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Contribution of Linoleic Acid to the Formation of Advanced Glycation End Products in Model Systems during Heat Treatment

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Abstract

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Advanced glycation end products (AGEs) are glycosylated metabolic products generated *in vivo* and are associated with aging-related diseases. They are also formed during heat treatment in food processing. In this work, we investigated the contribution of linoleic acid (LA) to AGE formation using a protein/glucose model. An electronic tongue, denaturing polyacrylamide gel electrophoresis, electron spin resonance spectroscopy, circular dichroism, and ultra-performance liquid chromatography-tandem mass spectrometry were used to analyse reaction intermediates and reactive radical formation. The results show that LA is the key factor responsible for the change in flavour including the rapid triggering of glycation reactions. The amount of lipid-induced reactive radicals was significantly higher than in the non-fat system, radical generation in the non-fat system was gradually quenched after a robust radical-yielding reaction in the first 25 minutes. Subsequent unsaturated lipid oxidation, and AGE accumulation surpass Maillard reaction-only outcomes. Initial LA-induced changes in protein structure are followed by glycation and are enhanced by hydrophobic interactions and increased carbonyl levels resulting from lipid oxidation. These findings implicate lipids and lipid oxidation as the main factors responsible for AGE formation during the processing of fat-rich unsaturated fatty acid-containing foods.

Keywords: glycation; linoleic acid; radical formation; flavour

Advanced glycation end products (AGEs) comprise a heterogeneous family of unavoidable by-products of endogenous sugar and lipid peroxidation that are generated by reactive metabolic intermediates. Non-enzymatic reaction between sugars and proteins, known as the Maillard reaction (MR), is a typical form of AGE formation. In addition, AGEs can be formed from sugar-derived carbonyls, with products including N^{ε}-(1-carboxyethyl)-lysine and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine, or by lipid peroxidation and oxidative stress, which can produce malondialdehyde or N^{ε}-(carboxymethyl)-lysine (CML). CML is a marker of (food) processing-induced or endogenously formed AGEs (Niquet-Léridon & Tessier 2011). It is a protein adduct generated by combined non-enzymatic glycation and oxidation (glycoxidation) reactions, or by oxidation of polyunsaturated fatty acids in the presence of proteins (Troise *et al.* 2015). Although MR chemistry has been a focus of intense research for more than a century and considerable progress has been made concerning the reaction mechanism, the AGE assortment is

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far from being completely characterised, especially in food processing.

Because lipids are important food ingredients, the interactions between lipids and non-enzymatic glycation have been of increasing concern in recent years (Matiacevich *et al.* 2005; Ahmad *et al.* 2014). Non-enzymatic oxidation or autoxidation of lipids proceeds via typical radical intermediate formation, with hydroperoxides as the possible initial reaction products (Zamora & Hidalgo 2005). Additionally, intermediates of MR can produce corresponding radicals (Shi *et al.* 2015). Thus, changes in radical levels can reflect changes of MR and LA. Electron spin resonance (ESR) spectroscopy is a recently developed method for analysing radical interactions, and we have successfully used it in our investigations (Hedegaard *et al.* 2015; Shi *et al.* 2015). However, advanced protein structure is likely affected by protein glycation, which can be observed and quantified by circular dichroism (CD), Fourier transform infrared spectroscopy, denaturing polyacrylamide gel electrophoresis (SDS-PAGE), high-performance size exclusion chromatography, and differential scanning calorimetry (Spada *et al.* 2015; Campbell *et al.* 2016; Wilde *et al.* 2016). Because the current studies have mainly been focusing on medical aspects of AGEs, some special methods for food analysis are rarely used in the study of AGEs. For example, although there are some correlations between AGE accumulation and flavour in food processing, flavour analysis has not been applied to investigations of AGEs. Electronic tongue (E-tongue) technology provides an effective method of investigating non-volatile flavour products. Isolation and identification of flavour peptides from puffer fish muscle, optimization of chicken flavouring, discrimination of orange juice, and flavour assessment of sweetsop have all been achieved using E-tongue technology (Baskaran *et al.* 2015; Ifeduba & Akoh 2015; Raithore *et al.* 2015; Wadehra 2016). Conventional technologies, such as gas chromatography, are used in measurements of Strecker reaction-derived volatiles formed in the course of non-enzymatic browning reactions. However, according to the current knowledge, AGEs are predominantly non-volatile components of larger molecules. Therefore, the E-tongue could provide a new and simple way of studying the AGE flavour arising from lipid oxidation and protein glycosylation.

Although the link between lipid oxidation and AGE accumulation has been gaining increasing attention in *in vivo* studies (Vistoli e*t al.* 2013), the details of this interaction remain largely unknown. Because of the complexity and heterogeneous nature of real food systems, polycondensation product formation is mainly studied in model systems comprising an amino acid and a carbonyl compound. In this study, to understand the effect of lipids on the generation of different glycation products, we established an MR system composed of linoleic acid (LA) reacting with bovine serum albumin (BSA) or lysine, and glucose. Moderate temperature (60°C) and 40 h heating time in solution were chosen as conditions representative of food processing. Various analytical methods, namely SDS-PAGE, E-tongue, ESR, CD, and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), were combined to characterise generated AGEs, radical levels, flavour, protein glycation, and protein structure changes, as affected by LA and heating.

MATERIAL AND METHODS

Chemicals. L-Lysine, D-glucose, monosodium phosphate, and disodium hydrogen phosphate were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). BSA, LA, and SDS-PAGE reagents were purchased from Dingguo Biotechnology (China). The CML standard (99%) was purchased from Toronto Research Chemicals Inc. (Canada). α-(4-Pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN) was purchased from Bailingwei Ltd. (China). Water was purified with an ultra-pure water system from Heal Force Bio-Meditech Holdings Ltd. (China). All other chemicals were of analytical grade or of the highest available purity.

LA-MR model systems. Model systems were prepared in 0.2 mol/l phosphate buffered saline (PBS), pH 8, the reactants were mixed with a magnetic stirrer, then sealed with sealing film and heated at 60°C for 40 h, unless specified otherwise. Samples were analysed at 10 h intervals. The following model systems were devised: GB, comprising 0.66 mol/l glucose and 0.22 mol/l BSA; GBY, comprising GB supplemented with 3.75 mol/l LA; GL, comprising 0.33 mol/l glucose and 0.10 mol/l lysine; GLY, comprising GL supplemented with 0.04 mol/l LA.

E-tongue analysis. A TS-5000Z E-tongue (Insent Inc., Japan) was used to profile GL and GLY system flavours. Eight sensors were assigned to specific taste sensations, namely bitterness, bitter aftertaste, astringency, astringent aftertaste, salty, sourness,

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umami, and richness. Test conditions were as follows: 80 ml sample volume, 3 min analysis time, 2 min acquisition time, room temperature. Ethanol (5%) was used as a sensor cleaning solution. Every sensory measurement was followed by a wash cycle (soaking in ethanol for 30 s). Three replicate measurements were conducted for each sample.

ESR spectroscopy. A miniscope MS 200 ESR spectrometer (Magnettech, Germany) was used according to a revised method (Hedegaard *et al.* 2015). The samples were heated at 70°C for up to 75 min before analysis. ESR parameters were set as follows: 3495.55 G centre field, 100.0 G sweep width, 40 s sweep time, 8 mW microwave power, and 1000 mG modulation amplitude. The radical activity signals were measured with Analysis 2.02 software (ESR application; Magnettech GmbH, Germany) and used for quantification. All measurements were performed in duplicate, and the average relative heights of middle peaks in the POBN ESR spectra were used for quantification.

Protein secondary structure determinations. Quantitative analyses of the effect of glycation on protein secondary structure elements were conducted in the far-UV CD spectrum region (190–250 nm) using a JASCO-810 spectropolarimeter (Jasco, Japan). Continuously heat-treated GB and GBY systems were monitored at 10 h intervals. The results are expressed as molar ellipticity $[\theta]$, defined as $[\theta] = \theta/(c \times l)$, where: l – light path length (mm); c – protein concentration (mmol/l); θ – measured ellipticity (mdeg) at the relevant wavelength.

The relative proportions of protein secondary structure elements were estimated as percentages using $SELCON_3$ software.

Protein glycation determinations. Glycation-induced protein fragmentation and cross-linking in GB and GBY systems were assessed by sodium dodecyl sulphate (SDS)-PAGE on 10% polyacrylamide gels, followed by Coomassie Brilliant Blue R staining (Ahmad *et al.* 2007). Protein samples were diluted 1 : 10 in 0.05 M Tris-HCl buffer (pH 6.8) supplemented with 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerin, and boiled for 5 minutes. Protein samples (10 µg) were mixed with 2 ml of bromophenol blue (0.2% in protein samples), loaded onto gels and subjected to electrophoresis on a Mini-Protean apparatus (Bio-Rad Laboratories, USA). The gels were stained in a solution containing 0.25% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol, and 10% (v/v) acetic acid, and were destained in a solution containing 25% (v/v) methanol and 7% (v/v) acetic acid. Gels were photographed and images were analysed with an AI600 Gel Imaging System (GE Healthcare Lifesciences, USA).

CML determinations. After being passed through 0.22-µm filters, GL and GLY mixtures were analysed by UPLC-MS/MS. The system comprised ACQUITY UPLC separation module (Waters, USA) and Waters Micromass Quattro Premier XE MS operated in a positive ESI multiple reaction-monitoring mode. CMLs were separated on a reversed-phase C_{18} column (2.1 × 75 mm, 2 µm; Shimadzu, Japan) with a linear gradient of acetonitrile and 0.1% FA (20 : 80). A previously method developed (Assar *et al.* 2008) was used, with a 0.25 ml/min flow rate and 10-µl injection volume. Optimal conditions for all parent and daughter ions were established by direct injection of standard CML solution (10 mg/ml). The electrospray source temperature was set to 110°C, with a 350°C desolvation temperature. Optimal ionisation was achieved with a 3 kV capillary voltage.

Fluorescent AGE determinations. The method reported by Skrt *et al.* (2011) was followed with slight modifications. The glycated samples were diluted 200 times with PBS to measure the amount of fluorescent AGEs (AGE-fl) by using a Hitachi FL-4600 fluorometer at excitation and emission wavelengths of 370 and 440 nm, respectively. All measurements were performed in duplicate.

Statistical analysis. All data were subjected to analysis of variance (ANOVA). The general linear model in the SPSS statistical package was used to evaluate the statistical significance of differences between groups.

RESULTS AND DISCUSSION

LA affects flavour and triggers glycation reactions during heating. Many literatures have reported that unsaturated fatty acids are more easily oxidised than saturated fatty acids in heating conditions (Liu *et al.* 2017). In order to understand the effect of oxidative lipid on AGEs, we choose linoleic acid, one kind of unsaturated fatty acids of simple structure and conventional components in edible oils, as the research target.

The effect of heating and LA on bitterness, bitter aftertaste, astringency, astringent aftertaste, saltiness, umami, sourness, and richness of the GL and GLY systems was determined using the E-tongue. The

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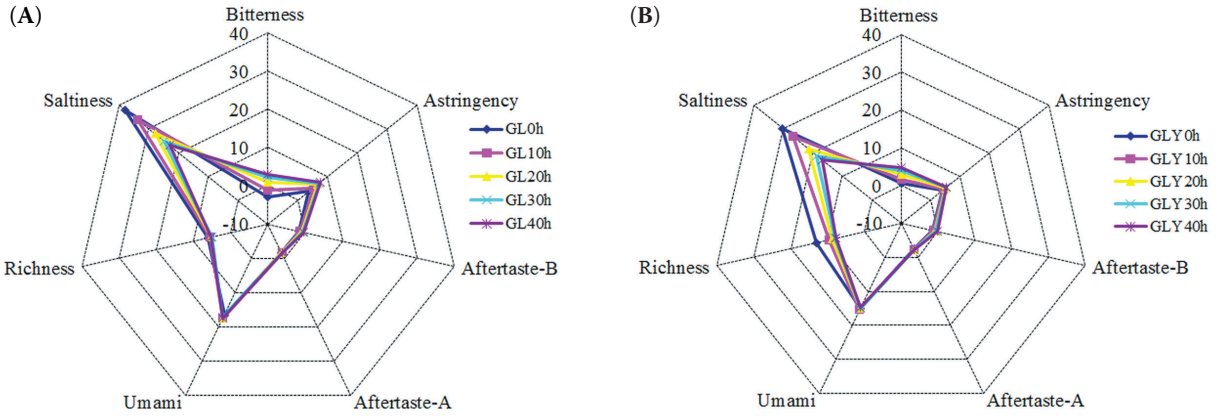

Figure 1. E-tongue data on GL system and GLY system heatedin range 0–40 h (interval every 10 h) at 60°C analysed using aroma sensory profiles: (**A**) GL system and (B) GLY system

data are presented in Figure 1, except for the data for bitterness aftertaste and astringent aftertaste, which did not significantly contribute to flavour, sourness, and saltiness mainly derived from PBS in these model systems. Heating exerted a significant effect on bitterness, saltiness, richness, and astringency ($P < 0.05$) in both systems. The two systems differed with regard to the effect of heat on astringency, richness, and bitterness (representative polymer flavours). Increased bitterness and astringency and decreased richness indicated increasing polymer content, e.g., increased content of melanin and cross-linked AGEs (Kobue-Lekalake 2009). Umami is mainly caused by the flavour of amino acids, and the richness of the flavour reflects the sustained perception of umami. As shown in Figure 1, the umami level was lower in the GLY system than in the GL system, and the richness of GLY system continued to decline as the heating time was extended. These results indicate that the amino acids in the GLY system were more extensively consumed than those in the GL system, and that the consumption of L-lysine increased quickly with heating extension, leading to greater polymer formation. This further indicates that lipids facilitate AGE formation.

E-tongue technology is an efficient method of documenting flavour variations and measuring the effect of heating on flavour. We modelled the difference between GL and GLY systems by performing Principal Component Analysis (PCA). PCA revealed that the contribution of the first principal component (x-axis) was 82.5% and that of the second principal component (y-axis) was 13.2% (Figure 2). The cumulative contribution of the two main components was 95.7%. After clustering analysis, the two systems were obviously divided into two categories, and the significant difference between them was associated with the first principal component (saltiness and bitterness), indicating that saltiness and bitterness are responsible for distinguishing the two systems. As is shown in Figure 2, compared with the saltiness of GLY system, the GL system showed an obvious initial maximum ($P < 0.05$), and the two systems showed a similar bitterness change ($P > 0.05$). Furthermore, the differences between ten samples collected at different heating times were mainly reflected in the second principal component (richness and umami). The GL system showed an extensive change in richness ($P > 0.05$), but the richness of the GLY system was significantly reduced ($P < 0.05$) with heating exten-

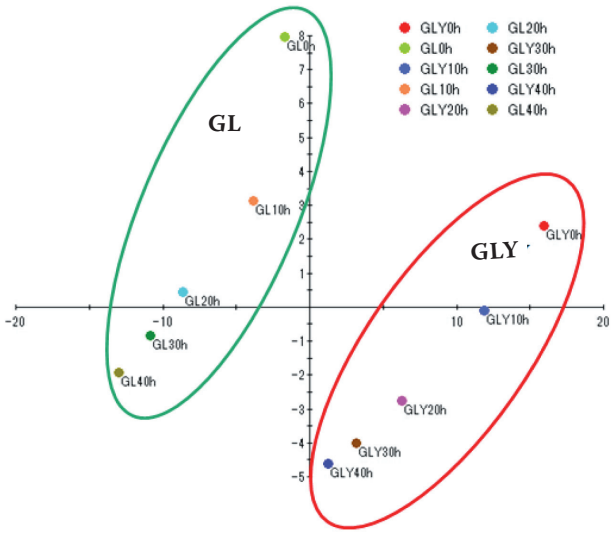

Figure 2. E-tongue data on GL system and GLY system heated 0–40 h (interval every 10 h) at 60°C analysed by Principal Component Analysis

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sion. Thus, PCA revealed a considerable difference between lipid-containing and lipid-devoid systems, that is to say, the lipid-containing system reduced the salt content, and moreover, with prolonged heating time, the richness continuously declined. These results confirm that LA is the key factor responsible for the change in flavour including the rapid triggering of glycation reactions.

LA promotes AGE formation via lipid peroxidation during heating. Previous studies have suggested that lipids might facilitate radical formation in MR systems (Lokuruka 2011; Han *et al.* 2013; Ifeduba & Akoh 2015). A recently developed ESR-based technique and spin trapping method have allowed structural information on radicals to be acquired (Yin *et al.* 2013). Due to low radical reactivity of the second structure of BSA at 60°C, we performed ESR spectroscopy experiments at 70°C. Highly reactive intermediary radicals were observed in the GLY system (Figure 3). Although the strength of the radical signal in the GLY system apparently slowly decreased, the amount of generated radicals was significantly higher ($P < 0.01$) than that in the GL system. Radical generation in the GL system was gradually quenched after a robust radical-yielding reaction of glucose and L-lysine in the first 25 minutes. This indicated that in the GL system, the highly reactive radicals mainly arise from the MR reaction component. Generation of these unstable radicals gradually decreased with the formation of MR polymers and MR antioxidation products (Yilmaz & Toledo 2005). In contrast, radical signal strength did not decrease in the GLY system and did not significantly change with heating time ($P > 0.05$). Because of the documented radical scavenging ability of MR (Morales & Jiménez-Pérez 2001; Sun & Luo 2011), radicals detected in the GLY system are probably generated by lipids and lipid peroxidation rather than MR. Moreover, a high reactive radical content results in protein oxidation and cross-linking (Saeed *et al.* 2006), and these oxidation products are considered to be intermediates of AGE formation (Renzone *et al.* 2015). Consequently, during heating, unsaturated fatty acids promote AGE formation mainly via the lipid peroxidation pathway, and not the MR pathway.

LA promotes participation of glucose in protein glycation. Protein glycation may lead to changes in the primary and secondary structures of BSA (Rondeau *et al.* 2010). Indeed, the BSA-AGE secondary structure transitions may be detected via CD spectropolarimetry (Figure 4). Analysis of the CD spectra using the SELCON$_3$ program enabled quantification of structural alterations in the model systems. The native BSA was detected before heating (0 h) and after heating (40 h), and the same conclusion was reached as follows: 19.0% α-helical, 29.8% β-sheet, 21.2% turn, and 30.0% unordered structure. Compared to native BSA, the α-helical contents of the GB and GBY systems at time 0 h were significantly lower ($P < 0.01$, GB vs. BSA and GBY vs. BSA), with

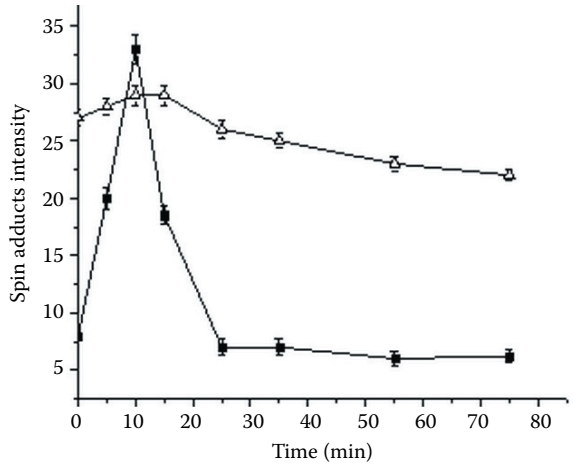

Figure 3. Intensity of spin adducts of POBN (0.1 M) generated in phosphate buffer (PBS) at pH 8.0 with L-lysine (0.1 M) together with glucose (0.33 M) (GL system) or L-lysine (0.1 M) together with glucose (0.33 M) and linoleic acid (0.04 M) (GLY system) when heated at 70°C for up to 75 min prior to recording the ESR spectra
(Δ) GLY system; (■) GL system

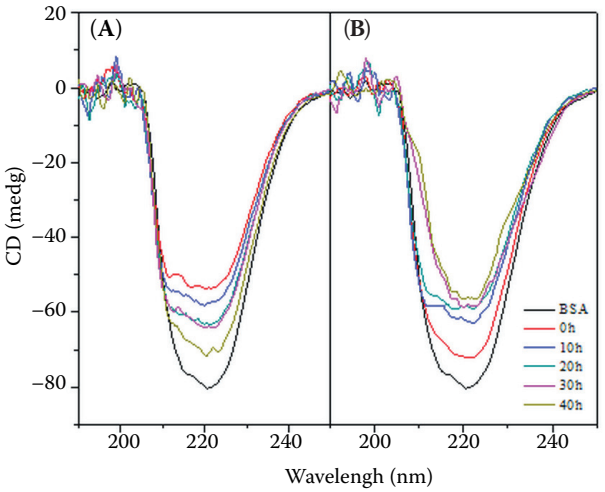

Figure 4. Far-UV CD spectra of glycated samples of BSA were obtained in the wavelength range of 190–250 nm in different heating time: (**A**) GB system and (**B**) GBY system

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significantly increased β-sheet contents ($P < 0.01$), which indicated that BSA in the GB and GBY systems formed aggregates and amyloid-like structures because these structural transitions are accompanied by a decrease in α-helical content and an increase in β-sheet content (Rondeau *et al.* 2010). Interestingly, the following secondary structure composition was observed before heating (0 h) for the GB system: 17.2% α-helical, 30.3% β-sheet, 21.7% turn, and 30.9% unordered structure. After heating at 60°C for 40 h, the α-helical content slightly increased to 17.8% (40 h) and the β-sheet content slightly decreased to 30.1%. At the same time, the α-helical content of the GBY secondary structure obviously decreased from 17.9% (0 h) to 16.8% (40 h), the β-sheet content increased from 30.5% (0 h) to 31.3% (40 h), and the turns and unordered structures showed slightly increased content. It is worth noting that the α-helical content in the GBY system decreased as the β-sheet, turn, and unordered structure increased, and the opposite effect was observed in the GB system, which indicates that the GBY system formed more aggregates and amyloid-like structures than the native BSA and GB system with heating extension. Under heating, the secondary structure of BSA will be looser, and glucose in the GB system could undergo a glycation reaction with fatty acids and exposed amino acids of BSA. The results of CD (Figure 4) indicated that the secondary structure of the GB system changed greatly before heating (0 h), but with the extension of heating time, polymerisation was decreased and the secondary structure was mainly loosened. This indicates that the glucose in the GB system is not strongly involved in glycation at this moderate temperature. However, the aggregate structure indicated that LA, and not glucose, participates in protein glycation from the start in the GBY system, and that intermediate polymers and AGEs are generated in large quantities with further heating. AGE formation partly results from protein-glucose interactions, as evidenced by secondary structure changes in both GB and GBY, but is mostly facilitated by the GBY system. The participation of LA, on the one hand, provided a more oxidative environment, but on the other hand, it increased the hydrophobicity of the GBY system, thereby promoting weak polar glycophore formation as the glucose molecules are bound to proteins.

LA accelerates protein polymerisation and AGE formation during heating. As shown in Figure 5, in both GB and GBY systems, non-polymerised BSA content

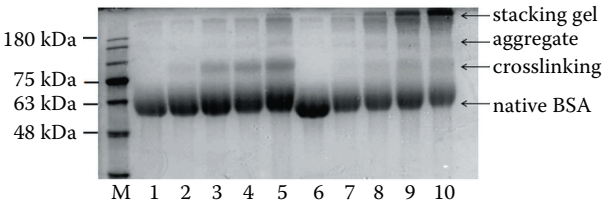

Figure 5. SDS-PAGE gels of GB system (lane 1–5) and GBY system (lane 6–10) were heated 0–40 h (interval every 10 h) at 60°C

gradually decreased whereas BSA/AGE polymer content increased with heating time. Protein glycation thus increased over time. High non-polymerised BSA content was observed in both systems at 0 h, but HMW compound forms, signifying aggregated BSA ($M_w > 180$ kDa) formation, gradually appeared in the GBY system. In the GB system, after 40 h of heating, the aggregated BSA content was significantly lower than that in the GBY system ($P < 0.01$), with (mostly) 100 kDa cross-linked BSA detected. This indicates that LA not only accelerates the cross-linking reaction, resulting in small molecular polymers and glycated intermediates ($M_w < 100$ kDa), but also promotes HMW aggregated BSA formation, especially after 30 hours.

These observations agree with previous reports. All conformational changes of BSA are reversible in the 42–50°C temperature range, whereas unfolding of secondary structures becomes irreversible at 52–60°C (Militello *et al.* 2004; Navarra *et al.* 2009). At approximately 60°C, BSA continues to unfold and begins to aggregate. Thereafter, prolonged heating induces irreversible structural alterations and also increases the protein susceptibility to glycation. When the heating time exceeds 30 h, the ordered structure of BSA is completely abolished, and it is likely that numerous glycation reactions involving exposed amino acids trigger AGE formation. Furthermore, when heating time is extended, self-oxidation and poly-oxidation of unsaturated fatty acid (Refsgaard *et al.* 2000) contribute to the formation of AGEs.

LA promotes CML and AGE formation as well as protein cross-linking in a complex system. Changes in the protein secondary structure lead to the exposure of amino acids. To determine whether or not protein polymerisation was triggered by cross-linking of exposed amino acid residues, we proceeded to analyse the CML content in our systems. CML is a representative glycation product of glycation-accompanying oxidation (Miyata *et al.* 1997). We examined the effect of LA on AGE formation by measuring CML levels by UPLC-MS and AGE-fl by

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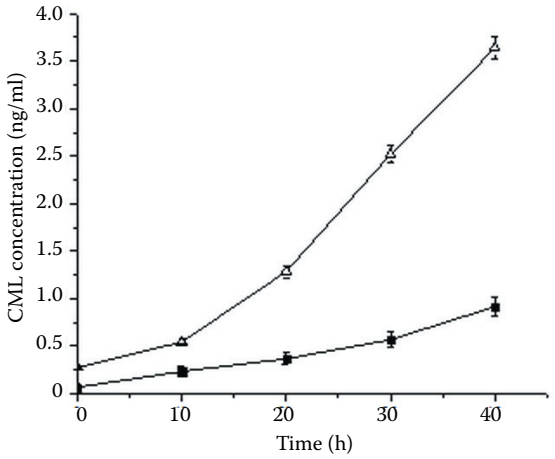

Figure 6. CML content generated in phosphate buffer (PBS) at pH 8.0 in (Δ) GLY system and (■) GL system, when heated at 60°C for up to 60 min to detect by UPLC-MS

the fluorescence spectrum. As shown in Figure 6, CML levels increased with heating time in the two systems, and CML formation was strongly promoted ($P < 0.01$) by LA in the GLY system. Figure 7 shows a similar trend. Linoleic acid has an obvious effect on the formation of AGEs, especially higher than that of the GL system ($P < 0.05$), but saturated stearic acid did not seem to have a pronounced effect on the Maillard reaction of the GL system ($P > 0.05$). Many studies have reported a significant increase in the amount of LA-derived hydroxy fatty acids followed by enhanced protein cross-linking (Udilova *et al.* 2003). Oxidation causes secondary structure unravelling and loosening, exposing basic amino acids

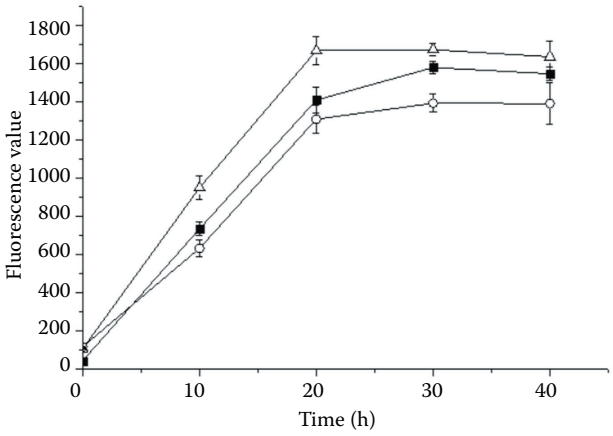

Figure 7. AGE-fl content generated in phosphate buffer (PBS) at pH 8.0 in (Δ) GLY system, (■) GL system, and (○) GL-stearic acid system, when heated at 60°C for up to 60 min to detect by fluorescence spectrum

that participate in glycation reactions, which leads to CML formation and cross-linking. In fact, both MR and lipid oxidation first result in the formation of Amadori compounds. These compounds then undergo fragmentation, rearrangement, and degradation reactions to produce low-molecular-weight volatile and non-volatile monomers (Hutapea *et al.* 2004; Zamora & Hidalgo 2005). In the GL system, CML can be formed by Strecker degradation during MR (Hull *et al.* 2012; Poulsen *et al.* 2013). In the presence of unsaturated fatty acids, however, the Strecker degradation of amino groups usually occurs through the lipid peroxidation pathway involving carbonyl groups (Delatour *et al.* 2009; Lu *et al.* 2012). Therefore, LA-triggered Strecker degradation products enhance the formation of CML. The abundant formation of CML and simple cross-linked AGE intermediates further facilitates HMW cross-linked protein formation.

CONCLUSIONS

This study details two main aspects of the contribution of unsaturated fatty acids to AGE formation: the effect on protein structure and the involvement of amino acids in glycation reactions. On the one hand, reactive radical content is obviously increased by triggered LA oxidation, which further leads to the formation of carbonyl compounds. On the other hand, LA oxidation promotes an alteration of the secondary structure of proteins, which leads to amino acid residue exposure. Carbonyl compounds are involved in degradation reactions, which trigger increased formation of low-molecular-weight AGEs. Furthermore, glycated amino acids and low-molecular-weight AGEs trigger cross-linking of proteins. Therefore, the cumulative outcome of lipid contribution to the formation of AGEs during heat treatment is the abundant formation of glycation cross-linked protein products.

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