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Nonenzymatic Browning of Lactose and Caseinate during Dry Heating at Different Relative Humidities

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Dry mixtures of lactose and caseinate were heated at 60 °C for up to 96 h at different relative humidities (RHs) ranging from 29 to 95%. The resulting nonenzymatic browning was studied by determining lactulosyl lysine formation in the caseinate (as measured by the conversion to furosine), amount of reacted lactose, loss of lysine, color formation, and fluorescent intensity. For each measurement, the maximum reaction occurred at intermediate RHs. While there is general agreement between the results obtained by different methods, discrepancies are understandable given the complex nature of nonenzymatic browning. It was shown that the degradation of the Amadori product, lactulosyl lysine, increased with RH. Moreover, the Maillard reaction, as opposed to caramelization of lactose, was the major pathway at all RHs. Visible browning occurred when the destruction of Amadori product became dominant, and interactions between sugar fragments and caseinate were not the rate-limiting steps in the nonenzymatic browning.

KEYWORDS: Nonenzymatic browning; Maillard reaction; Amadori product; water activity; furosine; lysine; lactose; caseinate

INTRODUCTION

Amino–carbonyl interactions contribute to the nonenzymatic browning of a wide range of foods (*1, 2*). Glycated proteins produced by such reactions give rise to fewer safety issues than chemically modified food proteins (*3–5*). Nonenzymatic browning may easily occur during the manufacture of milk powders because of the intimate mixing of lactose and protein. Indeed, careful control of the conditions is necessary to avoid browning when dry heating mixtures of caseinate and lactose or other reducing sugars, to generate novel glycoproteins (*2–5*). Conjugation of bacteria-inhibiting sialyl oligosaccharides to macromolecules can enhance their activity. When oligosaccharides are linked to a flexible polymer backbone to create a polyvalent macromolecule, they can simultaneously engage a number of adhesion molecules at a bacterial cell surface, forming a stable complex (*6*), which can markedly increase the affinity of the inhibitors and so prevent bacterial adhesion to host tissues (*7*). A common method for attaching a carbohydrate to a protein is by reductive amination with cyanoborohydride as the reducing agent (*8*). Due to the toxicity of cyanoborohydride, such an approach is not suitable for manufacturing food ingredients or nutraceuticals. In the present research, the possibility of linking a model oligosaccharide to a milk protein via the Maillard reaction was explored. Milk protein is rich in lysine and the ϵ-NH_2 groups of the lysine residues are the major contributors of free amine groups. In milk powder there are two nonenzymatic browning pathways: the Maillard reaction and lactose caramelization. The Maillard reaction is initiated by the reaction of an amine with a reducing sugar according to the following route: sugar + R–NH_2 → Amadori product → browning compounds (*1, 2, 9*). Lactose caramelization is initiated by enolization as part of the Lobry de Bruyn–Alberda van Ekenstein rearrangement, followed by inter alia β-elimination, dicarboxylic cleaving, retroaldolization, and aldol condensation (*10*). It normally happens at high temperatures. However, the degradation of lactose can occur under alkaline conditions at mild temperatures (*11*), while the presence of free amino groups in amino acids or proteins can catalyze the degradation (*12*).

Water activity (a_w) of a food system exerts a major influence on the food browning, and it is generally accepted that the maximum browning rate occurs at intermediate moisture content (*13*). In milk powder, browning reactions are maximal at a_w values of 0.6–0.7 (*14*). In the previous studies on the influence of a_w on the browning, the most commonly used indicator is color, measured as absorbance at 420 nm (*15*). Nonenzymatic browning is regarded as "one of the most complex and most investigated phenomena of food chemistry" (*9*). Because of the inherent complexity, one type of measurement would appear to be insufficient to give a complete understanding. However, few studies have been done using more than one procedure to investigate the effect of a_w on nonenzymatic browning. One previous study investigated the influence of a_w and storage temperature on lysine availability in a model milk system (*16*). In another study, whey protein concentrate and isolated soy protein were stored with glucose at different a_w values and temperatures, and the UV absorbance and fluorescence were measured (*17*). In a third, the changes in reactive lysine and

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lactulosyl lysine were measured when lactose and whey protein were heated at different a_w values (*18*).

To investigate the early stages of nonenzymatic browning, we have used a model system consisting of lactose and caseinate heated at seven relative humidities (RHs). Commonly used nonenzymatic browning indicators (furosine as a measure of lactulosyl lysine formation, reacted lactose, loss of lysine, color formation, and fluoresence intensity) at different time intervals were monitored to give a comprehensive understanding of the process. It is generally accepted that an excess of reducing sugar relative to amino compounds promotes the rate of Maillard browning (*13*, *19*). Therefore, in the design of the present study, the molarity of lactose was much lower than that of lysine (in caseinate) in order to simulate "mild" browning conditions.

MATERIALS AND METHODS

Materials. Sodium caseinate was manufactured by Fonterra Co-operative (New Zealand) under the commercial name of Alanate 180. Lactose monohydrate was purchased from Sigma (St. Louis, MO). Other chemicals used include ϵ-dinitrophenyl-L-lysine hydrochloride (ϵ-DNP-L-lysine hydrochloride; ICN Biomedicals Inc., Aurora, OH), furosine dihydrochloride (Neosystem Laboratoire, Strasbourg, France), 2,4-dinitrofluorobenzene (FDNB) (Aldrich, Castle Hill, NSW, Australia), and lactulose (Acros Organics, Geel, Belgium).

Relative Humidities (RHs). Saturated salt solutions of $MgCl_2$, NaBr, $NaNO_3$, NaCl, KCl, Na_2CO_3, and K_2SO_4 were used to give RHs (at 60 °C) of 29%, 50%, 67%, 75%, 80%, 90%, and 95%, respectively.

Reaction of Lactose and Caseinate. The procedures used were based on previous work (*4*, *5*) on the dry heating of caseinate and reducing sugars under controlled relative humidity. Lactose monohydrate (53 mg) and sodium caseinate (1 g) were dissolved in 100 mL of water, so that the molar ratio of lysine (in caseinate) to lactose was 3.6:1. An aliquot of 320 μL solution was added to each 2 mL vial. The aliquots were lyophilized and transferred to desiccators containing various saturated salt solutions. The desiccators were held at room temperature (17 °C) for 24 h to pre-equilibrate the samples and then heated at 60 °C in an oven to give 29%, 50%, 67%, 75%, 80%, 90%, and 95% RHs. Vials were removed after 12, 24, 48, and 96 h of heating and stored at –18 °C for HPLC analysis. Control samples containing the same amounts of caseinate and lactose were lyophilized without heating and stored at –18 °C for analysis. All samples were prepared in duplicate.

Preparation of Lactose-Modified Caseinate. Lactose can be covalently bonded to casein via the Maillard reaction, and it is assumed that lactulosyl-lysine (as part of the glycated caseinate) would be formed as a stable intermediate. To produce the lactose-modified caseinate, 64 mg of sodium caseinate and 3.4 mg of lactose monohydrate were dissolved in 10 mL of water and lyophilized. The lyophilized sample was transferred to a desiccator containing saturated $NaNO_3$ solution, which was held at room temperature for 24 h to allow the sample to pre-equilibrate, and then heated for 48 h at 60 °C, 67% RH. The reaction mixture was membrane-filtered with a Centricon YM-10 (10 kDa molecular weight cutoff) centrifugal filter device (Millipore, Bedford, MA) to remove the unreacted lactose and low molecular weight products. The residue was collected, lyophilized, and redissolved in 10 mL of water; it was regarded as the lactose-modified caseinate. The filtrate containing sugar fragments was collected for a separate experiment with caseinate (see next section).

Aliquots of 500 μL of the lactose-glycated caseinate solution were transferred to 2 mL vials which were heated in desiccators containing different saturated salt solutions and then lyophilized. The samples were treated and analyzed the same way as the caseinate and lactose mixture, except that only one time interval (96 h) was used for this model reaction. Samples without heating were used as controls. All samples were prepared in duplicate.

Reaction between Sugar Fragments and Caseinate. Sugar fragments of low molecular weight, such as α-dicarbonyl compounds, were formed as intermediates of nonenzymatic browning (*20*), and the reactions between sugar fragments and caseinate were investigated. The solution of sugar fragments obtained as mentioned above was mixed with sodium caseinate (64 mg) and the final volume adjusted to 20 mL with water. Aliquots of 1 mL were added to 2 mL vials which were lyophilized and equilibrated at 17 °C for 24 h in desiccators containing different saturated salt solutions and then heated at 60 °C for 48 or 96 h. All samples were prepared in duplicate.

Determination of Furosine. Lactulosyl lysine (Amadori product) is formed during the early stage of the Maillard reaction, and it can be quantitatively converted by acid hydrolysis to the stable ϵ-*N*-2-furoylmethyl-L-lysine, i.e., "furosine" (*21*, *22*), which can be used as an indicator of the extent of Maillard reaction. In the present study, furosine levels in the heated mixture of lactose and caseinate, and in the heated lactose-modified caseinate were determined using a modification of the method of Resmini et al. (*23*): 10 M HCl (3.2 mL) was added to the heated mixture of lactose and caseinate or heated lactose-modified caseinate samples, and the vials were flushed with nitrogen, capped, and hydrolyzed at 110 °C for 20 h. After hydrolysis, each was diluted to 10 mL with water, filtered through a 0.45 μm disk filter, and subjected to HPLC analysis. The HPLC system was the same as in the determination of the loss of lysine, and an Econosphere C8 column (250 × 4.60 mm, 5 μm, Alltech, Deerfield, IL) was used. The mobile phase consisted of 0.4% acetic acid in water as solvent A and 0.3% potassium chloride in solvent A as solvent B. The elution gradient was expressed as the proportion of solvent B: 0–13.5 min, 2%; 13.5–35 min, 50%; 35–36 min, 2%; 36–42 min, 2%. The flow rate was 1.2 mL/min, the chromatogram was recorded at 281 nm, and the injection volume was 30 μL.

Under 6 M HCl hydrolysis conditions, Finot et al. (*21*) established a factor of 3.1 to convert furosine to lactulosyl lysine, and such a factor was adopted by later researchers (*18*, *24*, *25*). However, this factor was affected by the molar ratio of HCl to protein and the concentration of HCl (*26*). In the present study, 10 M HCl was used for the hydrolysis, and the conversion of the furosine value to lactulosyl lysine content was not attempted.

For the quantitation of furosine in samples, furosine dihydrochloride (2.4 mg) was dissolved in 100 mL of 3 M HCl, and then 5 mL of the solution was diluted to 25 mL with 3 M HCl. The standard solution was prepared in triplicate.

Determination of Lactose. Water (1 mL) was added to each of the heated mixtures of lactose and caseinate, the solution was sonicated, heated at 50 °C with a water bath, shaken, and membrane filtered with a Centricon YM-10 centrifugal filter device. The filtrate (its weight was recorded and used for quantification) was lyophilized to reduce the volume; water (100 μL; the weight was recorded and used for quantification) was added to redissolve the sugar. The solution thus obtained was analyzed with the Shimadzu LC-10A HPLC system equipped with helium gas degasser, injection loop (20 μL), LC 10ATVP pump, refractive index detector (RID-10A), and column oven (CH-430). An Aminex Fast Carbo column (100 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA) was used at 85 °C with water as the mobile phase, and the flow rate was 0.6 mL/min. Control samples containing the same amounts of caseinate and lactose were lyophilized without heating. All samples were prepared in duplicate.

Lactulose is a product of lactose isomerization and is not generated by the Amadori product degradation (*27*). The degradation of lactulose occurs more readily than that of lactose, and it was used as an indicator of lactose caramelization (*28*–*30*). Under the above HPLC conditions the lactulose peak appeared as a shoulder of lactose if an excessive amount of lactose was present. To overcome the interference of lactose, a "standard addition" method was used to semiquantitatively estimate the lactulose content in the heated samples, i.e., various amounts of lactulose were added to lactose (1 mg/mL) solutions to give lactose/lactulose ratios of 10:1, 20:1, 50:1, and 100:1. By comparison of the shape of the lactulose peak of the sample with those of lactulose in the mixtures, the lactulose content can be estimated.

Determination of Loss of Lysine. FDNB (Sanger's reagent) reacts with the ϵ-NH_2 group of lysine residue in a protein, and the ϵ-DNP-L-lysine derivative formed can be analyzed by HPLC. The present method is a modification of the procedure of Albalá-Hurtado et al. (*31*): sodium bicarbonate solution (0.52 mL, 8%) was added to the heated mixture of lactose and caseinate. The sample solution was sonicated

for 40 min and heated at 50 °C for 10 min using a water bath and then shaken for 10 min. FDNB solution (0.32 mL; 3% in ethanol) was added to the vial (FDNB is a strong skin irritant and gloves were worn). The vial was capped and shaken for 2 h at 17 °C. The ethanol was evaporated at 95 °C using a heating block, until no further effervescence was produced. Then HCl (1.06 mL, 8 M) was added and the vial was capped and refluxed for 3.5 h at 133 °C using a heating block. After hydrolysis, the solution was transferred into a 50 mL volumetric flask while still warm and rinsed with 2 mL of methanol, which was combined with the hydrolyzate. Sodium bicarbonate solution (8 mL, 1 M) was added and the flask was shaken to release the carbon dioxide, then methanol (15.5 mL) was added and the volume made up to 50 mL with water. The sample solution was filtered through a 0.45 μm disk filter before HPLC analysis. The analysis was performed using a HP1100 HPLC (Agilent Technologies, Wilmington, DE) with a photodiode array detector and a Luna C18 column (250 × 4.60 mm, 5 μm, Phenomenex, Torrance, CA). An isocratic elution was used with a mobile phase composed of 35% methanol and 65% 0.01 M sodium acetate in water (which was adjusted to pH 4.5 with glacial acetic acid). The flow rate was 1 mL/min, the injection volume was 20 μL, and the chromatograms were monitored at 362 nm.

For the quantification, ϵ-DNP-L-lysine hydrochloride (10 mg) was dissolved in 100 mL of ethanol (95%), and then 10 mL of the solution was diluted to 100 mL with a mixture of water and methanol (65/35, v/v). The standard solution was prepared in triplicate. ϵ-DNP-L-lysine loss would occur during the hydrolysis, and a factor of 1.09 was introduced (*32*) to correct for the loss. This factor was adopted in the present study for the quantification.

To study whether the presence of lactose would affect the analytical results, a preliminary test was done to analyze the lysine content of 3.2 mg of sodium caseinate using the procedure described above. Results showed that lactose did not interfere.

To determine whether the loss of lysine was due solely to nonenzymatic browning, aliquots of sodium caseinate solution (320 μL, 1%) were transferred into each 2 mL vial. The vials were lyophilized and transferred into desiccators containing various saturated salt solutions. The desiccators were left at 17 °C for 24 h to come to equilibrium and then incubated at 60 °C for 48 h. The loss of lysine in the resultant samples was analyzed as above.

Color Formation. After the samples were heated, the mixtures of caseinate with lactose or caseinate with sugar fragments were dissolved in 4 mL of 2.5% sodium dodecyl sulfate (SDS). The samples were heated at 50 °C for 5 min, cooled, and measured with a Hewlett-Packard 8452A diode array spectrofluorophotometer (Agilent Technologies, Wilmington, DE). Absorbance at 420 nm was recorded and corrected for turbidity by subtracting the absorbance at 620 nm. SDS solution (2.5%) was used as a blank.

Determination of Fluorescence Intensity (FI). After the samples were heated, the mixtures of caseinate with lactose or caseinate with sugar fragments were dissolved in 4 mL of water, sonicated for 0.5 h, heated at 50 °C for 10 min, shaken for 20 min, and analyzed with a Shimadzu RF 540 spectrofluorophotometer. Samples were measured at 347 nm excitation and 415 nm emission. The slit width was 5 nm. Quinine sulfate solution (1 μg/mL) was used for instrument calibration.

RESULTS AND DISCUSSION

Formation of Lactulosyl Lysine, Reacted Lactose, and Loss of Lysine. The formation of lactulosyl lysine—as indicated by furosine content—at different RHs is shown in **Figure 1**, which suggests that at each time interval the maximal furosine formation occurred at intermediate RHs. In one study on milk powder, it was shown that the furosine level was higher when stored at 65% than at 45% or 85% RH (*22*), which agrees with the present study. In our work the maximum occurred at 67% RH after 12 and 24 h, but the maximum was at 50% RH after 48 and 96 h. In addition, for some samples, there is a decrease of lactulosyl lysine between 48 and 96 h. Such phenomena led us to investigate the rate of lactulosyl lysine destruction at different RHs, and **Table 1** shows that this destruction rate

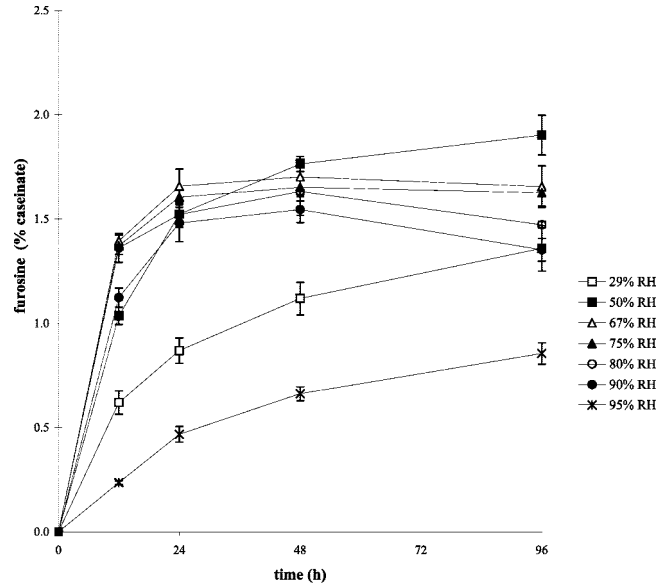

Figure 1. Formation of lactulosyl lysine as indicated by furosine, on dry heating the mixture of caseinate and lactose at 60 °C with different RHs and time intervals.

indeed increased with RH. So the rates of both generation and destruction of lactulosyl lysine were faster at 67% RH than at 50% RH.

The percentages of lactose that had reacted at different time intervals and different RHs are listed in **Table 2**. Results at 96 h are not shown because the lactose contents for most of the samples became extremely low and could not be distinguished from the interference peaks. **Table 2** shows that at 48 h, more than 90% of the lactose had been consumed at intermediate RH (67, 75, 80%) samples and the destruction of lactulosyl lysine became dominant, so gradually the lactulosyl lysine yield became higher at 50% RH, as mentioned above. **Table 2** also shows that as in formation of lactulosyl lysine, the maximum amounts of reacted lactose occurred at intermediate RHs.

In the presence of large amounts of lactose, lactulose was eluted as a shoulder of the lactose peak. With the semiquantitative "standard addition" method, it was determined that for any heated mixture of lactose and caseinate, the lactulose content was less than 5% of the lactose. Lactulose was used as an indicator of the caramelization pathway and the results from lactulose suggest that from a quantitative point of view, lactose caramelization plays an insignificant role in nonenzymatic browning under the experimental conditions. van Boekel's group (*28, 29*) has demonstrated that in heating the milk the Maillard reaction is much less important than lactose caramelization. Other studies on heating commercial milks also found that the lactulose content could be several times higher than the furosine level (*33, 34*). But in milk the molar ratio of lactose to lysine residue (in milk protein) is far greater than 1, and in the present experimental design, such a ratio is much smaller than 1. The Maillard reaction requires free NH_2 groups on the lysine residues as reactants. This reason alone can explain why lactose caramelization did not occur to a significant extent in the present study. In addition, sugar caramelization requires a high temperature (*10*). As reported by Pellegrino et al. (*34*), the lactulose/furosine ratio in milk increased with heating temperature. The present study was conducted at a mild temperature, which does not favor the caramelization pathway. Finally, studies have shown that furosine formation is due mainly to low a_w conditions (*22, 35*) and the Maillard reaction is more noticeable than lactose caramelization for dried products (*30, 32, 36*). In

Table 1. Formation of Lactulosyl Lysine (As Indicated by the Conversion to Furosine (μg)) in Lactose-Modified Caseinate Heated for 96 h

control[a]	29% RH	50% RH	67% RH	75% RH	80% RH	90% RH	95% RH
46.4 ± 0.4	45.7 ± 2.1	43.1 ± 3.1	37.3 ± 1.6	37.9 ± 2.0	34.7 ± 3.2	34.5 ± 1.8	32.6 ± 2.4

[a] Lactose-modified caseinate without heating was used as control.

Table 2. Amount of Reacted Lactose at Different RHs and Different Time Intervals

	percentage of reacted lactose (%)		
RH (%)	12 h	24 h	48 h
29	21.3 ± 1.4	31.2 ± 2.7	43.2 ± 4.2
50	34.7 ± 2.1	48.9 ± 1.5	75.0 ± 3.1
67	67.2 ± 1.8	82.3 ± 3.0	92.1 ± 2.6
75	70.9 ± 3.4	85.2 ± 1.2	98.4 ± 3.9
80	65.6 ± 1.0	81.5 ± 1.6	91.4 ± 1.4
90	48.4 ± 2.6	71.0 ± 1.6	81.0 ± 4.1
95	12.3 ± 1.1	24.2 ± 2.4	36.9 ± 2.9

Table 3. Ratios of Reacted Lactose to Furosine

	ratio of reacted lactose to furosine (mol/mol)		
RH (%)	12 h	24 h	48 h
29	1.29	1.34	1.45
50	1.26	1.20	1.60
67	1.81	1.86	2.03
75	1.94	1.99	2.23
80	1.81	2.01	2.10
90	1.61	1.80	1.96
95	1.95	1.94	2.09

the present study, the highest RH used is 95%, and this sample was liquid-like after equilibrium. However, only a small amount of lactulose was detected. So it can be concluded that for the whole range of RHs tested, the Maillard reaction is the predominant pathway.

The ratio of reacted lactose to furosine should be constant if the formation of lactulosyl lysine was the only reaction occurring. Instead it was observed (**Table 3**) that, with a few exceptions, at each RH value the ratio of reacted lactose/furosine increased with time. This result can be explained by the destruction of lactulose lysine (in the glycated caseinate) with time. Another less obvious trend was that at each time interval the ratio of reacted lactose/furosine was generally lower for low RH (29 and 50%) samples. It is possible that lactose caramelization occurred faster at high RH conditions. But the result could also be explained by the destruction of lactulose lysine (in caseinate), which increased with RH, as mentioned earlier.

The loss of lysine at different RHs is more complicated, and three patterns can be found as shown in **Figure 2**. At low RH, such as 29% and 50%, the loss of lysine increased for the whole 96 h period. At high RH such as 90% and 95%, the lysine content decreased rapidly at the beginning, as compared to the reacted lactose, but between 48 and 96 h, the decrease of lysine was not significant. At intermediate RHs such as 67%, 70%, and 80%, loss of lysine was rapid at first but between 48 and 96 h increased only slightly. This result agrees with a study on incubating ovalbumin and lactose at 50 °C and 65% RH (*37*). After 48 h, more than 90% of the lactose had been consumed and the degradation of the Amadori product began to be dominant. It has been reported that at this stage the amino compound is regenerated and a "no-loss" period can be observed (*38*). This might explain why samples at intermediate RHs have only a slight decrease in lysine between 48 and 96 h. It can also be seen in **Figure 2** that the maximum loss of lysine

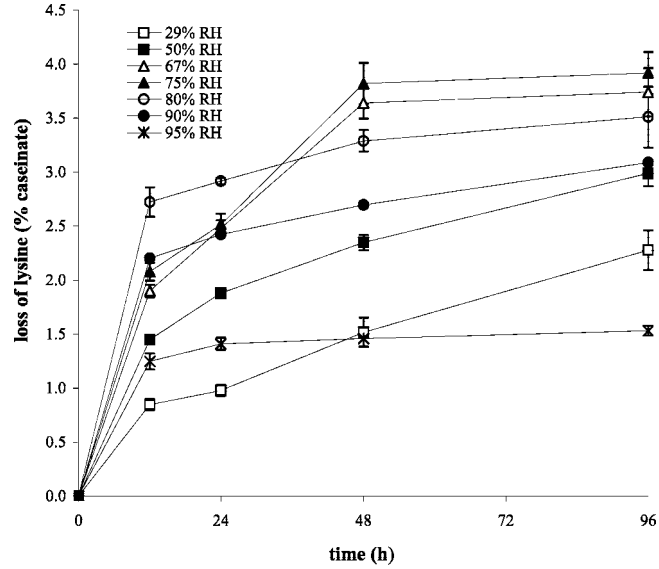

Figure 2. Loss of lysine on heating the dry mixture of caseinate and lactose at 60 °C at different RHs and time intervals.

occurred at intermediate RH. However, in contrast to reacted lactose and lactulosyl lysine formation, for 12 and 24 h, the maximal lysine loss was at 80% RH, and for 48 and 96 h, this maximum shifted to 75% RH. So a tentative conclusion can be made that compared with the reacted lactose and lactulosyl lysine formation, the loss of lysine occurred faster initially at higher RH.

Caseinate alone was incubated for 48 h at 60 °C and different RHs. No lysine loss was found in these samples (results are not shown). So it can be concluded that only amino-carbonyl interactions were solely responsible for the loss of lysine under the present experimental conditions.

Color and FI Results. The color and FI results of samples at different RHs and different time intervals are shown in **Figures 3** and **4**, respectively. For most samples, the UV absorbance at 12 h was smaller than the control; the only exception was the 95% RH sample. This demonstrates the existence of an induction period, as found in other studies (*38, 39*). We arbitrarily defined the induction period as being the period when the absorbance was smaller or very close to that of the unreacted mixture of lactose and caseinate. For samples at low RH conditions such as 29% and 50%, their UV absorbance was similar to that of the control even after 96 h. For samples at intermediate RH such as 67, 75, 80, and 90%, the increase of absorbance was slow between 12 and 48 h (induction period), and then there was a sharp increase between 48 and 96 h. The behavior of the 95% RH sample was abnormal, as its absorbance was comparably large at 12 h, but there was little increase for the following time period. At 12, 24, and 48 h, the maximal absorbance was at 95% RH, and 75% RH sample had the second largest absorbance. At 96 h, the maximal absorbance occurred at 75% RH.

Unlike UV absorbance, no induction period was found for FI in intermediate and higher RH (67% and above) samples (**Figure 4**). For low RH (29 and 50%) samples, the induction period was 48 h. Several other trends can be seen in **Figure 4**.

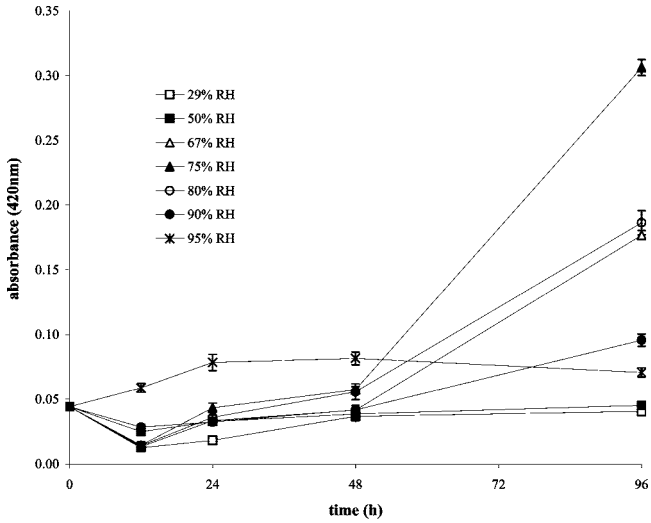

Figure 3. Time course of color development in the dry mixture of caseinate and lactose heated at 60 °C with different RHs.

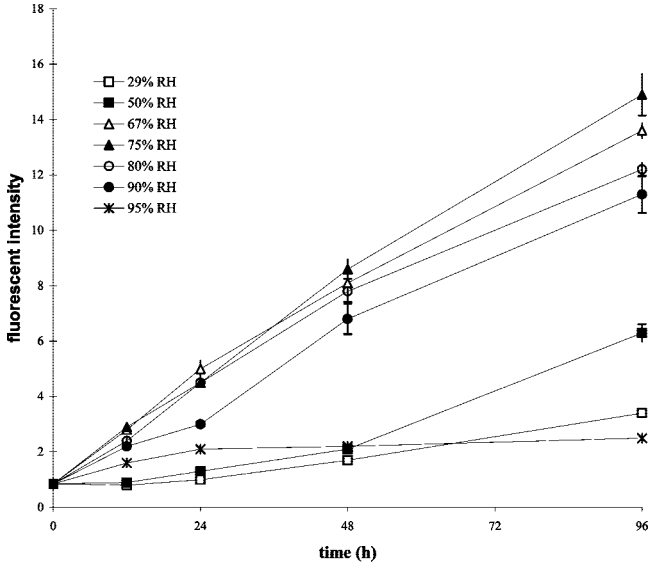

Figure 4. Time course of fluorescence intensity of the dry mixture of caseinate and lactose heated at 60 °C with different RHs.

First, the maximal FI was either at 67% or at 75% RH for every time interval. Second, the FI increased with time for all RHs. Finally, at 95% RH the increase of FI with time was insignificant compared to samples at other RHs.

From the color and FI results, it can be inferred that the browning occurred more rapidly between 67 and 80% RH with a maximum close to 75% RH. This conclusion is consistent with previous studies on milk powder (*14*).

After an induction period of 48 h, the UV absorbance of samples began to increase rapidly at intermediate RHs between 67 and 90%. This corresponds to the period when the lactulosyl lysine yield was decreasing (**Figure 1**). Therefore, we conclude that visible browning occurs when the destruction of Amadori product becomes dominant.

The browning rates due to advanced Maillard reaction were also examined with the incubation of a mixture of sugar fragments and caseinate for 48 or 96 h at different RHs. The UV absorbance and FI of the samples were determined, and the results are listed in **Table 4**, which shows that except for 95% RH samples the maximal color or FI still occurred at intermediate RH conditions (75% or 80%) for both 48 and 96 h time intervals. But the difference of color or FI between low and intermediate RH samples was not as great as that in the mixtures of lactose and caseinate. So it can be inferred that interactions between sugar fragments and caseinate were not the rate-limiting steps in the nonenzymatic browning. Another finding from **Table 4** is that in sharp contrast to the mixtures of lactose and caseinate, the UV absorbance and FI of the 95% RH sample was the largest in most cases. So although the overall nonenzymatic browning rate of 95% RH sample is slow, its advanced-stage browning rate is fast.

Both browning and the increase in fluorescence occur at advanced Maillard product stage (*38*). Previous studies have shown that the advanced Maillard reaction happens early on, and as rapidly as lactulosyl lysine (*40, 41*) formation, this was demonstrated by our results to be particularly true at 95% RH. From the present results, there is general agreement between the UV absorbance and FI results, except for 95% RH samples. However, fluorescence is a much more sensitive method and thus probably more reliable than UV absorbance for the evaluating the early stage of nonenzymatic browning.

Behavior of 95% RH samples. The behavior of 95% RH samples was quite different from that of others: color and FI were relatively high at 12 h but there was very little increase between 12 and 96 h. This trend also applied to the loss of lysine, which is shown by the disproportionally low lactulose lysine content. It is likely that the above finding can be explained in part by the fact that the slowest rate of lactulosyl lysine formation and the fastest rate of its destruction occur at 95% RH. Combined with the fact that the rate of sugar fragments and caseinate interaction at 95% RH is comparably fast, this may explain why the induction period of color and FI was not observed for 95% RH samples. The destruction of Amadori product was fast, and the sugar fragments thus formed quickly reacted with protein and caused browning. Another study (*42*) also found that induction time of UV absorbance decreased as moisture content increased.

In general the fastest rates of nonenzymatic browning occurred at intermediate RHs, but discrepancies existed if different indicators were used. For FI, color, lactulosyl lysine, loss of lysine, and reacted lactose, the maximum was at 67% (or 75%), 75% (or 95%), 50% (or 67%), 75% (or 80%), and 75% RH, respectively. Such discrepancies can be explained by the complexity of the nonenzymatic browning. The

Table 4. UV Absorbance (420 nm) and Fluorescence Intensity (excitation, 347 nm; emission, 415 nm) of Sugar Fragments–Caseinate Mixture

	control[a]	29% RH	50% RH	67% RH	75% RH	80% RH	90% RH	95% RH
48 h								
FI	1.8 ± 0.0	2.3 ± 0.5	3.7 ± 0.9	4.1 ± 0.3	4.7 ± 0.2	5.3 ± 0.4	4.4 ± 0.4	4.8 ± 0.6
UV	0.03 ± 0.00	0.03 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.09 ± 0.01
96 h								
FI	1.8 ± 0.0	2.7 ± 0.5	4.6 ± 0.3	4.8 ± 0.5	5.2 ± 0.5	6.1 ± 0.4	4.7 ± 0.2	8.4 ± 0.8
UV	0.03 ± 0.00	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.08 ± 0.02	0.10 ± 0.00	0.25 ± 0.01

[a] Sugar fragment–caseinate mixture without incubation was used as control.

observed rate was the result of a combination of different reactions. Such complexity makes the study of kinetics difficult. Moreover, the results showed that at every RH tested, the Maillard reaction is the major pathway. We conclude that the rate of Amadori product degradation increases with increasing RH and under our experimental conditions the formation of furosine is a better indicator of the extent of the Maillard reaction than the loss of lysine. In the present study, lactose was used because of its ready availability. However, the results may be applied to the conjugation of a bacterial-inhibiting sugar, such as sialyl oligosaccharides, with a protein using the Maillard reaction (*43*). A better understanding of the Maillard reaction should help to maximize the yield of sugar–protein conjugate and conserve valuable sialyl oligosaccharides.

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