Development of a Stable Isotope Dilution Assay for an Accurate Quantification of Protein-Bound N_ϵ-(1-Deoxy-D-fructos-1-yl)-L-lysine Using a ^{13}C-Labeled Internal Standard

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Syntheses of the labeled Amadori compound [$^{13}C_6$]-N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysine ([$^{13}C_6$]-DFLys) and the labeled glycated tetrapeptide Ala-[$^{13}C_6$]-DFLys-Leu-Gly are presented. The compounds were used in the development of stable isotope dilution assays for the quantification of the degree of glycosylation of bovine serum albumin treated for 20 min at 95 °C in the presence of glucose. The experiments revealed that the use of the labeled standards in combination with LC/MS allowed the exact quantification of protein-bound DFLys with the high recovery rate of 95% (at a spike level of 150 nmol/mg of protein) and a low detection limit of 5 nmol/mg of protein. The data revealed, however, that DFLys is significantly degraded during the enzymic hydrolysis of the protein backbone generally needed in the quantification procedure and, furthermore, incomplete digestion of the protein was observed. Both sources of errors were clearly overcome by using in particular the labeled peptide as the internal standard.

Keywords: *N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysine; fructosyl-L-lysine; protein glycation; stable isotope dilution assay; Maillard reaction; Amadori compound; LC/MS*

INTRODUCTION

Thermal treatment of foods as well as prolonged storage may lead to losses in nutritive value, especially by a reaction of the ϵ-amino group of protein-bound L-lysine with carbohydrates or their degradation products (Ledl and Schleicher, 1992). The unstable glucosylamine (I in Figure 1), formed as a first reaction product, is rapidly converted into the corresponding α-ketosylamine (II in Figure 1), which is known as Amadori rearrangement (Amadori, 1931; Kuhn and Weygand, 1937). The β-pyranose N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysine (DFLys) (Figure 1) is biologically not available on digestion (Finot et al., 1981), thereby reducing the quality of the respective food.

For this reason different methods have been proposed for the quantification of the extent of lysine losses based on glycosylation products formed during food processing. The so-called "furosine method" is based on the observation that acidic hydrolysis of proteins containing bound glucosylated L-lysine leads to the formation of a constant ratio of furosine (32%; I in Figure 2) and pyridosine (10%; II in Figure 2), besides regeneration of lysine (Finot et al., 1977). The HPLC analysis of furosine and pyridosine has been used in recent years to quantify L-lysine damage in thermally processed foods. Henle et al. (1991) showed that, due to the unreproducible formation of these hydrolysis products, the "furosine method" leads to a distinct underestimation of lysine modification by a factor of 4−5.

Henle and Klostermeyer (1993), therefore, developed a method for the direct measurement of DFLys in casein/glucose mixtures based on synthesized DFLys as external standard. Because Amadori products are known to be unstable in acidic as well as alkaline media and the digestion procedure was performed at pH 2.0 and 8.2, a significant degradation of DFLys can be expected to occur during this hydrolytic process. Because of this instability of DFLys and the fact that no internal standard was used by the authors, it is questionable whether the HPLC method yields exact results on protein-bound DFLys.

It might, therefore, be a more promising approach to develop a stable isotope dilution assay (SIDA) based on a carbon-13-labeled glycosylated peptide as the internal standard. Similar requirements in the analysis of food odorants (Sen et al., 1991), veterinary drugs (Suhre et al., 1981), food-packaging additives (Castle et al., 1988), and mycotoxins (Rychlik and Schieberle, 1998, 1999) have proven the superiority of the SIDA compared to conventional methods using internal standards of chemical structures different from the analyte.

The purpose of the present investigation was, therefore, to develop a SIDA for the quantification of protein-bound DFLys.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: N_α-9-fluorenylmethoxycarbonyl-N_ϵ-*tert*-butoxycarbonyl-L-lysyl-L-leucine hydrochloride [N_α-FMOC-Lys(N_ϵ-BOC)-Leu·HCl] and N_α-9-fluorenylmethoxycarbonyl-L-lysine hydrochloride [N_α-FMOC-Lys·HCl] (Bachem, Heidelberg, Germany); 1-hydroxybenzotriazol, dicyclohexylcarbodiimide, morpholine, ninhydrine, triethylamine, glycine *tert*-butyl ester hydrochloride (Gly-OtBu·HCl), and [$^{13}C_6$]-D-glucose (Aldrich, Steinhein,

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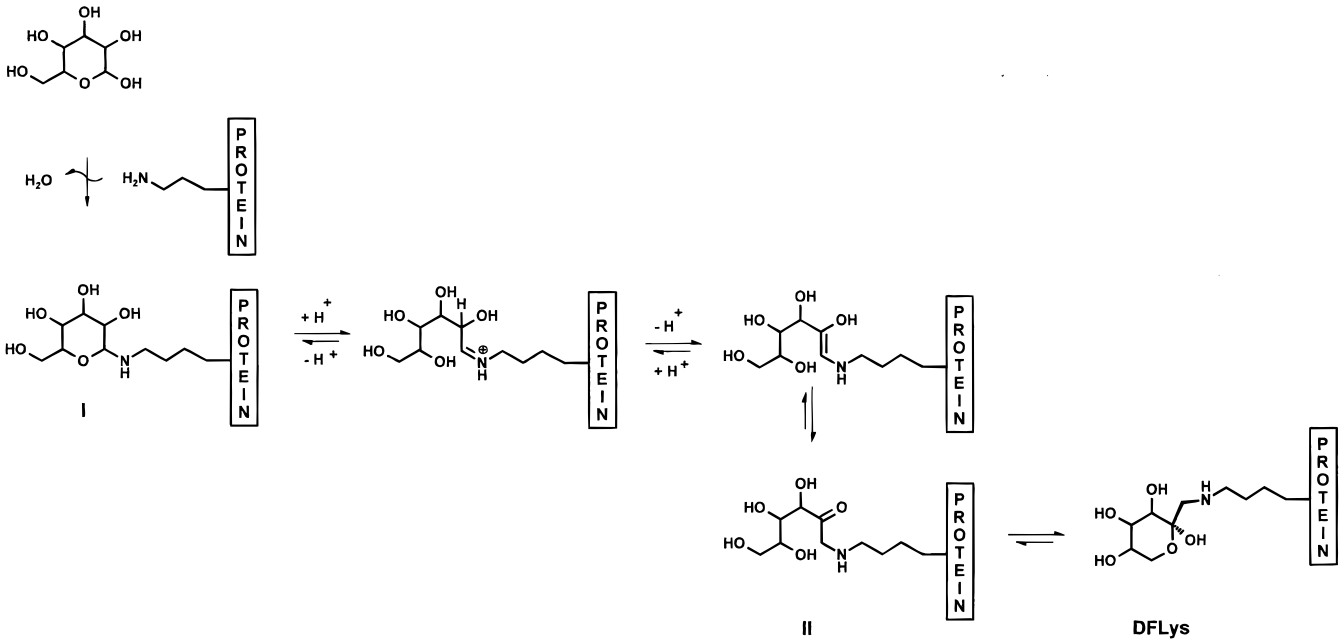

Figure 1. Formation of protein-bound DFLys by nonenzymic glucosylation of lysine side chains of proteins

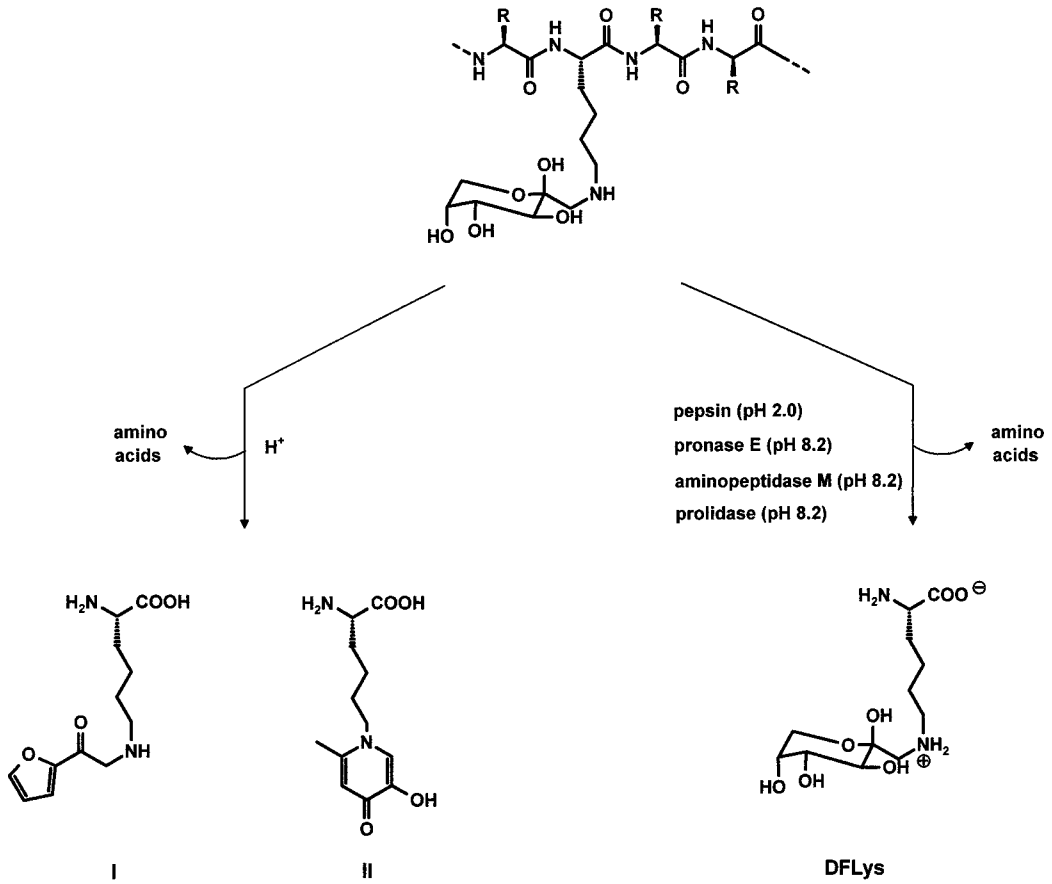

Figure 2. Reaction product formed by acid-catalyzed (A) or enzymic (B) hydrolysis of glucosylated proteins.

Germany), trifluoroacetic acid (TFA) and 9-fluorenylmethoxycarbonyl-L-alanine hydrochloride (Fluka, Buchs, Switzerland); dimethylformamide, anhydrous D-glucose (Merck, Darmstadt, Germany); bovine serum albumin (BSA) (Serva, Heidelberg, Germany); trishydrochloride, Pronase E (from *Streptomyces griseus*), pepsin (from porcine stomach mucosa), prolidase (from porcine kidney), and aminopeptidase M (from porcine kidney microsomes) were purchased from Sigma (Deisenhofen, Germany). Dichloromethane was distilled at 42 °C prior to use. Anhydrous methanol was prepared by distillation of a suspension of $LiAlH_4$ in methanol in vacuo. Dimethyl-d_6 sulfoxide (DMSO-d_6), methanol-d_4 (CD_3OD), and deuterium oxide (D_2O) were obtained from Isocom (Landshut, Germany).

Syntheses. *(A) N_ϵ-(1-Deoxy-D-fructos-1-yl)-L-lysine (DFLys) and N_ϵ-([$^{13}C_6$]-1-Deoxy-D-fructos-1-yl)-L-lysine ([$^{13}C_6$]-DFLys). (1) N_α-Fluorenylmethoxycarbonyl-N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysine (N_α-FMOC-DFLys) and N_α-9-Fluorenylmethoxycarbonyl-N_ϵ-([$^{13}C_6$-1-deoxy-D-fructos-1-yl)-L-lysine (N_α-FMOC-[$^{13}C_6$]-DFLys).* A solution of N_α-FMOC-Lys·HCl (0.5 mmol) and triethylamine (0.5 mmol) in methanol (2 mL) was stirred for 20 min and then either anhydrous D-glucose or [$^{13}C_6$]-D-glucose (2 mmol), respectively, was added and the reaction mixture was stirred

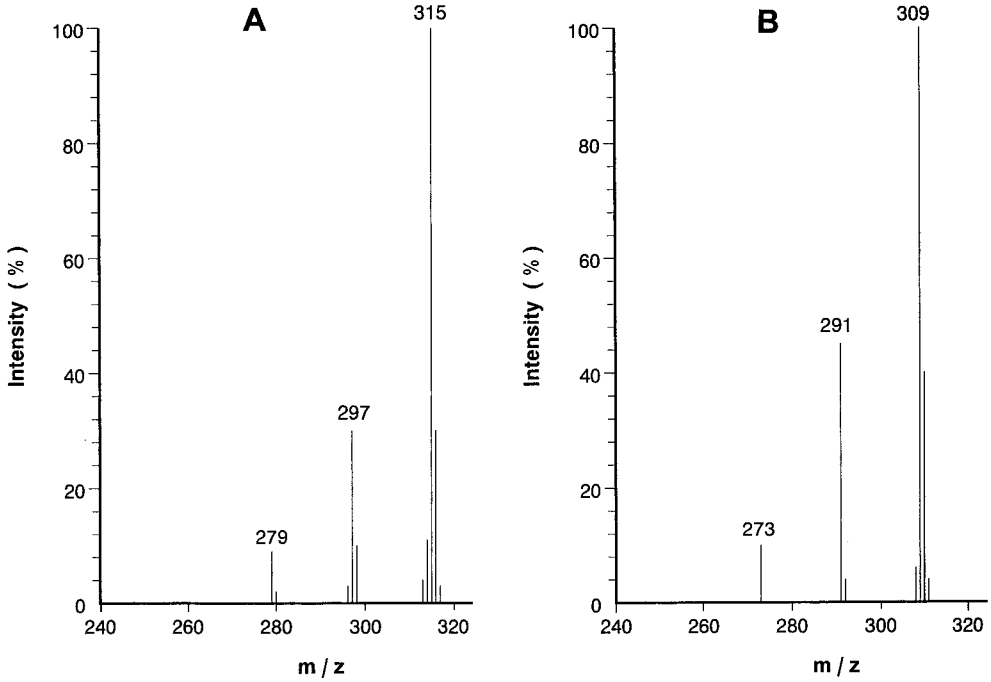

Figure 3. LC/MS spectra of (A) [$^{13}C_6$]-DFLys and (B) DFLys obtained by ESI.

for another 3 h at 64 °C under an atmosphere of nitrogen. After removal of the solvent in vacuo, the residue was taken up in water and applied onto a column (20 × 200 mm) filled with a slurry of RP-18 silica (Lichroprep 25−40 μm; 10.0 g; Merck) equilibrated in water. Chromatography was performed with the following solvent mixtures: water (50 mL; A), water/methanol (80:20, v/v; 70 mL; B), water/methanol (50:50, v/v; 50 mL; C), water/methanol (20:80, v/v; 50 mL; D), and methanol (100 mL; E). The fractions were analyzed by RP-TLC using a mixture of water/methanol (20:80, v/v) as the mobile phase. The target compound was detected in fraction C (R_f = 0.4) by spraying the plate with ninhydrin (5% in ethanol). After removal of the methanol in vacuo, fraction C was freeze-dried to give N_α-FMOC-DFLys or N_α-FMOC-[$^{13}C_6$]-DFLys, respectively, as a pale yellow powder (0.4 mmol, yield = 80%). The parameters from LC/MS operated in the electrospray ionization mode, as well as proton nuclear magnetic resonance data, are shown below.

LC/MS/ESI of N_α-FMOC-DFLys: *m/z* (%) 531 (100, $[M + 1]^+$), 513 (35, $[M - H_2O]^+$), 1061 (15, $[M_2 + 1]^+$), 495 (10, $[M - 2H_2O]^+$), 553 (9, $[M + Na]^+$), 477 (8, $[M + 3H_2O]^+$), 369 (5, $[M + 1 - Glu + H_2O]^+$), 180 (3, $[M + H - FMOCLys]^+$), 575 (2, $[M + 2Na]^+$).

LC/MS-ESI of N_α-FMOC-[$^{13}C_6$]-DFLys: *m/z* (%) 537 (100, $[M + 1]^+$), 559 (23, $[M + Na]^+$), 519 (18, $[M - H_2O]^+$), 501 (6, $[M - 2H_2O]^+$), 1067 (5, $[M_2 + 1]^+$), 1095 (5, $[M_2 + Na]^+$), 1117 (3, $[M_2 + 2Na]^+$), 483 (3, $[M + 3H_2O]^+$), 465 (3, $[M - 4H_2O]^+$).

^{1}H NMR (360 MHz, CD_3OD) of unlabeled and labeled N_α-FMOC-DFLys, respectively: δ 7.8 (d, 2H, J = 7.52 Hz, FMOC-aryl), 7.7 (dd, 2H, J = 2.27 Hz, FMOC-aryl), 7.4 (t, 2H, J = 7 Hz, FMOC-aryl), 7.3 (t, 2H, J = 7.52 Hz, FMOC-aryl), 4.35 (m, 2H, FMOC-CH_2), 4.33 (m, 1H, Glu-CH), 4.2 (t, 1H, J = 6.63 Hz, 1 H, FMOC-CH), 4.07 (m, 1H, Lys-CH), 3.86 (m, 1H_a, Glu-CH_2), 3.84 (m, 1H, Glc-CH), 3.8 (m, 1H_b, Glu-CH_2), 3.77 (m, 1H, Glu-CH), 3.3 (m, 2H, Lys-CH_2), 3.17 (s, 2H, Lys-CH_2-Glc), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.36 (m, 2H, Lys-CH_2).

(2) N_ϵ-(1-Deoxy-D-fructos-1-yl)-L-lysine (DFLys) and N_ϵ-([$^{13}C_6$]-1-Deoxy-D-fructos-1-yl)-L-lysine ([$^{13}C_6$]-DFLys). Morpholine (3 mmol) was added to N_α-FMOC-DFLys or N_α-FMOC-[$^{13}C_6$]-DFLys (0.38 mmol), respectively, dissolved in a mixture of dimethylformamide/methanol (9:1, v/v; 1.6 mL). After 2 h of stirring at 25 °C, the volatiles were removed in vacuo and the residue was suspended in diethyl ether (100 mL). Filtration yielded DFLys or [$^{13}C_6$]-DFLys, respectively, as a white powder (0.34 mmol, 90%) in a purity of ~95%. The mass spectra obtained by LC/MS in the electrospray ionization mode (LC/MS/ESI) of [$^{13}C_6$]-DFLys (A) and DFLys (B) are displayed in Figure 3.

^{1}H NMR (360 MHz, D_2O) of labeled and unlabeled DFLys, respectively: δ 4.33 (m, 1H, Glu-CH), 4.07 (m, 1H, Lys-CH), 3.86 (m, 1H_a, Glu-CH_2), 3.84 (m, 1H, Glu-CH), 3.81 (m, 1H_b, Glu-CH), 3.77 (m, 1H, Glu-CH_2), 3.31 (m, 2H, Lys-CH_2), 3.17 (s, 2H, Lys-CH_2-Glc), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.36 (m, 2H, Lys-CH_2).

(B) L-Alanyl-N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-DFLys-Leu-Gly) and L-Alanyl-N_ϵ-([$^{13}C_6$]-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-[$^{13}C_6$-]-DFLys-Leu-Gly). (1) N_α-9-Fluorenylmethoxycarbonyl-N_ϵ-tert-butoxycarbonyl-L-lysyl-L-leucyl-L-glycine tert-Butyl Ester [N_α-FMOC-Lys(N_ϵ-BOC)-Leu-Gly-OtBu]. Following a procedure for peptide-bond coupling reported by Chen et al. (1989) with some modifications, 1-hydroxybenzotriazole (1.5 mmol) and dicyclohexylcarbodiimide (1.5 mmol) were added to a solution of N_α-FMOC-Lys(N_ϵ-BOC)-Leu·HCl (1.2 mmol) in dichloromethane and dimethylformamide (5 mL; 4 + 1 by volume). After 30 min of stirring at 25 °C, Gly-OtBu·HCl (1.3 mmol) and triethylamine (1.3 mmol) were added and the reaction mixture was stirred at 25 °C. Monitoring the reaction by TLC on silica gel using pentane/diethyl ether (4:1, v/v) provided detection of the target compound at R_f = 0.4. After 4 h, the mixture was filtered, and the filtrate diluted with dichloromethane (60 mL) and, then, washed with aqueous hydrochloric acid (0.1 mol/L, 2 × 20 mL), followed by a $NaHCO_3$ solution (5% in water; 20 mL) and finally brine (2 × 20 mL). Drying over Na_2SO_4 and concentration in vacuo afforded N_α-FMOC-Lys(N_ϵ-BOC)-Leu-Gly-OtBu as a pure white solid (1.08 mmol, yield: 90%).

LC/MS/ESI *m/z* (%): 717 (100, $[M + Na]^+$), 661 (8, $[M + Na - C_4H_8]^+$), 1412 (7, $[M_2 + Na]^+$), 695 (5, $[M + 1]^+$).

^{1}H NMR (360 MHz, CD_3OD): δ 7.8 (d, 2H, J = 7.52 Hz, FMOC-aryl), 7.7 (dd, 2H, J = 2.27 Hz, FMOC-aryl), 7.4 (t, 2H, J = 7 Hz, FMOC-aryl), 7.3 (t, 2H, J = 7.51 Hz, FMOC-aryl), 4.45 (t, 1H, J = 7.52 Hz, Leu-CH), 4.35 (m, 1H, FMOC-CH_2), 4.2 (t, 1H, J = 6.63 Hz, FMOC-CH), 4.07 (m, 1H, Lys-CH), 3.88 (d, 1H_a, J = 18 Hz, Gly-CH_2), 3.74 (d, 1H_b, J = 18 Hz, Gly-CH_2), 3.03 (m, 2H, Lys-CH_2), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.64 (m, 2H, Leu-CH_2), 1.61 (m, 1H, Leu-CH), 1.4 (s, 9H, 3 × BOC-CH_3), 1.4 (s, 9H, 3 × t-Bu-CH_3), 1.36 (m, 2H, Lys-CH_2), 0.9 (m, 6H, 2 × Leu-CH_3).

(2) N_ϵ-tert-Butoxycarbonyl-L-lysyl-L-leucyl-L-glycine tert-Butyl Ester [N_ϵ-BOC-Lys-Leu-Gly-OtBu]. Following the classical procedure for removal of the FMOC protection group (Carpino and Han, 1972), morpholine (3.24 mmol) was added to N_α-FMOC-Lys(N_ϵ-BOC)-Leu-Gly-OtBu (0.86 mmol) dissolved in a mixture of dimethylformamide (2.1 g) and methanol (0.2 g). Cleavage was monitored by RP-TLC using methanol/water (8:2, v/v) as the mobile phase. After a reaction time of 2 h at room temperature, the target compound (R_f = 0.7) was detected upon spraying of the plate with ninhydrin solution (5% ninhydrin in ethanol). The volatiles were removed in vacuo, and the residual white solid was taken up in aqueous hydrochloric acid (0.1 mol/L; 60 mL) and washed with diethyl ether (3 × 30 mL). After the pH had been adjusted to 10.0 by addition of sodium hydroxide (30% in water), the precipitate formed was dissolved in diethyl ether (90 mL). Drying of the organic layer over Na_2SO_4, filtration, and concentration in vacuo afforded N_ϵ-BOC-Lys-Leu-Gly-OtBu as a white solid (0.72 mmol, yield = 83%).

LC/MS/ESI *m/z* (%): 495 (100, $[M + Na]^+$), 439 (7, $[M + Na - C_2H_4]^+$), 473 (5, $[M + 1]^+$), 968 (2, $[M_2 + Na]^+$).

^{1}H NMR (360 MHz, DMSO-d_6): δ 4.46 (t, 1H, J = 7.52 Hz, Leu-CH), 4.08 (m, 1H, Lys-CH), 3.88 (d, $1H_a$, J = 18 Hz, Gly-CH_2), 3.74 (d, $1H_b$, J = 18 Hz, Gly-CH_2), 3.03 (m, 2H, Lys-CH_2), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.65 (m, 2H, Leu-CH_2), 1.62 (m, 1H, Leu-CH), 1.4 (s, 9H, 3 × BOC-CH_3), 1.38 (s, 9H, 3 × t-Bu-CH_3), 1.36 (m, 2H, Lys-CH_2),0.9 (m, 6H, 2 × Leu-CH_3).

(3) N_α-9-Fluorenylmethoxycarbonyl-L-alanyl-N_ϵ-tert-butoxycarbonyl-L-lysyl-L-leucyl-L-glycine tert-Butyl Ester [N_α-FMOC-Ala-Lys(N_ϵ-BOC)-Leu-Gly-OtBu]. 1-Hydroxybenzotriazole (1 mmol) and dicyclohexylcarbodiimide (1 mmol) were added to a solution of FMOC-Ala (0.7 mmol) in a mixture of dichloromethane (2.2 mL) and dimethylformamide (0.3 mL). After 30 min of stirring at 25 °C, N_ϵ-BOC-Lys-Leu-Gly-OtBu (0.44 mmol) and triethylamine (0.40 mmol) were added and the mixture was stirred at 25 °C. Reaction was monitored by silica TLC using pentane/ethyl acetate (20:80, v/v) as the mobile phase. After 4 h, the educt was completely converted into the target compound (R_f = 0.6). The reaction was then filtered, and the filtrate was diluted with dichloromethane (60 mL) and was then washed with with aqueous hydrochloric acid (0.1 mol/L, 2 × 20 mL), followed by an $NaHCO_3$ solution (5% in water; 20 mL) and brine (2 × 20 mL). After drying over Na_2SO_4, concentration in vacuo yielded a white solid, which was washed with diethyl ether (3 × 50 mL). Crystallization of the residue from methanol afforded N_α-FMOC-Ala-Lys(N_ϵ-BOC)-Leu-Gly-OtBu as a white solid (0.35 mmol, yield = 80%).

LC/MS/ESI *m/z* (%): 788 (100, $[M + Na]^+$), 732 (15, $[M + Na - C_2H_4]^+$), 1554 (8, $[M_2 + Na]^+$), 676 (5, $[M + Na - 2C_2H_4]^+$), 767 (1, $[M + 1]^+$).

^{1}H NMR (360 MHz, CD_3OD): δ 7.8 (d, 2H, J = 7.52 Hz, FMOC-aryl), 7.7 (dd, 2H, J = 2.27 Hz, FMOC-aryl), 7.4 (t, 2H, J = 7 Hz, FMOC-aryl), 7.3 (t, 2H, J = 7.51 Hz, FMOC-aryl), 4.45 (t, 1H, J = 7.52 Hz, Leu-CH), 4.35 (m, 1H, FMOC-CH_2), 4.3 (m, 1H, Ala-CH), 4.2 (t, 1H, J = 6.63 Hz, FMOC-CH), 4.08 (m, 1H, Lys-CH), 3.87 (d, $1H_a$, J = 18 Hz, Gly-CH_2), 3.75 (d, $1H_b$, J = 18 Hz, Gly-CH_2), 3.3 (m, 2H, Lys-CH_2), 1.88 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.64 (m, 2H, Leu-CH_2), 1.61 (m, 1H, Leu-CH), 1.44 (s, 9H, 3 × BOC-CH_3), 1.43 (s, 9H, 3 × t-Bu-CH_3), 1.41 (d, 3H, Ala-CH_3), 1.36 (m, 2H, Lys-CH_2), 0.9 (m, 6H, 2 × Leu-CH_3).

(4) N_α-9-Fluorenylmethoxycarbonyl-L-alanyl-L-lysyl-L-leucyl-L-glycine (N_α-FMOC-Ala-Lys-Leu-Gly). Following a procedure of Kemp and Carey (1989), N_α-FMOC-Ala-Lys(N_ϵ-BOC)-Leu-Gly-OtB (0.27 mmol) was stirred in a solution of trifluoroacetic acid (16 mL) in dichloromethane (6 mL) under an atmosphere of nitrogen at room temperature. The cleavage was followed by RP-TLC using a mixture of methanol/water (80:20) as the mobile phase. After 2 h, the educt was completely converted into the target compound, which was detected at R_f = 0.4 upon spraying with ninhydrin (5% in ethanol). The volatiles were then removed under high vacuum, and the residual yellow oil was dissolved in water. Freeze-drying afforded a pure white solid of N_α-FMOC-Ala-Lys-Leu-Gly·CF_3-COOH (0.25 mmol, yield = 95%).

LC/MS/ESI *m/z* (%): 610 (100, $[M + H]^+$), 1219 (39, $[M_2 + H]^+$), 632 (12, $[M + Na]^+$), 535 (4, $[M + H - Gly]^+$), 517 (2, $[M + H - Gly - H_2O]^+$).

^{1}H NMR (360 MHz, CD_3OD): δ 7.8 (d, 2H, J = 7.52 Hz, FMOC-aryl), 7.7 (dd, 2H, J = 2.27 Hz, FMOC-aryl), 7.4 (t, 2H, J = 7 Hz, FMOC-aryl), 7.3 (t, 2H, J = 7.51 Hz, FMOC-aryl), 4.45 (t, 1H, J = 7.52 Hz, Leu-CH), 4.35 (m, 1H, FMOC-CH_2), 4.31 (m, 1H, Ala-CH), 4.2 (t, 1H, J = 6.63 Hz, FMOC-CH), 4.07 (m, 1H, Lys-CH), 3.86 (d, $1H_a$, J = 18 Hz, Gly-CH_2), 3.72 (d, $1H_b$, J = 18 Hz, Gly-CH_2), 3.34 (m, 2H, Lys-CH_2), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.63 (m, 2H, Leu-CH_2), 1.61 (m, 1H, Leu-CH), 1.41 (d, 3H, Ala-CH_3), 1.36 (m, 2H, Lys-CH_2), 0.9 (m, 6H, 2 × Leu-CH_3).

(5) N_α-9-Fluorenylmethoxycarbonyl-L-alanyl-N_ϵ-(1-deoxy-D-fructos-1yl)-L-lysyl-L-leucyl-L-glycine (N_α-FMOC-Ala-DFLys-Leu-Gly) and N_α-9-Fluorenylmethoxycarbonyl-L-alanyl-N_ϵ-([$^{13}C_6$]-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (N_α-FMOC-Ala-[$^{13}C_6$]-DFLys-Leu-Gly). N_α-FMOC-Ala-Lys-Leu-Gly (as trifluoroacetic acid salt; 0.1 mmol) and triethylamine (0.2 mmol) were dissolved in anhydrous methanol (2 mL) and stirred for 20 min. Anhydrous D-glucose or [$^{13}C_6$]-D-glucose (0.40 mmol), respectively, was then added, and the mixture was stirred at 64 °C under an atmosphere of nitrogen. The formation of the glycosylated, bound DFLys (R_f = 0.3) was monitored by RP-TLC using a mixture of water/methanol (20:80, v/v) as the mobile phase and spraying the plate with ninhydrin (5% in ethanol). After 3 h, the solvent was removed in vacuo, and the yellow residue was taken up in water and then fractionated by column chromatography (20 × 200 mm) on RP-18 silica (Lichroprep 25−40 μm; 10.0 g; Merck) suspended in water. After flushing with water (50 mL), the target compound was eluted with water/methanol (80:20, v/v; 100 mL). After removal of the methanol in vacuo, the aqueous solution was freeze-dried to give N_α-FMOC-Ala-DFLys-Leu-Gly or N_α-FMOC-Ala-[$^{13}C_6$]-DFLys-Leu-Gly, respectively, as a pale yellow powder (0.04 mmol, yield = 35%).

LC/MS/ESI of N_α-FMOC-Ala-DFLys-Leu-Gly *m/z* (%): 772 (100, $[M + H]^+$), 794 (51, $[M + Na]^+$), 754 (4, $[M + H - H_2O]^+$), 1543 (3, $[M_2 + H]^+$), 610 (3, $[M + H - Glu]^+$), 736 (2, $[M + H - 2H_2O]^+$), 718 (1, $[M + H - 3H_2O]^+$).

LC/MS-ESI of N_α-FMOC-Ala-[$^{13}C_6$]-DFLys-Leu-Gly *m/z* (%): 778 (100, $[M + H]^+$), 800 (46, $[M + Na]^+$), 1556 (8, $[M_2 + H]^+$), 616 (5, $[M + H - Glu]^+$), 760 (4, $[M + H - H_2O]^+$), 742 (3, $[M + H - 2H_2O]^+$), 724 (2, $[M + H - 3H_2O]^+$).

^{1}H NMR (360 MHz, CD_3OD) of labeled and unlabeled N_α-FMOC-Ala-DFLys-Leu-Gly, respectively: δ 7.8 (d, 2H, J = 7.52 Hz, FMOC-aryl), 7.7 (dd, 1H, J = 2.27 Hz, FMOC-aryl), 7.4 (t, 2H, J = 7 Hz, FMOC-aryl), 7.3 (t, 2H, J = 7.51 Hz, FMOC-aryl), 4.46 (t, 1H, J = 7.52 Hz, Leu-CH), 4.35 (m, 1H, FMOC-CH_2), 4.33 (m, 1H, Glu-CH), 4.3 (m, 1H, Ala-CH), 4.2 (t, 1H, J = 6.63 Hz, FMOC-CH), 4.07 (m, 1H, Lys-CH), 3.87 (m, $1H_a$, Glu-CH_2), 3.84 (m, 1H, Glu-CH), 3.82 (m, $1H_b$, Glu-CH_2), 3.80 (d, $1H_a$, J = 18 Hz, Gly-CH_2), 3.77 (m, 1H, Glu-CH), 3.74 (d, $1H_b$, J = 18 Hz, Gly-CH_2), 3.3 (m, 2H, Lys-CH_2), 3.1 (s, 2H, Lys-CH_2-Glu), 1.87 (m, 2H, Lys-CH_2), 1.72 (m, 2H, Lys-CH_2), 1.64 (m, 2H, Leu-CH_2), 1.61 (m, 1H, Leu-CH), 1.4 (d, 3H, Ala-CH_3), 1.36 (m, 2H, Lys-CH_2), 0.9 (m, 6H, 2 × Leu-CH_3).

(6) L-Alanyl-N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-DFLys-Leu-Gly) and L-Alanyl-N_ϵ-([$^{13}C_6$]-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-[$^{13}C_6$]-DFLys-Leu-Gly). A mixture of morpholine (2 mmol) and N_α-FMOC-Ala-DFLys-Leu-Gly or N_α-FMOC-Ala-[$^{13}C_6$]-DFLys-Leu-Gly (0.04 mmol), respectively, was stirred in dimethylformamide (1.2 mL)/methanol (0.8 mL). The cleavage was followed by RP-TLC using water/methanol (80:20, v/v) as the mobile phase and ninhydrin (0.5% in ethanol) as the detection reagent. After 2 h of stirring at room temperature, the starting material was converted into the target compound (R_f = 0.6). Solvent and morpholine were then removed under high vacuum, and the residue was suspended in diethyl ether (20 mL). Filtration afforded Ala-DFLys-Leu-Gly or Ala-[$^{13}C_6$]-DFLys-Leu-Gly, respectively, as a white solid (0.036 mmol, 91%) in a purity of ~96%.

Table 1. Concentrations of DFLys Formed in BSA[a]: Addition of [$^{13}C_6$]-DFLys (A) after and (B) before Enzymic Digestion

	DFLys[b] (μmol/mmol of L-lysine)		
expt	mean[b]	range	SD
A	93[b]	86−102	6.3
B	121[b]	116−125	4.0

[a] BSA (30 mg) and D-glucose (50 mg) were reacted in phosphate buffer (1 mL, pH 7.0; 0.1 mol/L) for 20 min at 95 °C. [b] The results are mean values of four replicates.

LC/MS-ESI of Ala-[$^{13}C_6$]-DFLys-Leu-Gly *m/z* (%): 556 (100, $[M + H]^+$), 557 (34), 538 (15, $[M + H - H_2O]^+$).

LC/MS/ESI of Ala-DFLys-Leu-Gly *m/z* (%): 550 (100, $[M + H]^+$), 551 (33), 532 (5, $[M + H - H_2O]^+$).

^{1}H NMR (360 MHz, D_2O) of labeled and unlabeled Ala-DFLys-Leu-Gly, respectively: δ 4.45 (t, 1H, $J = 7.52$ Hz, Leu-CH), 4.33 (m, 1H, Glu-CH), 4.3 (m, 1H, Ala-CH), 4.09 (m, 1H, Lys-CH), 3.86 (m, $1H_a$, Glu-CH_2), 3.84 (m, 1H, Glu-CH), 3.81 (m, $1H_b$, Glu-CH_2), 3.80 (d, $1H_a$, $J = 18$ Hz, Gly-CH_2), 3.78 (m, 1H, Glu-CH), 3.75 (d, $1H_b$, $J = 18$ Hz, Gly-CH_2), 3.3 (m, 2H, Lys-CH_2), 3.1 (s, 2H, Lys-CH_2-Glu), 1.88 (m, 2H, Lys-CH_2), 1.71 (m, 2H, Lys-CH_2), 1.36 (m, 2H, Lys-CH_2), 1.64 (m, 2H, Leu-CH_2), 1.62 (m, 1H, Leu-CH), 1.4 (d, 3H, Ala-CH_3), 0.9 (m, 6H, 2 × Leu-CH_3).

Preparation of Nonglycated BSA. Using a method of McPherson et al. (1988) with some modifications, commercial BSA was purified from glycosylated proteins by affinity chromatography using phenylboronate polyacrylamide gel (Affigel 601, Bio-Rad, München, Germany). A solution of BSA (60 mg) in aqueous ammonium acetate buffer (3 mL, 0.2 mol/L, pH 8.8) was applied onto the top of a column (15 × 200 mm), filled with a slurry of Affigel 601 (3.0 g) in aqueous ammonium acetate buffer (0.2 mol/L, pH 8.8). After a rinse with the same buffer solution (100 mL), the collected eluates were freeze-dried. The pure BSA afforded did not show any trace amounts of DFLys after enzymic digestion. For regeneration the column was flushed with an acidic aqueous ammonium acetate buffer (100 mL, 0.2 mol/L, pH 6.0), followed by an alkaline aqueous ammonium acetate buffer (100 mL, 0.2 mol/L, pH 8.8).

Reaction Mixture. A mixture of DFLys-free BSA (30 mg) and glucose (50 mg) dissolved in phosphate buffer (0.1 mol/L; pH 7.0; 1 mL) was reacted for 20 min at 95 °C in a closed vial.

Quantification procedure. *Removal of Unreacted Glucose from Reaction Mixture.* An aliquot (100 μL) of the reaction mixture was fractionated by ultrafiltration with a cutoff of 1000 Da (Centricon, Amicon, Witten, Germany) using a centrifuge equipped with a swinging-bucket rotor at 3000*g*. After the addition of water (5 × 100 μL) to the sample reservoir, residual glucose was removed by recentrifugation. The complete removal of glucose from the retentate was controlled by TLC (cellulose) of the filtrates using *n*-butanol/acetic acid/water (8:3:3 by volume) as the mobile phase and orcine/H_2SO_4 as the detection spray. The retentate (MG > 1000 Da) containing unreacted as well as glycated protein was then dissolved in water (100 μL).

Addition of Labeled Standards. An aqueous solution containing a defined amount of [$^{13}C_6$]-DFLys (50−250 nmol) was added to an aliquot (100 μL) of the enzymically hydrolyzed reaction mixture (A in Tables 1−3) or to the hexose-removed glycated protein solution (100 μL) prior to enzymic digestion (B in Tables 1 and 2). An aqueous solution containing Ala-[$^{13}C_6$]-DFLys-Leu-Gly (50−250 nmol) was added to the hexose-removed glycated protein solution (100 μL) prior to enzymic digestion (B in Table 3).

Enzymic Hydrolysis. An aliquot (100 μL) of the reaction mixture, equal to 3.0 mg of protein, was hydrolyzed enzymically using a procedure described in the literature (Henle et al., 1991; Hofmann, 1998a). After acidification with aqueous hydrochloric acid (0.02 mol/L; 2 mL), a solution of pepsin (200 μg) in aqueous hydrochloric acid (100 μL; 0.02 mol/L) and a trace amount of thymol were added. After incubation for 24 h at 37 °C, Tris buffer (2.0 mol/L; pH 8.2; 0.5 mL) and a solution of Pronase E (200 μg) in Tris buffer (100 μL; 2.0 mol/L; pH 8.2) were added, and the sample was incubated for another 24 h at 37 °C. Finally, aminopeptidase M (40 μL) and prolidase (20 μL) were added, and the mixture was maintained again for 24 h at 37 °C. After freeze-drying, the residue was taken up in water (2 mL), membrane-filtered (0.45 μm), and then analyzed by LC/MS.

Table 2. Concentrations of DFLys Determined in Model Mixtures Spiked with Synthetic DFLys (110.2 μg)[a]: Addition of [$^{13}C_6$]-DFLys (A) after and (B) before Enzymic Digestion

	DFLys[b] (μmol/mmol of L-lysine)		
expt	mean[b]	range	SD
A	85.0	79.1−92.5	5.7
B	108.1	104.2−111.0	3.4

[a] A solution of nonglucosylated BSA (30 mg) in water (1 mL) was spiked with synthetic DFLys and then analyzed by an SIDA. [b] The results are mean values of four replicates.

Table 3. Influence of the Use of either Free or Peptide-Bound [$^{13}C_6$]-DFLys on the Concentrations of DFLys Generated in a BSA/Glucose Mixture[a]

		DFLys[c] (μmol/mmol of L-lysine)		
expt	internal standard used[b]	mean	range	SD
A	[$^{13}C_6$]-DFLys	121	116−125	4.0
B	Ala-[$^{13}C_6$]-DFLys-Leu-Gly	132	126−138	4.5

[a] BSA (30 mg) and D-glucose (50 mg) were heated in phosphate buffer (1 mL, pH 7.0; 0.1 mol/L) for 20 min at 95 °C. [b] The internal standard was added prior to the enzymic digestion. [c] The results are mean values of four replicates.

High-Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS). A spectra series high-performance liquid chromatograph (Thermo Separation Products, San Jose, CA) equipped with a Nucleosil 100-5 NH_2 column (4.5 × 250 mm, Macherey-Nagel, Dürren, Germany) was coupled to an LCQ (Finnigan MAT GmbH, Bremen, Germany) ion trap mass spectrometer operating in the electrospray ionization (ESI) mode. After injection of the sample (2.0−20.0 μL), analysis was performed with a solvent flow of 0.6 mL/min using a gradient starting with water (100%) and increasing the methanol content to 1% within 20 min.

Determination of Recoveries and Detection Limits. To a solution of DFLys-free BSA (3.0 mg) in water (100 μL) were added the following amounts of Ala-DFLys-Leu-Gly: 10, 30, 90, and 150 nmol/mg of protein. Each sample was analyzed in triplicates, revealing a calibration line from which the detection limits were calculated according to a procedure reported in detail by Häderich and Vogelgesang (1996).

Liquid Chromatography/Mass Spectrometry (LC/MS). An LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) was used, operating in the electrospray ionization (ESI) mode. The sample (10 μg/mL) was dissolved in methanol and was then applied to the MS by using the loop injection technique (injection speed = 10 μL/min) described recently (Hofmann, 1998b).

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR measurements were performed by means of a Bruker-AM-360 spectrometer (Bruker, Rheinstetten, Germany) using the acquisition parameters described recently (Hofmann, 1997). Tetramethylsilane (TMS) was used as the internal standard.

RESULTS AND DISCUSSION

To develop a SIDA for quantification of protein-bound DFLys, first, N_ϵ-(1-deoxy-D-[$^{13}C_6$]-fructos-1-yl)-L-lysine ([$^{13}C_6$]-DFLys) was synthesized as the internal standard. Starting with N_α-FMOC-L-lysine, reaction with [$^{13}C_6$]-glucose in a mixture of methanol and dimethylform-

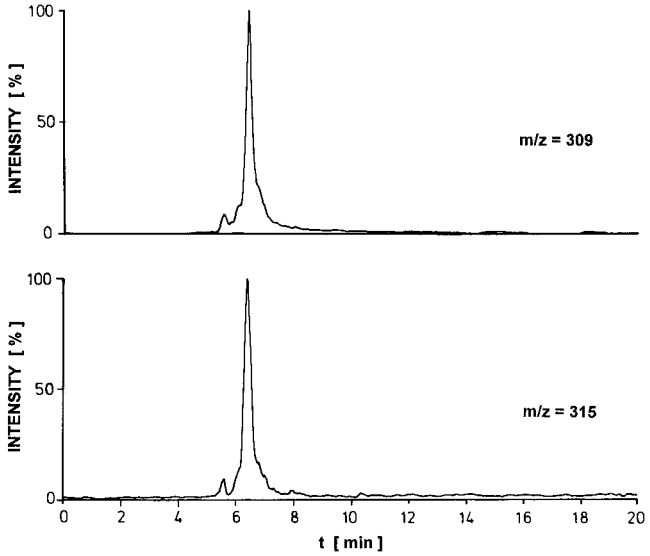

Figure 4. LC/mass chromatography of DFLys (*m*/*z* 309) and [$^{13}C_6$]-DFLys (*m*/*z* 315).

amide afforded N_α-FMOC-N_ϵ-(1-deoxy-D-[$^{13}C_6$]-fructos-1-yl)-L-lysine, which, after purification, was deprotected by exposure to morpholine yielding [$^{13}C_6$]-DFLys in a purity of ~95%. Using nonlabeled glucose afforded natural ^{13}C-abundant DFLys. In Figure 3, the LC/MS spectrum obtained for ^{13}C-labeled DFLys (A) is contrasted with the spectrum of the unlabeled Amadori product (B). In agreement with the incorporation of six ^{13}C atoms, the molecular mass was shifted by six units to give the $[M + 1]^+$ ion 315 in the labeled isotopomer (Figure 3A). Further ions showing the incorporation of six ^{13}C atoms were *m*/*z* 297 and 279, demonstrating the loss of one and two molecules of water, respectively.

To determine the response factor for HPLC/MS, mixtures of DFLys and [$^{13}C_6$]-DFLys in ratios of 5:1 to 1:5 were analyzed by HPLC/MS using aminopropylsilica as the stationary phase (Figure 4). Analysis in triplicates gave a response factor of 1.05 ± 7%, indicating that mass chromatography did not discriminate between the isotopomers.

To study the glucosylation of proteins, first, commercial BSA, which was chosen as a model food protein, was freed from glycated proteins. According to a procedure of McPherson et al. (1988), glycated protein was removed from nonglycated BSA by means of affinity chromatography on a phenylboronate polyacrylamide column utilizing the complex formation of the vicinal diol groups in the sugar moiety with the phenylboronate stationary phase. An aqueous solution of purified BSA and glucose was heated for 20 min. After cooling, unreacted glucose was removed from the heated mixture by ultrafiltration to prevent further reactions between nonmodified lysine residues and unreacted hexose. Because acidic hydrolysis of the protein is well-known to result in artifacts (Finot et al., 1977), such as furosine and pyridosine (I and II in Figure 2), protein-bound DFLys was liberated from the protein backbone by enzymic hydrolysis at 37 °C using a combination of two endoproteases and two exoproteases (Henle et al., 1991; Hofmann, 1998a).

For quantification of DFLys, in a first experiment, [$^{13}C_6$]-DFLys was added to the enzymically digested mixture (experiment A). To evaluate the extent of DFLys degradation during the enzymic cleavage procedure, in a second experiment (experiment B), the internal standard [$^{13}C_6$]-DFLys was added to the reaction mixture prior to digestion. Both hydrolysates were separated by HPLC and monitored by LC/MS. The

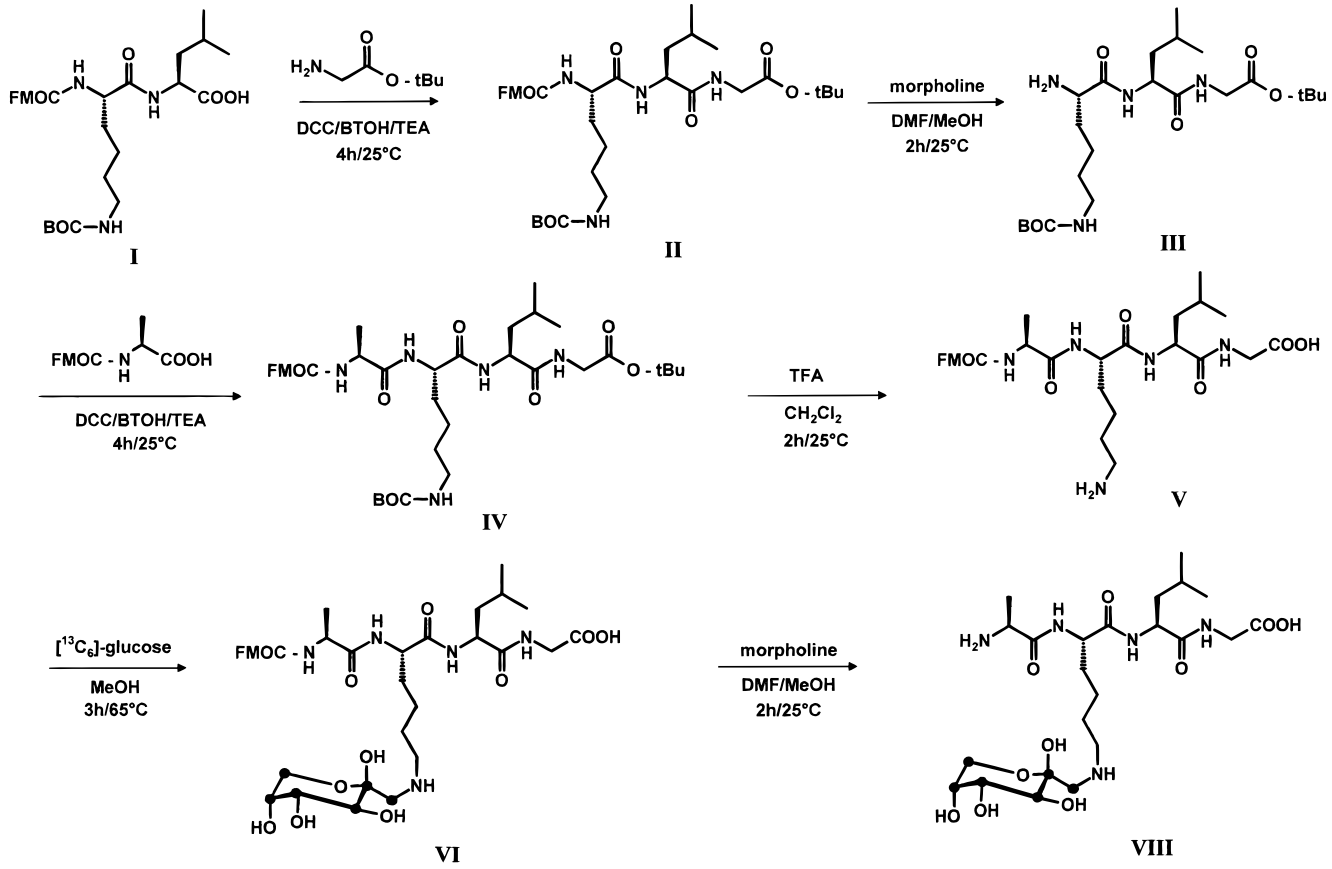

Figure 5. Synthetic route used in the synthesis of the labeled, glycosylated tetrapeptide Ala-[$^{13}C_6$]-DFLys-Leu-Gly.

$[M + 1]^+$ ions of labeled (*m/z* 315) and unlabeled DFLys (*m/z* 309) were scanned for 20 min (Figure 4). The results of four measurements (Table 1) showed that spiking the mixture with the labeled standard prior to enzymic protein digestion gave by a factor of 1.3 higher amounts of DFLys (cf. experiments A and B; Table 1), clearly demonstrating a significant degradation of DFLys during enzymic cleavage.

To determine the recovery of DFLys, a solution of nonglucosylated BSA was spiked with a certain amount of synthesized DFLys and the internal standard was added prior to or after protein digestion, respectively. The results demonstrated that adding the internal standard prior to digestion (experiment B in Table 2) gave a recovery (experiment A in Table 2) of 98% with a standard deviation (SD) of 3.4, whereas spiking after digestion gave much lower results (77%) that would lead to an underestimation of the glycosylation. This is undoubtedly caused by the instability of the Amadori product.

Besides the lability of the Amadori product, also a possible incompleteness of the enzymic hydrolysis of the glucosylated protein would lead to an underestimation of DFLys. It is well-known in the literature that enzymic protein digestion does not liberate the amino acids to the extent found after acidic hydrolyses; for example, enzymic digestion of wool was found to liberate the amino acid L-lysine only to a yield of ~70% (Köper et al., 1984). To further improve the accuracy of the method, it was, therefore, necessary to synthesize a labeled standard that also has to undergo enzymic digestion. To meet this demand, the $[^{13}C_6]$-DFLys was incorporated in the peptide L-alanyl-N_ϵ-($[^{13}C_6]$-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine, which was synthesized following the six-step reaction sequence displayed in Figure 5. Starting with N_α-FMOC-N_ϵ-BOC-protected L-lysyl-L-leucine (I), dicyclohexylcarbodiimide-catalyzed peptide-bond formation with L-alanine *tert*-butyl ester revealed N_α-FMOC-N_ϵ-BOC-L-lysyl-L-leucyl-L-glycine *tert*-butyl ester (II), from which the FMOC-protecting group was cleaved by exposure to morpholine, yielding the N_ϵ-BOC-L-lysyl-L-leucyl-L-glycine *tert*-butyl ester (III). Peptide-bond coupling between the deprotected amino group of III and FMOC-L-alanine afforded the N_α-FMOC-L-alanyl-N_ϵ-BOC-L-lysyl-L-leucyl-L-glycine *tert*-butyl ester (IV), which by exposure to trifluoracetic acid was cleaved to N_α-FMOC-L-alanyl-L-lysyl-L-leucyl-L-glycine (V). Glucosylation of the N_ϵ-lysyl amino group of V with $[^{13}C_6]$-glucose afforded the corresponding Amadori product N_α-FMOC-L-alanyl-N_ϵ-($[^{13}C_6]$-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine, which was then deprotected by morpholine to give the L-alanyl-N_ϵ-($[^{13}C_6]$-1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-$[^{13}C_6]$-DFLys-Leu-Gly) in a purity of ~96%. Reacting N_α-FMOC-tetrapeptide V with nonlabeled glucose, followed by FMOC deprotection, revealed the corresponding natural ^{13}C-abundant L-alanyl-N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysyl-L-leucyl-L-glycine (Ala-DFLys-Leu-Gly).

In Figure 6, the LC/MS spectrum obtained for Ala-$[^{13}C_6]$-DFLys-Leu-Gly (A) is contrasted with the data measured for the unlabeled glycated tetrapeptide (B). In agreement with the incorporation of six ^{13}C atoms, the molecular mass was shifted by six units to give the $[M + 1]^+$ ion 556 in the labeled isotopomer (Figure 6A). Further ions showing the incorporation of six ^{13}C atoms were *m/z* 538 and 520, demonstrating the loss of one or two molecules of water, respectively.

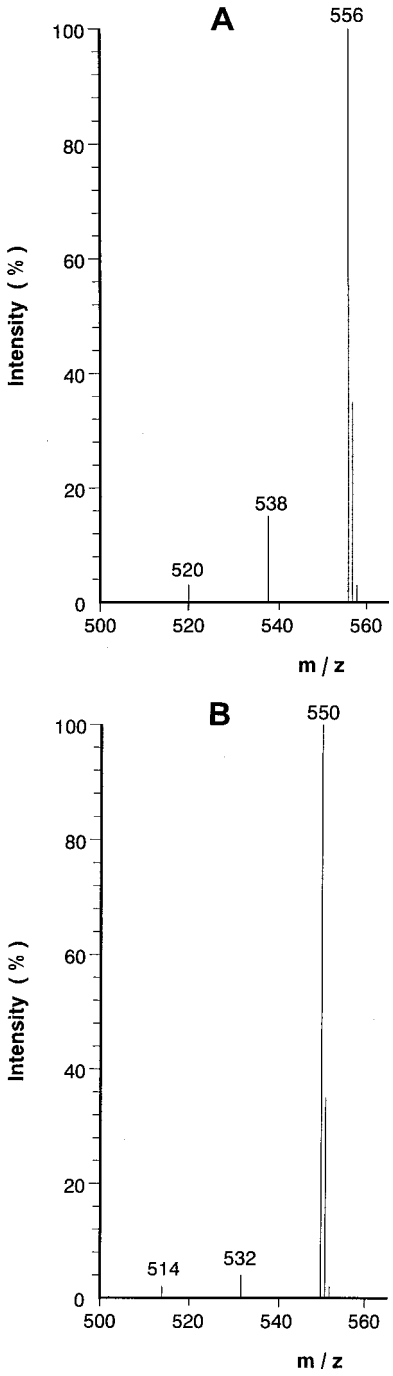

Figure 6. LC/MS-ESI spectra of (A) Ala-$[^{13}C_6]$-DFLys-Leu-Gly and (B) Ala-DFLys-Leu-Gly.

To determine the response factor for HPLC/MS, first, mixtures of Ala-DFLys-Leu-Gly and Ala-$[^{13}C_6]$-DFLys-Leu-Gly in ratios of 5:1 to 1:5 were enzymically digested and the hydrolysates were analyzed by HPLC/MS. Analysis in triplicates gave a response factor of 1.06 ± 5%, indicating that the enzymes did not discriminate between both isotopomers of glucosylated, peptide-bound lysine.

To determine the detection limit by the calibration line method, a solution of glycation-free BSA was spiked with increasing amounts of unlabeled Ala-DFLys-Leu-Gly prior to SIDA analysis. By LC/MS a detection limit of 5 nmol/mg of protein was calculated by means of a standard curve given in Figure 7. Calculating the

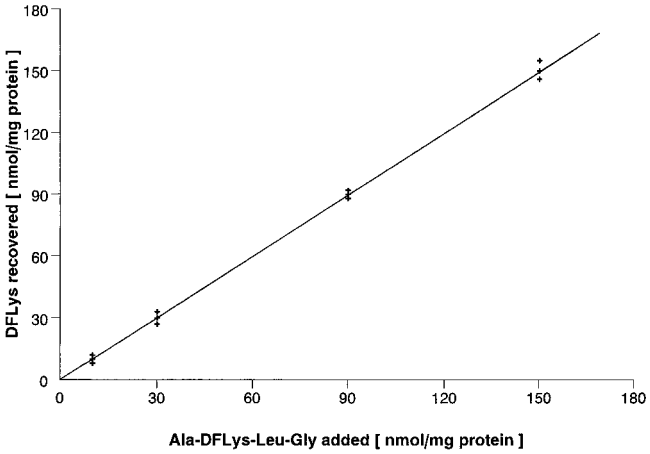

Figure 7. Quantification of protein-bound DFLys using Ala-[$^{13}C_6$]-DFLys-Leu-Gly as the internal standard.

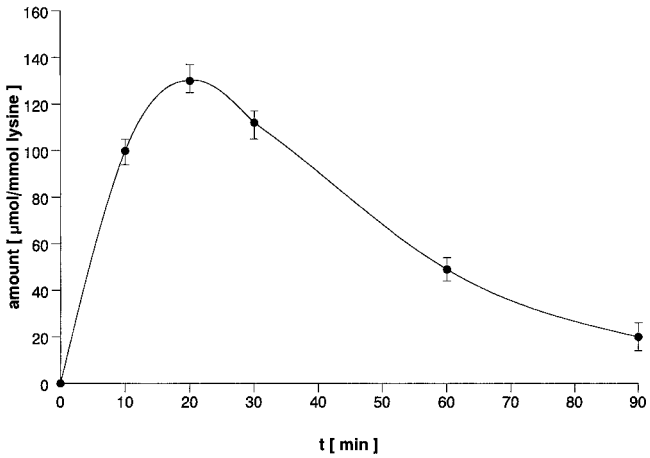

Figure 8. Time course of the formation of protein-bound DFLys in a thermally treated (95 °C) aqueous solution of BSA and glucose.

average of triplicates, a high recovery rate of 95% (at a spike level of 150 nmol/mg of protein) was determined.

To establish the effectivity of DFLys liberation by the enzymes, protein-bound DFLys was then determined in the aqueous BSA/glucose solution using Ala-[$^{13}C_6$]-DFLys-Leu-Gly as the internal standard. After enzymic digestion, the hydrolysate was analyzed by LC/MS. The results (Table 3) demonstrated that the use of the peptide-bound internal standard led to a further increase of DFLys (experiment B; Table 3) as compared to the use of the free [$^{13}C_6$]-DFLys (experiment A; Table 3). These data indicate that the enzyme mixture does not completely hydrolyze the protein backbone, being well in line with data reported by Köper et al. (1984). A 90% hydrolysis can be calculated by assuming a similar selectivity of the enzymes for BSA and the standard tetrapeptide. With the development of an SIDA using Ala-[$^{13}C_6$]-DFLys-Leu-Gly as an optimized internal standard, a powerful technique is now available enabling an accurate quantification of protein-bound N_ϵ-(1-deoxy-D-[$^{13}C_6$]-fructos-1-yl)-L-lysine with an SD of 4.5.

To gain insight into the time course of DFLys formation, an aqueous solution of BSA and glucose was heated, and after certain reaction times, aliquots were withdrawn and rapidly cooled to room temperature. After unreacted glucose was removed by ultrafiltration, the mixtures were analyzed by SIDA using Ala-[$^{13}C_6$]-DFLys-Leu-Gly as the internal standard. The results (Figure 8) showed that within the first 20 min the amounts of DFLys increased with increasing reaction time. However, after running through a maximum at 20 min, the amounts of DFLys decreased again, corroborating the assumption that DFLys is relatively unstable and is degraded to a significant extent during heating.

GENERAL CONSIDERATIONS

A SIDA using Ala-[$^{13}C_6$]-DFLys-Leu-Gly as the internal standard and LC/MS as the detection method was developed enabling an accurate quantification of protein-bound N_ϵ-(1-deoxy-D-fructos-1-yl)-L-lysine (DFLys) in protein/carbohydrate reactions. In comparison to methods such as the determination of furosine in acidic protein hydrolysates or of DFLys in enzymically digested proteins by means of an amino acid analyzer, this novel SIDA is a promising technique to overcome the problems of the hydrolytic instability of the target analyte as well as the incompleteness of the enzymic digestion procedure. Due to the sensitivity and selectivity of this SIDA, this method might be useful for physiological examinations, for example, in studying the glycation status in human serum as well as tissue proteins of diabetic patients. Studies aimed at applying this technique on physiological samples are now in progress.

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