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Short Communication

Solid-phase synthesis of glucose-derived Amadori peptides

ANDREJ FROLOV, DAVID SINGER and RALF HOFFMANN*

Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, Faculty of Chemistry and Mineralogy, Leipzig University, Deutscher Platz 5, 04103 Leipzig, Germany

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Abstract: Nonenzymatic glycosylation or glycation of amino groups in peptides and proteins by D-glucose is a universal reaction with important implications for the pathogenesis of many diseases including diabetes mellitus. Here a general approach is reported to synthesize site specifically glucose-derived *N*-glycated peptides. Therefore, model peptides H-AKASASFL-NH_2, H-AKASADFL-NH_2, H-ASKASKFL-NH_2, and H-AKDSASFL-NH_2 were synthesized on solid phase by Fmoc chemistry using Fmoc-Lys(4-methyltrityl)-OH in positions 2 or 3 to be glycated. After completion of the synthesis, the acid labile 4-methyltrityl-group was cleaved with 1% TFA in DCM and the free amino groups were glycated by the Lobry de Bruyn reaction using 2,3 : 4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose on solid phase. After TFA treatment, the crude peptides were obtained in high yields and purities above 80%. Minor by-products were well separated on reversed-phase HPLC. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Amadori product; glucose; glycation; glycoxidation; Lobry de Bruyn reaction; Maillard rearrangement

INTRODUCTION

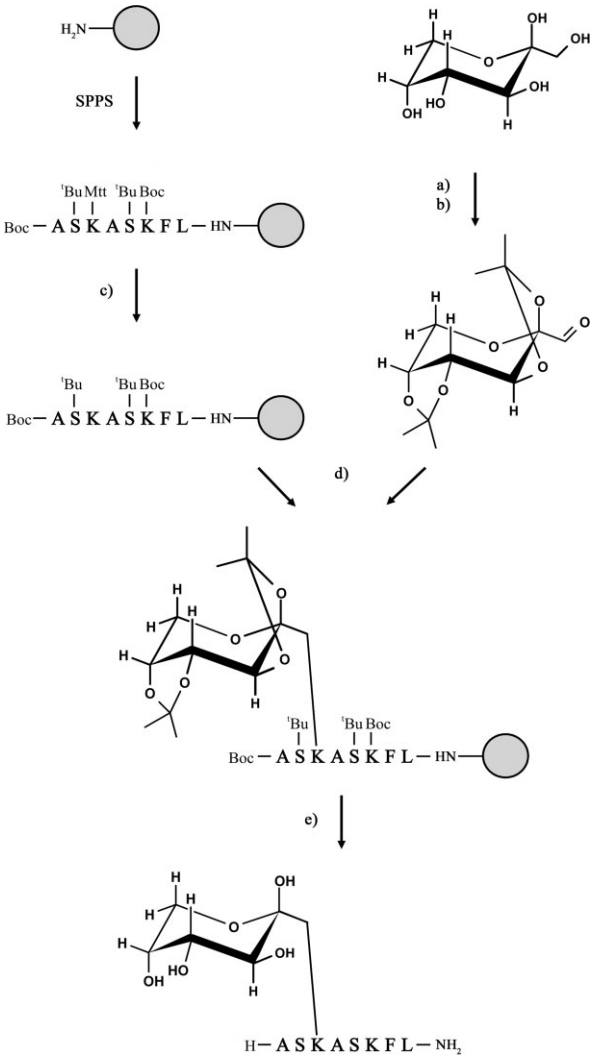
The amino groups of amino acids, peptides, and proteins react with reducing sugars under favorable conditions to form imines by reversible dehydration. These can undergo a further irreversible conversion, by the self-catalyzed Amadori rearrangement for aldoses or Heynes rearrangement for ketoses, to form relatively stable ketoamines or aldoamines respectively, such as *N*-(1-deoxy-D-fructos-1-yl) compounds in the case of D-glucose [1,2]. Early studies of these processes were triggered by formation of such modified amino acids in the browning, or Maillard reaction in foodstuff during heating [3] and more recently in the detection of acrylamide in fried food [4]. Similar processes modify especially long-lived proteins *in vivo*, which is typically referred to as *nonenzymatic glycosylation* or *glycation*. Glycated hemoglobin (HbA1c) in blood has been used as a clinical diagnostic marker for long-term control in diabetic patients [5]. The Amadori compounds can gradually undergo irreversible transformations yielding various advanced glycation end-products (AGEs) of more reactive, colored, and fluorescent compounds in different oxidation and degradation states [6], with some being considered as toxic and mutagenic [7]. AGEs in general are believed to play a more pathogenic role in the development of atherosclerosis and diabetic complications as well as aggregation in age-related neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases [8–11].

In order to understand the role of glycation and AGEs in the development of disorders, peptides and proteins glycated *in vitro* are essential models. They are typically synthesized in neutral aqueous phosphate buffers at 37 °C with reaction times of 24 h for peptides and up to 60 days for proteins [12–14]. However, these protocols are not site specific. In the best-case scenario, one or a few positions are kinetically favored, yielding the corresponding derivatives at a relatively high content. These problems were overcome for short peptides by glycating partially protected peptides in solution [15]. Two methods can be applied for this global strategy: direct glycation with D-glucose [16,17] and reductive amination with 2,3 : 4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose [18]. Whereas the reaction with D-glucose yields numerous by-products, the latter reagent yields predominantly the protected peptide. After cleavage of all protecting groups, the glycated peptide is typically obtained in high yields and purities. The major disadvantages of these in-solution strategies are the additional time-consuming handling steps after synthesizing the peptide on solid phase, the contamination of the peptide by the reagent excess, the solubility of partially protected peptides, and the need to optimize the reaction conditions for each sequence. Thus we have recently introduced the first general solid-phase strategy for glycated peptides [19,20]. The advantage of this strategy was that D-glucose, D-ribose, and D-fructose yielded the corresponding Amadori and Heynes peptides. However, the crude peptides contained many

*Correspondence to: Ralf Hoffmann, Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, Faculty of Chemistry and Mineralogy, Leipzig University, Deutscher Platz 5, 04103 Leipzig, Germany; e-mail: Hoffmann@chemie.uni-leipzig.de

by-products, including oxidized sugar moieties. A second solid-phase approach was introduced recently by Stefanowicz *et al.* [21] in parallel to our work [22]. These authors stopped the peptide synthesis at the lysine to be modified, and glycated this position after deprotecting the side chain. The peptide synthesis was continued after protecting the ε-amino group with a Boc group to complete the remaining *N*-terminal sequence.

In this work, we report a new solid-phase approach to site specifically glycate peptides on solid phase by a Lobry de Bruyn-type reaction to obtain 'osamines' [23], which are structurally identical to Amadori peptides derived by the reaction of D-glucose with the amino group of lysine (Scheme 1). Although the synthetic route does not rely on an Amadori rearrangement, we will use the term *Amadori peptide* for the products obtained, as this term is generally accepted in the literature for such compounds. The peptides were synthesized with the very acid labile 4-methyltrityl (Mtt) group on all amino groups to be modified. After completion of the peptide synthesis the Mtt group was selectively cleaved and the free amino groups were modified by 2,3 : 4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose in a Lobry de Bruyn reaction yielding the *N*-(1-deoxy-D-fructos-1-yl) peptides in high yields and purities.



Scheme 1 Reaction scheme for the synthesis of Amadori peptides. (a) H_2SO_4 in acetone, RT, 3 h; (b) DMSO, TFAA, TEA in DCM, −65 °C, 30 min; (c) 1% TFA in DCM; (d) $NaBH_3CN$ (25 equiv.), in methanol : isopropanol : water (2 : 2 : 1 by vol.), 70 °C, 18 h; (e) 5% H_2O in TFA for 1 and 2 h.

RESULTS AND DISCUSSION

2,3 : 4,5-Di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose was synthesized in high purity with an overall yield of approximately 38% including the final purification by normal phase chromatography. The reagent was stable for at least six months, if stored at 4 °C as a solution in dry methanol. Aliquots were added to the peptide resin carrying one lysine residue with a free ε-amino group to be glycated, whereas all other trifunctional amino acids were fully protected (Scheme 1). The tested peptide sequences were almost quantitatively modified over night, yielding the expected glucose-derived Amadori peptides as main products with purities above 80%, as calculated from the peak areas on reversed-phase high-performance liquid chromatography (RP-HPLC) (Table 1, Figure 1 panel A). The correct structures of the glycated peptides were confirmed by the b- and y-series ions using MALDI-TOF/TOF tandem mass spectrometry (Figure 2). Two by-products eluting later than the targeted peptide were detected on RP-HPLC at a relative amount of approximately 8% each (Figure 1, panel A). The unmodified peptide was not detected. The MALDI-TOF mass spectra of the crude peptides displayed also the mass corresponding to the glycated peptide as main peak accompanied by a strong sodium adduct ion (Figure 1, panel B), which is characteristic for peptides carrying sugar moieties. The signals corresponding to the unmodified peptides were also detected, but their intensities do not allow any conclusions to be drawn about their relative contents in the samples, as signal intensities in MALDI mass spectra depend on many parameters, especially the ionization behavior and the sample distribution in the matrix. Integration of the RP-HPLC data is more accurate, and indicated that the applied glycation strategy was very effective and can be applied to longer or more sterically hindered sequences. The low presence of the unmodified peptide could be explained by either an incomplete cleavage of the Mtt group or an incomplete glycation reaction, which was not further investigated.

The two additional fractions on RPC eluting after the Amadori peptide, i.e. peaks at 34.9 and 43.3 min for the ASKASKFL amide peptide (Figure 1, panel A), showed a mass increase of 40 and 242 u respectively, relative to the glycated peptide ion $[M + H]^+$. The mass

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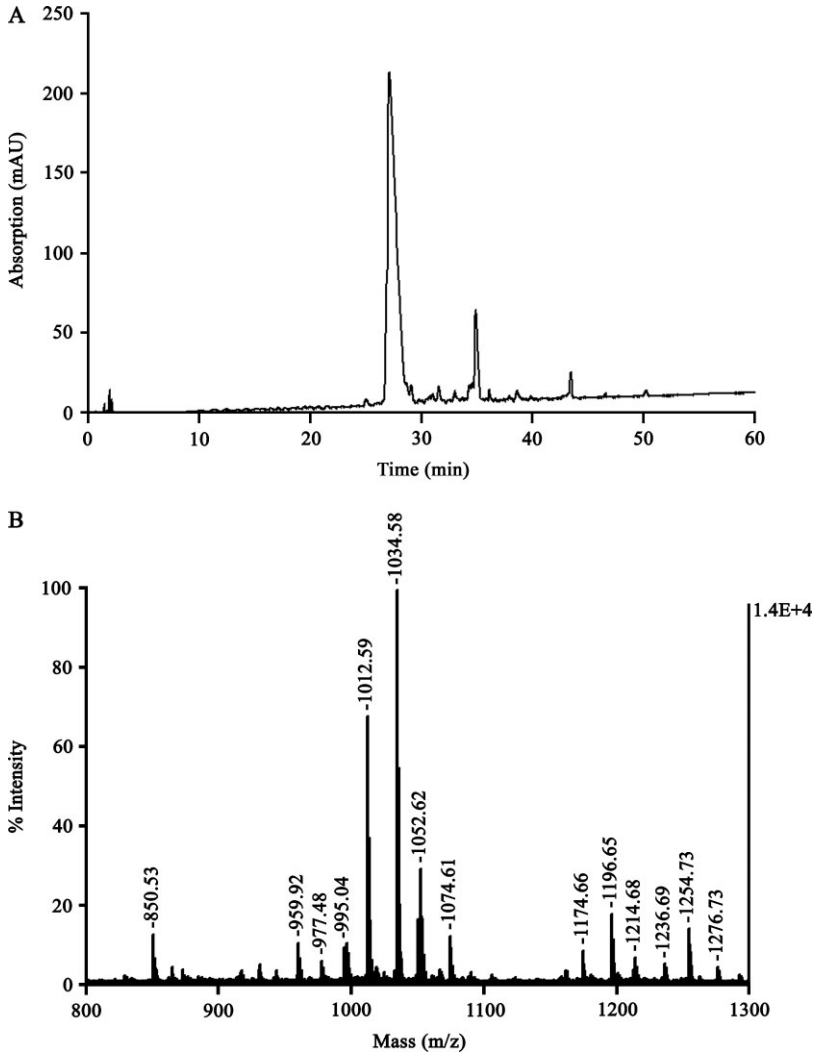

Figure 1 Reversed-phase chromatogram (A) and MALDI reflector TOF mass spectrum (B) of the crude H-ASKASKFL-NH_2 peptide after glycation of lysine in position 3 and TFA cleavage. The peaks at 27.1, 34.9, and 43.3 min displayed major peptide signals at m/z 1012.59, 1052.62, and 1174.66, respectively, as well as the corresponding sodium adducts. The mass spectrum was recorded in positive ion mode using α-cyano-4-hydroxy-cinnamic acid as matrix.

Table 1 Crude peptides glycated at the ε-amino groups of lysine on solid phase. The retention times were recorded on reversed-phase HPLC and the purities were calculated from the peak areas

Peptide sequence[a]	Retention time (min)	Purity (%)
H-AK* ASASFL-NH_2	30.2	83.7
H-ASK* ASKFL-NH_2	27.1	83.8
H-AK* ASADFL-NH_2	30.6	82.3
H-AK* DSASFL-NH_2	30.1	86.8

[a] K* denotes ε-*N*-(1-deoxy-D-fructos-1-yl)-lysine.

shifts of both by-products were linked to the modified lysine residue by the corresponding b- and y-ions of the MALDI-TOF/TOF tandem mass spectra (data not shown). The mass increase of 40 u corresponded to an isopropylidene protecting group of the sugar. For shorter TFA cleavage times the content of the singly protected peptide increased significantly and the doubly protected peptide was also detected in the chromatogram, as confirmed by a total mass shift of 80 u relative to the glycated peptide in MALDI-TOF-MS. Taken together, the two-step TFA cleavage resulted in the highest yields of the Amadori peptide with only minor quantities of the partially protected by-product, whereas a single cleavage step for up to 5 h was not very effective. As the singly protected peptide was only a minor impurity and well separated on RP-HPLC, stronger cleavage conditions using trimethylsilyl bromide (TMSBr) or trifluoromethanesulfonic acid (TFMSA) were not further investigated.

The second minor by-product eluted about 15 min later than the Amadori peptide (Figure 1(A)) with a

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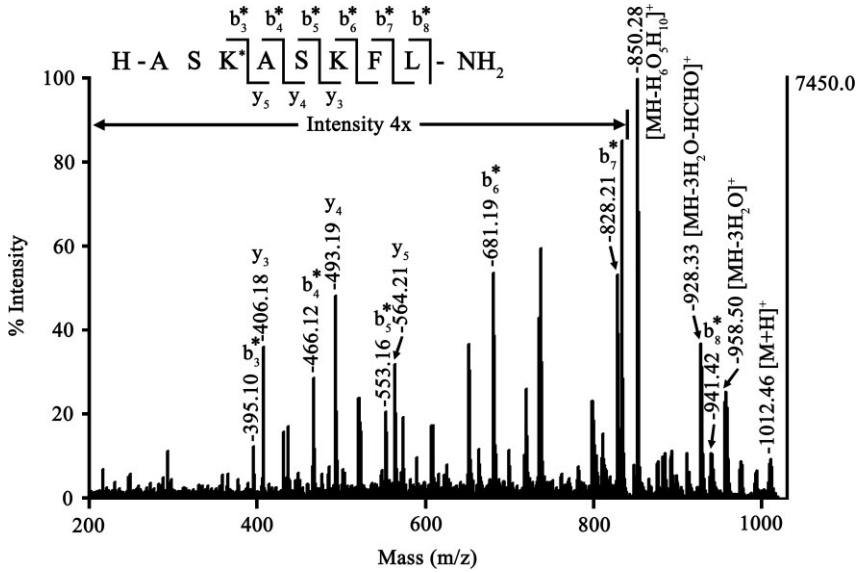

Figure 2 Fragment ion spectrum of peptide H-ASKASKFL-NH_2 glycated at the ε-amino group of lysine in position 3 recorded in the positive ion mode using the MALDI-TOF/TOF MS. The sequence was confirmed by the marked y- and furylium b*-fragment ions as illustrated above. The matrix was α-cyano-4-hydroxy-cinnamic acid. The intensities of the m/z range 200–840 have been enlarged 4 times.

mass increase of 242 u relative to the glycated peptide. The mass difference was explained by a second glycation site and two isopropylidene protecting group, probably one protecting at each sugar moiety. Most likely, the *N*-terminal Boc group slowly released during the reductive amination, yielding approximately 1–2% of the doubly glycated peptide. Owing to the low content of this by-product and its significantly higher retention times, it neither complicates the synthesis of longer peptides nor reduces the yields significantly. All other by-products can be neglected.

Taken together, the synthesis strategy described here using 2,3 : 4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose instead of unprotected D-glucose [19] appears superior, as fewer by-products were obtained and the yields for all studied peptides were much higher. The content of the glycated peptides in the crude products was above 80% compared to only 30% with free D-glucose [19].

CONCLUSIONS

N-(1-Deoxy-D-fructos-1-yl) peptides were automatically synthesized by standard Fmoc/^{t}Bu-chemistry in high yields and above 80% purities, superior to the recently reported solid-phase approaches [19,21]. Only minor by-products resulting from doubly glycated peptides and partially protected sugar moieties were obtained, which were well separated by RP-HPLC.

MATERIALS AND METHODS

Reagents

Standard Fmoc-amino acid derivatives were from Multi-SynTech GmbH (Witten, Germany) with the side chains being protected by *tert*-butyl-based protecting groups for serine, aspartic acid, and lysine. Fmoc-Lys(Mtt)-OH, Boc-Ala, and polystyrene-based 4-methylbenzhydrylamine (MBHA) resin (loading capacity 0.64 mmol/g), were obtained from Merck KGaA (Darmstadt, Germany). $NaBH_3CN$, trifluoroacetic anhydride (TFAA, p.a.), DCM (absolute), DMSO (absolute), TFA (UV-spectroscopy grade for eluents and purum for cleavage), DIC, and HOBt were from Fluka (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Triethylamine (TEA, $\geq$99%) was from Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). DMF (peptide synthesis grade) and acetonitrile (HPLC-S gradient grade) were from Biosolve V.B. (Valkenswaard, Netherlands). Water was purified on an ELGA system (LabWater Supplier, Bucks, Great Britain) in house. 2,3 : 4,5-Di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose was synthesized as described in the literature [24] using 2,3 : 4,5-di-*O*-isopropylidene-β-fructopyranose [25].

Solid-Phase Peptide Synthesis

Peptides were synthesized on a Syro2000 multiple peptide synthesizer (MultiSynTech GmbH) using eight equivalents of Fmoc-amino acids activated with DIC and HOBt in DMF as described recently [19]. The Mtt group was cleaved with 1% TFA in DCM at room temperature for 30 min [26]. The resin was washed with DCM and dried.

Synthesis of Amadori Modified Peptides

$NaBH_3CN$ (5.4 mg, 86 µmol) was dissolved in 20 µl of water and diluted with 40 µl of methanol and 40 µl of isopropanol.

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This mixture and 2,3 : 4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose (22.3 mg, 86 μmol) dissolved in 100 μl dry methanol were added to the peptidyl resin containing free amino groups to be modified. Both reagents were added at a 25-fold molar excess relative to the free amino groups on the peptidyl resin. The reaction mixture was shaken at 70 °C. After 18 h the reaction mixture was cooled to room temperature and 50 μl HCl (1 mol/l) were added to destroy the excess of $NaBH_3CN$. The solution was removed, and the resin was washed with DMF and DCM and dried under vacuum for 1 h. The peptides were cleaved with 5% water in TFA at room temperature for 1 h and precipitated with cold diethyl ether. After centrifugation (2000*g*) the pellet was washed twice with cold ether, dried and dissolved in 200 μl aqueous TFA (0.1%). The lyophilized samples were treated once more with 5% water in TFA to cleave the sugar protecting groups quantitatively and precipitated with cold diethyl ether after 2 h. The samples were washed twice with diethyl ether, dried, dissolved in 0.1% aqueous TFA, and stored at −20 °C.

Peptide Purification

Crude peptides were purified on a Zorbax Eclipse XDB-C8 column (internal diameter 4.6 mm, length 150 mm, particle size 5 μm, Agilent, Waldbronn, Germany) using a Beckman System Gold analytical HPLC system (Krefeld, Germany) equipped with a 125NM gradient pump, a 168NM diode array detector and a 508 autosampler (100 μl injection loop) controlled by the Gold 32 Karat Software (version 5.0). Eluent A was water and eluent B was 60% aqueous acetonitrile, both eluents containing 0.1% TFA as ion pair reagent. The column was equilibrated with 5% eluent B. After sample injection the starting conditions were held for 5 min before a linear gradient to 50% eluent B for 60 min and to 100% eluent B for 15 min was started using a flow rate of 1 ml/min. Absorption was monitored at 220 nm. Fractions were analyzed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS; 4700 proteomic analyzer, Applied Biosystems GmbH) operated in positive ion reflector TOF mode using α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics GmbH, Bremen, Germany) as matrix [19].

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