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Site-specific synthesis of Amadori-modified peptides on solid phase

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Abstract: Glycation of peptides and proteins is a slow chemical reaction of reducing sugars modifying the amino groups. The first intermediates of this nonenzymatic glycosylation are the Amadori products that can undergo further chemical reactions, finally leading to advanced glycation end products (AGEs). The formation of AGEs was not only linked to aging of tissues and organs in general but also to several diseases such as diabetes mellitus and Alzheimer's disease. Because of the importance of these modifications and their potential use as diagnostic markers, a global postsynthetic approach on solid phase was developed. The peptides were synthesized by Fmoc/^{t}Bu-chemistry, with the lysine residue to be modified being protected with the very acid-labile methyltrityl group. Incubation of the peptides with D-glucose in DMF at elevated temperatures resulted in product yields of 35%. Neighboring residues with bulky protecting groups reduced the yields only slightly. The major by-products were the unmodified peptide and an oxidation product. Whereas the unmodified peptide eluted before the glycated peptide, all other by-products eluted later in RP-HPLC, allowing simple purification. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Amadori product; glucose; glycation; glycoxidation; Maillard rearrangement

INTRODUCTION

In higher organisms, proteins are often co- or post-translationally modified by sequence-specific enzymatic or side-chain-specific nonenzymatic reactions. The latter modifications are typically slow chemical reactions that alter the side chains of some amino acid residues. Besides deamidation of Asn and Gln residues, glycation of lysines represents one of the most dominant modifications formed by the Maillard reactions of α-oxoaldehydes and reducing carbohydrates [1]. Initially, a carbonyl compound interacts with a free proteinogenic amino group yielding a Shiff base, which undergoes a rapid rearrangement forming more stable *N*-(1-deoxy-D-fructose-1-yl)-amino acid derivatives known as *Amadori products* [2]. In aqueous solutions, the open α-furanose, β-furanose and β-pyranose forms stay in equilibrium with prevalence for β-pyranose [3]. They gradually undergo irreversible transformations yielding various advanced glycation end products (AGEs) that are in different oxidation and degradation states [4]. AGEs are toxic and mutagenic [5] and their accumulation accompanies ageing, diabetes mellitus, renal failure and Alzheimer's disease [6,7]. Several reaction schemes leading to such compounds in aqueous solutions have been described: oxidation of monosaccharides in the presence of transition-metal ions [8], fragmentation of Shiff base [9] and degradation of fructosamines [10]. As the formation of Amadori products is an initial and reversible stage of the Maillard reaction, the increase of the fructosamine content in tissues can be used as an early diagnostic marker for related diseases [11]. For a valid diagnosis, it is important to identify the modified proteins and their modification sites by proteomic techniques [12]. However, standard compounds are currently not accessible by routine peptide synthesis.

Amadori derivatives of peptides and proteins 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 [10,13,14]. However, these protocols are not site-specific. In the best-case scenario, one or more positions, especially less basic lysine residues, are kinetically favored yielding the corresponding derivatives at a relatively high content with only minor impurities. The Amadori rearrangement is catalyzed by an acid–base mechanism and glycation is favored by the presence of neighboring imidazole groups of histidine residues [15] as well as carboxy and phosphate groups [16,17]. These conditions are not optimal for the synthesis of fructosamine derivatives, since the resulting Amadori products are rather unstable under these conditions and their degradation is accelerated by various metal ions [18]. Even traces of transition-metal ions and oxygen in the aqueous buffers initiate the Fenton reactions leading to the generation of hydroxy radicals and oxidative degradation of fructosamines [8]. Thus, it is a rule of thumb that the faster Amadori products are formed, the faster AGE products are formed. These numerous site reactions are responsible for the low yields of

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Amadori products and the often-irreproducible site reactions.

Organic solvents typically reduce such site-specific reactions by providing a higher stability of the Amadori compounds even at elevated temperatures [19]. Methanol may reduce the reaction time and minimizes AGE formation [20]. *N*-terminal Amadori products of leucine–enkephalin were obtained either by refluxing in the presence of reducing sugars or by reductive amination of fully protected hexodiulose in the presence of sodium cyanoborohydride [21,22]. Most recently, glycation was also reported in the solid state by mixing a protein with sugar and heating [23]. This approach was also applied to peptides dissolved in DMF [24].

All these strategies do not allow a position-specific synthesis of peptide-derived Amadori products for sequences containing more than one potential glycation site. Therefore, a general synthetic strategy was developed to synthesize site-specific 1-amino-1-deoxy-fructose-modified peptides on solid phase using Fmoc/^{t}Bu-chemistry without restrictions to activation reagents. The lysine to be glycated was introduced as Fmoc-Lys(Mtt)-OH allowing selective deprotection of the acid-labile side-chain-protecting group [25]. The optimized glycation on the solid phase was performed by adding a solution of the corresponding sugar in DMF and then heating the mixture. The reaction conditions were optimized for a short peptide-containing lysine in mid-chain position and tested afterwards for further peptide sequences containing other trifunctional amino acid residues.

MATERIALS AND METHODS

Solid-phase Peptide Synthesis

Amino acid derivatives were from MultiSynTech GmbH (Witten, Germany) using Fmoc-Ser(^{t}Bu)-OH, Fmoc-Asp(^{t}Bu)-OH and Fmoc-Lys(Boc)-OH for trifunctional amino acids. Fmoc-Lys(Mtt)-OH, Boc-Ala and polystyrene-based 4-methylbenzhydrylamine (MBHA) resin (loading capacity 0.64 mmol/g) were from Merck Biosciences (Schwalbach, Germany). Peptides were synthesized on a Syro2000 multiple peptide synthesizer (MultiSyn Tech GmbH) using 8 equiv. of amino acids activated with 1,3-diisopropylcarbodiimide DIC/HOBt (Fluka, Buchs, Switzerland) in DMF (Biosolve V.B., Valkenswaard, Netherlands) [26]. The Mtt-group was cleaved with 1% TFA (Fluka) in DCM (Roth, Karlsruhe, Germany) at room temperature for 30 min [25]. This deprotection of lysine was monitored by a ninhydrin assay [27].

Synthesis of Peptide Amadori Products

Typically, Mtt-deprotected, ninhydrin-positive peptide resin (5 mg) was reacted in a 1.5 ml Eppendorf tube with D-glucose monohydrate (500 µl, 0.25 mol/l) in dry DMF (stored over molecular sieve 4 Å), which was a 40-fold molar excess of D-glucose. Oxygen in the reaction mixture was removed by vacuum before 3 µl water was added and nitrogen was bubbled through the solution for at least 10 min. The tightly sealed tubes were incubated at 70 °C for 18, 45 or 100 h. Alternatively, glycation was performed at 110 °C using glass tubes, omitting water completely, for 5, 10, 15, 25, 40, 60 or 90 min. After cooling to room temperature, the peptide resin was washed thoroughly and dried under vacuum after a final DCM wash. The resin-bound peptides were cleaved and deprotected with 5% water in TFA at room temperature for 2 h. The peptides were precipitated with cold diethyl ether and centrifuged at 2000 *g*. The pellet was washed twice with cold ether, dried under air and dissolved in 0.1% aqueous TFA (400 µl; Fluka, UV-spectroscopy grade). The samples were stored at −20 °C.

Peptide Purification

The crude peptides and glycated peptides were purified on an Aqua C-18 column (2.0 × 150 mm, particle size 3 µm, Phenomenex Inc., Torrance, USA) using a Beckman System Gold analytical HPLC system (Fullerton, California, USA) equipped with a 125 NM gradient pump, a 168 NM diode array detector and a manual injector with a 20-µl injection loop (Rheodyne, Cotati, USA) controlled by the Gold 32 Karat Software (version 5.0). Eluent A was 0.1% aqueous TFA and eluent B was 80% aqueous methanol also containing 0.1% TFA as an ion pair reagent. The peptides were eluted with 1% eluent B for 15 min followed by a linear gradient to 100% eluent B for 50 min using a flow rate of 150 µl/min. The peptides were detected by absorption at 220 nm. Fractions were collected manually in 1.5 ml tubes and concentrated in vacuum. The fractions were analyzed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS; 4700 proteomic analyzer, Applied Biosystems GmbH, Darmstadt, Germany) operated in positive ion mode using α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics GmbH, Bremen, Germany) as matrix. The peptide masses were recorded in reflector TOF (reTOF) mode and the peptide sequence was additionally confirmed by collision-induced fragmentation (TOF/TOF) to confirm the glycated position. Some fractions were reduced with 500–1000 molar excess of $NaBH_4$ (Fluka) in aqueous solution at room temperature. After 4 h, the reaction was terminated by the addition of 1 mol/l HCl (50 µl) to destroy the excess of borohydride [28].

RESULTS AND DISCUSSION

The first experiments were carried out with the simple *N*-terminally Boc-protected peptide sequence AGGKAA. Then Gly and Ala were substituted with further trifunctional amino acids to study if (i) the used protecting groups were stable under the applied conditions, (ii) the yields were reduced by neighboring bulky side chains and (iii) these residues favored oxidation or degradation during the harsh glycation conditions. The sequences AKASAS, AKASAD, AKDSAS and ASKASK contained polar and charged residues near the glycation site. The last sequence was chosen to show whether a single Lys could be specifically

modified in sequences carrying other Lys residues in close proximity. The Mtt-protected lysine position to be modified was deprotected with 1% TFA in DCM, allowing the selective glycation of this position, since the Boc- and ^{t}Bu-protecting groups are stable against these cleavage conditions.

All unmodified peptides were synthesized in high yields and purities and the Mtt-group was quantitatively cleaved with 1% TFA. The on-resin glycation was performed in DMF because of its good swelling conditions and its relatively high boiling point. Furthermore, monosaccharides are well soluble in DMF. Owing to the long reaction times at 37 °C known from the literature [10,13,14], we did not further investigate these conditions but started at 70 °C, which was also described for glycation in methanol [20]. First, the AGGKAA sequence was modified. After 18 h, the peptide was not completely modified and more than 50% by-products were obtained on RP-HPLC, mostly hydrophobic contaminants (data not shown). This low yield and purity could be due to the known instability of the fructosamine derivatives, which are degraded and cross-linked in the presence of oxygen [8,9]. Indeed, evacuation of the reaction mixture to remove oxygen and performing the reaction under nitrogen atmosphere reduced the by-products by half in favor of the Amadori compound. On the basis of this result, oxygen was excluded from all further reaction mixtures as much as possible.

To optimize the reaction yields and reduce the number and content of by-products, the reaction times were increased from 18 h to 45 and 100 h, keeping all other reaction conditions similar. The MALDI-TOF mass spectra of the crude peptides glycated for 45 h displayed many and mostly weak signals spanning a mass range from the unmodified peptide mass up to about 1600 Da. The very complex mixture of different products is illustrated for peptide H-AKASAS-NH_2 in Figure 1. The MALDI mass spectrum of the glycated peptide displayed singly charged molecular ions at m/z 533.3 u for the unmodified peptide, 695.4 u for the Amadori peptide and 761.4 u for the major by-product (Figure 1). These masses were also detected for longer reaction times up to 100 h with a decreasing content of the Amadori peptide, although signal intensities in MALDI mass spectra do not necessarily reflect quantitatively the component ratios in a sample. Analysis of these short polar sequences selected to optimize glycation by RP-HPLC revealed their major drawback. The peptides eluted very early on C-18 columns using regular acetonitrile gradients starting at 5% in the presence of 0.1% TFA. Moreover, the unmodified and glycated peptides co-eluted even under isocratic conditions. Thus we replaced acetonitrile by methanol and used an Aqua-column, which can be used with pure aqueous eluents without collapse of the stationary phase. With these conditions, two peaks eluted under isocratic conditions at 0.8% aqueous methanol (0.1% TFA) corresponding to the unmodified and the glycated peptides. Furthermore, many more hydrophobic, only partially separated, by-products were obtained as well as one intense, very hydrophobic, well-separated compound, eluting at approximately 65% eluent B (Figure 2A).

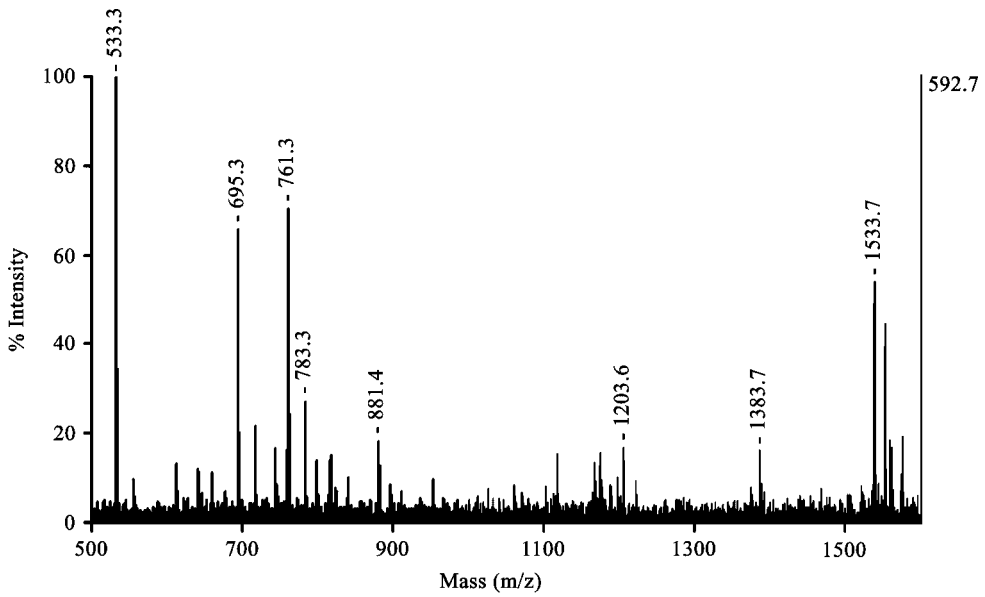

Figure 1 MALDI reflector TOF mass spectrum of the crude glycated H-AKASAS-NH_2 peptide. For glycation, 5 mg of the resin-bound peptide was incubated with 0.25 mol/l D-glucose in DMF/water under nitrogen atmosphere at 70 °C for 45 h. After cleavage with 5% water in TFA at room temperature for 2 h, the peptide was precipitated with diethyl ether, lyophilized and dissolved in 0.1% aqueous TFA. This solution (0.5 µl) was mixed with the same volume of 2 mg α-cyano-4-hydroxy-cinnamic acid dissolved in 500 µl of a water–acetonitrile mixture (1 : 1 v/v) containing 0.1% TFA on the target. The mass spectrum was recorded with a 4700 proteomic analyzer MALDI-TOF/TOF mass spectrometer.

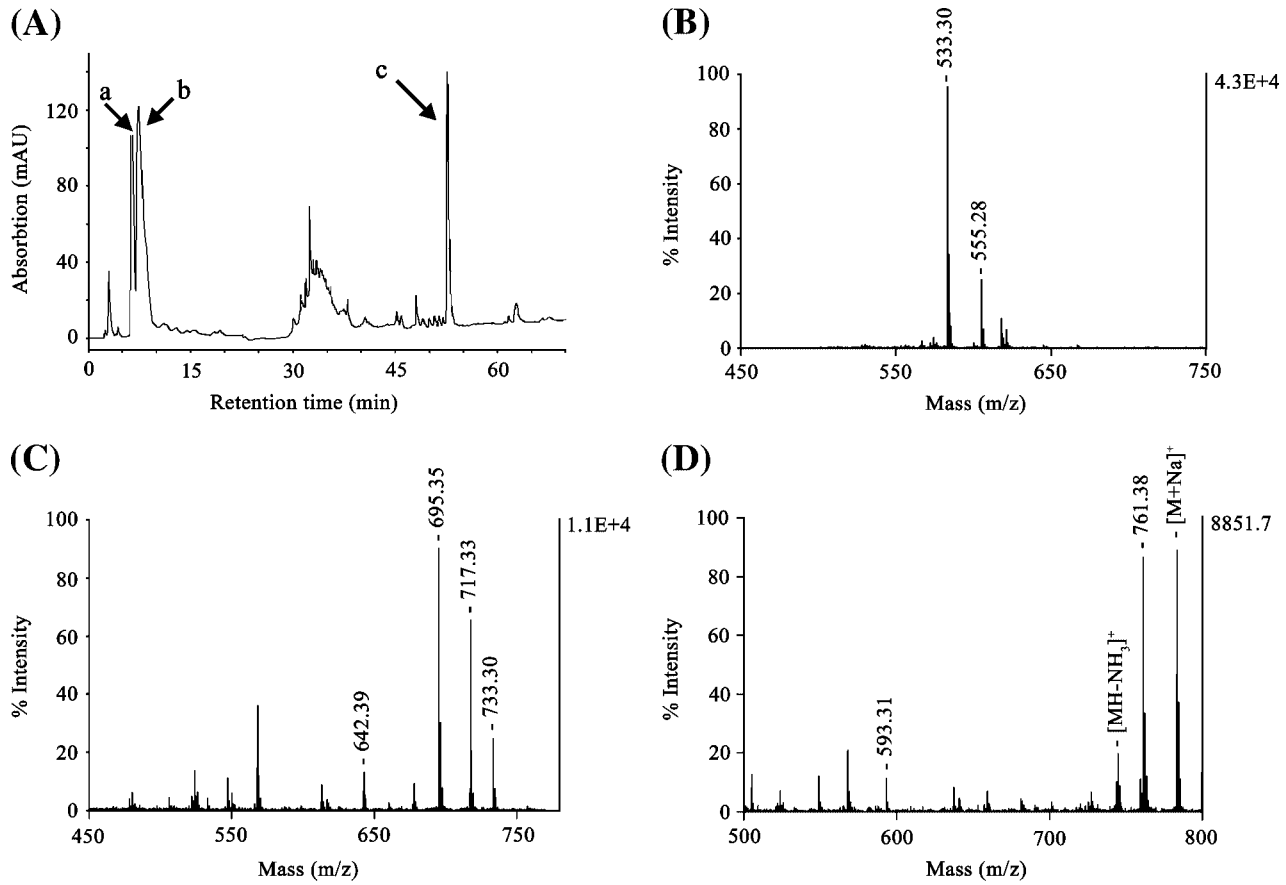

Figure 2 (A) Reversed-phase chromatogram of the crude H-AKASAS-NH_2 peptide after glycation of the lysine residue using 0.25 mol/l D-glucose in dry DMF under nitrogen atmosphere (70 °C, 45 h) and cleavage with a mixture of 5% water in TFA at room temperature for 2 h. The three main fractions a, b and c were further characterized by MALDI-reTOF-MS using α-cyano-4-hydroxy-cinnamic acid as matrix. The fractions corresponded to the unmodified peptide (B) (m/z 553.30), Amadori product (C) (m/z 695.35) and the presumed final oxidation product (D) (m/z 761.38).

Mass spectral analysis of the fractions identified the first abundant peak at 6.4 min as the unmodified peptide H-AKASAS-NH_2 (Figure 2B). The next peak with a retention time (RT) of 7.4 min (Figure 2C) was the targeted Amadori product with the characteristic mass shift of 162 Da corresponding to 1-deoxyfructosamine. This elution order can be explained by the stronger interaction of the sugar with the stationary phase compared to the protonated ε-amino group of lysine, which is in contrast to the typical elution order of *O*- and *N*-glycosylated peptides *versus* unmodified peptides. It is important to note that this elution order might be sequence or eluent dependent, as for longer peptides a reversed elution order was described [11].

Considering reaction times of 18, 45, and 100 h, it appeared that the Amadori product was formed first and reached a maximum relative content at approximately 45 h, when most of the peptide was modified. At longer reaction times up to 100 h, the content of the unmodified peptide decreased until it was below the detection limit on RP-HPLC. The yield of the Amadori product decreased also with longer reaction times, whereas the peak areas of the later-eluting by-products increased, indicating that they were formed by successive reactions, most likely oxidation. This might be a result of slow oxygen diffusion through the polypropylene vials into the solution.

The chromatogram of the crude glycated product after a reaction time of 100 h was dominated by a hydrophobic peptide with m/z 761.4 u, which eluted at 53 min in RP-HPLC. The fact that both the mass and the RT of this by-product depended on the peptide sequence indicated that this by-product indeed was a modified peptide carrying some kind of chemically modified, most probably oxidized, sugar. Between the two peaks of the Amadori peptide and the presumed final oxidation product, again a complex elution pattern of further peptidic by-products was obtained, which most likely represented intermediate oxidation and degradation products or peptides containing several glucose units on a single Lys residue due to a condensation reaction under dry conditions at elevated temperatures. At least the high masses above 800 u indicate such a condensation probably in conjunction with oxidation and degradation reactions. The elucidation of the composition and structure of these by-products was

outside the focus of this report. For the other four sequences tested, the highest yields of the Amadori products were also obtained after 45 h with yields of approximately 30%. Overall, the data were very well reproducible and consistent among the different studied pentapeptides. No peptide loss by prolonged heating was observed, indicating the stability of the peptide linker. Furthermore, the side-chain-protecting groups were stable and effectively prevented glycation at other residues.

The structure of the glycated peptide was further confirmed by tandem mass spectrometry using MALDI-TOF/TOF-MS (Figure 3). The mass losses of 54 u and 84 u could be explained by cleavage of three water molecules (54 u) or three water molecules together with one molecule of formaldehyde (84 u). The resulting pyrylium and furylium (or immonium tautomer) ions are characteristic for the pyranose moiety [29] of the parent ion at m/z 695.4 u. Furthermore, the b_2–b_5 and the y_5-ions confirmed unambiguously that only the ε-amino group of Lys was modified. All these fragment ions displayed also mass losses representative of the presumed formation of pyrylium and furylium derivatives (Figure 3).

The assumption that the hydrophobic by-product represented an oxidation product was further confirmed by addition of a 500–1000-fold molar excess of $NaBH_4$ to reduce this by-product. Indeed a mass loss of 64 u was observed, which might correspond to four oxygen atoms. The molecular mass of 696.37 Da is 2 Da higher than the mass of the corresponding Amadori peptide, most likely because of hydrogenation of the keto function of the sugar moiety to a hydroxy group.

The slow reaction rates at 70 °C as well as the increased oxidation at longer reaction times prompted us to further increase the reaction temperatures. As described for glycation in dry solid state [23,24], we glycated the sequence AKASAS at 110 °C in DMF. This sequence was chosen, as intermediate yields of the Amadori peptide were obtained at 70 °C. The yields of the Amadori product relative to the unmodified peptide and the most dominant by-product, i.e. the final oxidation product, were determined after reaction times of 5, 10, 15, 25, 40, 60 and 90 min (Figure 4). The relative peptide content decreased very fast at the beginning and continuously, whereas the Amadori product increased for the first 40 min to a maximum of approximately 55% and decreased afterwards slowly to 50% at 90 min. The content of the unmodified peptide and the oxidized product at 40 min was about 20% each. The intensity of the hydrophobic by-product increased very slowly at the beginning. It basically followed the intensity curve of the Amadori product with a time delay of about 20 min. The data suggested two concurrent reactions. First, formation of the Amadori product followed by successive oxidation, degradation or other modifications yielding unstable intermediates that finally lead to the hydrophobic peptidic by-product (Figure 4). It should be stressed that these numbers represent only the relative content of the three main products and do not consider all intermediates detected by RP-HPLC. If these additional peak areas of the RP chromatogram are included, the maximum yield was 37% at 25 min, which decreased to 33% at 40 min. Compared to the results at 70 °C, this high-temperature reaction appears superior, yielding the Amadori peptides relatively faster and at reasonable

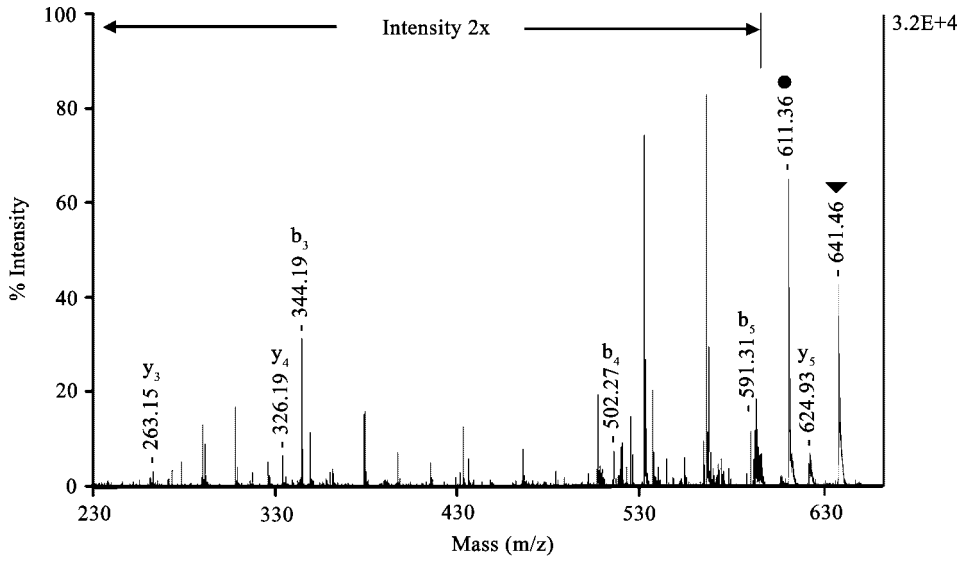

Figure 3 Fragment ion spectrum recorded in MALDI-TOF/TOF mode for peptide H-AKASAS-NH_2 glycated on the lysine residue. Marker ions at m/z 611.36 and 641.45 most probably corresponded to furylium and pyrylium ions that are formed by neutral losses of 84 u and 54 u from the parent peptide. The sequence was confirmed by the marked b- and y-fragment ions, especially the mass difference between y_4 and y_5, which corresponded to the expected mass increment of glycated lysine.

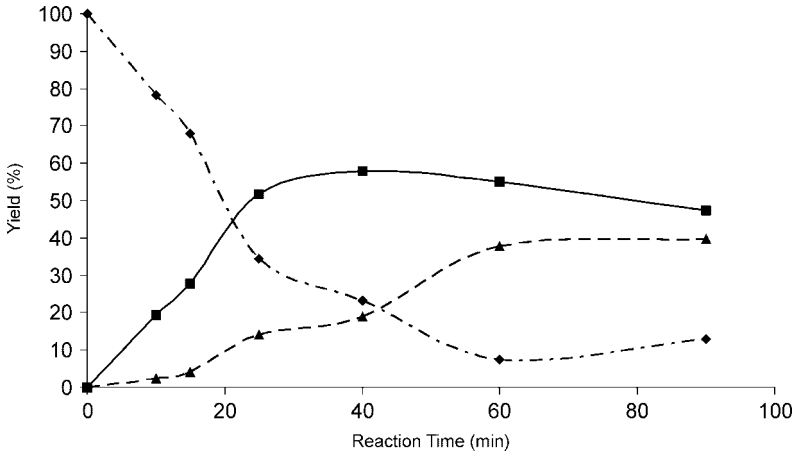

Figure 4 Relative contents of peptide H-AKASAS-NH_2 (diamonds, dashed and dotted line), the corresponding glycated sequence (squares, solid line) and the final oxidation product (triangles, dashed line) in the crude peptide obtained after glycation of the lysine residue in resin-bound peptide using 0.25 mol/l D-glucose in dry DMF under nitrogen atmosphere (110 °C) for 5, 10, 15, 25, 40, 60 or 90 min. Measurements were performed in duplicates.

yields. The by-products of the pentapeptides were well separated from the targeted compound, indicating that they can be separated by RP-HPLC also for longer sequences. Another advantage of this protocol was that the glucose solution did not alter its color, whereas at 70 °C the solutions turned yellow to light brown because of the long reaction times. This was probably due to caramelization of the sugar, which, however, did not negatively affect the peptide yields and purities.

Another concern might be racemization of the peptide due to the elevated temperatures. We have not tested this possible side reaction, as it was already shown a few years ago for microwave-assisted peptide synthesis that peptides can be synthesized effectively at elevated temperatures up to 110 °C without detectable racemization [30]. In contrast, saccharides induce partial racemization of free L-amino acids at elevated temperatures [31].

CONCLUSIONS

Glycated peptides bearing a 1-deoxyfructosamine moiety can be specifically synthesized on solid phase using Fmoc/^{t}Bu-chemistry with DIC/HOBt-activation. After selective deprotection of the position to be modified, the Amadori compound was synthesized using the monosaccharide dissolved in DMF and heating to 110 °C for 40 min. After cleavage and deprotection of the peptide with TFA, the glycated peptide was obtained at reasonable yields of approximately 30 to 35% with only minor influence of the neighboring residues including their bulky side-chain-protecting groups. However, many by-products were obtained that all eluted much later than the Amadori peptide and could therefore be separated by RP-HPLC. This synthetic approach appears advantageous over the syntheses performed in solution so far, mostly because of its site-specificity, high reproducibility, short reaction times and superior yields. This will allow further studies based on synthetic peptides to elucidate oxidation, degradation and cross-linking of Amadori compounds finally yielding AGE products. Furthermore, analytical techniques including monoclonal antibodies can be developed with well-defined peptides of high purities. Further studies currently underway in our laboratory target the limitations of this approach for synthesizing medium-sized peptides and its compatibility with all proteinogenic amino acids.

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