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ORIGINAL ARTICLE

Methods of the site-selective solid phase synthesis of peptide-derived Amadori products

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Abstract Two procedures of glycated peptides' synthesis have been developed. The first method involves reductive alkylation of the ε-amino groups of lysine with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose in the presence of sodium cyanoborohydride on solid support. The second one uses a new fully protected lysine derivative, which is a building block designed for direct introduction of the glycated lysine moiety into a peptide, according to the standard solid phase synthesis protocol. The applicability of the proposed methods for the synthesis of peptide-derived Amadori products is discussed. The structure of the synthesized glycated peptides was confirmed by high-resolution mass spectrometry and enzymatic hydrolysis. Circular dichroism studies, performed in water solution, revealed that the formation of the Amadori rearrangement product in the lysine side chain does not influence significantly the conformational preferences of the peptides studied. However, when the solvent was changed to trifluoroethanol, the glycated peptides preferred β-turn conformation.

Keywords Solid phase peptide synthesis · Amadori rearrangement · Glycation · Enzymatic stability

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Introduction

Amadori compounds—*N*-substituted (1-deoxy-ketos-1-yl) amines—are representatives of an important class of Maillard intermediates. These compounds, formed in non-enzymatic reactions of proteins' amino groups with glucose and other reducing sugars, may decompose and initiate further reactions. The final result of that process is the formation of complex mixtures of advanced glycation end products (AGEs) (Ahmed and Thornalley 2003) involved in aging and pathological processes such as diabetes and (Thorpe and Baynes 1996; Mosier et al. 1986) Alzheimer's (Moreira et al. 2005) and Parkinson's (Munch et al. 1998) diseases. The proteolytic processing of glycated proteins produces a mixture of glycated peptides, which can be considered as the markers for diabetes and other diseases. The defined and analytically pure peptide-derived Amadori products could be useful as model compounds for studying the formation of AGEs. Therefore, the search for new methods of synthesis of Amadori products is intense (Frolov et al. 2006b, 2007; Stefanowicz et al. 2007).

The first successful methods of peptide-derived Amadori products' synthesis were based on the solution phase approach. The peptides were directly glycated in the presence of reducing sugars, such as glucose, mannose or galactose (Roscic and Horvat 2006) or, alternatively, a protected aminofructose moiety was incorporated into the peptide chain by reductive alkylation of the amino group by 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose (Forrow and Batchelor 1990; Horvat and Jakas 2004). Recently, several new procedures of the site-selective synthesis of Amadori-modified peptides on solid support appeared. The protocol developed by Frolov et al. (2006b) is based on the direct reaction of a deprotected ε-amino group of lysine with the solution of glucose in DMF.

The Frolov's procedure is simple and general (allows the synthesis of conjugates of fructose, ribose and other reducing sugars (Frolov et al. 2006a), and potentially may be useful for the synthesis of izotopicaly labeled compounds). However, its low yield (30–35%), difficulties with the product purification and a serious risk of side reactions limit its application. Moreover, the process is rather slow and requires relatively high temperature (70–110°C). Our recent paper (Stefanowicz et al. 2007) described a new method of solid phase synthesis of peptide-derived Amadori products involving the reductive alkylation of ε-amino groups of lysine with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose in the presence of sodium cyanoborohydride on solid support. The reagent used for this procedure was previously used for solution phase synthesis of Amadori products.

A similar approach was also applied by Frolov et al. (2007). Our recent studies revealed that 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose can be replaced by its stable and crystalline hydrate, which facilitates the procedure.

In this paper we describe the synthesis of a series of model peptides—the fragments of bovine serum albumine (BSA).The synthetic fragments of BSA may be used in our further studies on the formation of the advanced glycation products. The obtained compounds were characterized by ESI-MS/MS and CD methods. The structures of glycated peptides were also confirmed by their proteolytic digestion combined with direct ESI-MS measurement. This method of analysis of Amadori modified peptides is efficient for the determination of the glycation sites and gives more comprehensive and easier for interpretation results then those from a direct MS/MS analysis. We also developed a new fully protected lysine derivative (*N*α-9-fluorenylmethoxycarbonyl-*N*ε-*tert*-butyloxycarbonyl-*N*ε-*N*-(2,3:4,5-di-*O*-isopropyliden-1-deoxy-β-D-fructopyranose-1-ylo)lysine, Fmoc-Lys(*i,i*-Fru,BOC)-OH, which is a building block useful for incorporating the glycated lysine moiety into the peptide chain. Application of this derivative allows a facile synthesis of peptide-derived Amadori products according to the standard solid phase synthesis protocol.

Materials and methods

Reagents

The derivatives of amino acids for peptide synthesis and the coupling reagent (TBTU) were purchased from NovaBiochem. Fmoc-Lys-OH × TFA was obtained from Fmoc-Lys(Boc)-OH by treatment with TFA + 5% H_2O solution followed by evaporation in vacuo. The preloaded Wang resin (0.50–0.70 mmol/g) was purchased from NovaBiochem. Sodium cyanoborohydride was purchased from Aldrich. The 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose (**1**) was prepared according to the procedure reported by Cubero (1990). The solvents for peptide synthesis (analytical grade) were obtained from Riedel de Haën (DMF) and J. T. Baker (methanol). Other solvents used in this work were obtained from Aldrich.

Synthesis[1] of Fmoc-Lys(*i,i*-Fru,Boc)-OH (**2**)

Fmoc-Lys(Boc)-OH (0.7 g, 1.494 mmol) was dissolved in trifluoroacetic acid (10 ml) containing 5% of water. After 1 h the solvent was evaporated. Obtained Fmoc-Lys-OH × TFA was dissolved in THF (50 ml) containing 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose (**1**) (3.735 mmol, 2.5 eq). After the addition of sodium cyanoborohydride (3.735 mmol, 2.5 eq) the solution was refluxed for 2 h and the mixture was kept for 5 h at room temperature. After removing THF, the crude product was acylated overnight with $(Boc)_2O$ (3.212 mmol, 2.15 eq) in dioxane (25 ml) containing triethylamine (5.378 mmol, 3.6 eq) at room temperature. After the evaporation of the solvent, the crude product was mixed with water and the mixture was brought to pH 2 using potassium hydrogen sulfate. Then it was extracted with ethyl acetate (25 ml). The organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. Finally the reaction product was purified by chromatography on a silica gel. After washing the column with chloroform containing 5% of isopropanol, the reaction product was eluted with 10% isopropanol in chloroform.

Yield: 42%, mp 79.5–84.5°C, $[\alpha]_D^{25}$ −14.10 (c 1.0, MeCN). HPLC: retention time (min) 42.83 (conditions for HPLC are given in "Materials and methods", "Purification and characterization of peptides"). ^{1}H NMR ($CDCl_3$), δ (ppm) = 1.32–1.52 (12H, m), 1.45 (4H, m), 1.48 (9H, s), 1.74 (1H, m), 1.92 (1H, m), 3.35 (2H, m), 3.50 (2H, s), 3.72 (1H, m), 3.85 (1H, m), 4.19–4.24 (1H, 1H, m two signals overlap), 4.39 (1H, 2H, m two signals overlap), 4.57 (1H, m), 5.41 (1H, m), 5.58 (1H, m), 7.19–7.77 (8H, m). HR-MS: Found 711.3487 calculated for $(C_{38}H_{50}N_2O_{11} + H)^+$ 711.3492; MS/MS (parent 711.35): 369.2, 495.2, 535.2, 553.3, 611.3, 655.3.

Preparation of peptides

Peptides were prepared by manual solid-phase techniques, on solid support, using the standard Fmoc synthetic procedure (Chan Weng and White 1999). The following side chain protecting groups for Fmoc-amino acids were used:

[1] This procedure should be performed in an efficient fume hood because of the toxicity of hydrogen cyanide.

t-butyl for Thr, Asp, Glu, and Ser, Mtt for lysine moieties intended for glycation and Boc for unglycated lysine. The consecutive amino acid residues were coupled using TBTU in DMF. After the removal of the Mtt group (using the 1% solution of TFA in dichloromethane), the ε-amino group of lysine was reductively alkylated with (**1**) in the presence of sodium cyanoborohydride. Then the obtained secondary amino group was protected with Boc_2O, and the synthesis of the peptide was continued according to standard solid phase synthesis protocol as described previously (Stefanowicz et al. 2007).

In the second synthetic protocol, which utilizes fully protected lysine derivative Fmoc-Lys(*i,i*-Fru,BOC)-OH (**2**), the standard Fmoc procedure with TBTU as a coupling reagent was applied. The peptide was cleaved from the resin using TFA/water/TIS (90:5:5, v/v) for 8 h at room temperature and precipitated with cold diethyl ether.

Purification and characterization of peptides

The crude product was characterized by HPLC on a C18 column. Solvent systems: S1 0.1% aqueous TFA, S2: 80% acetonitrile + 0.1% TFA, linear gradient from 0 to 100% of S2 for 60 min, flow rate 1.0 ml/min, UV detection at 220 nm. The main reaction product was purified by a preparative reversed-phase HPLC on a Vydac C18 column (22 mm × 250 mm), using solvent systems: S1 0.1% aqueous TFA, S2 40% acetonitrile + 0.1% TFA, linear gradient from 50 to 100% of S2 for 60 min, flow rate 7.0 ml/min, UV detection at 220 nm. The fractions were collected and lyophilized. Their identities were confirmed on the basis of molecular weights measured with a micrOTOF-Q mass spectrometer equipped with an electrospray ionization source.

MS/MS spectra

Mass spectrometric measurements were performed on a quadrupole time-of-flight (micrOTOF-Q) instrument (Bruker, Germany) equipped with an electrospray source. Spectra were recorded using aqueous solutions of acetonitrile (50%) and formic acid (1%) at the peptide concentration of typically 5 μM. The potential between the spray needle and the orifice was set to 4.5 kV. In the MS/MS mode, the quadrupole was used to select the precursor ions, which were fragmented in the hexapole collision cell generating product ions that were subsequently mass analyzed by the orthogonal reflectron TOF mass analyzer. For collision-induced dissociation (CID) MS/MS measurements, the voltage over the hexapole collision cell varied from 25 to 70 V and argon was used as the collision gas.

Enzymatic analysis of glycated peptides

Peptides were dissolved in 10 mM ammonium bicarbonate buffer to the final concentration of 10 μM. 1 ml samples of peptide solution, after addition of 20 μl 0.1% trypsin in water, were incubated for 30 min at room temperature and then diluted with 5% formic acid in MeCN (1 ml). Obtained solutions were analyzed directly by the ESI-MS method.

CD spectra

Circular dichroism spectra were recorded on a Jasco J-700 spectropolarimeter. Peptides were dissolved at concentrations of 100 μg/ml and the spectra were recorded in water and TFE. The pH of water solutions was adjusted to 7 using 0.1 M NaOH. A rectangular quartz cuvette of 1 mm pathlength was used. Each spectrum represents the average of eight scans. Data are presented as molar ellipticity [Θ].

Results and discussion

Synthesis of peptide-derived Amadori products by reductive alkylation on solid support

In the recent paper we proposed a new method of site-specific synthesis of glycated peptides on solid support (Stefanowicz et al. 2007). Briefly, the peptides were assembled on a Wang resin by the standard Fmoc strategy. After the removal of the Mtt group, using 1% TFA in dichloromethane, the obtained free ε-amino group of the lysine side chain was alkylated by 2,3:4,5-di-*O*-isopropylidene-*β*-D-arabino-hexos-2-ulo-2,6-pyranose in the presence of sodium cyanoborohydride. The obtained secondary amino group was protected using Boc_2O and the peptide synthesis could be continued according to a standard procedure. After completing the synthesis of the peptide chain, the N-terminal Fmoc group was removed from the resin using the solution of piperidine in DMF, and a peptide was cleaved from the resin using the TFA–water–TIS (90:5:5) mixture. The cleavage was monitored by ESI-MS. The deprotection of the hydroxyl groups in the aminofructose moiety required 8 h at room temperature to remove more than 95% of diisopropylidene groups. Cleaved peptides were precipitated using cold diethyl ether, dried in vacuum, and purified with a preparative HPLC. The fractions containing monoglycated peptides were collected. In some cases, diglycated compounds (IIb, and IVb) were also isolated as byproducts. Additionally, the unglycated fragments of BSA were synthesized (not shown in the experimental section) for comparison of their enzymatic stability and conformational properties with the peptide-derived Amadori products.

The obtained compounds are presented in Table 1. Their purity and identity was tested by HPLC and HR-MS. All the compounds' *m/z* values were consistent with the calculated ones based on their chemical formulas. Selected compounds (I, Ia, Ic) were subjected for MS/MS analysis, which confirmed their sequences and allowed the localization of the site of glycation (Stefanowicz et al. 2007).

Yields (60–95%) and HPLC purities (30–75%) of crude peptides prepared by the reductive alkylation on solid support are comparable to those reported in our previous paper (Stefanowicz et al. 2007). The main side products were unglycated peptides and peptides containing two moieties of fructose attached to the ε-amino group of lysine as judged by HR-MS analysis. Unfortunately, the retention times of these compounds were close. Therefore, a careful optimization of the separation process was required. This observation is consistent with the results published by Frolov (Frolov et al. 2006b; Frolov and Hoffmann 2008). The purification of peptides glycated at two lysine residues was even more difficult because numerous byproducts with very similar chromatographic characteristics were formed.

Synthesis of peptide-derived Amadori products using the novel lysine derivative: Fmoc-Lys(*i,i*-Fru,Boc)-OH

The purity of peptide-derived Amadori products can be improved using a suitable building block containing the protected fructose residue attached to the ε-amino group of lysine. Therefore, we decided to synthesize and test the novel lysine derivative Fmoc-Lys(*i,i*-Fru,Boc)-OH (**2**), which is compatible with the Fmoc protocol of solid phase peptide synthesis. The main advantage of this approach is the possibility of purification of the lysine derivative before the solid phase synthesis. The compound (**2**) was obtained as presented in Scheme 1. The obtained trifluoroacetate, which in contrast to free Fmoc-Lys-OH is well soluble in THF, was alkylated using 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose in the presence of sodium cyanoborohydride. After the glycation, the secondary amino group of lysine was protected using Boc_2O. The final product was purified on a silica-gel column. The purity and identity of the obtained derivative (**2**) was confirmed by analytical HPLC, TLC, ESI-MS, ESI-MS/MS and NMR. The usefulness of the obtained building block was tested in the standard peptide synthesis. A series of the modified fragments of BSA has been prepared.

After the purification, the peptides were characterized in the same way as compounds prepared by the reductive alkylation performed directly on the resin. Analytical data are shown in Table 2. According to the presented data, the last method gave a purer product. The average purity of peptides obtained by glycation on the resin (Table 1, only monoglycated peptides were taken in consideration) is 55%, while the average purity of compounds synthesized according to the new procedure is 83.5%. The direct comparison of products IIIa, IIIb and IIIc obtained by two

Table 1 Analytical data for glycated peptides obtained by direct reductive alkylation of lysine ε-amino group on solid support

Glycated peptide	R_t (min)[a]	Crude yield (%)	HPLC purity (%)[b]	Predominant ion	*m/z* calc/found[c]
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu-OH[e] **(I)**	16.6	65	62	$[MH_2]^{2+}$	574.8120/574.8322
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Fru)-Glu-OH[e] **(Ia)**	17.3	93	56	$[MH_2]^{2+}$	655.8385/655.8351
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys(Fru)-Leu-Lys-Glu-OH[e] **(Ib)**	16.8	74	58	$[MH_2]^{2+}$	655.8385/655.8361
H-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys-Leu-Lys-OH **(II)**	18.0	78	63	$[MH_2]^{2+}$	564.3013/564.3064
H-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys(Fru)-Leu-Lys-OH **(IIa)**	18.1	81	52	$[MH_2]^{2+}$	645.3277/645.3325
H-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys$(Fru)_2$-Leu-Lys-OH[d] **(IIb)**	18.6	–	15	$[MH_2]^{2+}$	726.3541/726.3864
H-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys-Leu-Lys(Fru)-OH **(IIc)**	19.6	87	50	$[MH_2]^{2+}$	645.3277/645.3136
H-Asp-Thr-Glu-Lys-Gln-Ile-Lys-Lys-Gln-Thr-OH **(III)**	12.6	82	55	$[MH_3]^{3+}$	406.8950/406.8958
H-Asp-Thr-Glu-Lys(Fru)-Gln-Ile-Lys-Lys-Gln-Thr-OH **(IIIa)**	13.1	70	40	$[MH_2]^{2+}$	690.8650/690.8623
H-Asp-Thr-Glu-Lys-Gln-Ile-Lys-Lys(Fru)-Gln-Thr-OH **(IIIb)**	12.9	78	53	$[MH_2]^{2+}$	690.8650/690,8624
H-Asp-Thr-Glu-Lys(Fru)-Gln-Ile-Lys(Fru)-Lys-Gln-Thr-OH **(IIIc)**	12.8	67	30	$[MH_3]^{3+}$	514.9302/514.9320
H-Lys-Ala-Ala-Phe-OH[e] **(IV)**	16.6	80	91	$[MH]^{+}$	436.2560/436.2563
H-Lys(Fru)-Ala-Ala-Phe-OH[e] **(IVa)**	16.3	77	80	$[MH]^{+}$	598.3088/598.3091
H-Lys$(Fru)_2$-Ala-Ala-Phe-OH[d] **(IVb)**	16.0	–	10	$[MH]^{+}$	760.3616/760.3609

[a] C18 column Vydac (250 × 4.6 mm): gradient 0–80% B in A in 60 min, A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA

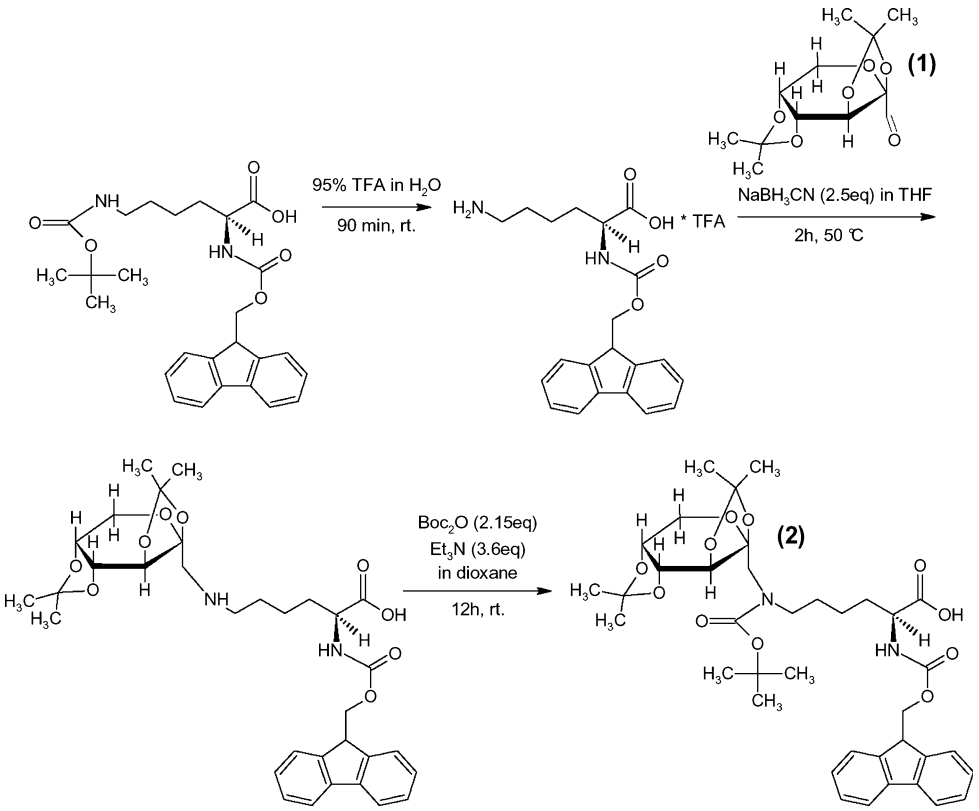
[b] Purity based on the integral of the crude product absorption at λ 220 nm on RP-HPLC

[c] ESI-MS spectrum, micrOTOF-Q (Bruker Daltonics)

[d] Diglycated peptides isolated as byproducts

[e] Synthesis of these peptides was described previously (Stefanowicz et al. 2007)

Scheme 1 The synthesis of Fmoc-Lys(*i*,*i*-Fru,Boc)-OH



methods and presented in Tables 1 and 2 also revels that the new procedure provides significantly purer peptides. The most striking example is diglycated peptide IIIc. The reductive alkylation on the resin gave a material containing 30% of the desired product, while the synthesis based on Fmoc-Lys(*i*,*i*-Fru,Boc)-OH provided the purity of 83%. The main advantage of this approach is that the obtained glycoconjugates are free of unglycated and diglycated peptide impurities, which are difficult to separate because of their similar retention times on RP-HPLC (Frolov et al. 2006b). The method utilizing derivative (**2**) is especially convenient for the synthesis of long and difficult sequences or sequences containing more than one glycation site. Because this new approach does not require any nonstandard reagents and procedures at the stage of peptide synthesis, it is recommended for the automatic synthesis of glycated peptides.

Enzymatic analysis of peptide-derived Amadori products

Recently, a tandem mass spectrometry (MS/MS) was applied to the glycated peptide sequencing (Frolov et al. 2006a, b; Stefanowicz et al. 2007). The studies were performed on instruments that utilize CID, in which the intramolecular vibrational energy redistribution occurs prior to the bond cleavage (Zhang et al. 2007). Thus, the weakest bonds in the modified side-chain tend to dissociate preferentially, resulting in a high abundance of ions corresponding to various degrees of water loss. A neutral loss of formaldehyde and of a whole hexose was also observed (Frolov et al. 2006a; Stefanowicz et al. 2001). The abundance of peptide backbone fragments is low, which makes the sequencing of glycated peptides difficult, especially those containing more than one glycation site. Although the electron transfer dissociation (ETD) method, performed on specialized ion-trap mass spectrometers only, has recently been applied to overcome these difficulties (Zhang et al. 2007), the sequencing of the glycated peptides still remains a challenge.

In our work we decided to use the enzymatic hydrolysis as a tool to analyze the obtained conjugates. Peptides dissolved in ammonium bicarbonate buffer were treated with the water solution of trypsin, and after a defined time, the samples were analyzed by ESI-MS. The glycated moiety remains intact permitting the localization of the carbohydrate attachment site. The obtained results are presented in Fig. 1.

There are three groups of isomeric compounds among peptides subjected to the enzymatic hydrolysis: (Ia; Ib), (IIa; IIc) and (IIIa; IIIb; IIIc). Those isomers differ by location of the Lys(Fru) moiety (abbreviated as X) within the peptide sequences. A comparison of the masses of the hydrolysis products reveals distinct differences between the isomeric

Table 2 Analytical data for glycated peptides obtained using Fmoc-Lys(*i*,*i*-Fru,BOC)-OH

Glycated peptide	R_t (min)[a]	Crude yield (%)	HPLC purity (%)[b]	Predominant ion	*m/z* calc/found[c]
H-Asp-Thr-Ile-Ser-Ser-Lys(Fru)-Leu-Lys-Glu-OH	16.50	78.9	85	$[MH_2]^{2+}$	591.8092/591.8110
Ac-Gln-Asp-Thr-Ile-Ser-Ser-Lys(Fru)-Leu-Lys-Glu-OH	17.17	78	88	$[MH_2]^{2+}$	676.8438/676.8457
H-Asp-Thr-Glu-Lys-Gln-Ile-Lys(Fru)-Lys-Gln-Thr-OH	13.00	88.1	92	$[MH_2]^{2+}$	690.8650/690.8623
H-Asp-Thr-Glu-Lys(Fru)-Gln-Ile-Lys-Lys-Gln-Thr-OH **(IIIa)**	13.05	81	75	$[MH_2]^{2+}$	690.8650/690.8630
H-Asp-Thr-Glu-Lys-Gln-Ile-Lys-Lys(Fru)-Gln-Thr-OH **(IIIb)**	12.95	88.5	78	$[MH_2]^{2+}$	690.8650/690.8641
H-Asp-Thr-Glu-Lys(Fru)-Gln-Ile-Lys(Fru)-Lys-Gln-Thr-OH **(IIIc)**	12.83	87.1	83	$[MH_2]^{2+}$	771.8914/771.8913

[a] C18 column Vydac (250 × 4.6 mm): gradient 0–80% B in A in 60 min, A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA

[b] Purity based on the integral of the crude product absorption at λ 220 nm on RP-HPLC

[c] ESI-MS spectrum, micrOTOF-Q (Bruker Daltonics)

peptides. For example, peptide Ia (QDTISSKLXE) produced fragments: LXE (*m/z* 551.3; $z+1$) and QDTISSK (*m/z* 778.4; $z+1$), while Ib (QDTISSXLKE) produced the fragment QDTISSXLK (*m/z* 591.3; $z+2$). According to the presented results, the peptide chain was cleaved only at the carboxyl end of unmodified lysine, but not at lysine with a glycated or diglycated side chain, even at the enhanced enzyme concentration (fivefold higher) and incubation time (up to 5 h). This observation is consistent with the previous reports indicating that the enzymatic activity of trypsin is inhibited by the lysine glycation (Brock et al. 2003; Lapolla et al. 2004). Analysis of the formed fragments allows the identification of the hydrolysis sites and in a comprehensive way confirms the localization of Lys(Fru) residues in the peptide chain. The pattern of tryptic hydrolysis unambiguously confirmed the regioselectivity of the proposed synthetic procedures.

Conformational preferences of peptide-derived Amadori products

Circular dichroism spectra measured in water solution at pH 7 are presented in Fig. 2. The spectra of peptides I, Ia, Ib, II, IIa, IIb, IIc, III, IIIa, IIIb, IIIc exhibit a strong, negative band at 198 nm. The molar ellipticity of the investigated peptides ranged from −170,000 to −400,000. These spectra strongly suggest the unordered (open) conformation of the peptides studied. According to the literature data this type of conformation is characterized by a negative band near 200 nm with the mean molar ellipticity of 40,000 deg cm^2/dmol (Woody 1985). The CD spectra are presented in three groups (Fig. 2a–c). Each group of spectra is based on the same sequence. The Lys (Fru) moiety is not present in peptides I, II, III, whereas peptide IIb has two Fru groups attached to the same side chain of Lys. Other compounds differ by location of the glycated lysine. A comparison of spectra of the same sequences, glycated in a different way, revealed significant similarity. Therefore, in water solution, the influence of glycation on the conformational preferences of the investigated peptides determined by the CD method is negligible. Selected peptides (I, Ia and Ib) were also investigated in TFE. Results are shown in Fig. 3. In TFE three bands were observed: the positive at 185 nm and two negative at 200 and 220 nm, respectively. This type of CD spectrum (C class) usually is interpreted as α-helix, but in short peptides may correspond to β-turn conformation of I or III type (Perczel et al. 1991). This influence of trifluoroethanol on the peptide conformation is well known in the literature (Woody 1985). The CD spectra of glycated peptides Ia and Ib are similar. The molar ellipticity at 220 nm for these compounds is significantly higher in comparison to nonglycated peptide I. This may suggest that in trifluoroethanol glycated compounds contain more β-turn conformers in the conformational equilibrium. This result is consistent with previous data (Vass et al. 2000) evidencing that N-terminally glycated opioid peptides prefer β-turn conformation. It was also observed, using the NMR analysis, that the glycation of synthetic human serum albumin α-helix 28 can influence or change the local protein secondary structure (Howard and Smales 2005). Similar studies performed on the helical fragment of lysozyme also revealed that the glycation of lysine results in the disruption of the local secondary structure (Povey et al. 2008).

The literature shows examples of strong influence of the glycation on the peptide conformation as well as examples (Mendez et al. 2005; Stefanowicz et al. 2001) of non-significant influence of the glycation on the conformational preferences of the peptide. In this study, the effect of the peptide modification on the conformation is different in water and in trifluoroethanol. The presented data suggest that the conformational consequences of the lysine moiety glycation depend on the sequence and on the solvent.

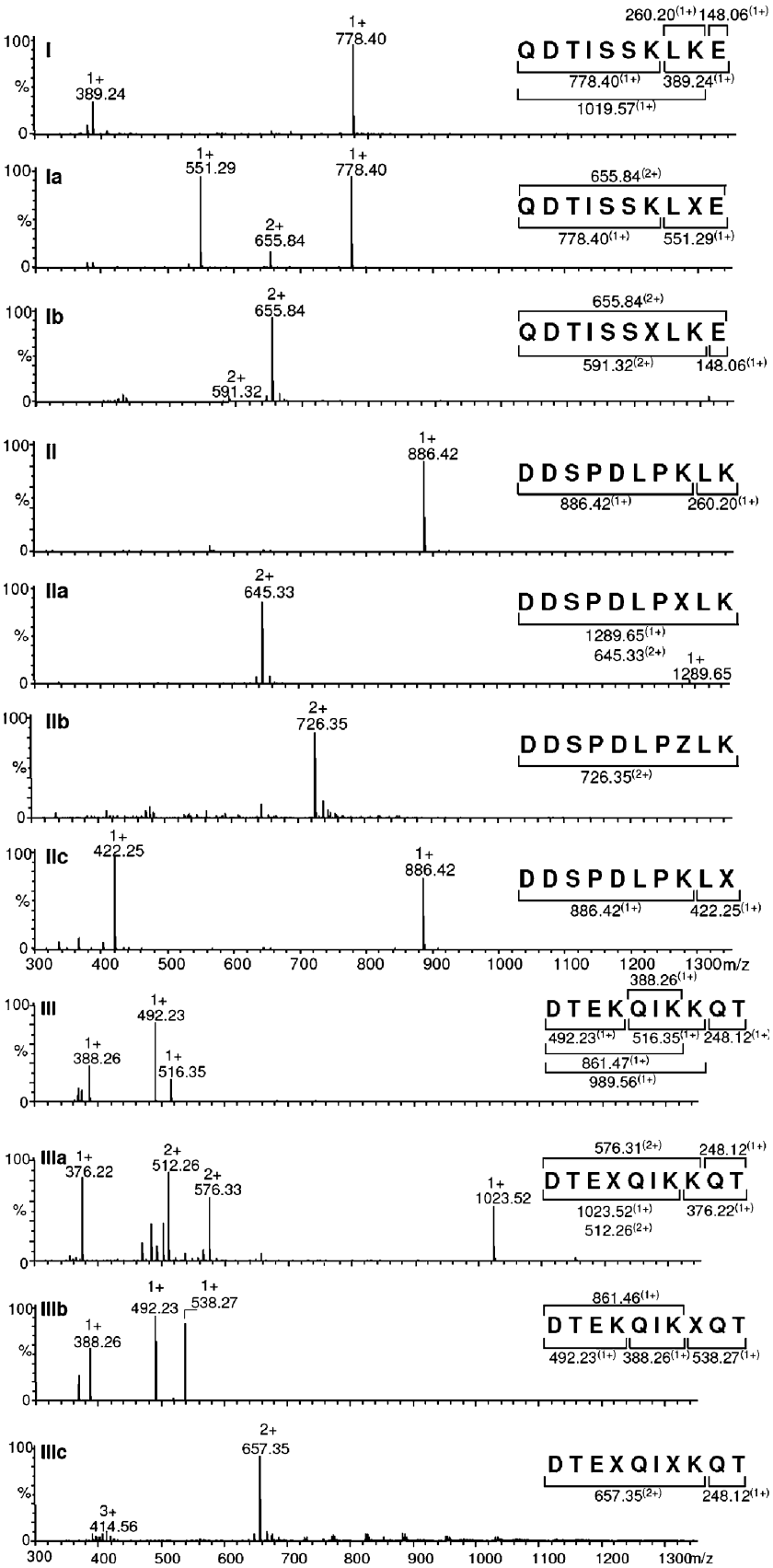


Fig. 1 The ESI-MS spectra of tryptic hydrolysis products of peptides. Peptide charges were calculated based on the distribution of the isotopic patterns. Cleavage sites together with *m/z* values of fragments are also presented. X: monoglycated Lys residue, Z: diglycated Lys residue

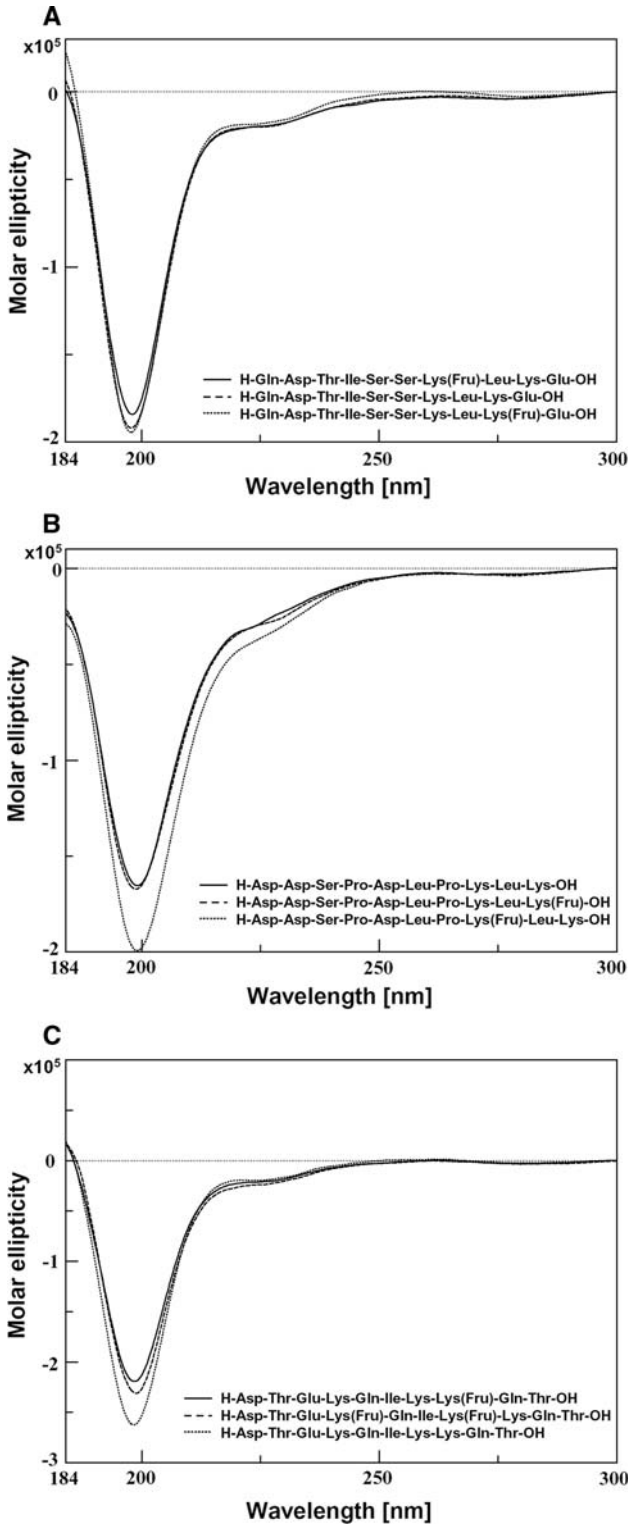


Fig. 2 CD spectra of the glycated albumin fragments: A: peptides derived from the sequence H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu-OH, B: peptides derived from the sequence H-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys-Leu-Lys-OH and C: peptides derived from the sequence H-Asp-Thr-Glu-Lys-Gln-Ile-Lys-Lys-Gln-Thr-OH. All spectra were measured in water at pH 7, details are given in "Materials and methods"

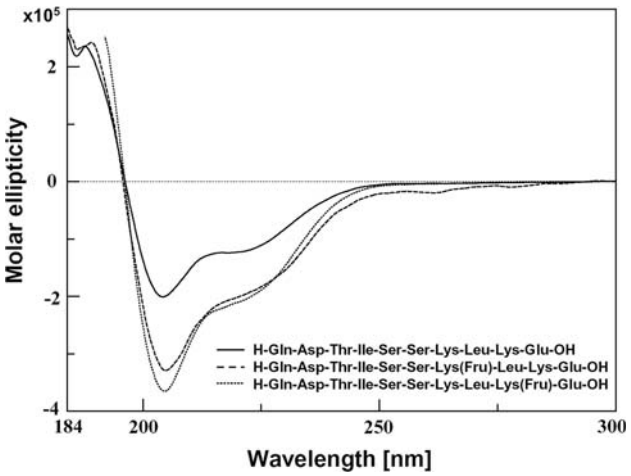


Fig. 3 CD spectra of the glycated peptides: H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu-OH, H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Fru)-Glu-OH and H-Gln-Asp-Thr-Ile-Ser-Ser-Lys(Fru)-Leu-Lys-Glu-OH. Spectra were measured in TFE, details are given in "Materials and methods"

Conclusion

Two efficient procedures for the solid-phase synthesis of site-selectively glycated peptides were developed. Both proposed methods are compatible with the Fmoc strategy of the solid-phase peptide synthesis. The second method based on the new lysine derivative gives purer products, which are free from diglycated and unglycated peptides. This last approach does not require any nonstandard procedures therefore its application for the automatic synthesis of glycated peptides is promising.

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