Convenient Synthesis of Lactuloselysine and Its Use for LC-MS Analysis in Milk-like Model Systems§

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The synthesis of the Amadori product lactuloselysine [N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine] was obtained starting from FMOC-lysine-OH (N_α-9-fluorenylmethoxy-carbonyl-$N_\epsilon H_2$-L-lysine-OH) and lactose. Compound identity was confirmed by MALDI-ToF, electrospray, and NMR analysis. A selective LC-MS procedure which allowed the detection of lactuloselysine up to 10 ng mL^{-1} was set up and used to follow the formation of the compound in a lactose-lysine model system; quantification of this molecule after complete enzymatic hydrolysis of whey-proteins from milk samples was also performed.

Keywords: *Maillard reaction; lactuloselysine; LC-MS; milklike model systems.*

INTRODUCTION

During food processing the Maillard reaction (MR) strongly affects the quality of food products. In milk, the first step of the Maillard reaction is the condensation between lactose and lysine residues of proteins (van Boekel, 1998). After the formation of the Schiff's base and the subsequent Amadori rearrangement, protein-bound lactuloselysine is formed. At this stage, the main consequence is a loss in nutritive value (Mauron, 1981) due to blockage of lysine residues which are not available for digestion (Finot, 1990) and to inhibition of enzymes (Friedman, 1996a, 1996b).

The identification and quantification of products which can be considered chemical markers of the early stage of MR in milks is a relevant task of food chemistry (van Boekel, 1998). The measure of lactuloselysine could be particularly useful to find out the first step of the MR since its quantification in milk and milk products has been attempted by several authors using different analytical techniques.

A direct method of measuring lactuloselysine after complete enzymatic hydrolysis by amino acid analyzer was proposed by Henle et al. (1991). More recently an immunological approach to directly detect protein bound Amadori compound was proposed by Fogliano et al. (1997). Pizzano et al. (1998) developed a competitive ELISA using antibodies against a glycated peptide of caseins. A linear relationship between antibodies recognition and milk thermal treatment was obtained.

Other studies have proposed the measure of lactuloselysine derivatives such as determining the formation of CML (carboxymethyllysine) formed after oxidation by periodic acid and acid hydrolysis of the Amadori compound (Badoud et al., 1991). The amount of unavailable lysine was measured by Berg and Van Boekel (1994), while the formation of HMF (hydroxymethylfurfural) from the Amadori compound by boiling in oxalic acid has been considered by Burton (1984) and by Pellegrino and co-workers (1995). An improved method for the evaluation of lysine modification was recently proposed by Morales and Jemenez Perez (1998) who determined protein-bound HMF by HPLC.

So far, the most widely used method to evaluate the Amadori compound in thermally treated milk and milk products is the furosine method by ion exchange chromatography (Finot et al., 1981; Erbersdobler, 1986). Furosine is formed after strong acid hydrolysis of the Amadori compound and can be also estimated by HPLC (Resmini et al., 1990). The main drawbacks of this procedure are the time of analysis and the fact that only part of lactuloselysine is converted into furosine (Furth, 1988).

One of the problems related to the direct quantification of lactuloselysine is the lack of a pure standard. In fact, the synthetic strategies proposed so far (Schreuder and Welling, 1983; Henle et al., 1991) are rather time-consuming and do not yield a consistent rate of pure Amadori compound.

This paper proposes a new method for the synthesis of lactuloselysine that is easier to handle and gives a high yield of pure lactuloselysine. In addition, a LC-MS method for its detection and quantification has been set up. This method was used to quantify lactuloselysine in a milk-like model system and in whey-proteins purified from skim milk.

MATERIALS AND METHODS

D-(+)-Lactose monohydrate, morpholine, *N,N*-dimethylformamide, ninhydrin, Pronase E from *Streptomices griseus* (EC no. 2329666), prolidase from *Lactococcus lactis* (EC no. 2327915), aminopeptidase M microsomal from hog kidney (EC no. 2326183), trifluoracetic acid, and trichloroacetic acid were of analytical grade and purchased from Fluka (Fluka Chemie AG, Buchs, CH). FMOC-lysine-OH was of analytical grade and purchased from Novabiochem (Calbiochem-Novabiochem AG-CH-4448 Laufelfingen). HPLC-grade methanol, acetonitrile, diethyl ether, and water were from Merck (Darmstadt,

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§ In memory of Prof. Giacomino Randazzo.

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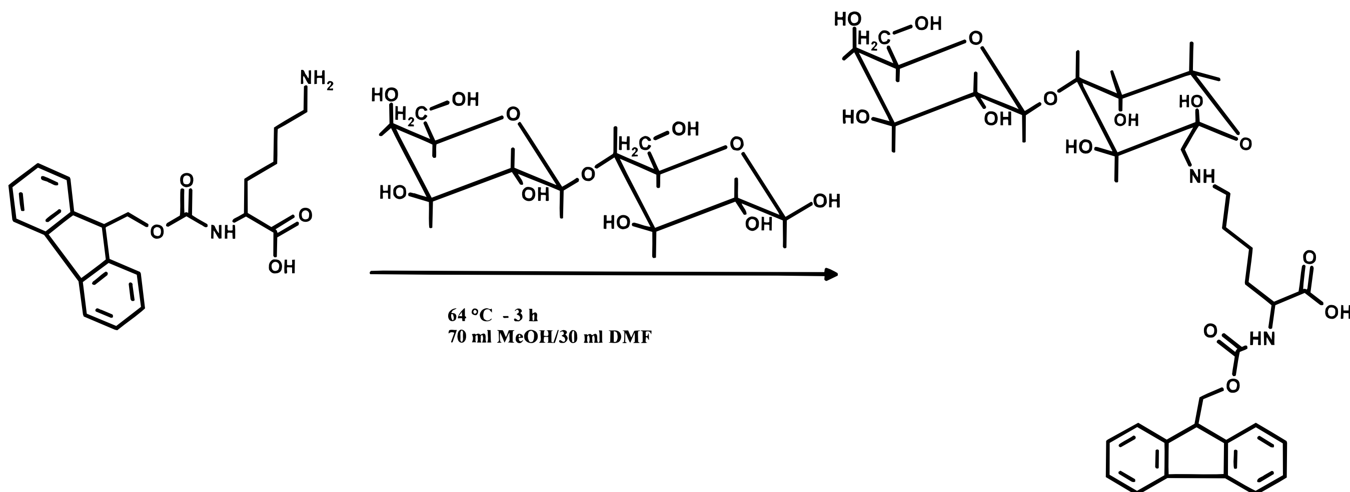

Figure 1. Synthesis of N_α-9-fluorenylmethoxy-carbonyl-N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine.

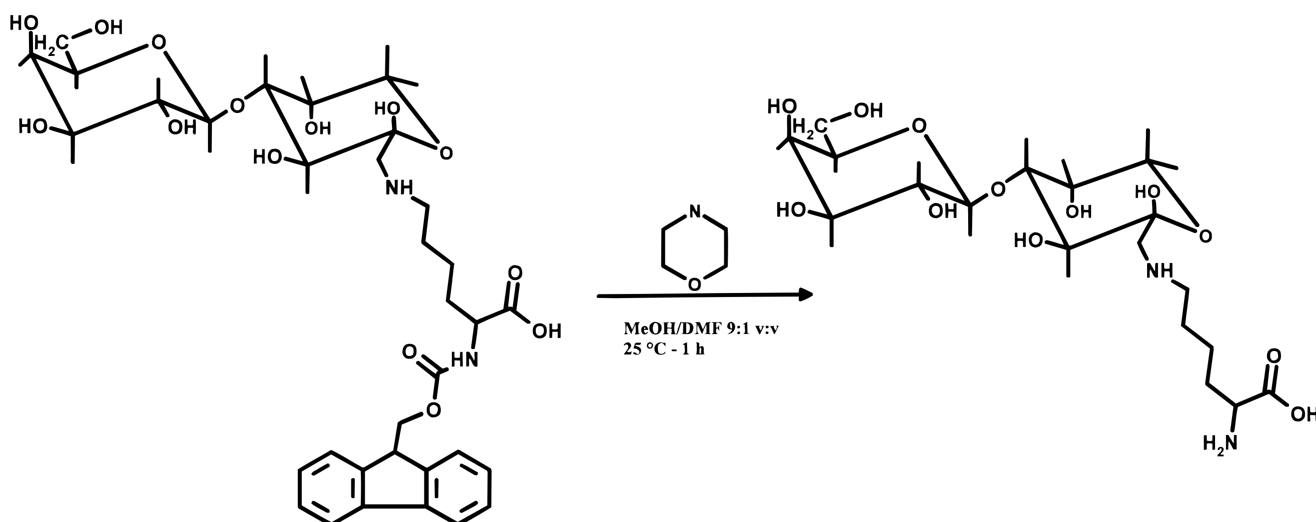

Figure 2. Synthesis of N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine.

Germany) and were filtered through disposable 0.2 μm filters from Acrodisc (Gelman Sciences, Ann Arbor, MI).

Synthesis of H-Lys(Lact)-OH [N_ϵ-(1-Deoxy-D-lactulosyl-1)-L-lysine]. *Synthesis of FMOC-Lys(Lact)-OH [N_α-9-Fluorenylmethoxy-carbonyl-N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine] (Figure 1).* The synthesis of FMOC-Lys(Lact)-OH was carried out at 64 ± 2 °C for 3 h under continuous stirring of a mixture consisting of 70 mL of MeOH (methanol), 30 mL of DMF (*N,N*-dimethylformammide), 2.18 g of FMOC-Lys-OH (6 mmol), and 4.3 g of lactose monohydrate (12 mmol) (Finot and Mauron, 1969). The solvent was evaporated under reduced pressure, and the residue was dissolved in MeOH and filtered. MeOH was removed, and the residue was taken up in 5 mL of deionized water and applied onto a C_{18} column (Varian 3CC/500 MG, Varian Associates, Harbor City, CA). C_{18} column was extensively washed with deionized water to remove the excess of lactose and FMOC-Lys(Lact)-OH eluted with MeOH. Solvent was immediately evaporated. Reaction was monitored by RP-TLC using a mixture of MeOH/water (8:2 v:v). Products were detected by UV light and ninhydrin vapors.

FMOC-Lys(Lact)-OH has a λ_{max} at 254 nm and IR absorption at 700 cm^{-1} (aryl), 1000−1100 cm^{-1} strong (C−O stretching), 1400 cm^{-1} (O−H bending), 1600 cm^{-1} (−NH−CO−O-), 1700 cm^{-1} (−CO−OH), 3000 cm^{-1} (−NH_2^+- broad), and 3200−3600 cm^{-1} broad and strong (O−H stretching).

LC/MS [electrospray ionization] of FMOC-Lys(Lact)-OH m/s showed the following signals: $[M + 1]^+$ 693, $[M + Na]^+$ 715, $[M + 2Na]^+$ 737, $[M + 3Na]^+$ 759, $[M_2 + 1]^+$ 1385, $[M - H_2O]^+$ 675.

MALDI-ToF of FMOC-Lys(Lact)-OH m/s: 693 (M), 1384 (2M).

^{1}H NMR (400 MHz, D_2O) of FMOC-Lys(Lact)-OH: δ 7.8 (d, J = 7 Hz, 2 H, FMOC aryl), δ 7.7 (dd, J = 2 Hz 2 H, FMOC aryl), δ 7.4 (t, J = 7 Hz, 2 H, FMOC aryl), δ 7.3 (t, J = 7 Hz, 2 H, FMOC aryl), δ 4.35 (m, 2 H, CH_2 FMOC), δ 4.50 (m, 1 H, CH Lact), δ 4.2 (t, J = 6 Hz, 1 H, CH FMOC), δ 4.17 (m, 1 H, CH Lact), δ 4.07 (m, 1 H, CH Lact), δ 4.08 (m, 1 H, CH Lys methine), δ 3.88 (m, 1 H, CH Lact), δ 3.82 (m, 1 H, CH Lact), δ 3.8 (m, 1 H_a, CH_2 Lact), δ 3.7 (m, 1 H_b, CH_2 Lact), δ 3.69 (m, 1 H, CH Lact), δ 3.66 (m, 1 H, CH Lact), δ 3.59 (m, 1 H, CH Lact), δ 3.3 (s, 2 H, CH_2 Lact-Lys), δ 3,07 (m, 2 H, CH_2 Lys), δ 1.7 (m, 2 H, CH_2 Lys), δ 1.6 (m, 2 H, CH_2 Lys), δ 1.38 (m, 2 H, CH_2 Lys).

Synthesis of H-Lys(Lact)-OH [N_ϵ-(1-Deoxy-D-lactulosyl-1)-L-lysine]. Elimination of FMOC Group (Figure 2). FMOC was eliminated dissolving 60 mg of FMOC-Lys(Lact)-OH in 10 mL of a solution containing 20% of morpholine in DMF/MeOH (9:1 v:v) (Carpino and Han, 1972). After 1 h under continuous stirring at room

temperature the mixture constituted of DMF, MeOH, and morpholine was evaporated under vacuum. The resulting residual product was filtered with 200 mL of diethyl ether to eliminate secondary products of the reaction. Reaction was monitored by TLC using a mixture of buthanol/water/acetic acid (60:25:15 v:v:v). The dried product was stored at 4 °C before use.

H-Lys(Lact)-OH has a λ_{max} at 210 nm and IR absorption at 1000−1100 cm^{-1} strong (C−O stretching), 1400 cm^{-1} (O−H bending), 1600 cm^{-1} (−NH−CO−O-), 1700 cm^{-1} (−CO−OH), 3000 cm^{-1} ($-NH_2^+$- broad), and 3200−3600 cm^{-1} broad and strong (O−H stretching).

LC/MS [electrospray ionization] of H-Lys(Lact)-OH: $[M + 1]^+$ 471.2, $[M + H_2O]^+$ 489, $[M + Na]^+$ 493, $[M_2 + 1]^+$ 834, $[M - H_2O]^+$ 453, $[M - 2H_2O]^+$ 435.

^1H NMR analysis indicated the presence of only minor impurities. ^1H NMR (400 MHz, D_2O) of H-Lys(Lact)-OH: δ 4.50 (m, 1 H, CH Lact), δ 4.17 (m, 1 H, CH Lact), δ 4.07 (m, 1 H, CH Lact), δ 4.08 (m, 1 H, CH Lys methine), δ 3.88 (m, 1 H, CH Lact), δ 3.82 (m, 1 H, CH Lact), δ 3.8 (m, 1 H_a, CH_2 Lact), δ 3.7 (m, 1 H_b, CH_2 Lact), δ 3.69 (m, 1 H, CH Lact), δ 3.66 (m, 1 H, CH Lact), δ 3.59 (m, 1 H, CH Lact), δ 3.3 (s, 2 H, CH_2 Lact-Lys), δ 3,07 (m, 2 H, CH_2 Lys), δ 1.7 (m, 2 H, CH_2 Lys), δ 1.6 (m, 2 H, CH_2 Lys), δ 1.38 (m, 2 H, CH_2 Lys).

^{13}C NMR (400 MHz, D_2O) of H-Lys(Lact)-OH: δ 176 (COOH, Lys), δ 101 (CH, Lact), δ 96 (C, Lact), δ 78 (CH, Lact), δ 76 (CH, Lact), δ 73 (CH, Lact), δ 72 (CH, Lact), δ 69.5 (CH, Lact), δ 69 (CH, Lact), δ 67 (CH, Lact), δ 64 (CH_2, Lact), δ 62 (CH, Lact), δ 55 (CH, Lys), δ 54 (CH_2, Lact-Lys), δ 32 (CH_2, Lys), δ 26 (CH_2, Lys), δ 23 (CH_2, Lys), δ 22.5 (CH_2, Lys).

Characterization of FMOC-Lys(Lact)-OH and H-Lys(Lact)-OH. The UV spectra were measured on a UV-vis Shimadzu UV-2100 recording spectrophotometer in water solution. The IR spectra were carried out with a Jasco FT/IR-430.

^1H NMR ^{13}C NMR Analysis. The ^1H NMR ^{13}C NMR spectra were recorded on a Brucker 400 MHz spectrometer in D_2O; chemical shifts (δ) are expressed in ppm and coupling constants (J) are expressed in Hz (hertz).

Mass Spectrometry Analysis. MALDI-ToF. Spectra were recorded on a Kratos Kompact MALDI 3 spectrometer (Shimadzu). Sample (0.5 μL) (solvent: D_2O), 0.5 μL of matrix solution (3,5-dimethoxy-4-hydroxycinnamic acid − sinapic acid − 10 mg/mL in CH_3CN/TFA 0.1% 2/3 v:v), and 0.5 μL of TFA 0.1% were overlaid on the sample slide, and the solvent was evaporated. The instrument was calibrated using substance P and insulin as standards.

Electrospray. Mass spectra were obtained on an API-100 single quadrupole mass spectrometry (Perkin-Elmer Sciex Instruments, Canada) equipped with an atmospheric pressure ionization source. A probe voltage of 4700 kV and a declustering potential of 70 V were used. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol.

Analyses of H-Lys(Lact)-OH and of FMOC-Lys(Lact)-OH intermediates were performed by injecting directly into the ion source at a flow rate of 10 $\mu L\ min^{-1}$ a 0.1 mg/mL solution of purified compound. Acquisition was made in positive ion mode in the range of 300−1500 uma with a dwell time of 4 ms and a step size of 0.2 amu. Data were processed through the Bio Multi View software (Sciex, PE, Foster City, CA).

Preparation of the Model System. The lactose-lysine model system was prepared and analyzed according to Monti et al. (1998) with slight modifications. Briefly, lysine monohydrochloride (7.3 g, 0.04 mol) and lactose (14.4 g, 0.04 mol) were added to distilled water (40 mL), in a 50 mL Quickfit round-bottom two-necked flask, equipped with a double-coil water condenser, a lid, and a magnetic stirrer bar. The solution was heated for up to 4 h at 100 °C under reflux. Samples were collected after each hour and cooled in crushed ice before analysis, and the reaction was monitored by measuring the pH and the optical density at 420 nm, H-Lys(Lact)-OH and Lact-Lys-OH.

Preparation of Heated Skim Milk and Whey-Proteins Hydrolysis. A sample of 250 mL of pasteurized commercial skim milk was heated at 70 °C for 7 h. Caseins were precipitated according to Aschaffenburg and Dewry (1959). Whey-proteins were precipitated with trichloroacetic acid (TCA) 10%. An aliquot of 3 mg of whey-proteins (150 μL) was added to 200 μL of a solution of Pronase E (0.02 mg in 1 mL of PBS with 0.025% NaN_3), 100 μL of prolidase solution (0.02 mg in 1 mL of PBS with 0.025% NaN_3), and 100 μL of amminopeptidase solution (0.02 mg in 1 mL of PBS with 0.025% NaN_3). The same enzyme cocktail was added again for other 24 h of digestion. The buffer with NaN_3 was filtered through a 0.2 μ filter before dissolving the enzyme. Incubation was carried out at 37 °C for 24 h. After hydrolysis the sample was stored at −20 °C.

Measurement of H-Lys(Lact)-OH and (Lact)Lys-OH in Lactose-Lysine Model System and of H-Lys-(Lact)-OH in Skim Milk Sample by LC/MS. HPLC analyses were performed on a Perkin-Elmer series 200 liquid chromatography pump, using a Brownlee C18 column (4.6 mm internal diameter 3.3 cm length) and a 20 μL loop. The chromatographic column was equilibrated in water at a flow rate of 0.5 mL min^{-1}, and elution was achieved with the following steps: 2 min 100% water, 1 min 50% water 50% MeOH, 4 min 100% MeOH. Twenty microliters min^{-1} of the mobile phase was split into the ion spray source. Acquisition of the signal was performed at 471.2 amu corresponding to the molecular ion $[MH]^+$ of the Lys(Lact)-OH using single ion monitoring parameters. Peaks integration and quantification of the compounds were performed using Mac Quan software (PE, Foster City, CA). Each determination, of standard and of unknown samples, was performed in triplicate.

RESULTS AND DISCUSSION

The synthesis of Amadori compound H-Lys(Lact)-OH is complicated by further reaction of the final product. Using Nα-formyl-L-lysine Finot and Mauron (1969) obtained a good yield of the reaction (48%). Unfortunately the formyl group is removed in strong acid conditions, this causing a degradation of the molecule. Henle et al. (1991) obtained H-Lys(Lact)-OH starting from Nα-acetyl-L-lysine-OH. To remove the acetyl group acid conditions have been used.

The method here proposed (Figures 1 and 2) is carried out under mild heating conditions, this allowing a better control of the reaction system and limiting the formation of the advanced MRPs. In addition, the use of FMOC-Lys-OH rather than Nα-acetyl-L-lysine-OH allowed the removal of the protective group into an alkaline solution, thus avoiding the hydrolysis of the disaccharide and/or the hydrolysis of the Amadori product.

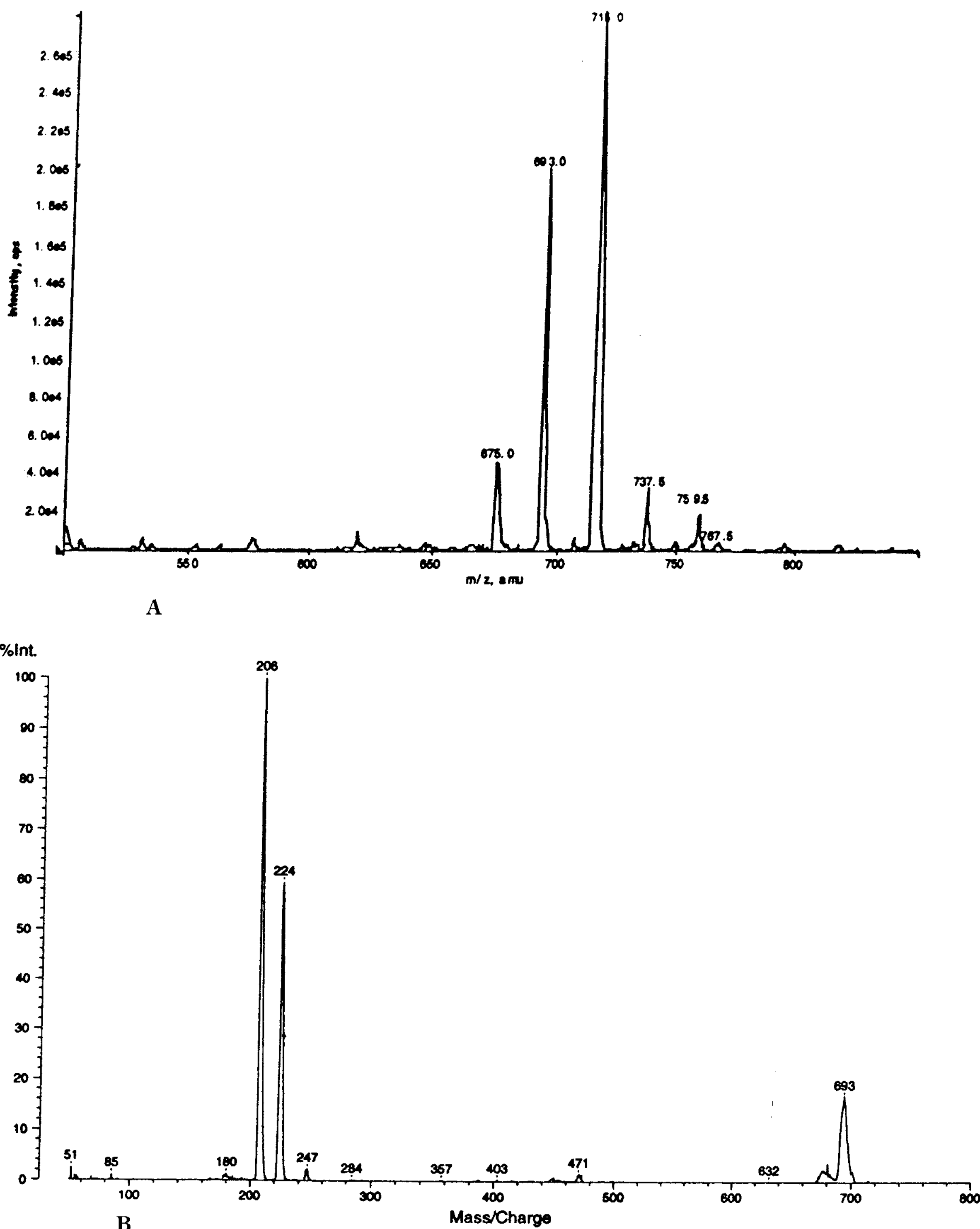

Figure 3. Mass spectra of N$_{\alpha}$-9-fluorenylmethoxy-carbonyl-N$_{\epsilon}$-(1-deoxy-D-lactulosyl-1)-L-lysine; panel A, electrospray; panel B, MALDI-ToF. In the MALDI-ToF spectrum the signals at 206 and 224 amu are due to the matrix (sinapic acid).

The presence of FMOC group allowed a rapid and quantitative recovery by RP-column of the intermediate product FMOC-Lys(Lact)-OH. The final yield of this coupling step was 60%. The product was detectable by RP-TLC at 254 nm and showed an R_f value of 0.37. The FMOC-Lys(Lact)-OH was characterized by MALDI-ToF-MS, electrospray-MS, and ^{1}H NMR (Figure 3 panel A electrospray-MS, panel B MALDI-ToF-MS). In both mass spectra the molecular ion at 693 *m/z* was detectable, while on the electrospray spectrum the Na$^+$-adducts are clearly evident. In the ^{1}H NMR spectrum the signal at 3.3 ppm is evident. This singlet is characteristic of Amadori products and is due to the methylene group placed between the carbonyl and the amino group.

The step regarding the elimination of FMOC group was performed in a basic solution. Secondary products were eliminated by filtration with Et_2O (TLC detected with ninidrine − $Rf_{\text{H-Lys(Lact)-OH}} = 0.2$). The yield of the final product H-Lys(Lact)-OH was 96%. The compound was obtained as a white amorphous powder that became brown in the presence of small quantities of water. Electrospray mass spectrum (Figure 4) gave a molecular ion at 471 amu and signals due to the loss of water from

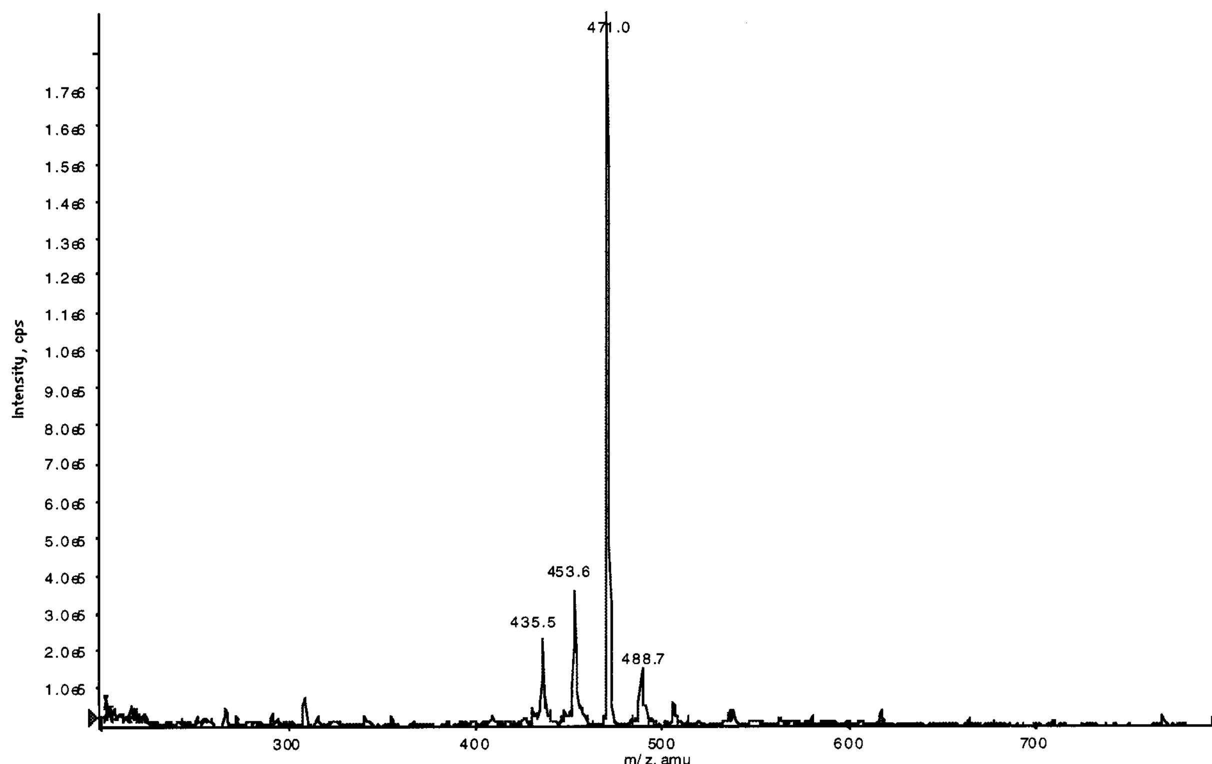

Figure 4. Mass spectrum (electrospray) of N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine.

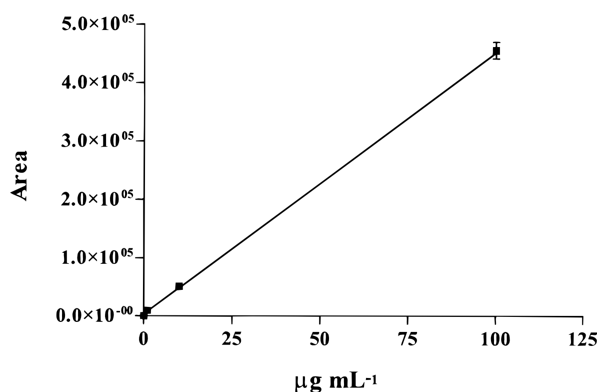

Figure 5. Reference curve obtained by LC-MS (electrospray) in single ion monitoring using different concentration of H-Lys(Lact)-OH.

lactose. MALDI spectrum did not give considerable signals.

Data obtained by ^{1}H NMR and ^{13}C spectra confirmed the molecular structure of the compound and are fully compatible with those reported by Olano et al. (1991). The presence of four different singlets at around 3.3 ppm are due to the four possibilities of ring closure: α- and β-furanose and -pyranose, respectively.

The synthetic H-Lys(Lact)-OH was used as authentic standard to attempt its quantification by LC-MS. Being that 471.2 amu was the highest peak detected by the mass spectrum (Figure 4), this mass was selected for a SIM detection to enhance the sensitivity. This value corresponds to the molecular ion of the product, while the water and the sodium adduct showed lower intensity. The reference curve obtained using different concentration of H-Lys(Lact)-OH is shown in Figure 5. A wide range of linearity between 1 μg mL^{-1} and 1 mg mL^{-1} was obtained; the detection limit was 10 ng mL^{-1}, and the standard deviation of the measure was lower than 5%.

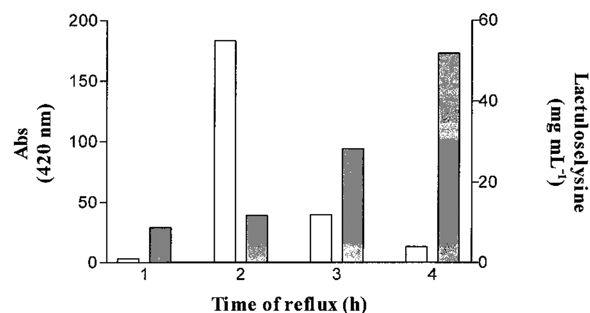

Figure 6. Amount of browning (420 nm, grey bars) and H-Lys(Lact)-OH (mg mL^{-1}, white bars) in a milklike model system.

H-Lys(Lact)-OH was then monitored in a lactose-lysine model system heated at different time on reflux (Monti et al., 1999). In this system the glycation on the Nα of the free lysine can also occur. Therefore the value measured is the sum of the two forms Lact-Lys-OH and H-Lys(Lact)-OH. As reported in Figure 6 the amount of the two molecules was maximal after 2 h of heating (56 mg mL^{-1}), whereas it decreased in the following 2 h (4 mg mL^{-1}). This result is in good agreement with previous data observed in the lactose-lysine model system. In fact, a consistent browning monitored at 420 nm was observed after 2 h of heating, and it is known that colored compounds are due to the degradation of the Amadori product (advanced and final stages of the MR). As pointed out by Porretta (1992) the Amadori product is a useful molecular marker for the first stages of the MR, whereas other compounds, such as pyrraline, are more suitable to monitor the advanced stages of the reaction.

In addition the synthetic H-Lys(Lact)-OH was used as the authentic standard for the quantification of protein-bound lactuloselysine. This compound represents a rather stable form of modified lysine in mild thermal treated milk samples (Mauron, 1981). Whey-

proteins from a commercial pasteurized skimmed milk were isolated and heated as described above and subjected to enzymatic hydrolysis. Quantification was carried out by LC-MS analysis using the same procedure adopted for the lactose-lysine model system. Two different experiments were performed: (i) direct injection into the ion source of the hydrolyzed solution and (ii) separation by HPLC followed by splitting of mobile phase into the ion spray source. The first experiment enabled the identification of the signals due to H-Lys-(Lact)-OH and its adducts confirming its presence in the raw sample. The second experiment enabled to quantify H-Lys(Lact)-OH using the single ion monitoring (SIM) detection of the signal at 471.2 amu corresponding to the molecular ion $[MH]^+$ of H-Lys(Lact)-OH. The concentration of H-Lys(Lact)-OH in the hydrolyzed sample was 13.3 μg/mL. Considering that the amount of lysines of β-lactoglobulin and α-lactalbumin is about 10% (Corradini, 1995), 50% of lysine residues were converted in lactuloselysine. This result, obtained on a severely heated sample, is in good agreement with that reported by Henle et al. (1991) who found a value of H-Lys(Lact)-OH in different heated milk ranging from 5 to 51%.

In conclusion, the use of FMOC amino acid and mild heating condition are used for the first time to synthesize lactuloselysine. This method can be applied for the synthesis of other compounds formed in the first stage of Maillard reaction.

The LC-MS technique proved to be a powerful tool for the detection of low amount of hydrophilic compounds such as those formed in the first stage of MR. The bottleneck of the proposed method is the hydrolysis of the protein measure which is time-consuming and fairly reproducible.

ABBREVIATIONS USED

MR, Maillard reaction; MRPs, Maillard reaction products; FMOC, 9-fluorenylmethoxy-carbonyl; MeOH, methanol; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DMF, *N,N*-dimethylformamide; TLC, thin-layer chromatography; H-Lys(Lact)-OH, N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine-OH; FMOC-Lys(Lact)-OH, $N\alpha$-9-fluorenylmethoxy-carbonyl-N_ϵ-(1-deoxy-D-lactulosyl-1)-L-lysine-OH; Lact-Lys-OH, N_α (1-deoxy-D-lactulosyl-1)-$N_\epsilon H_2$-L-lysine; HPLC, high performance liquid chromatography; UV, ultraviolet; LC-MS, liquid chromatography mass spectrometry; SIM, single ion monitoring; MALDI-ToF, matrix assisted laser desorbtion ionization—time-of-flight.

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