to induce functional arterial aging in wild-type mice (Grossin et al., 2015), and to increase the markers which are related to the increasing risk of type II diabetes mellitus and cardiovascular diseases in healthy subjects (Birlouez-Aragon et al., 2010). Identifying strategies that reduce the concentration of CML in processed foods is a matter of great importance for the improvement of food quality and safety. *o*-Benzoquinones, which are generated by oxidation of catechols, are highly electrophilic molecules (Bittner, 2006), and can react with nucleophilic groups, such as thiol and amine groups on free amino acids, peptides and proteins. Pierpoint (1969) found that chlorogenoquinone (an *o*-benzoquinone) would modify the lysine residues of BSA to form red coloured adducts in excess of BSA. The quinone of epicatechin has been found to react with both the α- or ε-amino group of lysine (Yin, Hedegaard, Skibsted, & Andersen, 2014). In free CML, the α-amino group of lysine is available, while the ε-amino group is modified to a more reactive secondary amino group with higher nucleophilicity (Brotzel, Chu, & Mayr, 2007). Consequently, we hypothesize that *o*-benzoquinones will trap CML by reacting with both its primary and secondary amine.

In the present study, 4-methylcatechol (4MC) was used as a model compound for catechols to produce a relatively stable *o*-benzoquinone (4-methylbenzoquinone, 4MBQ) (Boots, Haenen, den Hartog, & Bast, 2002) for investigation of the reaction mechanism between CML and an *o*-benzoquinone compound. Adducts formed by the reaction of 4MBQ

* Corresponding authors at: School of Food Science and Technology, South China University of Technology, Guangzhou 510640, China.
E-mail addresses: felinli@scut.edu.cn (L. Li), bli@scut.edu.cn (B. Li).

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with CML were identified by LC-ESI-MS/MS, and the involved kinetics were determined by stopped-flow UV–Vis spectrophotometry.

2. Materials and methods

2.1. Chemicals and reagents

Nε-(carboxymethyl)lysine (CML, 98%) was purchased from TRC (Toronto, Canada). 4-Methylcatechol (≥95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Oceanpak Alexative Chemical (Gothenburg, Sweden). All other reagents were of analytical grade.

2.2. Generation of 4MBQ

A solution of 2 mM 4MC was prepared in 0.2 M phosphate buffer (pH 4.5). 4MBQ was generated by bulk electrolysis of the 4MC solution as described previously (Li, Jongberg, Andersen, Davies, & Lund, 2016) and used within 1 h after generation. The theoretical yield of 4MBQ was 100% at the end of the electrolysis as determined by the amount of charge passed described in a previous study (Li et al., 2016). The actual yield of 4MBQ was ~80%, which was determined by UV–Vis spectrophotometry with $\varepsilon_{395} = 1350\ M^{-1}\ cm^{-1}$ (Whitaker, Voragen, & Wong, 2003). The 4MBQ solution was stable at 25 °C with ~16% loss over 60 min as determined by UV–Vis spectrophotometry.

2.3. Kinetic experiments

CML was dissolved in 0.2 M phosphate buffer to reach different concentrations and the desired pH values and was kept in at least 10-fold excess of 4MBQ concentration to obtain pseudo first-order conditions during the kinetic experiments. Specific concentrations for each experiment are given in Table 1. 4MBQ and CML were placed in each syringe of a SX20 stopped-flow spectrophotometer (Applied Photophysic, London, UK), and the reaction was recorded by absorbance measurements in the UV/Vis range (200–750 nm) at 25 °C. For each measurement, equal volumes (60 µl) of 4MBQ and CML were mixed in the optical cell to obtain pH 5, pH 7 and pH 8. Spectra were recorded at different time intervals depending on the reaction rate.

Table 1
Observed pseudo-first-order rate constants (k_{obs}) and second-order rate constant (k_2) for the reaction of 4MBQ with CML at pH 5, pH 7 and pH 8, obtained by following the loss of 4MBQ at 401 nm or the adduct formation at 531 nm.

pH	CML concentration (mM)	k_{obs} (s^{-1})	k_2 ($M^{-1}s^{-1}$)
5	0.00	$(78.2 \pm 1.7) \times 10^{-3}$	~0
(401 nm)	0.50	$(81.0 \pm 2.0) \times 10^{-3}$	
	1.25	$(80.2 \pm 2.2) \times 10^{-3}$	
	2.50	$(87.6 \pm 6.6) \times 10^{-3}$	
	3.75	$(81.4 \pm 1.5) \times 10^{-3}$	
	5.00	$(78.6 \pm 1.0) \times 10^{-3}$	
7	0.00	$(83.1 \pm 1.3) \times 10^{-3}$	9.5 ± 1.4
(401 nm)	0.50	$(92.8 \pm 4.4) \times 10^{-3}$	
	1.25	$(105.8 \pm 10.8) \times 10^{-3}$	
	2.50	$(101.6 \pm 1.9) \times 10^{-3}$	
	3.75	$(122.1 \pm 13.1) \times 10^{-3}$	
	5.00	$(135.8 \pm 6.4) \times 10^{-3}$	
8	0.00	$(76.7 \pm 6.5) \times 10^{-3}$	164.5 ± 5.0
(401 nm)	0.50	$(179.0 \pm 21.6) \times 10^{-3}$	
	1.25	$(249.8 \pm 41.4) \times 10^{-3}$	
	2.50	$(472.7 \pm 64.0) \times 10^{-3}$	
	3.75	$(678.3 \pm 98.6) \times 10^{-3}$	
	5.00	$(910.8 \pm 46.1) \times 10^{-3}$	
8	0.50	$(209.9 \pm 17.3) \times 10^{-3}$	107.7 ± 8.7
(531 nm)	1.25	$(298.3 \pm 45.3) \times 10^{-3}$	
	2.50	$(377.4 \pm 36.9) \times 10^{-3}$	
	3.75	$(585.9 \pm 41.5) \times 10^{-3}$	
	5.00	$(684.4 \pm 26.5) \times 10^{-3}$	

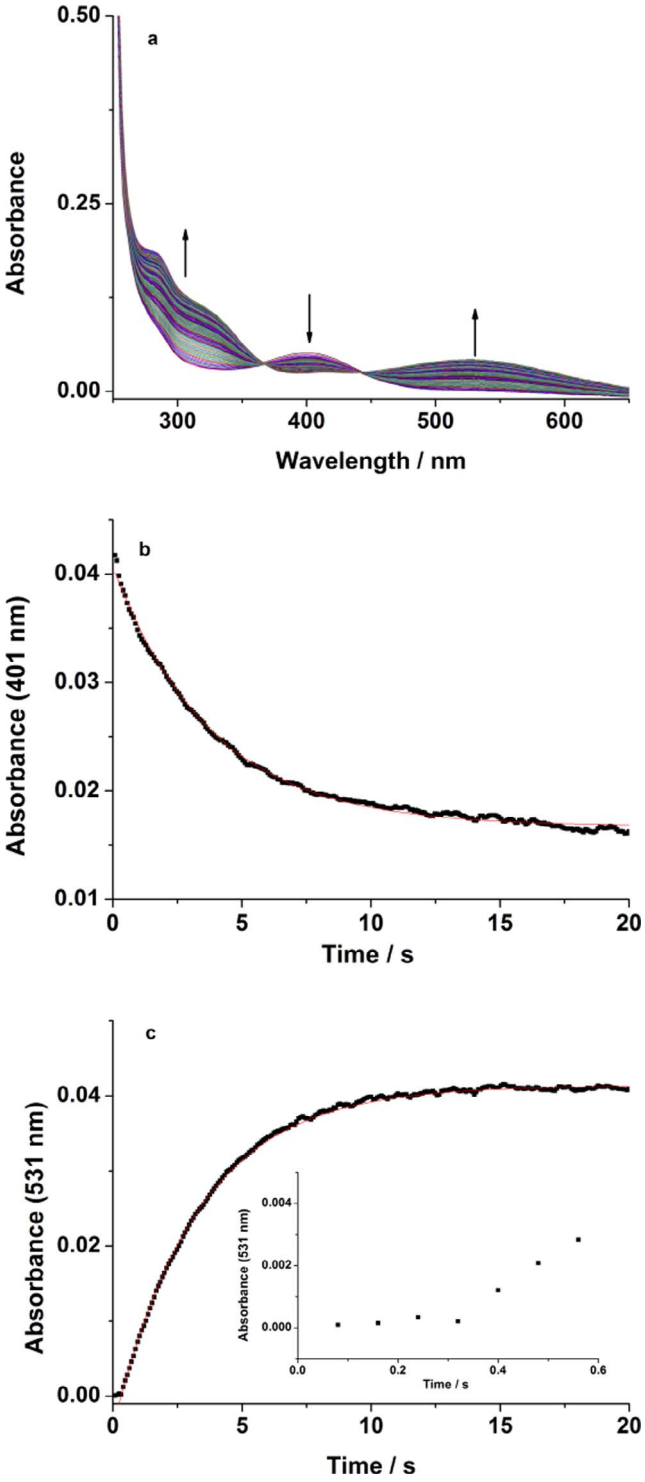

Fig. 1. a) Absorption spectra of 1.25 mM CML and 0.04 mM 4MBQ in 0.2 M aqueous phosphate buffer (25 °C, pH 8), the arrows indicate the decrease of 4MBQ at 401 nm and the adduct formation at 531 nm; b) Exponential fit of absorption recorded at 401 nm for 20 s to calculate the pseudo-first-order rate constant; c) Exponential fit of absorption recorded at 531 nm for 20 s to calculate the pseudo-first-order rate constant, the insert shows the lag phase occurring within the first 0.4 s.

2.4. Identification of the 4MBQ-CML reaction product by bulk electrolysis and LC-ESI-MS/MS

The reaction product was prepared by the reaction of 4MBQ (0.5 mM) with CML (5.0 mM) in phosphate buffer (pH 5, 7 and 8, 25 °C). After 1 min of stirring, the mixture was reduced by bulk