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Modification of Casein by the Lipid Oxidation Product Malondialdehyde

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The reaction of malondialdehyde with casein was studied in aqueous solution to evaluate the impact of this lipid oxidation product on food protein modification. By using multiresponse modeling, a kinetic model was developed for this reaction. The influence of temperature and pH on protein browning and malondialdehyde degradation was evaluated. The hypothesis that one malondialdehyde unit leads to the cross-linking of two casein-bound lysine residues was in accordance with the data. At higher malondialdehyde concentrations, a different reaction mechanism was operative, probably involving a dihydropyridine cross-link. The results obtained were compared with the reaction of casein with 2-oxopropanal, a well-studied α-dicarbonyl compound. The reaction of casein with 2-oxopropanal followed a different reaction pathway. Comparison of the degree of browning of casein by reaction with malondialdehyde and 2-oxopropanal showed a considerably higher degree of browning induced by malondialdehyde. This research has shown that kinetic modeling is a useful tool to unravel reaction mechanisms. Clearly, the contribution of lipid oxidation products, such as malondialdehyde, to protein modification (both in food and in vivo) can be substantial and needs to be taken into account in future studies.

KEYWORDS: Malondialdehyde; protein browning; kinetic modeling; Maillard reaction

INTRODUCTION

Lipid oxidation and the Maillard reaction are probably the two most important chemical reactions in food. They are largely responsible for the formation of flavor, color, and texture and for the resulting nutritional value of processed foods. In complex food products, composed of lipids and carbohydrates as well as proteins, both reactions are closely related and involve common intermediates and polymerization pathways. In addition, lipid oxidation products as well as carbohydrates or their degradation products can induce protein browning in a comparable manner (*1*). However, whereas many publications describe browning as a result of the Maillard reaction, much less is known on the impact of lipid oxidation products on protein browning.

Lipid oxidation yields a large variety of reactive carbonyl compounds. Most of these possess only one functional group, whereas malondialdehyde possesses two functionalities and therefore a higher reactivity. Malondialdehyde is a secondary product of lipid peroxidation and has been widely used as a marker compound to measure the extent of oxidative deterioration of lipids in food and biological systems (*2, 3*).

The reactivity of various proteins with malondialdehyde under physiological conditions has been shown, leading to the identification of various amino acid modifications in model reactions or in vivo modifications (**Figure 1**). Reaction of malondialdehyde with primary amino groups leads to the formation of resonance-stabilized imines, that is, 3-amino-2-propenal derivatives or enaminals **1**. Formation of these compounds is of little nutritional concern because they are most

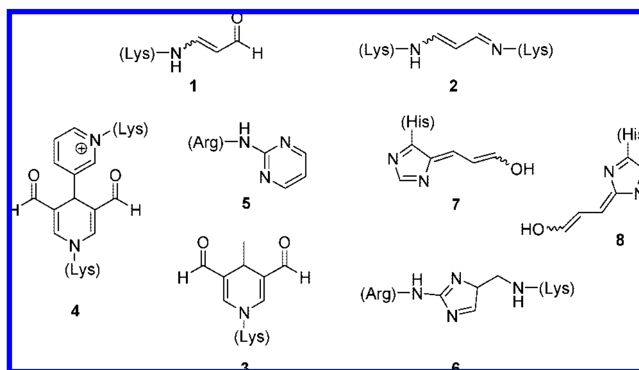

Figure 1. Malondialdehyde-induced modifications of amino acid residues identified in various proteins and model systems.

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probably hydrolyzed at the acidic pH of the stomach. Nucleophilic addition of a second free amino functionality leads to the formation of a 3-amino-1-iminopropene cross-link **2**. Another type of structure containing bound malondialdehyde concerns 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde derivatives (e.g., **3**). These are formed when malondialdehyde reacts with an amino compound in the presence of aldehydes (*4*). Because of the presence of two additional formyl groups in **3**, these lysine modifications can induce further cross-linking. Synthesis and bioavailability studies of *N*,*N'*-di-(4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde)lysine showed that this compound represents a form of unavailable lysine, because it is not metabolized to free lysine and cannot be absorbed from the gut (*5*). From model reactions of *N*-acetylglycyl-L-lysine methyl ester with malondialdehyde, an N-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde substituted at the 4-position with a pyridinium unit **4** was identified (*6*). A modification of collagenous arginine with the formation of 2-aminopyrimidine derivative **5** has also been described (*7*). Other investigations showed the possibility of an imidazole cross-link formed in the reaction of malondialdehyde with lysine and arginine **6**. Modifications of the imidazole functionality of histidine as a result of the reaction with malondialdehyde have also been observed (**7** and **8**) (*8*). Malondialdehyde has been shown to cross-link bovine serum albumin, forming dimers, and to modify RNase, crystallin, and hemoglobin (*3*). Malondialdehyde can also react with DNA and is as such both mutagenic and carcinogenic (*3*). Requena et al. developed an analytical method for the analysis of lysine−malondialdehyde adducts and proved them to be useful biomarkers of lipid peroxidative protein modification and of oxidative stress in vivo and in vitro (*9*). Investigations of the antioxidant activity of the soluble reaction products of beef sarcoplasmic proteins with malondialdehyde showed that light brown pigments were produced with antioxidant properties, in analogy with nonenzymatic browning (*10*).

Because of the importance of malondialdehyde as a lipid oxidation product in vivo, most studies have been carried out on the reaction of this β-dicarbonyl compound with human proteins in physiological conditions. However, the reaction of malondialdehyde with food proteins during food processing is also of interest. In this respect, it has been shown that the decrease of free lysine in milk as a result of heating can be due in part to the reaction of proteins with malondialdehyde, and, as a result, the determination of heat-induced damage in milk-based products by furosine measurements can lead to an underestimation (*11*). Therefore, this research was undertaken to study the browning of casein with malondialdehyde in comparison with the browning of casein induced by the well-known α-dicarbonyl compound 2-oxopropanal (often referred to as methylglyoxal), resulting from sugar degradation as well as from lipid peroxidation (*12*). Kinetic modeling was used to quantitatively describe the changes observed during the reaction.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Sodium caseinate (91% protein, 4% water, 0.1% lactose, 3.8% ash, and 1.1% fat) was obtained from DMV (Veghel, The Netherlands). 2-Oxopropanal (methylglyoxal) was obtained as a 40% solution in water (Sigma-Aldrich, Bornem, Belgium). A 100 mM solution of malondialdehyde (MDA) was prepared by dissolving 1100 mg of 1,1,3,3-tetraethoxypropane (Acros Organics, Geel, Belgium) in 50 mL of 0.1 M HCl. This solution was stirred and heated for 60 min at 50 °C in a water bath, upon which a dark yellow color developed. The concentration of malondialdehyde was determined by spectrophotometric measurements of the dilution 10^{-3} at 245 nm (acid solutions; $\epsilon = 13700$) or at 267 nm (basic solutions; $\epsilon = 31500$). Aliquots of this stock solution were used to prepare model mixtures of various concentrations. After MDA addition, the pH was adjusted with 1 M NaOH when necessary.

Heating of Samples. Ten milliliter aqueous solutions of 3% (w/w) casein and various concentrations of MDA or 2-oxopropanol in 0.1 M phosphate buffer were heated in screw-capped pressure-resistant metal tubes (1.35 cm × 11.0 cm) in an aluminum heating block for a certain period of time and temperature. After heating, the samples were rapidly cooled in an ice bath. The samples were transferred to plastic 10 mL tubes (Greiner) and stored at −18 °C. Samples of 0.5 mL were diluted in 1.5 mL of 16% sodium dodecyl sulfate (SDS) and stored overnight at 4 °C. All samples were heated and analyzed in duplicate.

Determination of Lysine Residues. Available lysine residues were determined by derivatization with *o*-phthaldialdehyde (OPA). Fresh OPA reagent was prepared daily by dissolving 40 mg of *o*-phthaldialdehyde in 1 mL of ethanol, adding 25 mL of 0.1 M sodium tetraborate buffer (pH 9.8), 2.5 mL of 20% SDS, and 0.1 mL of 2-mercaptoethanol in demineralized water, and adjusting the volume to 50 mL. The SDS-diluted reaction mixtures were diluted 25 times in 0.1 M sodium tetraborate buffer, and to 80 μL of diluted sample was added 2.4 mL of OPA reagent. The sample was vortexed for 10 s, and the fluorescence was measured after 2 min (or as close to 2 min as practically achievable). A fluorescence spectrophotometer (LS50B, Perkin-Elmer, Beaconsfield, U.K.) was used at excitation and emission wavelengths of 340 and 430 nm, respectively, with slit widths of 2.5 μm. The OPA reagent was used as the blank.

Determination of Malondialdehyde. Concentrations of malondialdehyde were determined by using the thiobarbituric acid (TBA) assay. Reaction mixtures were diluted 10 times with demineralized water, and 50 μL of this dilution was added to 3 mL of TCA solution (7.5% trichloroacetic acid, 0.1% propyl gallate, and 0.1% EDTA in water). To these samples was added 3 mL of 0.02 M TBA, and the solutions were heated for 30 min in a boiling water bath. After cooling of the samples to room temperature, the amount of the TBA−MDA complex was determined by measuring the absorbance at 532 nm, reduced by the absorbance at 600 nm to correct for turbidity.

Separation of High and Low Molecular Weight Compounds. To determine the absorbance of the high molecular weight fraction and thus the melanoidin concentration, the low molecular weight compounds were separated from the protein via Sephadex G25 disposable columns (PD-10, Sigma-Aldrich). A sample of 2.5 mL was brought on the column and was eluted with 3.5 mL of water to collect the protein fraction. With another 7.0 mL of water, the low molecular weight fraction was eluted.

Analysis of Brown Compounds. The browning intensity of the heated reaction mixtures was determined by measuring the absorbance of the SDS-diluted samples at 420 nm with a spectrophotometer (Cary 50, Varian). The browning of the protein-free fraction was measured without dilution and subtracted from the browning of the complete reaction mixture to obtain protein browning.

Analysis of 2-Oxopropanal by HPLC. 2-Oxopropanal was derivatized with *o*-phenylenediamine (OPD), according to a known procedure (*13*), by adding 1 mL of Milli-Q water and 2 mL of 0.1 M OPD/methanol to 1 mL of sample (80 mM samples were first diluted 10 times). This reaction was kept at room temperature overnight after adjustment of the pH to 6.5. The samples were analyzed by HPLC using a Lichrospher column (60 RP-Select B; 5 μm; 60 A; 250 × 4 mm) and a solvent gradient (A, water; B, acetonitrile; start 80/20 to 40/60 in 45 min). Standards of 2-methylquinoxaline (10–500 μg/mL) were analyzed to make the calibration curve ($t_R = 9.5$ min).

Kinetic Modeling. On the basis of the proposed reaction pathways a kinetic model was built and translated into a mathematical model by deriving coupled differential equations for each reaction step, as explained previously (*14*). These equations contain rate constants as parameters. For the studies done at various temperatures, the rate constants were coupled to the reparametrized Arrhenius equation. Reparametrization is necessary for statistical reasons. The advantage of coupling the rate constants directly to the Arrhenius equation is that all data at all temperatures studied are used at once to estimate activation energies, resulting in much more precise parameter estimates (*14*). The software package Athena Visual Workbench (www.athenavisual.com)

was used for numerical integration of the coupled differential equations as well as for parameter estimation. The model parameters, that is, the rate constants and activation energies, were estimated by nonlinear regression using minimization of the so-called determinant criterion. This criterion replaces the commonly used least-squares minimization, to comply with the statistical demands typical for multiresponse modeling (*14*).

RESULTS AND DISCUSSION

The reaction of casein with different concentrations of MDA was studied in a phosphate buffer at various conditions of pH (6, 7, 8) and temperature (90, 110, 120, 130 °C). Samples were taken at regular time intervals (0–120 min). In the reaction mixtures, the remaining free lysine residues (after derivatization with *o*-phthaldialdehyde), the remaining malondialdehyde (measured as thiobarbituric acid reactive compounds) and protein browning were determined. To quantify protein browning, an approach used for melanoidin quantification was used. The concentration of brown high molecular weight Maillard reaction products, named melanoidins, can be quantified by spectrophotometrically measured browning. Brands et al. (*15*) have shown that, by means of the molar extinction coefficient, the browning of the melanoidins resulting from heated glucose−casein and fructose−casein model systems could be converted into the concentration of sugar incorporated. The molar extinction coefficient was calculated by measuring the concentration of ^{14}C-labeled sugar incorporated into the browned protein and remained constant in time. This experimental value was not significantly different for the melanoidins resulting from the reaction of glucose or fructose with casein and was determined as 500 L/(mol·cm) ($\lambda = 420$ nm). Calculation of the extinction coefficient of melanoidins derived from glucose with various amino acids showed that this extinction coefficient is dependent on the amino acid involved (*16*) and varied roughly between 500 and 1000 L/(mol·cm). The browned protein resulting from the reaction of casein with malondialdehyde is a so-called advanced lipoxidation end product (ALE) but can to some extent be considered as melanoidin-like. Therefore, the quantification approach described was applied for the quantification of casein browning after reaction of casein with malondialdehyde or 2-oxopropanal. A molar extinction coefficient of 1.000 L/(mol·cm) was applied, supposing that for one C6-sugar (glucose or fructose), two C3-compounds can be incorporated into the casein structure. Solutions of 3% of caseinate (corresponding to 15 mM of lysine residues) in a phosphate buffer (pH 7) were reacted with 8 mM of malondialdehyde at 120 °C. In such a simple model system of casein and MDA it can be assumed that 1 mol of malondialdehyde (containing two reactive carbonyl functions) reacts with 2 mol of lysine residues and as such leads to a cross-linking of the protein (*2*). In addition, malondialdehyde can also undergo self-condensation by aldol reactions. Therefore, a degradation reaction was included in the model to account for additional losses of malondialdehyde (model A, **Figure 2**). In the headspace of the reaction mixtures and in extracts of control MDA heating experiments, various condensation products of MDA were detected such as benzene, benzaldehyde, traces of acetaldehyde, and some unidentified compounds. To be able to determine browning of the high molecular weight fraction, a separation of high and low molecular weight compounds was carried out. Because the absorbance at 420 nm of the low molecular weight fraction did not change during the course of the reaction, browning could not be used to monitor the formation of low molecular weight reaction products. Because of their multitude and low amounts, the malondialdehyde degradation products were not further characterized or quantified. In the literature, it has been reported that malondialdehyde slowly undergoes self-condensation or cleavage in aqueous solution at room temperature with the formation of various reaction products, such as 2,4-bishydroxymethylene-3-methylpentanedial (*17*).

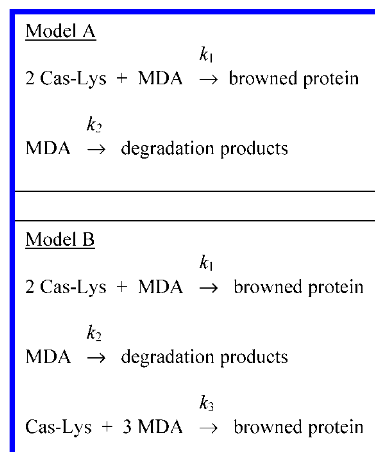

Figure 2. Kinetic models used for the reaction of lysine residues of casein (Cas-Lys) with malondialdehyde (MDA).

Influence of Temperature. The reaction of casein with malondialdehyde in the described reaction conditions occurred quite rapidly, and the results and model fits were well in line with the hypothesis that 1 mol of malondialdehyde reacts with 2 mol of lysine residues. This hypothesis was not disproved at the different temperatures investigated (90, 110, 130 °C). In **Figure 3**, the experimental data and the model fits are shown. The model was fitted to all of the data at the same time, taking into account the temperature dependency by including the reparametrized Arrhenius equation at the four heating temperatures simultaneously. As can be seen in **Figure 3B**, the decrease of malondialdehyde with time was described well by the model and showed a temperature dependency as expected. In contrast, the courses of the decrease of lysine were quite similar at 110, 120, and 130 °C (**Figure 3A**). Also, the browning of casein was not significantly different for these higher temperatures (**Figure 3C**). The fact that the same behavior was noted in both cases is reassuring because in the kinetic model the two are directly linked. One remark to be made is that also at t_0 some protein browning was measured, which could not be excluded but developed in the time period between the mixing of the reagents and the analytical measurements (storage at 0 °C). In **Table 1**, the different rate constants for this set of reactions are shown. The degradation reaction of malondialdehyde showed a temperature dependency (k_2) that is in accordance with normal chemical reactions. The value of the activation energy found for lysine decrease and casein browning (32 kJ/mol) is, however, quite low for a chemical reaction, reflecting that the reactants lysine and malondialdehyde react readily already at lower temperatures. The higher temperature dependency of the second reaction, the degradation of malondialdehyde, must thus be due to reactions other than with casein. It can be deduced that the estimate of rate constant k_1 is much more precise than the estimate of k_2, which is due to the fact that the concentration of the degradation products was not experimentally measured.

The browned casein displayed fluorescence ($\lambda_{exc} = 377$ nm; $\lambda_{em} = 459$ nm) that was in accordance with the values reported for 3-amino-1-iminopropene cross-links ($\lambda_{exc} = 370-400$ nm; $\lambda_{em} = 450-470$ nm), which supports the hypothesis of model A in **Figure 2** (*6*).

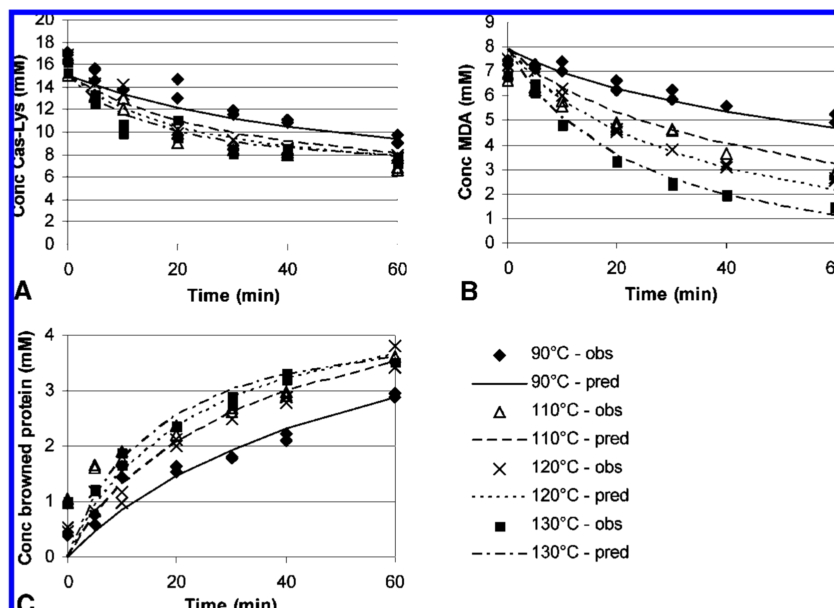

Figure 3. Model fits (drawn lines) based on kinetic model A (**Figure 2**) (pred = predicted) and experimental data (obs = observed) for the model reaction of 3% casein with 8 mM malondialdehyde at different temperatures and pH 7.

Table 1. Rate Constants of the Reaction of 8 mM Malondialdehyde with 3% Casein as a Function of Temperature and pH, As Calculated with Kinetic Model A (**Figure 2**)[a]

	rate constants	
reaction conditions	k_1 (L^2 $mmol^{-2}$ min^{-1})	k_2 (min^{-1})
90 °C, pH 7	$(0.57 \pm 0.08) \times 10^{-4}$	$(0.86 \pm 0.67) \times 10^{-3}$
110 °C, pH 7	$(0.99 \pm 0.07) \times 10^{-4}$	$(4.1 \pm 1.4) \times 10^{-3}$
120 °C, pH 7	$(1.3 \pm 0.1) \times 10^{-4}$	$(8.5 \pm 1.5) \times 10^{-3}$
130 °C, pH 7	$(1.6 \pm 0.2) \times 10^{-4}$	$(17 \pm 3.2) \times 10^{-3}$
E_a (kJ/mol)	32 ± 5.9	91 ± 26
pH 6, 120 °C	$(4.3 \pm 0.57) \times 10^{-4}$	$(6.8 \pm 4.1) \times 10^{-3}$
pH 7, 120 °C	$(1.3 \pm 0.1) \times 10^{-4}$	$(8.5 \pm 1.5) \times 10^{-3}$
pH 8, 120 °C	$(1.7 \pm 0.49) \times 10^{-4}$	$(12 \pm 6.1) \times 10^{-3}$

[a] The precisions indicated are 95% higher posterior densities (HPD), the Bayesian equivalents of 95% confidence intervals.

Influence of pH. Study of the reaction between 8 mM malondialdehyde and 3% casein at 120 °C at different pH values showed that the reaction proceeded more rapidly at lower pH (pH 6), but also to a lesser extent at higher pH values (pH 8); the pH values given are the values at room temperature, not at the heating temperature (**Figure 4**). The nucleophilic addition of the ϵ-amino group of lysine toward the MDA carbonyl function can be acid as well as base catalyzed. At pH 8, however, the model did not fit the observed data points very well. Study of the residuals showed that not all of the information which the data points contain was explained by the model. A possible explanation can be related to the preferential tautomerization at higher pH of compound **1**, resulting from the first step of the reaction of lysine with malondialdehyde, which reduces the electrophilicity of the second carbonyl function and thus the susceptibility for a second nucleophilic attack of lysine. However, an alternative model, in which 1 mol of malondialdehyde was supposed to react with 1 mol of lysine residues, did not result in a better fit of the data. Also, a reversible formation of malondialdehyde degradation products and browned protein did not improve the fit of the model to the experimental data at pH 8.

Table 1 shows the values of the rate constants at the different pH values. This table shows that the rate constant k_2 of the malondialdehyde degradation reaction was not significantly different for the three pH values tested. Here as well, a high imprecision of the estimate of the rate constant k_2 is noted.

Influence of Concentration of Malondialdehyde. To evaluate the kinetic model at different malondialdehyde concentrations, the reaction between malondialdehyde was performed at 120 °C, pH 7, and 3% of caseinate with malondialdehyde concentrations of 1, 8, and 15 mM. At 1 mM malondialdehyde the model fitted the data quite well, considering that 1 mol of malondialdehyde reacted with 2 mol of lysine residues, as was the case for 8 mM malondialdehyde. However, at higher malondialdehyde concentration (15 mM), this assumption was no longer valid, as the experimentally measured final concentration of browned protein was higher than the maximum concentration possible as simulated by model A (being half the initial lysine concentration or 7.5 mM). At elevated malondialdehyde concentrations, it is more likely that 1 mol of lysine residues reacts preferably with 1 mol of malondialdehyde (formation of structure **1**), rather than the cross-linking reaction occurring at lower malondialdehyde concentrations. However, the experimental concentrations of browned casein exceeded even the initial lysine concentration, indicating that other reactions, for example, with other amino acid residues such as arginine or histidine, may contribute to protein browning. Incorporating these assumptions in the model still did not result in a good fit, because the experimental lysine concentration decreased very quickly, whereas the experimental melanoidin concentration increased much more slowly. This might indicate the formation of an intermediate, which is nevertheless difficult to explain theoretically, and also did not improve the model fit.

Such high malondialdehyde concentrations are in fact not realistic, neither physiologically nor related to food systems. As many other reactive carbonyl compounds are present in real food systems, these will compete with malondialdehyde for the reaction with nucleophilic amino groups. In this respect it has been shown that the reaction of malondialdehyde and alkanals with proteins proceeds synergistically with the formation of a dihydropyridine cross-link. Such a dihydropyridine cross-link, **7**, can be formed from the reaction of 1 mol of lysine residues, 2 mol of malondialdehyde, and 1 mol of acetaldehyde, being a hydrolytic degradation product of malondialdehyde (*4*). To

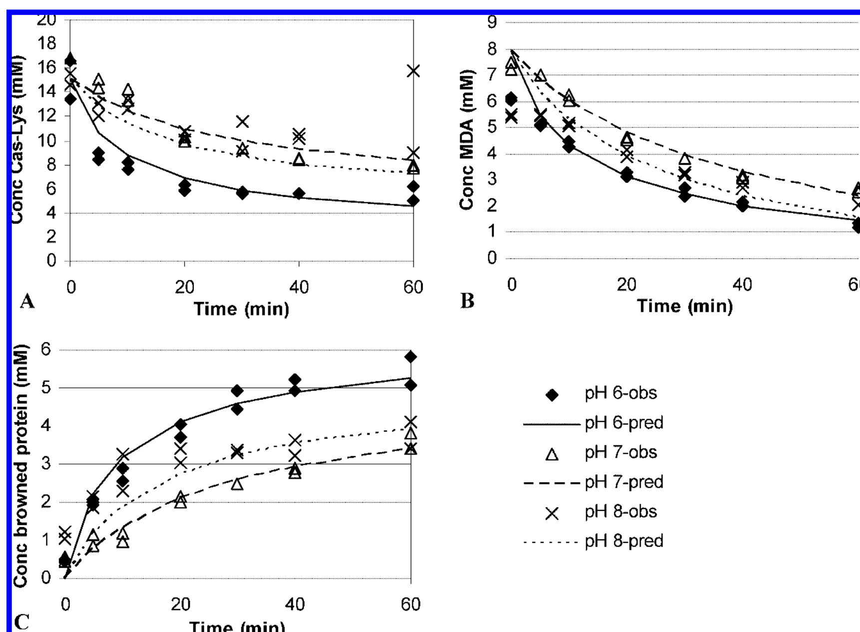

Figure 4. Model fits (drawn lines) based on kinetic model A (**Figure 2**) (pred = predicted) and experimental data (obs = observed) for the reaction of 3% casein with 8 mM malondialdehyde at different pH values and 120 °C.

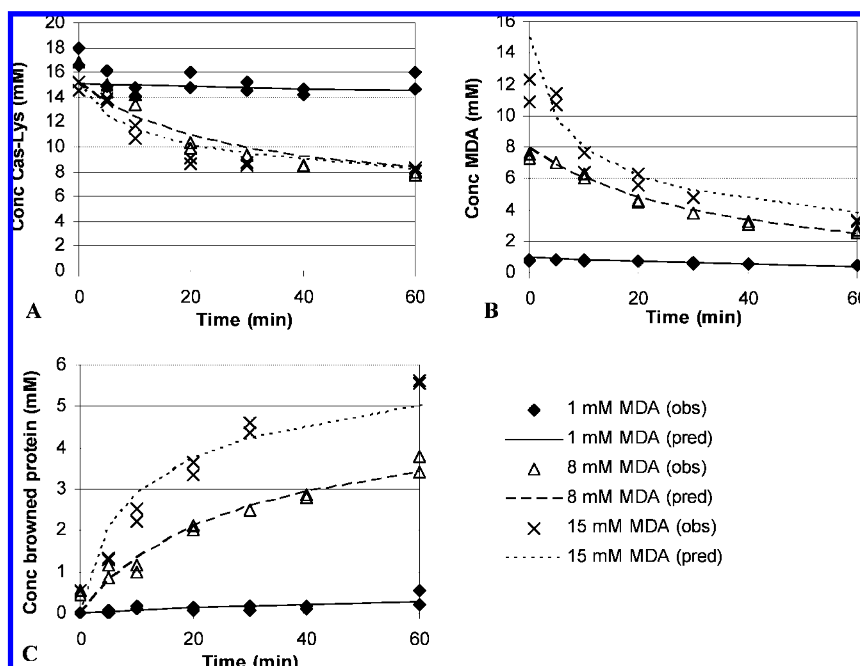

Figure 5. Model fits (drawn lines) based on kinetic model B (**Figure 2**) (pred = predicted) and experimental data (obs = observed) for the reaction of 3% casein with different concentrations of malondialdehyde at 120 °C and pH 7.

model this reaction the kinetic model was extended with a third reaction (model B, **Figure 2**). Applying this model to the data obtained from the reaction of casein with 15 mM malondialdehyde showed a considerably improved fit (**Figure 5**). The model fit deviated still slightly from the observed protein browning. When kinetic model B was applied to the reaction of casein with 8 mM malondialdehyde, the quality of the fit and the values of k_1 and k_2 did not differ significantly from model A, and a very low value of k_3 was found (7×10^{-9} L^3 mmol^{-3} min^{-1}) with a confidence interval that was much higher than the estimate itself. This means that this parameter is redundant for the data obtained at 1 and 8 mM. It makes sense only for the results obtained with 15 mM. The degradation rate constant k_2 was not significantly different for the three malondialdehyde concentrations (**Table 2**).

Table 2. Rate Constants of the Reaction of Malondialdehyde with Caseinate at 120 °C, pH 7, as a Function of MDA Concentration, Calculated with Kinetic Model B (**Figure 2**)

	rate constants		
MDA (mM)	k_1 (L^2 mmol^{-2} min^{-1})	k_2 (min^{-1})	k_3 (L^3 mmol^{-3} min^{-1})
1	$(3.2 \pm 1.0) \times 10^{-5}$	$(9.6 \pm 4.0) \times 10^{-3}$	
8	$(11 \pm 2.1) \times 10^{-5}$	$(8.2 \pm 4.0) \times 10^{-3}$	$6.8 \times 10^{-9} \pm 5.0 \times 10^{-6}$
15	$(4.8 \pm 1.7) \times 10^{-5}$	$(0.2 \pm 5.9) \times 10^{-3}$	$(1.3 \pm 0.5) \times 10^{-5}$

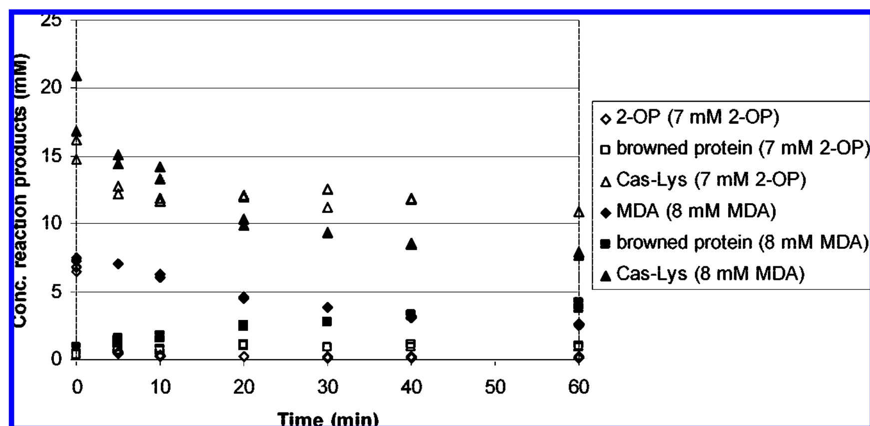

Figure 6. Comparison of the experimental data for the reaction of 3% casein with malondialdehyde (8 mM MDA) and 2-oxopropanal (7 mM 2-OP) at 120 °C and pH 7.

For all of these experiments, translation of protein browning into concentrations of C3-compounds incorporated by means of the molar extinction coefficient was used to estimate molar concentrations, which are needed for kinetic modeling. Earlier investigations have shown the usefulness of this approach, calculating extinction coefficients between 500 and 1000 L/(mol·cm) ($\lambda = 420$ nm) for various model systems. Apparently, the chromophores formed in the nondialyzable melanoidins in the early stages of the Maillard reaction are similar to those at the later stages, and their formation is not very sensitive to the reaction conditions (*18*). However, in the course of this study of the casein–malondialdehyde condensation, two different chromophores, namely, the 3-amino-1-iminopropene cross-link **2** and the dihydropyridine cross-link **3**, were hypothesized, resulting from the condensation of one lysine residue with one and three malondialdehyde units, respectively. On the other hand, the second cross-link becomes significant only at concentrations above 8 mM. This is not completely consistent with the background of using the molar extinction coefficient, but enabled a successful prediction of casein browning induced by malondialdehyde. Although the value of the extinction coefficient that was used to transform the browning of casein into number of moles of product remains an uncertain element, this was the best approach available to tackle the formation of brown color quantitatively in a kinetic model. Modifying the numerical value of the extinction coefficient resulted in differences in the numerical values of the rate constants, but not in the general trend. In other words, the general conclusions drawn remained valid.

Reaction of 2-Oxopropanal with Casein. In comparison with the reaction of casein with malondialdehyde, the reaction of casein with 2-oxopropanal was studied, applying similar reaction conditions. At 120 °C, the concentration of 2-oxopropanal was depleted almost immediately (data not shown). Therefore, the reaction between casein and 2-oxopropanal was studied at 60 °C. At this temperature, the concentration of lysine residues decreased only slightly (about 2 mM), whereas the concentration of 2-oxopropanal (initial concentrations of 10 and 20 mM) decreased steadily and continuously until 120 min. Protein browning was also very low at 60 °C and corresponded to a concentration of 2 mM of C3-compound incorporated. These results suggest that an important part of 2-oxopropopanal is lost in the formation of low molecular weight degradation products.

The different models evaluated for the reaction of casein with malondialdehyde could not be applied to the model reaction of casein with 2-oxopropanal, which followed a different reaction mechanism. It is well-known that 2-oxopropanal reacts preferably with arginine residues as compared to lysine residues (*19*), but the concentration of arginine residues was not determined in this study. This reaction may be partly responsible for casein browning at 120 °C (where browning was more significant). The fluorescence recorded for the 2-oxopropanal modified casein ($\lambda_{exc} = 318$ nm; $\lambda_{em} = 389$ nm) was in agreement with fluorescence values reported for the formation of the so-called "argpyrimidine" cross-link, resulting from the modification of arginine residues by the condensation product of two 2-oxopropanal molecules ($\lambda_{exc} = 320$ nm; $\lambda_{em} = 382$ nm) (*20*).

Comparison of the reaction course of casein with malondialdehyde and 2-oxopropanal at 120 °C (pH 7) showed that the decreases of lysine were the same in both systems (**Figure 6**). The decrease in 2-oxopropanal concentration, however, was considerably faster than the decrease of malondialdehyde. This can be explained by the stabilization of the β-dicarbonyl structure by resonance at a pH higher than the pK_a (4.46), making malondialdehyde less prone to self-condensation and other degradative reactions than is the case for 2-oxopropanal. Casein browning, on the other hand, occurred considerably more quickly with malondialdehyde than with 2-oxopropanal. This illustrates the importance of this lipid oxidation product in the induction of protein browning as compared to the better known Maillard browning caused by reactive sugar degradation products such as 2-oxopropanal.

Considering that the characteristic fluorescence of advanced glycation endproducts (AGEs) ($\lambda_{exc} = 370$ nm; $\lambda_{em} = 440$ nm) has been widely used as an indicator of the level of AGE-modified proteins (*21*), the contribution of malondialdehyde to these fluorescence values cannot be neglected. Whereas the fluorescence resulting from the modification of casein by 2-oxopropanal in this research was substantially different from the typical values reported for AGEs, the fluorescence of the MDA-modified casein (ALE) contributes to the so-called AGE browning. Casein-bound fluorescence reported as a result of the advanced Maillard reaction of lactose/caseinate or glucose/caseinate was characterized by maximum excitation and emission wavelengths of 347 and 415 nm, respectively (*22*).

In conclusion, this study has shown that kinetic modeling is a useful tool to unravel reaction mechanisms. Clearly, the contribution of lipid oxidation products, such as malondialdehyde, to protein browning (both in food and in vivo) can be substantial and needs to be taken into account in future studies.

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