Formation of an Aminoreductone during the Maillard Reaction of Lactose with N^{α}-Acetyllysine or Proteins

Monika Pischetsrieder,* Christiane Schoetter, and Theodor Severin*

Institut für Pharmazie und Lebensmittelchemie der Universität München, Sophienstrasse 10, 80333 München

When lactose is heated with N^{α}-acetyllysine or other amines for a short time, e.g. 15 min under reflux, a main product can be detected by HPLC/DAD. The product has a UV maximum at 319 nm, indicating an aminoreductone structure (AR). AR was isolated and identified as 1-[N^{ϵ}-(N^{α}-acetyllysinyl)]-1,2-dehydro-4-deoxy-3-hexulose. The analogous derivative of butylamine has previously been obtained from maltose. The product is the main UV absorbing material for several hours of heating and at various reaction temperatures. When lactose is heated with milk proteins, such as β-lactoglobulin, formation of AR can be observed by measuring a new UV maximum of the modified protein at 319 nm. Since AR can also be found after a short heating time and since it is the main product at various reaction conditions, it was concluded that the new product can be of importance for the Maillard reaction during milk processing.

Keywords: *Maillard reaction; lactose; milk products; aminoreductone*

INTRODUCTION

The Maillard reaction, which occurs during food processing, has great influence on the quality of food products. Reducing sugars react with amino acids or proteins, resulting in the formation of brown or flavor-generating products, which can also have antioxidative, mutagenic, or antimutagenic properties (Ledl and Schleicher, 1990). Especially during milk processing the Maillard reaction between lactose and milk proteins causes problems due to the generation of yellow and brown color and off-flavor.

Therefore, the Maillard reaction of lactose has been thoroughly investigated. It was found that disaccharides form other products compared to monosaccharides, and several compounds (Figure 1) were identified as disaccharide specific Maillard products (Kramhöller et al., 1993; Pischetsrieder and Severin, 1994, 1996, and literature cited therein). Furthermore, lactose-derived Amadori product (Henle et al., 1991) and, in smaller amounts, pentosidine (Henle et al., 1997) and pyrraline (Henle and Klostermeyer, 1993) were detected in milk products.

However, with exception of the Amadori product these compounds can only be detected in Maillard reaction mixtures heated for long periods. Since during milk processing milder conditions are usually applied, we have investigated short-time-heated reaction mixtures of lactose and N^{α}-acetyllysine or β-lactoglobulin and identified the main product formed.

MATERIALS AND METHODS

Reagents. Methanol LiChrosolv of chromatography grade was purchased from Merck (Darmstadt, Germany), β-lactoglobulin from Sigma (St. Louis, MO), and N^{α}-acetyllysine from Acros (Geel, Belgium). Deionized water was distilled before use for HPLC.

* Address correspondence to either author (telephone ++49-89-5902-387; fax ++49-89-5902-447; e-mail pischets@pharmchem.uni-muenchen.de).

HPLC. Analytical HPLC was performed with a Merck L-7100 gradient pump and a Merck L-7450 photodiode array detector including Merck-Hitachi Model D-7000 chromatography data station software. The mixtures were separated on a column packed with LiChr Select B (RP 18, 125 × 3 mm i.d., 5 μm particle size) from Macherey & Nagel (Oensingen, Switzerland). For elution a gradient was used of 0−70% B from 0 to 20 min and 100% B from 20.1 to 30 min at a flow rate of 0.4 mL/min (solvent A, 5 mM triethylammonium acetate, pH 7.0; solvent B, methanol). The substances were detected in a wavelength range from 200 to 400 nm. For preparative HPLC a Merck L-6250 pump, a Merck L-4000 UV detector, and a Merck D-2500 chromatointegrator were used and separation was performed on a SP 250/2 Nucl 7 C18 column (Macherey & Nagel).

Relative quantification was achieved with a DAD-System-Manager Software D-7000 chromatography data station (Merck-Hitachi) and manual baseline correction. Caffeine was used as internal standard, and a chromatogram was plotted at 319 nm from 0 to 13 min and at 278 nm from 13.1 to 30 min. The relative yield was calculated in relation to the standard concentration and was not corrected for the ϵ value of aminoreductone (AR), which could not be determined because AR is unstable in its pure and undissolved form.

Isolation of 1-[N^{ϵ}-(N^{α}-Acetyllysinyl)]-1,2-dehydro-4-deoxy-3-hexulose. Lactose (800 mg) and N^{α}-acetyllysine (120 mg) were dissolved in 4 mL of phosphate buffer (1.28 M, pH 7.0) and heated for 75 min at 100 °C. The mixture was directly separated by preparative HPLC with an eluent of 2.5% methanol in 5 mM ammonium formate and a flow rate of 9.0 mL/min. Peaks were detected at 320 nm, and the fraction at 27 min was collected and lyophilized. The analytically pure product was used for spectral analyses. Since all multiplets represent higher order spin patterns, coupling constants cannot be extracted (Bible, 1965). ^{1}H NMR (CD_3OD) δ 1.29−1.41 (m, 2H, H_2C_{Lys-4}), 1.59−1.67 (m, 3H, H_2C_{Lys-5} and $H_AH_BC_{Lys-3}$), 1.94 (m, 1H, $H_AH_BC_{Lys-3}$), 1.98 (s, 3H, CH_3), 2.56 (m, 2H, O=C−CH_2), 3.2 (m, 2H, H_2C_{Lys-6}), 3.5 (m, 1H, H_2COH), 4.0 (m, 1H, HCOH), 4.2 (m, 1H, H C_{Lys-2}), 7.1 (s, 1H, HC=C); UV (H_2O) λ_{max} 319.2 nm.

Isolation of 1-(Butylamino)-1,2-dehydro-4-deoxy-3-hexulose. Lactose (180 mg) and butylamine (170 mg) were dissolved in 2 mL of phosphate buffer (1.28 M, pH 7.0), and the pH value was adjusted to 7.0 with phosphoric acid. The

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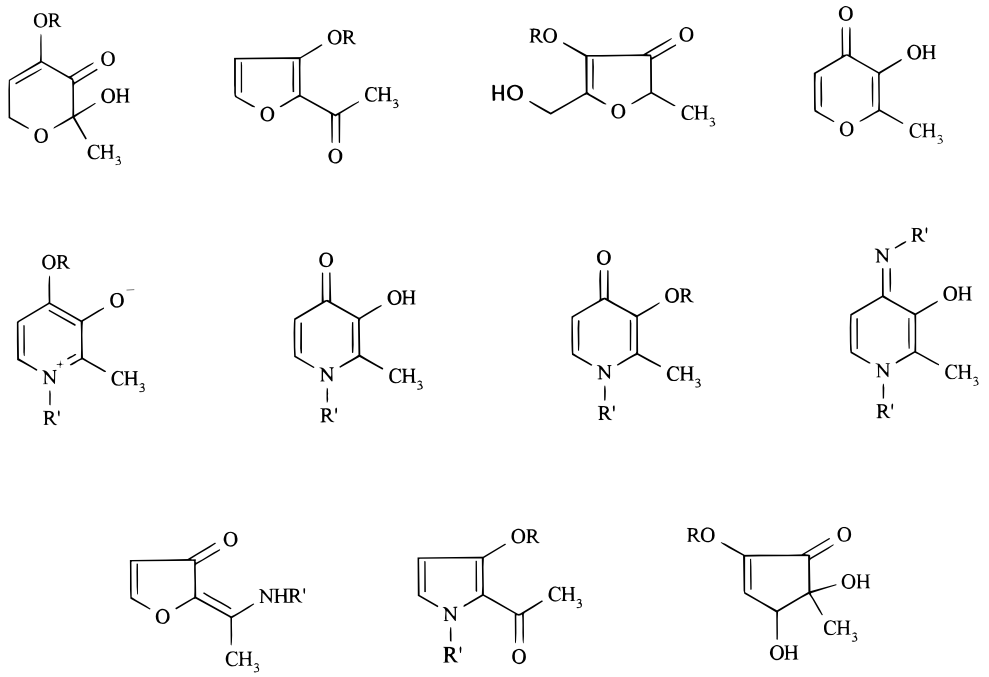

Figure 1. Disaccharide specific Maillard products. R = D-galactose; R′ = e.g. propylamine or N^{α}-acetyllysine.

mixture was heated for 30 min at 100 °C, and after cooling, it was extracted three times with ethyl acetate. The solvent was evaporated from the unified organic layers and the residue was directly used for NMR analyses. The spectral data were identical with those of the reference compound (Schoetter et al., 1994).

Synthesis of the Reference Compound. 1-(Butylamino)-1,2-dehydro-4-deoxy-3-hexulose was synthesized by alkaline degradation of the maltose−butylamine Amadori product (Schoetter et al., 1994). Briefly, 1200 mg of maltose−butylamine Amadori product was dissolved in 12 mL of 2 N NaOH, and the mixture was allowed to stand overnight at room temperature. The solution was then neutralized with phosphoric acid and separated by preparative HPLC as described.

Transformation of AR into the Analogous Butylamine-Derived Product. AR (10 mg), butylamine (219 mg), and acetic acid (180 mg) were dissolved in 1 mL of water and heated for 30 min at 100 °C. The resulting 1-(butylamino)-1,2-dehydro-4-deoxy-3-hexulose was identical to the synthesized reference compound.

Preparation of the Samples. *Short-Term-Heated Reaction Mixture.* Lactose (360 mg) and N^{α}-acetyllysine (47 mg) were dissolved in 4.3 mL of phosphate buffer (1.28 M, pH 7.0) and heated for 15 min under reflux. The sample was immediately (without dilution) injected into HPLC.

Time Course at 70 °C. Lactose (400 mg) and N^{α}-acetyllysine (60 mg) were dissolved in 2 mL of phosphate buffer (1.28 M, pH 7.0) and heated at 70 °C. Samples (100 μL) were taken as indicated and diluted with 600 μL of caffeine standard solution to obtain a final caffeine concentration of 72.6 μg/mL.

Time Course at 100 °C. The mixtures were prepared as described above, with the exception that each time 100 μL of the sample was diluted with 1.65 mL of caffeine solution to a final concentration of 94.3 μg of caffeine/mL.

β-Lactoglobulin−Lactose Reaction Mixtures. β-Lactoglobulin (38 mg) and lactose (260 mg) were dissolved in 2 mL of phosphate buffer (22 mM, pH 7.0) by ultrasonication and heated for 90 min at 100 °C.

Photometric Analyses. UV spectra were recorded on a Perkin-Elmer UV−vis Lambda 20 spectometer from 250 to 500 nm. UV absorbance of the samples was measured after they were passed through a 0.2 μm filter without dilution. Reference spectra of β-lactoglobulin or purified AR in water were obtained.

RESULTS

To investigate Maillard products of lactose in short-time-heated mixtures, lactose and N^{α}-acetyllysine were heated for 15 min under reflux. The reaction mixtures were then analyzed by HPLC with a diode array detector (HPLC/DAD). It was found that one main compound is formed, whereas the previously identified lactose products played only a minor role (Figure 2). The main compound displays a characteristic UV maximum at 319 nm, indicating that the new lactose product has an AR structure.

AR was isolated by preparative HPLC, and the UV and ^{1}H NMR sprectra were recorded. The spectra that were obtained were similar to those of a previously identified compound which is formed during the decomposition of maltose in the presence of butylamine (Schoetter et al., 1994). Thus, the analogous AR, which is derived from butylamine, was synthesized by heating lactose and butylamine for 30 min at 100 °C and purified. It was found that this product and the authentic reference compound were identical with regard to spectra and retention time of HPLC analyses. It can therefore be concluded that AR is 1-[N^{ϵ}-(N^{α}-acetyllysinyl)]-1,2-dehydro-4-deoxy-3-hexulose (Figure 3). For further confirmation of this result, the isolated AR was also transformed by transamination into the analogous butylamine-derived AR.

In the next step the significance of AR was evaluated by performing time course experiments of the reaction of lactose with N^{α}-acetyllysine at 70 and 100 °C. AR is the main product detected over the investigated time range (Figure 4A). When the mixture was heated at 100 °C, AR was found to be the main product for up to 3 h of reaction time. However, the maximum yield is obtained after 1.5 h, and after 4 h, AR cannot be detected anymore (Figure 4B) and other products dominate the chromatogram.

Furthermore, formation of AR at 37 °C was investigated to determine if AR can also be formed at lower temperature and might play a role during storage of milk products. It was found that AR can be detected

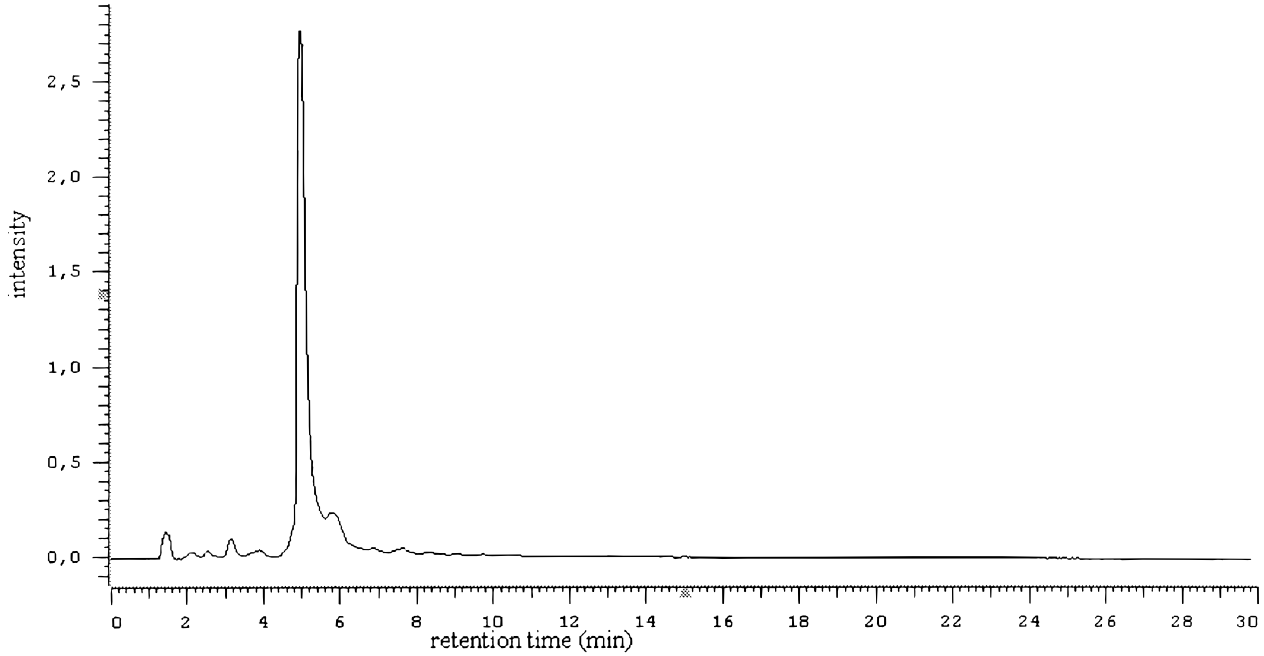

Figure 2. HPLC chromatogram (detection at 319 nm) of a reaction mixture of lactose and N^{α}-acetyllysine, which was heated for 15 min under reflux.

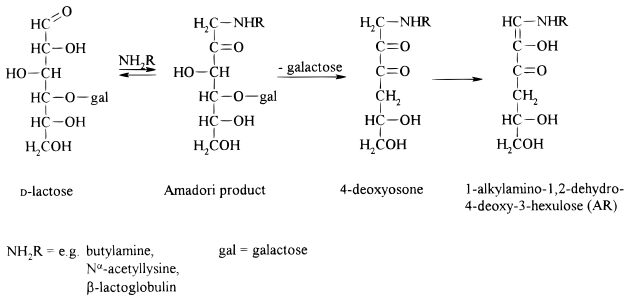

Figure 3. Formation of AR from lactose.

after 5 days of incubation, whereas other products were found in trace amounts only.

So far, all reactions were carried out with N^{α}-acetyllysine as amino component. However, in foods, free amino acids occur only in lower quantities and most of the amino groups are bound to proteins such as lysine side chains or the N terminus.

Therefore, lactose was reacted with milk proteins, such as β-lactoglobulin and formation of AR from proteins was determined. Since AR has a labile aminoreductone structure, it is not stable under the conditions of alkaline or acidic hydrolysis. Therefore, a photometric test was used for detection. AR has a characteristic UV maximum at 319 nm which does not interfere with the UV absorbance of unmodified protein. Thus, β-lactoglobulin was incubated with lactose for 70 min at 100 °C and a UV spectrum of the protein was recorded. It was found that in addition to the maximum at 280 nm of β-lactoglobulin a second one appeared at 319 nm (Figure 5A). When the UV spectra of β-lactoglobulin and the purified AR were superimposed, a curve was obtained that is identical to the product spectrum (Figure 5B). This result indicates that the aminoreductone is also formed as a derivative of lysine side chains of proteins.

DISCUSSION

With the identification of AR an important Maillard product of lactose was obtained, which is of relevance

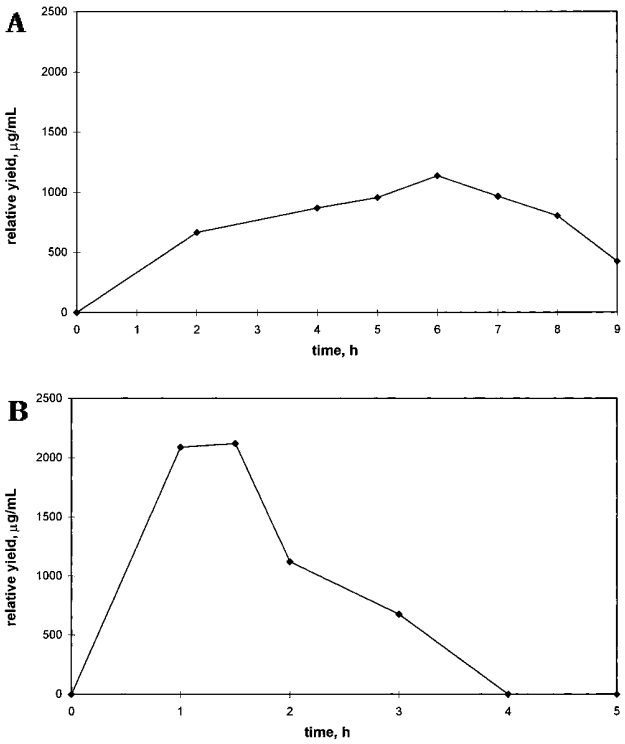

Figure 4. Time course for the formation of AR from lactose and N^{α}-acetyllysine at 70 °C (A) and 100 °C (B).

in heated or processed milk products. In contrast to other Maillard compounds that are derived from lactose, AR can be detected after short time heating, e.g. after 15 min at 100 °C, and over a relatively long time range it is the main product. Additionally, AR is formed at various conditions such as incubation at 37 °C or heating at 100 °C. However, if the mixtures are reacted under more vigorous conditions, AR is degraded and other products (Figure 1) become important. Finally, it was shown that AR can be formed from various primary amines, such as simple alkylamines (butyl-

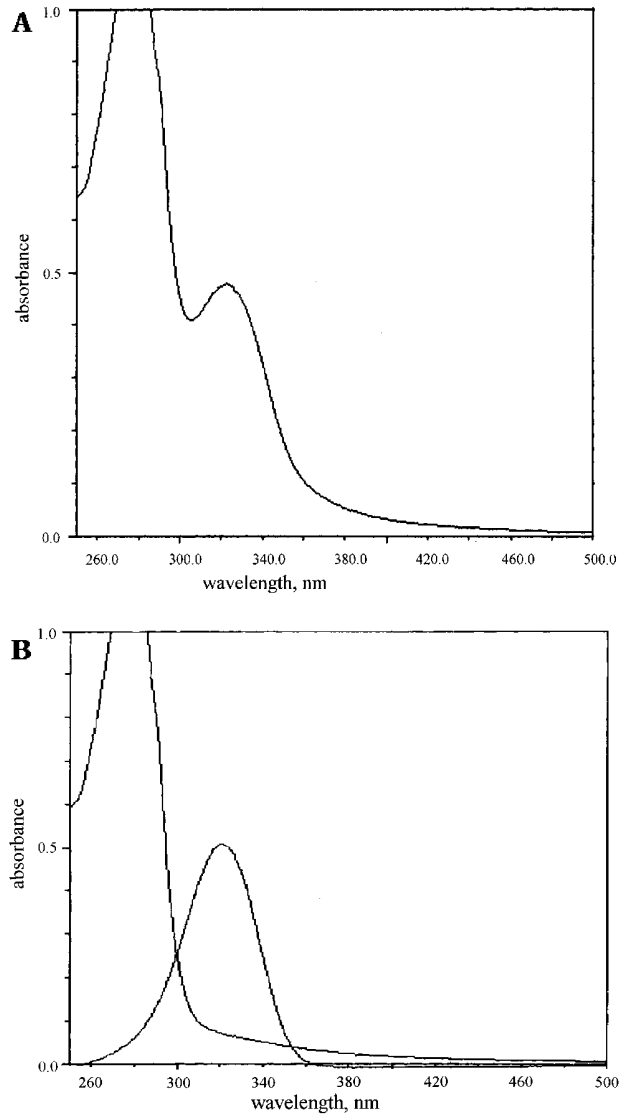

Figure 5. UV spectra of β-lactoglobulin that was incubated with lactose (A) and the spectra of unreacted β-lactoglobulin and purified AR (N^{α}-acetyllysine derivative) (B).

amine), amino acids (N^{α}-acetyllysine), and side chains of proteins. Therefore, it can be assumed that AR is also formed in milk products as a major nitrogen-containing Maillard compound of lactose.

It can be assumed that for the formation of AR lactose reacts in the first step with the alkylamine to give the Amadori product (Figure 3). In the next step the galactose residue is eliminated and the 4-deoxyosone is formed, which then isomerizes and AR is obtained. The 4-deoxyosone has not been isolated so far, but is was trapped as the quinoxaline derivative after derivatization with *o*-phenylenediamine (Beck et al., 1989). It was found that the 4-deoxyosones and AR are mainly formed from disaccharides, such as maltose or lactose. On the other hand, glucose or other monosaccharides yield both products only in minor amounts (Estendorfer et al., 1990). It can be predicted that the 4-deoxyosone with its β-amino−dicarbonyl structure is not stable, but the equilibrium is shifted toward the β-aminoreductone structure (Euler and Eistert, 1957). Therefore, AR can be considered the first stable tautomer of the 4-deoxyosone that has been isolated so far.

Besides its widespread occurrence, AR can be important because it can undergo further reactions due to its aminoreductone structure. First, it is known that aminoreductones possess antioxidative properties which can exceed even those of reductones, such as ascorbic acid, suggesting that the formation of AR can prevent oxidative spoilage and rancidity. On the other hand, in the presence of trace amounts of metal ions AR can also have a pro-oxidative effect, resulting in damage of proteins or fatty acids. Since AR is usually protein bound, and therefore in close vicinity to the proteins, antioxidative and pro-oxidative properties can be of particular relevance for the impact of reactive oxidative species on proteins (Pischetsrieder and Severin, 1997, and literature cited therein).

To confirm these assumptions, the role of aminoreductones, such as AR on redox reactions, is currently under detailed investigation.

ABBREVIATIONS USED

AR, aminoreductone; DAD, diode array detection; HPLC, high-performance liquid chromatography.

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