JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Formation of Peptide-Bound Heyns Compounds

RENÉ KRAUSE, KATHRIN SCHLEGEL, EVELYN SCHWARZER, AND THOMAS HENLE*

Institute of Food Chemistry, Technische Universität Dresden, D-01062 Dresden, Germany

The reaction of the N^{α}-hippuryllysine (BzGK) with fructose was investigated in two model systems to obtain an insight in fructose-induced modification of lysine in bakery products. After BzGK and fructose had been heated in a buffered low-moisture model system (80 °C, 60 min, $a_W = 0.86$, pH 7.4), formation of epimeric Heyns compounds N^{α}-hippuryl-N^{ϵ}-glucosyl-lysine (BzGGlcK) and N^{α}-hippuryl-N^{ϵ}-mannosyl-lysine (BzGManK) was clearly demonstrated using RP-HPLC with UV as well as MS detection. The Amadori compound N^{α}-hippuryl-N^{ϵ}-fructosyl-lysine (BzGFruK) was formed in glucose-containing samples. When BzGK was added to the dough of fructose-containing biscuits, the Heyns compounds were detectable after baking at 175 °C for 7 min. The yields of the Heyns compounds in the fructose-containing biscuits and the yield of the Amadori compound in the glucose-containing biscuits were determined to 33 and 63%, pointing to the fact that formation of substantial amounts of Heyns products is very likely in fructose-containing bakery products.

KEYWORDS: Maillard reaction; glycation; Heyns compound; Amadori compound; fructose; lysine

INTRODUCTION

Thermal processing of food containing both reducing sugars and proteins leads to the formation of pleasant flavors and a desirable color intermediate between yellow and dark brown in the course of the Maillard reaction. On the other hand, this reaction can adversely affect the nutritional value of food by the blocking of the essential amino acid lysine. The derivatization of protein-bound lysine by aldoses and the formation of Amadori compounds are well documented and widely used to evaluate the extent of the early stage of the Maillard reaction in food (*1*). In comparison to this, the derivatization of lysine by ketoses is generally recognized (*2, 3*), but very little is known about the occurrence of the so-called Heyns compounds as the first stable products of this reaction in food. Heyns compounds in particular were described within synthetic studies (*4*), and low amounts of Heyns compounds of free amino acids were found in long-stored lyophilized apricots (*5*), raw licorice (*6*), tomato powder (*7*), and tobacco (*8*) in earlier work. Thin layer chromatography (*6, 8*), paper chromatography (*5, 8*), and cation exchange HPLC and refractive index detection (*7*) were used for detection. In addition, protein-bound Heyns compounds were identified in lens protein in vivo using a RP-HPLC method (*9*). To the best of our knowledge, however, direct analysis of protein-bound Heyns compounds in food samples has not been described yet. As Heyns compounds could not be detected in aqueous ketose−casein systems heated for up to 40 min at 120 °C, the occurrence of this type of Maillard products in food was questioned generally (*3*).

However, as fructose is often used as sweetening agent, in particular, in food for diabetics, we decided to study the reaction of fructose with the ϵ-amino group of protein-bound lysine and the formation of Heyns compounds during thermal processing. To examine this hypothesis, the reactions of the lysine-containing dipeptide N^{α}-hippuryllysine (BzGK) with fructose and glucose were investigated in two model systems in detail with regard to bakery products.

* Corresponding author (telephone ++49-351-463-34647; fax ++49-351-463-34138; e-mail Thomas.Henle@chemie.tu-dresden.de).

MATERIALS AND METHODS

Chemicals and Reagents. N^{α}-Hippuryllysine (BzGK, N^{α}-benzoyl-glycyl-L-lysine) was obtained from Bachem (Weil, Germany). HPLC gradient grade methanol was from Sigma-Aldrich (Taufkirchen, Germany). Carboxypeptidase B (EC 3.4.17.2) (DFP-treated, 133 units/mg of protein, 5 mg of protein/mL), and all other chemicals used were of analytical grade and purchased from Fluka (Taufkirchen, Germany). The water used for the preparation of buffers and solutions was obtained using a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany).

High-Pressure Liquid Chromatography (HPLC). All analytical HPLC analyses were performed with a gradient pump system from Knauer (Berlin, Germany) with an online degasser, a K1500 solvent organizer, a K1001 pump, a K2501 Knauer variable-wavelength detector, a dynamic mixing chamber, and a column oven.

HPLC Analysis of Synthesis Mixtures and Samples of the Low-Moisture Model System. The analytical gradient pump system described above was used. Analytical separation was achieved using a 250 × 4.6 mm i.d., column filled with Eurosphere 100 RP 18, 5 μm, with an integrated guard column, 5 × 4 mm, filled with the same material (Knauer). The injection volume was 50 μL, the column temperature was set to 20 °C, and ultraviolet detection was performed at 230 nm. The mobile phase consisted of 0.01 M sodium phosphate buffer, pH 7.0 (solvent A), and methanol (solvent B). A linear gradient from 5 - 20 % B in 45 min at a flow rate of 0.5 mL/min was used.

HPLC Analysis of the Samples of Baked Biscuits. The analytical gradient pump system described above was used. Analytical separation was achieved using a stainless steel column, 250 × 3 mm, filled with

10.1021/jf073256y CCC: $40.75
Published on Web 03/05/2008

Knauer Eurospher 100, RP18-material of 5 μm particle size, with an integrated guard column, 5 × 3 mm, filled with the same material (Knauer). The injection volume was 50 μL, the column temperature was set to 20 °C, and ultraviolet detection was performed at 230 nm. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and 0.1% (v/v) formic acid in methanol (solvent B). A linear gradient from 10 to 25% B in 45 min at a flow rate of 0.2 mL/min was used.

Semipreparative HPLC. Semipreparative HPLC was performed with a gradient pump system from Knauer consisting of two K1001 pumps with 50 mL pump heads, an online degasser, a K1500 solvent organizer, a dynamic mixing chamber, a column oven, and a K2501 Knauer variable-wavelength detector. All semipreparative separations were performed using a stainless steel column, 250 × 16 mm, with a guard column, 30 × 16 mm, both filled with Knauer Eurospher 100 RP18-material of 15−25 μm particle size (Knauer). The flow rate was 6 mL/min, the temperature was set to 20 °C, and ultraviolet detection was at 280 nm. The first chromatographic stage was isocratic with a mixture of 0.01 M sodium phosphate buffer, pH 7.0, and methanol (94:6, v/v). The second stage (desalting) was realized using 0.05 M acetic acid (solvent A) and methanol (solvent B) as well as a linear gradient from 5 to 15% B in 45 min.

Mass Spectrometry. Mass spectrometric analysis was performed with a PerSeptive Biosystems Mariner time-of-flight mass spectrometry (TOF-MS) instrument equipped with an electrospray ionization source (ESI) working in the positive mode (Applied Biosystems, Stafford, TX). Calibration of the mass scale was established using a mixture of bradykinin, angiotensin I, and neurotensin.

After appropriate dilution with 1% acetic acid in 50% methanol, the sample was injected at a flow rate of 5 μL/min into the ESI source, using a syringe pump for direct ESI-TOF-MS analysis. In LC-ESI-MS experiments the outlet of the HPLC system was coupled to the ESI interface.

The monoisotopic molecular masses were determined using the peak with the lowest *m*/*z* ratio (monoisotopic peak) from prominent multiple-charged ions and the equation $M_r = z \times M_z - 1.0078z$, where M_r is the monoisotopic molecular mass, M_z is the *m*/*z* ratio, z is the number of charges, and 1.0078 is the mass of a proton.

LC-ESI-MS Analysis of the Samples of the Low-Moisture Model System and Baked Biscuits. LC-ESI-MS analysis was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a high-pressure gradient pump system, column oven, and diode array detector, which was coupled to a Mariner ESI-TOF-MS instrument (see above). A sample volume of 100 μL was applied. Separation was achieved using the same column, temperature, gradient, and solvents as described for HPLC analysis of the baked biscuits.

Nuclear Magnetic Resonance Spectroscopy. NMR spectra were recorded on a Bruker DRX 500 instrument (Reinstetten, Germany) with 500 MHz for ^{1}H and 125 MHz for ^{13}C experiments. Proton chemical shifts are given relative to internal HOD signal (4.70 ppm) for deuterium oxide solutions. Carbon chemical shifts are given relative to the signal of the external standard tetramethylsilane for deuterium oxide solutions. Assignments of ^{1}H and ^{13}C signals were based on ^{1}H−^{1}H COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), HMBC (heteronuclear multiple bond correlation), and DEPT (distortionless enhancement by polarization transfer) experiments.

Elemental Analysis. Elemental analysis data were obtained on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy).

Synthesis and Isolation of N^{α}-Hippuryl-N^{ϵ}-(1-deoxy-D-fructosyl)lysine (BzGFruK). The Amadori compound N^{α}-hippuryl-N^{ϵ}-(1-deoxy-D-fructosyl)lysine (BzGFruK) was prepared as described previously, using N^{α}-hippuryl-lysine as starting material (*10*). N^{α}-Hippuryl-lysine (0.62 g, 2.0 mmol) and 2.16 g (12.0 mmol) of anhydrous glucose were refluxed in 84 mL of methanol for 4 h. The reaction mixture was evaporated to dryness at 20 °C under reduced pressure, and the residue was dissolved in 9.0 mL of 0.2 M *N*-ethylmorpholine/acetic acid buffer, pH 8.0. The pH value was adjusted to 8.0 with *N*-ethylmorpholine. Twenty-seven microliters of a solution of carboxypeptidase B (665 units/mL) was added, to a final activity of 2 units/mL. The solution was incubated in a screw-cap culture tube at 25 °C. Hydrolysis of unreacted N^{α}-hippuryl-lysine was followed by analytical HPLC and was complete after 24 h. After that, the solvent was evaporated at 40 °C under reduced pressure and the solid was dissolved in 9 mL of 0.01 M sodium phosphate buffer, pH 7.0. Isolation of BzGFruK was achieved by semipreparative HPLC as described above. After desalting, the BzGFruK-containing fractions were lyophilized and stored at –20 °C. Purity and identity were checked using analytical RP-HPLC, ^{1}H NMR, ^{13}C NMR, and mass spectrometry as well as elemental analysis. Analytical data were consistent with those reported previously (*10*).

Synthesis and Isolation of N^{α}-Hippuryl-N^{ϵ}-(2-deoxy-D-glucosyl)lysine (BzGGlcK) and N^{α}-Hippuryl-N^{ϵ}-(2-deoxy-D-mannosyl)lysine (BzGManK). A mixture of the Heyns compounds BzGGlcK and BzGManK was synthesized according to the method of Heyns et al. (*4, 11*), using N^{α}-hippuryl-lysine as starting material. The used HPLC procedure for purification of BzGFruK (*10*) was adapted for isolation of the Heyns compounds. N^{α}-Hippuryl-lysine (0.62 g, 2.0 mmol) and 3.61 g (20.0 mmol) of anhydrous fructose were heated at 75 °C in 25 mL of dry dimethyl sulfoxide for 4 h. The reaction mixture was evaporated to dryness at 35 °C in vacuo (0.25 mbar), and the residue was dissolved in 9.0 mL of 0.2 M *N*-ethylmorpholine/acetic acid buffer, pH 8.0. The pH value was adjusted to 8.0 with *N*-ethylmorpholine. Twenty-seven microliters of a solution of carboxypeptidase B (665 units/mL) was added, to a final activity of 2 units/mL. The solution was incubated in a screw-cap culture tube at 25 °C. Hydrolysis of unreacted N^{α}-hippuryl-lysine was followed by analytical HPLC and was complete after 24 h. After that, the solvent was evaporated at 40 °C under reduced pressure and the solid was dissolved in 9 mL of 0.01 M sodium phosphate buffer, pH 7.0, and subjected to semipreparative HPLC as described above. The desalted mixed fractions containing BzGGlcK and BzGManK were lyophilized and stored at –20 °C.

BzGGlcK: ESI-MS, positive mode, $[M + H]^+$ *m*/*z* 470.2; ^{1}H NMR (500 MHz, D_2O, α-pyranose), δ 1.29 (2H, m, K−H4), 1.61 (1H, m, K−H3A), 1.61 (2H, m, K−H5), 1.75 (1H, m, K−H3B), 3.02 (2H, m, K−H6), 3.13 (1H, dd, H2′), 3.34 (1H, dd, H4′), 3.65 (1H, dd, H6A′), 3.72 (1H, dd, H5′), 3.72 (1H, d, H6B′), 3.80 (1H, dd, H3′), 3.97 (1H, d, G-H2A), 4.03 (1H, d, G-H2B), 4.15 (1H, dd, K−H2), 5.41 (1H, d, H1′), 7.42 (1H, t, Bz-Hm), 7.52 (2H, t, Bz-Hp), 7.70 (2H, d, Bz-Ho); (β-pyranose), δ 4.91 (1H, d, H1′), 2.90 (1H, dd, H2′): ^{13}C NMR (125 MHz, D_2O, α-pyranose), δ 21.93 (t, K−C4), 24.81 (t, K−C5), 30.77 (t, K−C3), 43.00 (t, G−C2), 45.36 (t, K−C6), 54.28 (d, K−C2), 59.75 (d, C2′), 60.17 (t, C6′), 69.40 (d, C3′), 69.52 (d, C4′), 71.14 (d, C5′), 87.66 (d, C1′), 127.11 (d, Bz−Co), 128.74 (d, Bz-Cm), 132.40 (d, Bz−Cp), 132.68 (s, Bz−Ci), 170.80 (s, G−C1), 171.08 (s, Bz−C1), 178.27 (s, K−C1); (β-pyranose), δ 91.9 (d, C1′). Elemental analysis: $C_{21}H_{31}N_3O_9 \times 0.6\ CH_3COOH \times 1.6\ H_2O$ (MW = 534.34); calcd, C 49.90%, H 6.90%, N 7.86%; found, C 49.94%, H 6.92%, N 7.81%. Yield = 222 mg (molar yield = 20.8%).

Studies Related to the Formation of Heyns Compounds in a Low-Moisture Model System. Phosphate buffers (0.1 M), pH 6.0 or 7.4, and 1.78 g of disodium hydrogen phosphate were dissolved in 90 mL of water, the pH value was adjusted using hydrochloric acid, and the volume was adjusted to 100 mL.

The model mixtures were prepared with a lysine-to-sugar ratio of 1:6 as follows: 30.7 mg of BzGK and 108.0 mg of fructose (or glucose) were dissolved in 20 mL of 0.1 M phosphate buffer, pH 6.0 or 7.4. After the addition of 3.861 g of microcristalline cellulose (Avicel), the mixture were intensively mixed using an Ultra Turrax (Janke & Kunkel GmbH, Staufen, Germany). The samples were then deep frozen, lyophilized, and homogenized. The water activity of the powdered model mixtures was adjusted to an a_W value of 0.52 or 0.86 by storing them in Petri dishes placed in desiccators over saturated solutions of magnesium nitrate hexahydrate ($a_W = 0.52$) or potassium chloride ($a_W = 0.86$) at ambient temperature (22 °C) for 6 days (*12*).

Aliquots of 100 mg of the equilibrated model mixtures were heated in sealed screw-cap vials for up to 4 h at 80 or 100 °C in a sand bath in a drying oven. After heating, the samples were extracted with 1.0 mL of 0.2 M *N*-ethylmorpholine/acetic acid buffer, pH 8.0, and centrifuged. Seven hundred microliters of the supernatant was mixed with 2.1 μL of a solution of carboxypeptidase B (665 units/mL), to a final activity of 2 units/mL. After incubation at 25 °C for 24 h, an aliquot of 600 μL was diluted with 600 μL of solvent A (10 mM sodium phosphate buffer, pH 7.0) and subjected to HPLC analysis.

The same procedure of analysis was applied for an additional aliquot of 100 mg of the equilibrated model mixture but without heating and treatment with carboxypeptidase B to determine the initial content of BzGK. Each experiment, beginning with or without heating, was made in triplicate.

The identities of the two product peaks in the chromatogram of the heated BzGK−fructose mixtures were elucidated using LC-ESI-MS. Therefore, the fractions of the two peaks were collected manually using HPLC with the phosphate-containing eluent system (six runs), concentrated, and subsequently analyzed using LC-ESI-MS with the formic acid containing eluent system (see above).

Studies Related to the Formation of Heyns Compounds in Biscuits. A biscuit dough was prepared from 10.00 g of wheat flour, 5.00 g of butter, 5.00 g of fructose (or another sugar component: glucose, sucrose, or sorbitol), 0.30 g of commercial baking powder (sodium bicarbonate, sodium diphosphate), 25 mg of sodium chloride, and 60 mg of BzGK (dissolved in 1.0 mL of water). The dough was rolled out to a thickness of about 5 mm, and biscuits were cut out with a diameter of about 5 cm. The biscuits were baked in a laboratory drying oven at 175 °C for 7 min. After cooling, the biscuits were ground using a mill. A sample of 6.5 g was defatted using 20 mL of petroleum ether (boiling point range = 30−50 °C) in a screw-cap centrifuge tube. The sample were centrifuged, and the petroleum ether was discarded. After this procedure had been repeated, residual petroleum ether was removed with a nitrogen stream. The residue was transferred in a 100 mL volumetric flask and extracted with 70 mL of 40% ethanol (v/v) under stirring (magnetic stirrer) for 60 min and using ultrasonic treatment for 5 min. After that, the volume was adjusted to 100 mL. The extract was filtered and centrifuged (15000*g*, 10 min). Two milliliters of the purified extract was evaporated to dryness at 40 °C under reduced pressure, and the residue was dissolved in 2.00 mL of 0.01 M sodium phosphate buffer, pH 7.0. An aliquot of 500 μL was mixed with 500 μL of 0.4 M *N*-ethylmorpholine/acetic acid buffer, pH 8.0 (= solution E). An aliquot of 500 μL of solution E was mixed with 1.5 μL of a solution of carboxypeptidase B (665 units/mL), to a final activity of 2 units/mL (= solution F). After incubation at 25 °C for 24 h, an aliquot of 68 μL was diluted with 932 μL of solvent A (0.1% formic acid) and subjected to HPLC analysis for determination of the content of BzGGlcK and BzGManK. An aliquot of 68 μL of solution E was diluted with 932 μL of solvent A (0.1% formic acid) and subjected to HPLC analysis for determination of the total content of BzGK, BzGGlcK, and BzGManK. Each experiment, starting with the preparation of the dough, was made in triplicate.

An aliquot of 200 μL of carboxypeptidase B-treated solution F was diluted with 800 μL of solvent A (0.1% formic acid) and subjected to LC-ESI-MS analysis.

RESULTS AND DISCUSSION

The reaction of the ϵ-amino group of lysine with fructose or glucose and the formation of Heyns and Amadori compounds was studied in two model systems to investigate a possible formation of these lysine derivatives in bakery products. The peptide derivative BzGK was used as model for protein-bound lysine because the N^{α}-hippuryl moiety allowed a convenient direct analysis of the samples by RP-HPLC and UV detection. The putative derivatives resulting from the reaction with fructose are the epimeric Heyns compounds BzGGlcK and BzGManK, whereas the reaction with glucose leads to the Amadori compound BzGFruK (**Figure 1**). These compounds were synthesized as reference materials for chromatographic analysis. Isolation from the synthesis mixtures was achieved using a two-step semipreparative RP-HPLC method after hydrolysis of unreacted BzGK with carboxypeptidase B (EC 3.4.17.2) as described previously (*10*). Because the reaction of BzGK and fructose leads to the formation of both Heyns compounds, which could not be separated by HPLC, a mixture of the epimeric Heyns compounds was obtained. As heating experiments with the fructose-containing model systems resulted also in the formation of both compounds, it was concluded that the isolate was sufficient as reference material. The Amadori compound BzGFruK was prepared using BzGK and glucose as starting material in chromatographically pure form. The structural identity of the desired compounds was confirmed by mass spectrometry and NMR spectroscopy. Elemental analysis and NMR spectroscopy indicated residual acetic acid from the HPLC eluent and some water after lyophilization. Because chromatography and NMR analysis revealed no byproduct, it was deduced that the purity of the isolates was adequate for the purposes of this study.

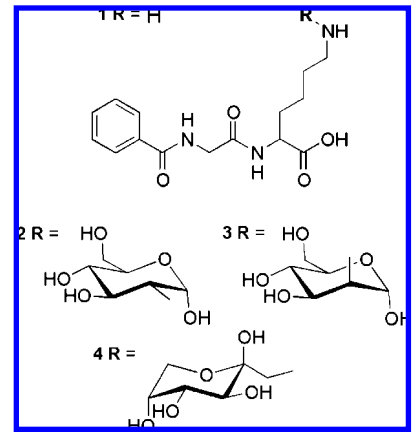

Figure 1. Structures of BzGK (**1**), BzGGlcK (**2**), BzGManK (**3**), and BzGFruKe (**4**).

To compare the formation of Heyns and Amadori products under reduced water activity, first the reaction of BzGK with fructose or glucose was investigated in a buffered low-moisture model system with microcrystalline cellulose as support. Mixtures consisting of BzGK and the sugar component (molar ratio 1:6) in phosphate buffer (pH 6.0 or 7.4) as well as microcrystalline cellulose were prepared and lyophilized. After that, the water activity was adjusted to $a_W = 0.52$ or 0.86 at 20 °C. Water activities and pH values were chosen with respect to bakery products. It is interesting that commercial biscuits have a relatively high pH value of 7.4, if a 10% suspension is measured. The reason may be the bicarbonate salt in the baking powder. The molar ratio between BzGK and fructose, namely, 1:6, was chosen to keep the Maillard reaction in an "early stage" and to avoid caramelization and intense browning reactions.

The model mixtures were heated and the degradation of BzGK and formation of peptide bound rearrangement products were monitored using RP-HPLC with UV detection. Chromatographic separation of the modified lysine derivatives from the starting peptide was optimized by treatment with carboxypeptidase B (EC 3.4.17.2) before chromatography. This enzyme is used in sequence analysis of peptides and hydrolyses the basic amino acids arginine and lysine from the C terminus of peptides (*13*). Thus, unreacted BzGK was hydrolyzed to hippuric acid (BzG) and lysine, whereas the modified lysine peptides remained unchanged. On this basis, separation of the rearrangement products was possible (**Figure 2**). The epimeric Heyns compounds BzGGlcK and BzGManK were detected in the fructose-containing samples and the Amadori compound BzGFruK was detected in that with glucose. Identity of the products was examined and confirmed by co-injection of sample and reference material in the HPLC system as well as by LC-ESI-MS. **Figure 3** shows the time dependency of the product concentrations in the samples at pH 7.4 and $a_W = 0.86$. As can be seen from **Figure 3**, the formation of Heyns or Amadori compounds, respectively, is significant in both model systems. Lysine

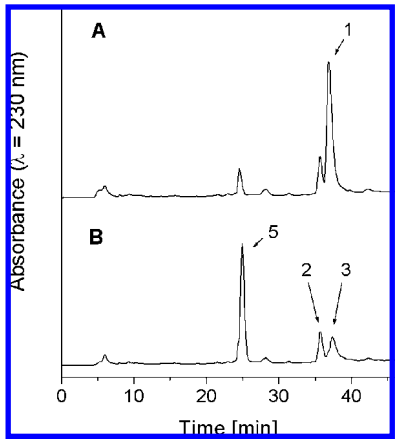

Figure 2. HPLC analysis of heated mixtures of BzGK (peak 1) with fructose (molar ratio 1:6, pH 7.4, $a_w = 0.52$, 60 min, 100 °C) (**A**) before and (**B**) after hydrolysis with carboxypeptidase B. Peaks: 2, BzGGlcK; 3, BzGManK; 5, hippuric acid.

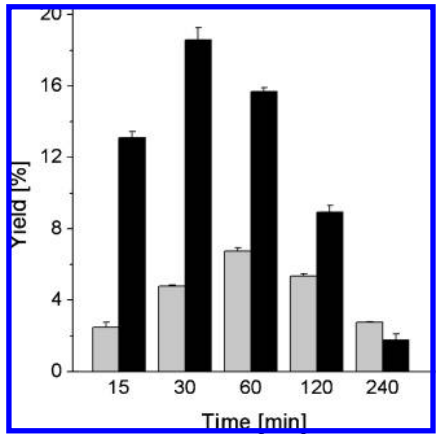

Figure 3. Yield of Heyns compounds in heated mixtures of BzGK and fructose (gray bars) and of Amadori compound in heated mixtures of BzGK and glucose (black bars) (molar ratio of BzGK to sugar 1:6, pH 7.4, $a_W = 0.86$, 80 °C). Mean value ± standard deviation of three separate determinations is shown.

derivatization was calculated by comparing peak areas of BzGK before and after heating. For example, with respect to the initial lysine content, the yields were measured with 8% for the Heyns compounds and with 17% for the Amadori compound (pH 7.4, $a_W = 0.86$, 100 °C, 60 min). Lysine derivatization was calculated by comparing peak areas of BzGK before and after heating. The total derivatization of lysine, however, was considerably higher: 48 and 70% were measured in the fructose-containing and in the glucose-containing samples, respectively. According to these results, besides the formation of the rearrangement products, degradation of the Maillard products takes place at the same time and. consequently, the measured contents are the result of these two processes.

Figure 4 shows the yields of rearrangement products at different a_W and pH values after heating at 80 or 100 °C for 60 min. When compared with the Amadori compound, lower amounts of Heyns compounds are formed at the same reaction conditions. This may be attributed to the specific properties of Heyns compounds, their lower rate of formation, the reversibility of the formation reaction, and significantly faster conversion to other products. To interpret the different yields of the rearrangement products at the chosen a_W and pH values, both the formation reactions and degradation reactions must be considered as mentioned above. At $a_W = 0.52$, the yields are higher than at $a_W = 0.86$ for the same product. This might be explained by a higher stability and a certain accumulation of the rearrangement products at $a_W = 0.52$, as is known for Amadori compounds (*14*). Furthermore, the samples at pH 7.4 show after heating at 80 °C a higher content than the samples at pH 6.0. Consequently, heating of the samples at 80 °C for 60 min at pH 7.4 versus pH 6.0 favors more the formation of the rearrangement products. The results have reversed after heating at 100 °C for 60 min, samples with pH 6.0 show a higher content than the samples with pH 7.4 under these conditions. At the increased temperature of 100 °C it may by of importance that the higher pH value accelerates not only the formation of the rearrangement products but also its degradation.

In conclusion, the studies clearly revealed the formation of Heyns or Amadori compounds for the low-moisture model systems used. According these results, the formation of Heyns compounds could be expected in fructose-containing bakery products. To examine this hypothesis, the formation of the rearrangement products was investigated under conditions more closely resembling baked food. Biscuits were baked for this purpose. A usual recipe for rolled biscuits was used with the ingredients of wheat flour, butter, fructose or an other sugar component, baking powder, and salt. Additionally, BzGK dissolved in a small amount of water was added to the dough as a model for protein-bound lysine. Therewith, the detection of rearrangement products was possible using RP-HPLC with UV detection as in the model systems used before. The molar ratio of added BzGK to lysine from the ingredients was 1:1. The temperature of the baking oven was set to 175 °C, and a baking time of 7 min was chosen to obtain sensorially pleasant, slightly browned biscuits.

Figure 5 shows the HPLC analysis of the biscuits containing different sugar components using 0.1% formic acid and 0.1% formic acid in methanol as eluents. In comparison to the phosphate buffer containing eluent system used for analysis of cellulose-containing model mixtures, this eluent system improved the peak form of the BzGFruK peak in the biscuit samples containing glucose. HPLC analysis revealed the formation of the epimeric Heyns compounds BzGGlcK and BzGManK in the fructose-containing biscuits and of the Amadori compound BzGFruK in the glucose-containing biscuits. Compared with this, no rearrangement product was detected in the biscuits with sorbitol or sucrose as ingredient (**Figure 5**). This was expected, because the sugar alcohol sorbitol does not contain a reactive aldehyde or keto group. Sucrose is a nonreductive disaccharide and apparently was not hydrolyzed under the baking conditions used. As no Heyns compounds were detectable in the fructose-containing biscuit samples before heating (data not shown), it was clearly proven that its formation is exclusively due to heat-induced reactions between peptide-bound lysine and fructose.

The detection of the rearrangement products in the biscuits with fructose or glucose was confirmed by co-injection of sample and reference material in the HPLC system as well as by mass spectrometric measurement via LC-ESI-MS. **Figure 6** shows the mass spectrometric verification of BzGGlcK detection in a the sample of the fructose-containing biscuits. As can be seen, the mass spectrum of the product peak of a sample of the fructose-containing biscuits was dominated by a $[M + H]^+$ ion with m/z 470.42, which led to a monoisotopic mass M_r of 469.41 (**Figure 6**). This result was consistent with the expected value for BzGGlcK (M_r of 469.49). Accordingly, BzGFruK was identified by LC-ESI-MS (**Figure 7**).

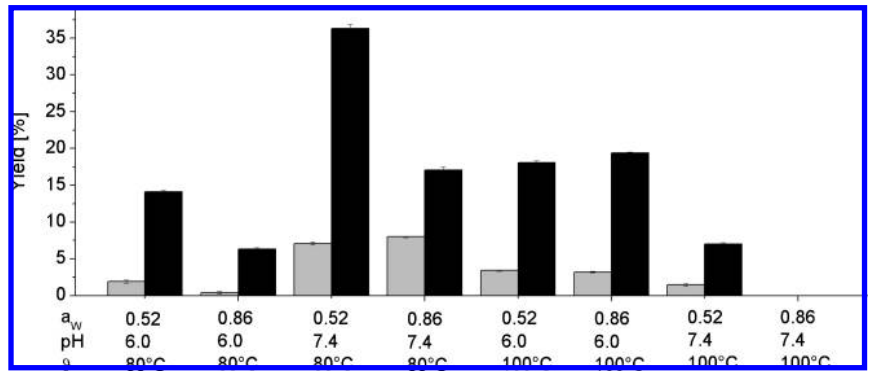

Figure 4. Yield of Heyns compounds in heated mixtures of BzGK and fructose (gray bars) and of Amadori compound in heated mixtures of BzGK and glucose (black bars) (molar ratio of BzGK to sugar 1:6, heating time = 60 min). Mean value ± standard deviation of three separate determinations is shown.

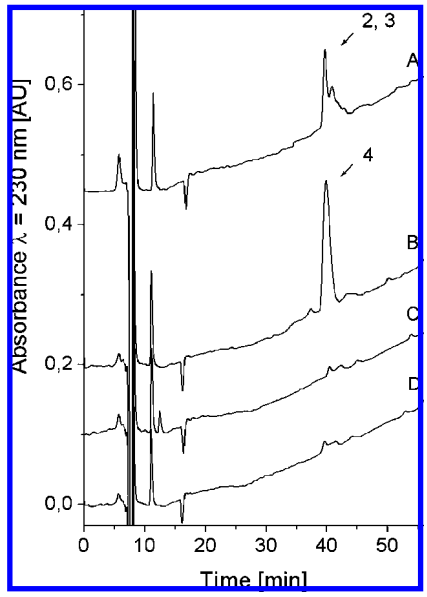

Figure 5. HPLC analysis of baked biscuits containing BzGK as well as fructose (**A**), glucose (**B**), sorbitol (**C**), or sucrose (**D**) after hydrolysis with carboxypeptidase B. Peaks: 2, BzGGlcK; 3, BzGManK; 4, BzGFruK.

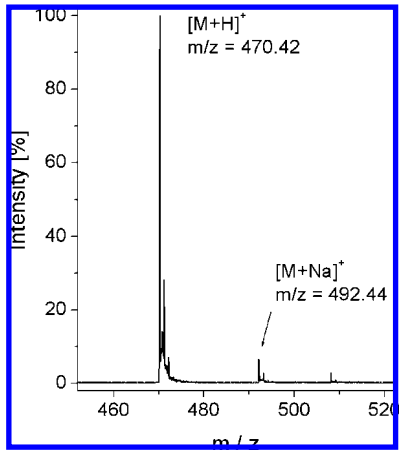

Figure 6. Mass spectrometric analysis of the product peak (BzGGlcK) in the fructose-containing biscuits using LC-ESI-MS in positive mode.

Quantitative studies demonstrated high yields of the rearrangement products under the baking conditions used in the baked biscuits containing fructose or glucose. The yields of the Heyns compounds in the fructose-containing biscuits and the yield of the Amadori compounds in the biscuits with glucose were 33 and 63%, respectively. Consequently, the baking

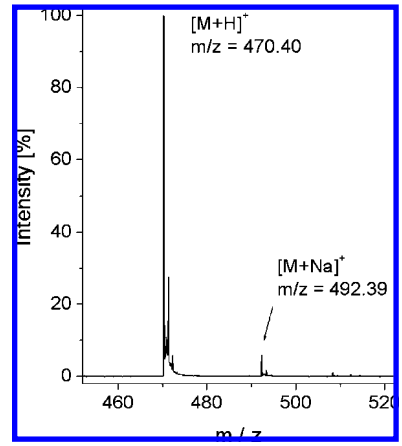

Figure 7. Mass spectrometric analysis of the product peak (BzGFruK) in the fructose-containing biscuits using LC-ESI-MS in positive mode.

conditions used for the biscuits, for example, oven temperature (175 °C) and pH value (pH 7.4, if a 10% suspension is measured), favor the formation of the rearrangement products. Due to the short baking time, degradation reactions apparently are not of substantial importance.

Whereas the formation of Amadori compounds in the model systems is well-documented for baked food, very little is known about the occurrence of Heyns compounds in food. Because the model studies unequivocally showed the formation of Heyns compounds, it can be concluded that they are also formed in substantial amounts in fructose-containing bakery products. In particular, the formation of Heyns compounds may be of importance in bakery products for diabetic patients, in which saccharose as sweetener is substituted by fructose. Furthermore, products containing honey, high-fructose corn syrup, or corresponding sweeteners may contain Heyns compounds depending on the heating conditions applied during food processing. With that, a fructose-induced lysine derivatization could have nutritional importance. It is well-known that Amadori compounds of lysine are not available as lysine source during digestion (*1*). Furthermore, a possible antinutritional role for Maillard compounds is under debate (*15*). With that in mind, further studies must deal with analytical approaches for the identification and quantification of protein-bound Heyns compounds in food to clarify a possible impact of these lysine derivatives on the nutritional quality of relevant food items.

ABBREVIATIONS USED

BzGFruK, *N*-α-hippuryl-N^{ϵ}-(1-deoxy-D-fructosyl)lysine; BzGGlcK, *N*-α-hippuryl-N^{ϵ}-(2-deoxy-D-glucosyl)lysine; BzGK, *N*-α-

hippuryllysine; BzGManK, *N*-α-hippuryl-N^{ϵ}-(2-deoxy-D-mannosyl)lysine; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; RP, reversed phase.

ACKNOWLEDGMENT

We thank Dr. Uwe Schwarzenbolz, Institute of Food Chemistry, for acquisition of the mass spectrometric data. We are grateful to the members of the Institute of Organic Chemistry, Technische Universität Dresden, namely, Dr. Margit Gruner and Annett Rudolf, for recording the NMR spectra and Anke Peritz for performing the elemental analysis.

LITERATURE CITED

(1) Henle, T. Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids* **2005**, *29*, 313–322.

(2) Matsuda, T.; Kato, Y.; Nakamura, R. Lysine loss and polymerization of bovine β-lactoglobulin by amino carbonyl reaction with lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). *J. Agric. Food Chem.* **1991**, *39*, 1201–1204.

(3) Brands, C. M. J.; Van Boekel, M. A. J. S. Reactions of monosaccharides during heating of sugar−casein systems: building of a reaction network model. *J. Agric. Food Chem.* **2001**, *49*, 4667–4675.

(4) Heyns, K.; Breuer, H.; Paulsen, H. Darstellung und Verhalten der 2-*N*-Aminosäure-2-desoxy-glucosen ("Glucose-Aminosäuren") aus Glycin, Alanin, Leucin und Fructose. *Chem. Ber.* **1957**, *90*, 1374–1386.

(5) Anet, E. F. J. L.; Reynolds, T. M. Reactions between amino acids, organic acids, and sugars in freeze-dried apricots and peaches. *Aust. J. Chem.* **1957**, *10*, 193–197.

(6) Vondenhof, T.; Glombitza, K. W.; Steiner, M. Lösliche Kohlenhydrate in Rohlakritzen (*Succus Liquiritiae*). *Z. Lebensm. Unters. Forsch.* **1973**, *152*, 345–347.

(7) Van den Ouweland, G. A. M.; Peer, H. G.; Tjan, S. B. Occurrence of Amadori and Heyns rearrangement products in processed foods and their role in flavor formation. In *Flavor of Foods and Beverages*; Charalambous, G., Inglett, G. E., Eds.; Academic Press: New York, 1978; Vol. 1, pp 131–143.

(8) Wahl, R. Aminozucker in Tabak. *Z. Lebensm. Unters. Forsch.* **1972**, *148*, 94–96.

(9) McPherson, J. D.; Shilton, B. H.; Walton, D. J. Role of fructose in glycation and cross-linking of proteins. *Biochemistry* **1988**, *27*, 1901–1907.

(10) Krause, R.; Knoll, K.; Henle, T. Studies on the formation of furosine and pyridosine during acid hydrolysis of different Amadori products of lysine. *Eur. Food Res. Technol.* **2003**, *216*, 277–283.

(11) Heyns, K.; Noack, H. Die Umsetzung von D-Fructose mit L-Lysin und Arginin und deren Beziehung zu nichtenzymatischen Bräunungsreaktionen. *Chem. Ber.* **1962**, *95*, 720–727.

(12) Rockland, L. B. Saturated salt solutions for static control of relative humidity between 5 and 40 °C. *Anal. Chem.* **1960**, *32*, 1375–1376.

(13) Ambler, R. P. Enzymatic hydrolysis with carboxypeptidases. *Methods Enzymol.* **1972**, *25*, 143–154.

(14) Ciner-Doruk, M.; Eichner, K. Bildung und Stabilität von Amadori-Verbindungen in wasserarmen Lebensmitteln. *Z. Lebensm. Unters. Forsch.* **1979**, *168*, 9–20.

(15) Henle, T. Dietary advanced glycation end products—a risk to human health. *Mol. Nutr. Food Res.* **2007**, *51*, 1075–1078.

Received for review November 7, 2007. Revised manuscript received January 24, 2008. Accepted January 29, 2008.

JF073256Y