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ARTICLE

Formation of N^{ε}-(Carboxymethyl)lysine and Loss of Lysine in Casein Glucose−Fatty Acid Model Systems

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Advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs) form when proteins are heated with reducing sugar or lipid. N^{ε}-(Carboxymethyl)lysine (CML) is the most commonly studied AGE/ALE in foods, but the relative importance of dietary sugar and lipid as its precursors is uncertain. The aim of this study was to determine the relative amounts of CML formed from fatty acid and glucose in a model food system. Model systems were prepared by heating casein (3.2%) with glucose or fatty acid (oleic, linoleic, linolenic, or arachidonic acid) (200 mM) or a mixture of glucose and linolenic acid (200 mM of each precursor) at 95 °C for up to 8 h. CML was determined by ultrapressure liquid chromatography−tandem mass spectrometry. The amount of CML formed from casein and glucose incubated at 95 °C for 8 h was 15-fold higher than that obtained when casein was heated with arachidonic acid under the same conditions. However, the loss of lysine in the casein−arachidonic acid incubations was 83% compared to 54% loss in the casein−glucose incubations. The loss of lysine in casein−fatty acid model systems increased with degree of unsaturation of the fatty acid. The formation of lipid peroxidation products during oxidation of fatty acids might be a potent factor for loss of lysine in the casein−fatty acid systems.

KEYWORDS: Advanced glycation end-products; advanced lipoxidation end-products; N^{ε}-(carboxymethyl)-lysine; ultrapressure liquid chromatography−tandem mass spectrometry; CML precursors

INTRODUCTION

The Maillard reaction (MR) occurs in almost all foods that undergo heat treatment. The ε-amino group of lysine residues and the guanidino group of arginine residues in protein are particularly susceptible to glycation (*1*). The Amadori product, N^{ε}-(fructosyl)lysine (FL), is formed when glucose is the carbonyl reactant. FL subsequently undergoes oxidation to form the advanced glycation end-product (AGE), or glycoxidation product, N^{ε}-(carboxymethyl)lysine (CML) (*2*). α-Dicarbonyls such as glyoxal (GO), formed during the oxidation of the sugar, the Schiff base, or the ARP, are immediate precursors of CML (*3*). Lipid peroxidation is another route to CML, and GO has been suggested as an intermediate (*4*). Glyoxal is one of several aldehydes and ketones formed during lipid peroxidation and, for example, malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and hexanal may also form adducts, known as advanced lipoxidation end-products (ALEs), on protein (*5*, *6*).

Various studies have quantified CML in a range of foods including milk and milk products (*7*−*11*). It remains uncertain whether sugars or fatty acids are the main precursor of CML in dietary items, but the degree of fatty acid unsaturation may play a role in the relative importance of these two components. Thus, the aim of this study was to use ultrapressure liquid chromatography−tandem mass spectrometry (UPLC-MS/MS) to determine the formation of CML and loss of lysine in model systems comprising bovine casein and glucose and/or fatty acid at a temperature relevant to food processing.

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EXPERIMENTAL PROCEDURES

Materials. The following chemicals were obtained from Sigma (Gillingham, U.K.): boric acid, 99.5%; sodium hydroxide, 98%; sodium borohydride, lysine, trifluoroacetic acid (TFA), and nonafluoropentanoic acid (NFPA) 97%. Other chemicals and their suppliers were as follows: methanol free from acetone (analytical reagent), hydrochloric acid, 37%, (J. T. Baker, Devender, The Netherlands); acetonitrile for HPLC (Chromanorm, Leuven, Belgium); chloroform (GPR) (Bios Europe, Skelmersdale, U.K.); trichloroacetic acid (TCA) (BDH, Poole, U.K.); CML and d_2-CML (NeoMPS, Strasbourg, France); d_4-lysine and $^{13}C_6$-glucose (Cambridge Isotopes, Andover, MA). d_4-CML was kindly provided by Professor John Baynes and Professor Suzanne Thorpe (Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC).

Isolation of Casein from Bovine Milk. Casein was precipitated from raw bovine milk according to the method of Gomez-Ruiz et al. (*12*). Milk (250 mL) was placed into Nalgene bottles and placed in a preheated water bath (37 °C) for 30 min. Following incubation, samples were centrifuged at 3000*g* and 5 °C for 30 min. The milk was drained into a beaker through a

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Table 1. Codes for Casein−Glucose−Fatty Acid Model Systems

model system	code
casein + oleic acid	C/18:1
casein + linoleic acid	C/18:2
casein + linolenic acid	C/18:3
casein + arachidonic acid	C/20:4
casein + glucose	C/G200
casein + $^{13}C_6$-glucose + linolenic acid	C/G/18:3

funnel with glass wool to remove any visible solid fat. Casein from milk was precipitated by slowly reducing the pH of the milk to 4.5, by the addition of HCl (0.1 M) with constant stirring. The bottles were left at room temperature for 30 min and centrifuged at 3500*g* and 5 °C for 20 min. The whey was discarded, and the casein precipitate was washed three times with sodium acetate buffer (1 M, pH 4.6, 200 mL) by centrifuging at 3500*g* and 5 °C for 20 min. A mixture of sodium acetate buffer and dichloromethane (1:1) was added to the casein precipitate and centrifuged at 3500*g* and 5 °C for 20 min. This washing procedure was repeated two times with sodium acetate buffer/dichloromethane, and the casein precipitate was finally washed three times with Purite water to remove any traces of dichloromethane. The bottles were covered with Parafilm, pierced, and left at −80 °C overnight. The contents were freeze-dried and stored in an airtight container at −20 °C.

Incubation of Casein with Fatty Acids and/or Glucose. Solutions of casein (3.2% protein) and either D-(+)-glucose or fatty acid (200 mM) in phosphate buffer (0.2M; pH 6.7) were heated at 95 °C for 0, 2, 4, and 8 h. Model systems were prepared in triplicate. Oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), and arachidonic acid (20:4) were the fatty acids. A model system containing $^{13}C_6$-glucose (200 mM) and 18:3 (200 mM) was also prepared. All fatty acid-containing systems were prepared by dissolving the fatty acid 20-fold with chloroform. Solutions of 18:1 (126.8 μL), 18:2 (123 μL), 18:3 (122 μL), and 20:4 (133 μL) were aliquoted into glass vials, and the solvent was removed under a stream of nitrogen. Casein solution (3.2% protein in phosphate buffer, 0.2 M, pH 6.7; 100 μL) was added to each vial. For the mixture containing casein, $^{13}C_6$-glucose, and 18:3, $^{13}C_6$-glucose (0.037 g, 200 mM) and casein (0.032 g, 3.2%) were dissolved in phosphate buffer (0.2 M; pH 6.7, 1 mL). 18:3 diluted in chloroform (122 μL as described above) was aliquoted into a series of glass vials. The solvent was removed under nitrogen, and casein−$^{13}C_6$-glucose solution (100 μL) was added. For the casein−glucose system, D-(+)-glucose (0.036 g, 200 mM) and casein (0.032 g, 3.2%) were dissolved in phosphate buffer (0.2 M; pH 6.7, 1 mL), and 100 μL aliquots were placed into glass vials. All vials were incubated at 95 °C for up to 8 h. During incubation, vials were removed every 2 h, and lids were opened for 30 s to replenish the air, tightly closed, and left in the shaking water bath for the appropriate time. All model systems were prepared in triplicate. Samples were stored at −20 °C, prior to analysis. The codes of the model systems are summarized in **Table 1**.

Folch Extraction of the Protein. Following incubation, protein was isolated according to the method of Folch et al. (*13*). The entire model system was transferred into a 2 mL screw closure polypropylene O-ring sealed vial (Sarstedt, Leicester, U.K.). Chloroform−methanol (2:1, v/v, 1.5 mL) was added, and protein was isolated by centrifugation at 5000*g* for 10 min. The protein pellet was washed once with chloroform−methanol (2:1, v/v, 1 mL), and residual solvent was completely removed under a fume hood.

Sodium Borohydride Reduction. Sodium borate buffer (0.5 M, pH 9.2, 100 μL) was added to protein solution (100 μL) containing 2 mg of protein and dissolved. Sodium borohydride solution (2 M in 0.1 M NaOH, 10 μL) was added to the sodium borate−protein mixture to attain a final concentration of ~0.1 M sodium borohydride. Samples were reduced overnight at 4 °C.

Protein Isolation−TCA Precipitation. Following reduction, 1−2 drops of acetone were added to each reduced sample to consume any unreacted sodium borohydride. Samples were allowed to stand for 5 min at room temperature. TCA solution (60%, 110 μL) was added to each sample to achieve a final TCA concentration of 20% in the protein solution. The samples were kept on ice for 10 min before centrifugation at 5000*g* for 10 min. The recovered protein pellet was washed once with TCA solution (20%, 500 μL) by centrifuging at 5000*g* for 10 min.

Acid Hydrolysis. Following TCA precipitation, isolated protein was hydrolyzed in 6 M HCl (1 mL) at 110 °C for 24 h in 2 mL screw closure polypropylene O-ring sealed vials (Sarstedt). Acid was removed under vacuum (Speed Vac, Thermo Electron Corp., Milford, MA), and the dried protein hydrolysate was reconstituted in 1% aqueous TFA (1 mL) and stored at −20 °C.

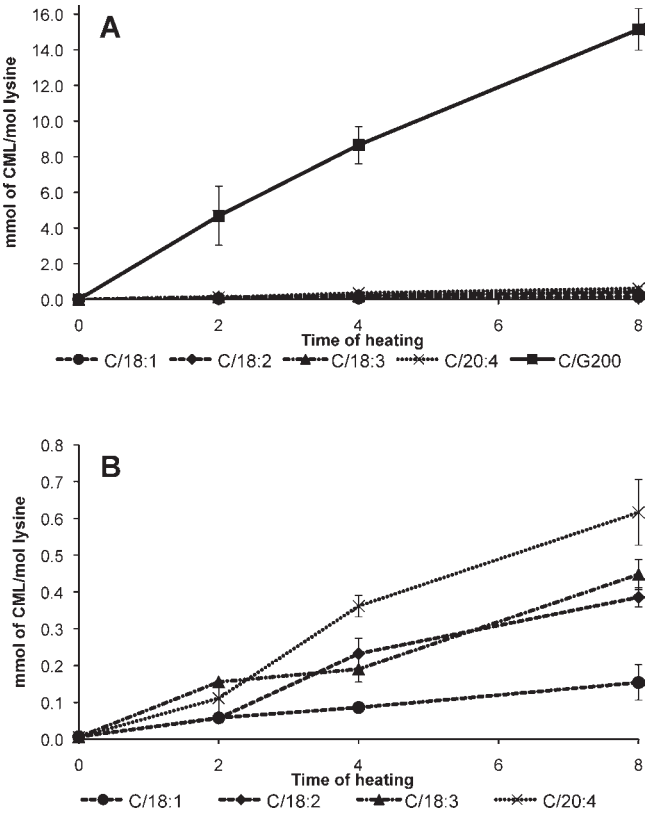

Figure 1. (**A**) Kinetics of formation of CML in casein−glucose and casein−fatty acid model systems and (**B**) expansion of (**A**) to show differences between casein−fatty acid model systems. Data are the mean of triplicate incubations ± SD.

Solid Phase Extraction. Aliquots of reconstituted protein hydrolysate (equivalent to 200 μg of protein) were extracted on a 3 mL Supelco C_{18} cartridge (Sigma, Gillingham, U.K.). The C_{18} cartridge was conditioned with methanol (3 mL) followed by 1% aqueous TFA (3 mL). The protein hydrolysate solution was then placed onto the cartridge, and amino acids were eluted with 1% TFA in methanol−water (20:80, v/v, 3 mL). The eluate was dried under vacuum and reconstituted in aqueous 5 mM NFPA (200 μL), prior to analysis by UPLC-MS/MS.

UPLC-MS/MS. Samples were fractionated on a Waters (Manchester, U.K.) Acquity UPLC system coupled to a Waters Premier triple-quadrupole mass spectrometer. Separations were conducted on a Waters UPLC (2.1 × 50 mm) column. The details of the method are provided by Assar et al. (*12*). Multiple reaction monitoring (MRM) was conducted by operating the MS in positive ion electrospray mode. Sample peaks corresponding to lysine, d_2-lysine, CML, d_2-CML, d_4-CML, and valine were integrated. Data were analyzed using MassLynx software (version 4.1), supplied by Waters. Analytes were quantified by reference to an external standard calibration curve by plotting MS area ratio against amount ratio (unlabeled compound/deuterated compound). A typical external standard curve was constructed by plotting the MS area ratio of lysine and CML against the amount ratio. Sample peaks corresponding to lysine, d_4-lysine, CML, d_4-CML, d_2-CML, and valine were integrated, and the concentration of each compound was calculated by using the equation of the relevant standard curves. The correlation coefficient (R^2) value was ≥0.98 for all calibration curves. Loss of lysine was quantified by dividing the peak area of lysine by valine and expressing it as a percentage loss of lysine. The amount of CML was expressed as millimoles per mole of lysine.

RESULTS

Figure 1A shows the kinetics of formation of CML in all of the casein−carbonyl model systems incubated for up to 8 h.

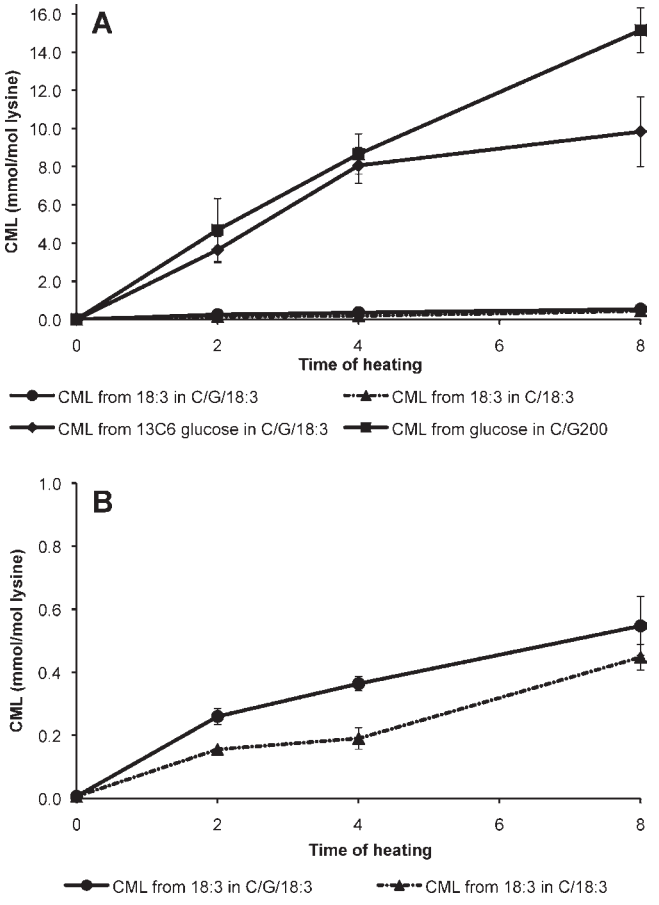

Figure 2. (**A**) Kinetics of formation of CML from glucose and/or 18:3 in C/G/18:3, C/18:3, and C/G200 and (**B**) expansion of (**A**) to show differences between kinetics of CML formation from 18:3 in C/G/18:3 and C/18:3. Data are the mean of triplicate incubations ± SD.

Figure 1B depicts the same data for the casein−fatty acids systems on an expanded scale. The level of CML in C/G200 heated for 8 h was 15.1 ± 1.17 mmol/mol of lysine. In contrast, the concentration of CML in C/20:4 (the fatty acid producing the highest amount of CML) increased from 0.11 ± 0.05 mmol/mol of lysine at 2 h to only 0.62 ± 0.09 mmol/mol of lysine after 8 h of heating. 18:1 produced the lowest amount of CML, ranging from 0.06 ± 0.00 mmol CML/mol of lysine at 2 h to 0.13 ± 0.05 mmol CML/mol of lysine at 8 h. CML formed from C/18:2 increased from 0.06 ± 0.01 mmol of CML/mol of lysine at 2 h to 0.38 ± 0.03 mmol of CML/mol of lysine at 8 h. C/18:3 produced 0.16 ± 0.00 mmol of CML/mol of lysine at 2 h, and the yield rose to 0.45 ± 0.04 mmol of CML/mol of lysine at 8 h. At 8 h the order of reactivity for the formation of CML was glucose ≫ 20:4 > 18:3 > 18:2 > 18:1.

The kinetics of formation of CML formed from glucose and 18:3 in C/G/18:3 is shown in **Figure 2**. Although both carbonyls were used at the same concentration (200 mM), the yield of CML (measured as $^{13}C_2$-CML) formed from $^{13}C_6$-glucose was higher than that formed from 18:3 in the same model system. After 2 h of heating, CML formed from $^{13}C_6$-glucose was 14-fold higher than CML formed from 18:3. The amounts of CML from $^{13}C_6$-glucose were 22- and 18-fold greater than that formed from 18:3 after 4 and 8 h, respectively.

The kinetics of formation of CML obtained from C/G/18:3 was compared to the systems containing casein heated with either 18:3 (C/18:3) or glucose (C/G200) (**Figure 2**). Throughout incubation, the amount of CML formed from 18:3 in C/G/18:3 was similar to that formed in C/18:3. In contrast, amounts of CML formed from glucose in C/G/18:3 and C/G200 were similar after 2 and 4 h but, at 8 h, the amount of CML formed from glucose in C/G200 was 1.5-fold higher compared to CML originating from glucose in C/G/18:3.

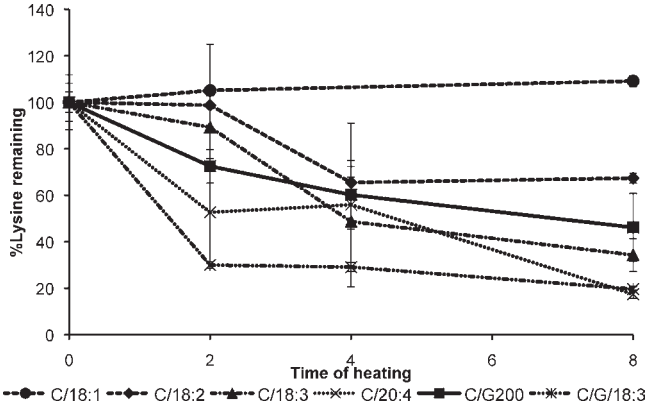

Figure 3. Loss of lysine in casein carbonyl model systems. Data points are the mean of triplicate incubations ± SD.

The loss of lysine in all model systems was calculated by ratioing the peak area of lysine to that of valine, an amino acid that, when in the peptide form, does not react with carbonyl compounds. Data are expressed as a percentage of the ratio at time zero. The data are presented in **Figure 3**. Although the yield of CML was 15-fold greater in C/G200 compared to all of the systems containing only fatty acid (**Figure 1**), with the exception of C/18:1, the loss of lysine increased in all of the model systems with time of incubation and was the same order of magnitude, regardless of whether the carbonyl was glucose or fatty acid. There was no measurable loss of lysine in C/18:1, even after 8 h. In C/18:2, between 0 and 2 h, only 1% of lysine was lost, but the loss increased to 36% by 4 h, after which it remained fairly constant. There was an initial 11% loss of lysine in C/18:3 between 0 and 2 h, which increased to 52% by 4 h and to 66% at 8 h. For C/20:4, 47% of the lysine was lost within the first 2 h of incubation, after which it increased to 85% at 8 h. Loss of lysine in C/G200 was 28% by 2 h of incubation, followed by increases to 40 and 54% losses at 4 and 8 h, respectively. For C/G/18:3, the loss was 70% between 0 and 2 h, after which the percent loss gradually increased to 80% at 8 h. C/G200 suffered a 54% loss of lysine after heating for 8 h. To summarize, after 8 h of heating, the order of loss of lysine in the casein carbonyl model systems was C/18:1≪ C/18:2 < C/G200 < C/18:3 < C/G/18:3 ≈ C/20:4.

DISCUSSION

The concentrations of glucose and each fatty acid were the same in all of the model systems, but the yield of CML in the casein−fatty acid systems was much lower compared to C/G200. CML may be formed in C/G200 via various pathways (*2*). These are (1) the Wolff pathway involving autoxidation of glucose to GO (*14*), (2) the Namiki pathway involving degradation of Schiff base to glycoladehyde and GO (*15*), and (3) the Hodge pathway involving oxidative cleavage of the Amadori product (FL) leading directly to CML (*16*). In routes 1 and 2, the carbonyl intermediates GO and glycolaldehyde would subsequently react with lysine residues to form CML. When fatty acids are the carbonyl precursor, the pathway to CML may involve oxidation of fatty acids to GO or glycolaldehyde and subsequent CML formation (*4*).

GO and glycoladehyde are some of the abundant intermediates formed during the autoxidation of glucose (*3*, *17*).

Previous studies have been undertaken under in vitro physiological conditions and have shown that RNase incubated with either glucose (400 mM) or GO (5 mM) leads to ready carboxymethylation of four lysine residues in RNase−glucose following incubation at 37 °C, compared to only one lysine residue being modified in RNA−GO (*18*). Although GO is a precursor of CML, only 0.1% of glucose is oxidized to GO under physiological conditions (*3*, *19*). In HSA−glucose incubations, very low levels (< 0.5 μM) of GO were detected, but this was attributed to the high reactivity of GO formed toward arginine adducts on protein (*3*). Thus, these reports suggest that GO generated during the autoxidation of glucose may play a minor role in CML formation in protein−sugar model systems and would support the case for FL being the main precursor of CML in C/G200. However, the observed high yield of CML in C/G200 may be attributed to direct oxidation of FL or via GO from FL, the Schiff base, or glucose because the relative importance of the different routes at 95 °C (used in the current study) may be different compared to 37 °C.

Other factors, such as pH, ionic strength (*20*), and temperature (*21*), are known to influence glycation. However, the concentration of phosphate ions is likely to play an important role in the current study because phosphate catalyzes the rate of glycation and oxidation reactions through various mechanisms (*2*, *3*, *20*, *22*, *23*). In a collagen−glucose system the conversion of FL to CML increased 3-fold, and the yield of CML from glucose increased 30-fold when the phosphate concentration was increased from 10 to 200 mM (*19*). Because the casein−carbonyl model systems used in the current study contain a high phosphate buffer concentration (200 mM), the formation of CML from sugar is likely to be promoted in C/G200 and C/G/18:3.

Around 20-fold more CML was formed from sugar ($^{13}C_6$-glucose) than from lipid (18:3) in C/G/18:3. Also, the amount of CML formed from glucose in C/G200 was around 15-fold higher than from 20:4 in C/20:4. Fu et al. (*4*) reported that RNase (1 mM) incubated with either glucose (100 mM) or 20:4 (100 mM) in PBS at 37 °C for up to 6 days resulted in a 23-fold higher yield of CML from 20:4 compared to glucose at day 6, indicating that PUFAs are a more efficient source of CML in systems incubated under in vitro conditions. These results differ from the current findings that glucose is a preferred precursor for CML compared to lipid. Whereas a large fraction of 20:4 was oxidized in the RNase−20:4 system (*4*), ≤2% of the glucose was oxidized in RNase−glucose at the same time period (*19*). The phosphate concentration was relatively low (10 mM) in the study reported by Fu et al. (*4*), compared with 200 mM in the current work, and this might account for the different observations, as discussed above. In support of this, Miyata et al. (*24*) reported that BSA incubated at 37 °C in 100 mM phosphate buffer with 20:4 (10 mM) produced a 4-fold lower amount of CML compared to BSA incubated with glucose (100 mM) under the same conditions. These results are in line with our findings that the formation of CML from glucose is favored over CML formation from lipid when the phosphate concentration is high.

In the current study, after 8 h of heating, 1.5% of lysine residues were modified to CML in C/G200 compared to < 0.07% in C/20:4. Similarly, in the RNase−20:4 system of Fu et al. (*4*), CML accounted for < 0.1% of the lysine residues. Furthermore, Refsgaard et al. (*5*) reported that only trace amounts of CML were detected in iron-catalyzed oxidation of BSA−20:4 incubations. Thus, lipoxidation of fatty acids in protein-containing systems may not lead to CML as a major product. In milk products and infant formulas, neither lipoxidation nor oxidation of the Amadori product was the main source of CML, because no correlation was observed between levels of CML and those of either furosine or secondary lipid peroxidation products (MDA, hexanal) (*25*). However, in the current study, using a model system containing the milk protein fraction, casein, CML is mainly formed via glycoxidation rather than lipoxidation.

Loss of lysine in the C/18:3 and C/20:4 model systems was greater than that in C/G200. The loss of lysine in the casein−fatty acid model systems was strongly dependent on the degree of unsaturation of the fatty acid and increased in the order C/18:1 ≪ C/18:2 < C/18:3 < C/20:4, in agreement with a study involving incubation of BSA with PUFA (*5*). In the current study, although the loss of lysine after 8 h in C/20:4 was higher (85%) than that in C/G200 (54%) or C/18:3 (65%), the model system containing casein, sugar, and lipid (C/G/18:3) showed a similar loss of lysine (∼80%) to that for C/20:4. Free radical intermediates are formed during oxidative degradation of the Schiff base and FL or autoxidation of glucose (*26*) and also during lipid peroxidation (*6*, *27*). Thus, in C/G/18:3, free radicals and reactive carbonyl compounds (RCCs) will be formed from both the sugar and the lipid, resulting in a greater loss of lysine (80%), compared to C/18:3 (65%) or C/G200 (54%).

A range of RCCs, such as aldehydes and dicarbonyls, are formed when PUFA undergo peroxidation. Carbonyl compounds, for example, MDA, HNE, and acrolein, are formed in abundance and react with amino acid residues, including lysine, cysteine, and histidine, to form an array of ALEs and protein cross-links (*6*, *27*, *28*). As a result, structures such as MDA−lysine and HNE−lysine are formed (*6*, *29*). The higher loss of lysine in the casein−fatty acid model systems compared to C/G200 may be due to the high reactivity of lipid peroxidation products such as HNE and MDA with lysine residues, because reactive carbonyl derivatives of protein are formed by MDA (*30*). Previous studies (*19*, *29*) show that MDA and HNE adducts are formed in protein−fatty acid systems, and MDA−protein adducts account for 55−80% loss of amino groups. Thus, there is ample evidence to support the contention that the loss of lysine in casein−fatty acid incubations is mainly due to secondary LPP-derived adducts on lysine.

In conclusion, although CML was formed in ∼15-fold lower amounts in the casein−fatty acid model systems compared to C/G200, the loss of lysine was greater in C/18:3 (65% loss) and C/20:4 (80% loss) compared to 54% loss of lysine in C/G200. The loss of lysine in casein−fatty acid model systems increased with degree of unsaturation of the fatty acid. The formation and subsequent reaction of LPPs are probably potent determinents of the loss of lysine in the casein−fatty acid-containing systems.

ABBREVIATIONS USED

AGEs, advanced glycation end-products; ALEs, advanced lipoxidation end-products; CML, N^{ε}-(carboxymethyl)lysine; MR, Maillard reaction; MDA, malondialdehyde; HNE, 4-hydroxy-2-nonenal; LPP, lipid peroxidation products; NFPA, nonafluoropentanoic acid; UPLC-MS, ultraperformance liquid chromatography−tandem mass spectrometry; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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