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ORIGINAL ARTICLE

The efficient synthesis of isotopically labeled peptide-derived Amadori products and their characterization

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Abstract Protein glycation is often a cause of diabetes-associated complications. The isotopically labeled peptide-derived Amadori products may serve as standards for quantitative determination of the glycated proteins. In this paper, we discussed various approaches to the synthesis of Amadori products labeled selectively with stable isotopes ^{2}H, ^{13}C and ^{18}O.

Keywords Amadori rearrangement · Peptides · Glucose · Fructose · Glycation · ^{13}C · ^{2}H · ^{18}O · Isotopes · Labeling · Regioselective · Non-enzymatic protein modifications · Microwave synthesis · NMR studies

Introduction

The reducing sugars, mainly glucose, interact with amino groups of proteins and other biomolecules forming the glycation products. This reaction is significantly accelerated in diabetes, since this condition results in elevated level of blood glucose. Consequently, the formation of protein–sugar conjugates is often considered as a possible cause of the diabetes-associated complications (Baynes and Thorpe 1999; Thorpe and Baynes 1996). The glycation process is initiated by the interaction of glucose aldehyde group with the amino group in the protein molecule. The Schiff base formed in the first, reversible reaction stage rearranges to *N*-substituted (1-deoxy-ketos-2-yl)amines (Amadori 1929; Kuhn and Weygand 1937)—the known products of the Amadori rearrangement. The further oxidation and dehydration reactions give rise to advanced glycation end products (AGEs) which, according to many reports, mediate diabetes-related pathological processes (Ulrich and Cerami 2001) and aging (Monnier 1989). Moreover, Amadori-modified proteins themselves, mainly serum albumin, are recently considered as factors playing a key role in the development of cardiovascular complications of diabetes (Cohen et al. 2006; Zhang et al. 2006).

Monitoring the level of glycation of selected proteins (mainly hemoglobin) is nowadays widely applied as a method for monitoring diabetic patients. The reports confirming that the serum albumin glycation is strongly correlated with the probability of cardiovascular complications suggest that the quantitative determination of glycation level of particular proteins may serve as a useful diagnostic method.

The most efficient approach to proteins quantification is the combination of enzymatic hydrolysis and the isotopic dilution method. This method helps in overcoming the matrix effects affecting the response factors of the analyzed compounds (Ikonomou et al. 1990; Choi et al. 2001; Delatour 2004). However, the use of the mentioned method requires the preparation of an isotopomer (Delatour et al. 2006)—the target peptide labeled with the stable isotopes.

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Isotopically labeled glycated peptides are also useful tool in studying mechanisms of AGE formation (Huyghesy-Despointes and Yaylayan 1996) as well as in investigations on gas-phase fragmentation of peptide-derived Amadori products (Stefanowicz et al. 2009).

The isotopically labeled peptides are formed during enzymatic hydrolysis of proteins modified by the treatment with $^{13}C_6$ glucose. The comparison of the in vivo glycated protein with the protein glycated in vitro using $^{13}C_6$ glucose allows proteomic profiling of glycated proteins (Priego-Capote et al. 2010) in glycation isotopic labeling (GIL) method. The modification of the protein with equimolar mixture of glucose and $^{13}C_6$ glucose, combined with enzymatic hydrolysis, allows the selective detection of glycated peptides and, as a consequence, the localization of glycation sites in the protein molecule (Stefanowicz et al. 2010b). The isotopic labeling method was also applied to the determination of 6-*N*-carboxymethyllysine, one of AGEs (Delatour et al. 2009).

The presented examples demonstrate that isotopically labeled peptide-derived Amadori products may be useful research tools. However, there is no convenient method of preparation of these compounds. In the last few years, several methods of peptide-derived Amadori products syntheses have been published. To date, there are four main methods of preparation of glycated peptides:

- solution phase methods (Forrow and Batchelor 1990; Horvat and Jakas 2004);
- direct glycation on solid support (Frolov et al. 2006b);
- glycation on solid support by reductive alkylation (Stefanowicz et al. 2007; Frolov et al. 2007);
- synthesis of fully protected building block and its application in the solid-phase synthesis of peptide-derived Amadori products. (Stefanowicz et al. 2010a; Carganico et al. 2009).

Although each of the above methods may be used in the Amadori products synthesis, the price and availability of required labeled sugars may limit their applicability. The economic reasons make the direct method more attractive, as there is no need to prepare labeled intermediates and the price of the variety of isotopically labeled glucoses is moderate. This method, however, needs the optimization to improve the yield and the purity of products.

Materials and methods

Reagents

The amino acid derivatives, the coupling reagent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and the preloaded Wang resins (0.50–0.70 mmol/g) were purchased from NovaBiochem. The Rink resin (0.65 mmol/g) was purchased from Iris Biotech. Sodium cyanoborohydride ($NaBH_3CN$) and deuterated sodium cyanoborohydride (NaB^2H_3CN—99 atom %), D-glucose-6,6-$^2H_2$ (99 atom %) and $H_2^{18}O$ (97 atom %) were purchased from Aldrich. D-glucose-$^{13}C_6$ was purchased from ISOTEC. 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.

Preparation of peptides

Ac-Lys(Fru)-Ala-Ala-Phe-OH (I)

Ac-Lys($[1-^2H]$Fru)-Ala-Ala-Phe-OH (II) (Fru-fructose)

Peptides I and II were prepared manually on the solid support according to the procedures described previously (Stefanowicz et al. 2010c, 2007). The details of the synthesis are given as supplementary material.

H-Lys(Fru)-Ala-Ala-Phe-OH (III)

H-Lys($[6,6-^2H_2]$Fru)-Ala-Ala-Phe-OH (IV)

H-Lys($[^{13}C_6]$Fru)-Ala-Ala-Phe-OH (V)

H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VI)

H-Lys($[^{13}C_6]$Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VII)

Peptides III–VII were prepared on the solid support by microwave-assisted glycation of corresponding precursors according to (Frolov et al. 2006b). The procedure is given as supplementary material. The purity of obtained peptides was checked by analytical HPLC. The identity of peptides was verified based on their HR-MS spectra (data given in Table 1). The structures of peptides I–V were additionally confirmed by ^1H and ^{13}C NMR spectroscopy (chemical shifts and coupling constants were given as supplementary material).

H-Lys($[2-^{18}O]$Fru)-Ala-Ala-Phe-NH_2 (VIII)

The peptide H-Lys(Fru)-Ala-Ala-Phe-NH_2 was synthesized on the Rink resin using fully protected lysine residue Fmoc-Lys(*i*,*i*-Fru,Boc)-OH (Stefanowicz et al. 2010a). 1 mg of the purified peptide was dissolved in 50 µl of $H_2^{18}O$ and incubated at 25°C for 200 h (Stefanowicz et al. 2009).

H-Lys($[2-^{18}O]$Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (IX)

H-Lys($[1,1-^2H_2]$Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (X)

Table 1 Analytical data and yields for peptide-derived Amadori products

Petide	Rt (min)	Crude yield (%)	Yield of isolated, purified product (%)	Predominant ion	*m/z* calc/found
Ac-Lys(Fru)-Ala-Ala-Phe-OH (I)	16, 73	52	42	$[MH]^+$	640,3194/640,3199
Ac-Lys([1-^{2}H]Fru)-Ala-Ala-Phe-OH (II)	16, 50	59	49	$[MH]^+$	641,3256/641,3248
H-Lys(Fru)-Ala-Ala-Phe-OH (III)	16, 53	82	29	$[MH]^+$	598,3090/598,3073
H-Lys([6,6-$^2H_2$]Fru)-Ala-Ala-Phe-OH (IV)	16, 51	89	30	$[MH]^+$	600,3214/600,3179
H-Lys([$^{13}C_6$]Fru)-Ala-Ala-Phe-OH (V)	16, 53	77	21	$[MH]^+$	604,3289/604,3257
H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VI)	19, 76	56	13	$[MH]^+$	1290,7520/1290,7583
H-Lys([$^{13}C_6$]Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VII)	19, 26	56	11	$[MH_2]^{2+}$	1296,7722/1296,7877
H-Lys([2-^{18}O]Fru)-Ala-Ala-Phe-NH_2 (VIII)	14, 82[a]	96[a]	53[a]	$[MH]^+$	599,3290/599,3255
H-Lys([2-^{18}O]Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (IX)	[b]	[b]	[b]	$[MH_2]^{2+}$	1292,7563/1292,7729
H-Lys([1,1-$^2H_2$]Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (X)	[b]	[b]	[b]	$[MH]^+$	1292,7646/1292,7837

[a] Before isotopic exchange

[b] Values the same as given for peptide VI

1 mg of purified peptide H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VI) was dissolved in 60 µl of $H_2^{18}O$ (peptide IX) or ^2H_2O (peptide X). 20 µl of peptide solution was sealed in a glass capillary and placed in microwave vessel containing 5 ml of distilled water. The vessel was irradiated on CEM Discover reactor at 75°C for 30 min and 90 min, at 100°C for 30 min and at 150°C for 5 min in the temperature control mode of the reactor with stirring and external gas cooling. The power of approximately 10 W was needed to maintain the temperature of 75°C, 60 W of 100°C and 300 W of 150°C. The products microwaved with $H_2^{18}O$ were diluted with aqueous solution of acetonitrile (50%) and formic acid (0.1%) and characterized directly by ESI-MS. The products microwaved with ^2H_2O were lyophilized, dissolved in 500 µl of water, incubated for 1 h to completely exchange the deuterated amide protons. The sample after dilution with aqueous solution of acetonitrile (50%) containing formic acid (0.1%) was characterized by ESI-MS.

MS

All mass spectrometric experiments were performed on an Apex-Qe Ultra 7T instrument (Bruker Daltonic, Germany) equipped with ESI source. The instrument was operated in the positive-ion mode and calibrated with the Tunemix™ mixture (Bruker Daltonic, Germany). The mass accuracy was better than 5 ppm. The samples for MS and MS/MS experiments (approx. 0.05 mg) were dissolved in 1 ml of acetonitrile–water–formic acid mixture (50:50:0.1; v:v:v) and infused at the flow rate 3 µl/min. The obtained mass spectra were analyzed using the Biotools (Bruker Daltonic, Germany) software. The instrumental parameters were as follows: scan range 300–2,500 *m/z*; drying gas: nitrogen; temperature of drying gas: 200°C; the potential between the spray needle and the orifice was set to 4.5 kV, source accumulation 0.5 s, ion accumulation time 0.5 s. In MS/MS mode, the precursor ions were selected in the quadrupole and subsequently fragmented in the hexapole collision cell. Argon was used as a collision gas. The obtained fragment ions were directed to the ICR mass analyzer and registered as a MS/MS spectrum. The collision energy in the hexapole collision cell was set at 20 V.

NMR

Each peptide (5 mg) was dissolved in 99% D_2O (650 µL). The pH values were in the range of 4.3–4.8. All measurements were done at 30°C. The 2D NMR experiments were recorded at 11.7 T on Varian Unity + 500 spectrometer. The 1D ^{1}H NMR spectra were performed at 9.4 T on Varian INOVA 400 spectrometer and analyzed using VnmrJ software (Varian Inc., PaloAlto, USA), while 2D dimensional spectra (homo- and heteronuclear) were processed by NMRPipe (Delaglio et al. 1995) and analyzed with Sparky (Goddard and Kneller 2003) programs. The complete assignments of non-exchangeable ^{1}H and ^{13}C resonances for all peptides were performed by application of a standard procedure (Wuthrich 1986) based on 2D homonuclear TOCSY (mixing times 10 and 90 ms) and ROESY (mixing time 200 and 500 ms) experiments supplemented by 2D heteronuclear ^{1}H-^{13}C HSQC spectra, recorded on natural abundances of ^{13}C nuclei. For sugar moiety only assignments for the major tautomeric form

Table 2 Averaged chemical shifts list of ^{13}C resonances in ppm from four different major tautomeric forms of aminofructose: β-D-pyranose (β–p), β-D-furanose (β–f), hydrate and α-D-furanose (α–f)

	Averaged ^{13}C chemical shifts of tautomers in studied glycopeptides (abundancies)/$\sigma \pm 0.5$ ppm			
	β–p (65–70%)	β–f (13–18%)	α–f (12–16%)	Hydrate (2–4%)
C1	55.6	53.7	55.1	50.8
C3	72.2	83.5	83.4	72.9
C4	72.1	76.6	78.4	74.2
C5	71.5	84.9	85.0	68.4
C6	66.6	64.4	63.4	65.3

C–H correlations from other tautomers were also observed, but with total abundances below 5%

(β-D-pyranose) are listed, while ^{13}C chemical shifts of other forms with their abundances over 5% are listed in Table 2. For $^{13}C_6$-glucose-labeled peptide V and its non-labeled equivalent peptide III, the two 2D ^{1}H-^{13}C HSQC spectra were recorded on the Varian Unity + 500 spectrometer with 1 and 32 scans, respectively. A single scan experiment shows predominantly signals, corresponding to labeled sugar moiety bound to the peptide. The experiment with 32 accumulated scans shows expected peptide and sugar moiety resonances in the 2D ^{1}H-^{13}C HSQC spectra. The accuracies of estimated proton and carbon chemical shifts and $^{3}J_{HH}$ coupling constants are 0.02, 0.2 and 0.4 Hz, respectively.

Results and discussion

We tested three methods of isotopic labeling of peptide-derived Amadori products.

1. Direct reaction of the lysine side chain ε-amino group of the resin-bound peptide with glucose labeled with the stable isotope (^{13}C or ^{2}H). This method is a modification of Frolov's approach. We applied microwave activation instead of heating the resin immobilized peptide with glucose solution in DMF.
2. Reductive alkylation of amino groups in the peptide immobilized on the resin with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose hydrate in the presence of $NaB^{2}H_3CN$, as a source of deuterium. This reaction resulted in regioselective deuteration at the [1-^{2}H]Fru position. The applied protocol is a modification of our method published previously (Stefanowicz et al. 2007).
3. Regioselective labeling of peptide-derived Amadori products by $^{16}O/^{18}O$ or $^{2}H/^{1}H$ exchange in solution using $H_2^{18}O$ or $^{2}H_2O$.

The microwave activation

Several years ago Frolov and Hoffman reported solid-phase synthesis of peptide-derived Amadori products. The procedure was simple, since it did not require any nonstandard reagents. On the other hand, reaction was slow and needed high temperature. The Frolov's method, however, seems promising in the synthesis of isotopically labeled Amadori products, because various labeled glucoses are commercially available.

To improve the yield and purity of peptide-derived Amadori products, we applied the microwave activation. A series of peptides: III–VII was obtained to test the microwave method of glycation.

Compounds I–V and VIII were obtained by modification of short model peptide H-Lys-Ala-Ala-Phe-OH. We also obtained the HSA fragment-H-Lys([$^{13}C_6$]Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH (VII) as well as its non-labeled precursor VI, to show that proposed synthetic procedure can be applied to fragments of natural proteins. The peptides were assembled on the solid phase by standard Fmoc method, only the Lys moiety designed for glycation was protected with acid-labile Mtt group. After completing the synthesis, the Mtt group was removed using 1% TFA in DCM, the resin was washed and treated with glucose or labeled glucose in the microwave reactor. After deprotection of the *N*-terminal amino group, the peptide was relased from the resin. The obtained samples were studied by analytical HPLC and ESI-MS. The results given in the Table 1 show that yields and purities of products obtained by MW method are better than these obtained using original Frolov's procedure. The microwave method was directly compared with thermal glycation on the solid support. Two similar peptides Lys(Fru)-Ala-Ala-Phe and Ac-Lys(Fru)-Leu-Leu-Phe were synthesized using microwave activation and conventional heating, respectively. The ESI-MS spectra of crude products (presented as supplementary material) indicated that the purity of peptide Lys(Fru)-Ala-Ala-Phe obtained using microwave method was higher. In addition, the reaction time was significantly (15 times) shorter.

The reaction products were further purified using preparative HPLC. In all cases, the yield of purified peptide obtained from 100 mg of resin was in the range of 10–20 mg which corresopnds to 11–53% of theoretical yield calculated based on the loading of the resin used for the synthesis. The purified compounds were analyzed by HR ESI-MS. In addition, for peptides I–V, we performed the NMR measurements to show the chemical integrity and the isotopic labelling of obtained peptides. All non-exchangable in D_2O resonances ^{1}H and ^{13}C were assigned for all studied peptides (I–V) and their sugar moieties. Owing to the relatively small overlap of the signals in the standard 1D ^{1}H NMR spectra of investigated peptides, the

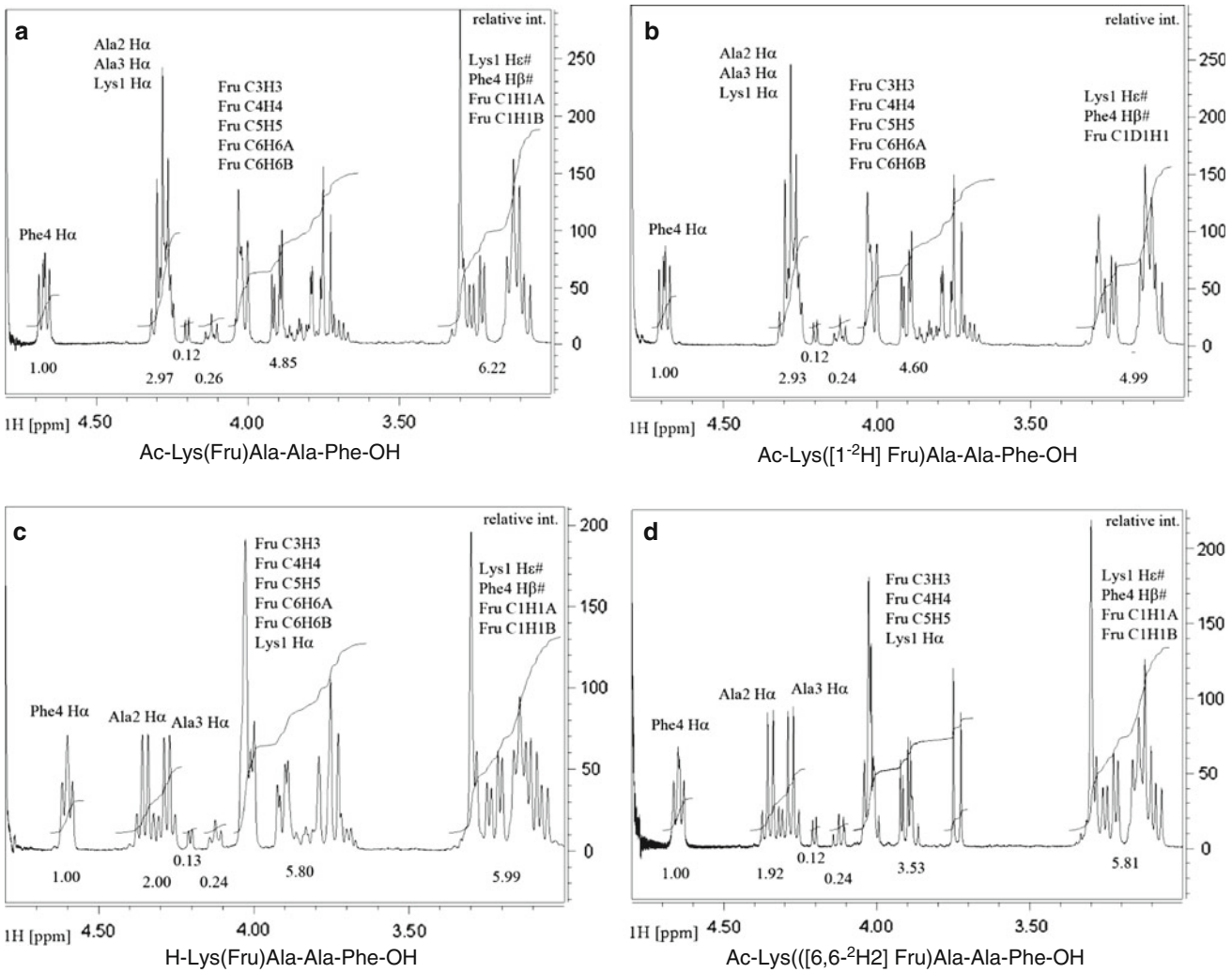

Fig. 1 **a–d** The 1D ^{1}H NMR spectra of diagnostic region for regioselective labeling of ^{2}H in sugar moieties in investigated glycopeptides with integrated and assigned resonance peaks

following regioselectvity of substitution of ^{1}H by ^{2}H by peak integration was relatively easy. In case of the peptides II and IV, one can observe the decrease in relative peaks integrals in adequate spectrum regions equal to the number of deuterium substituted protons, for II one proton at 3.1–3.3 ppm and for IV two protons at 3.7–4.1 ppm, respectively. The peptide IV with uniformly-^{13}C-labeled glucose exhibits all cross peaks characteristic for all glucose tautomeric forms, which are present in solution in 2D ^{1}H-^{13}C HSQC experiment (Table 2). Carbon and proton chemical shifts, which are observed in this spectrum correspond, as expected, to amino-glucose moiety peaks only. The remaining C–H correlations from isotopically non-labeled peptidic part are of ca. 70 times smaller intensity or are not visible at all, while the same experiment with 32 scans recorded for peptide II with natural isotope abundance shows all C–H correlations from both peptide and sugar fragments of the compound (Figs. 1, 2).

The MS and NMR data confirmed chemical composition of obtained products. We synthesised peptide-derived Amadori products regioselectively labeled in hexose moiety with two ^{2}H atoms at position 2 and completely labeled with ^{13}C as well as their non-labeled analogs. Because many analogs of glucose labeled with stable isotopes are available, this method makes possible the synthesis of almost any regioselectively labeled analogs of peptide-derived Amadori products. The $^{13}C_6$-glucose analogs are the best candidate for analytical purpose, since 6 Da shift reduces the overlapping the labeled compound with isotopic peaks of non-labeled analog.

Reductive alkylation

The reductive alkylation approach to the isotopic labeling of peptide-derived Amadori product is based on our procedure published previously. (Stefanowicz et al. 2007, 2010a). In

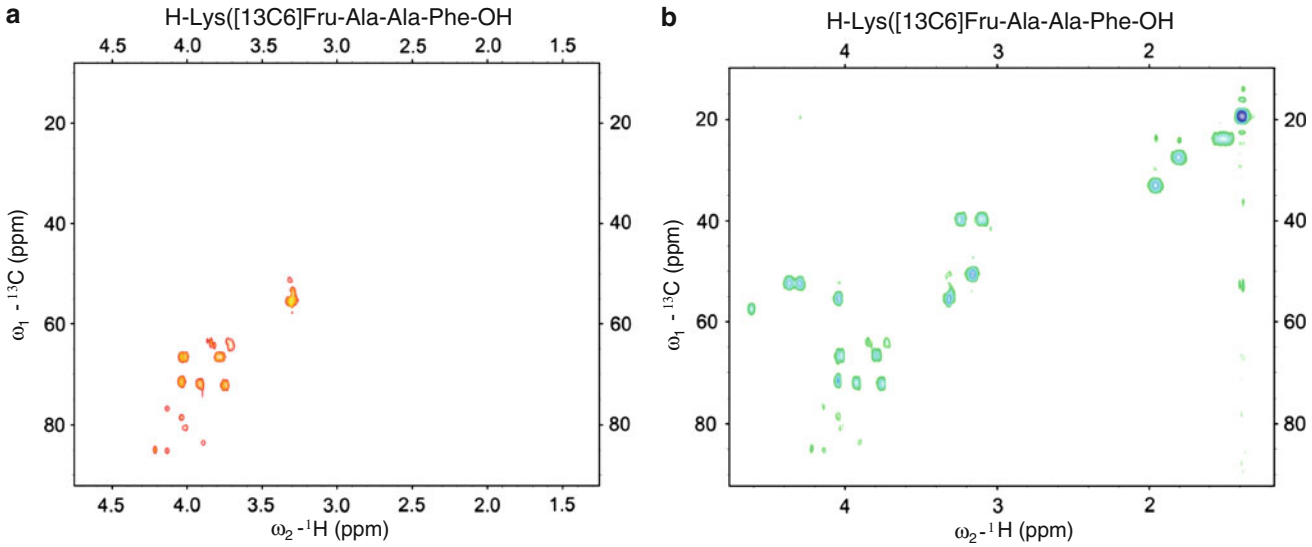

Fig. 2 The 2D ^{1}H-^{13}C HSQC experiments of **a** H-Lys([$^{13}C_6$]Fru)-Ala-Ala-Phe-OH (1 scan) and **b** H-Lys(Fru)-Ala-Ala-Phe-OH (32 scans). In case of H-Lys([$^{13}C_6$]Fru)-Ala-Ala-Phe-OH peptide with ^{13}C uniformly labeled glucose correlations from aminoglucose moiety are visible. The same peptide with natural abundance of ^{13}C nuclei exhibits all expected C–H correlations

this method, the deprotected ε-amino group of resin-bound peptide is modified using 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose hydrate in presence of $NaB^{2}H_3CN$. The sodium cyanoborohydride used as a reducing agent, incorporates 1 deuterium atom into obtained Amadori product. Using this method, we synthesized peptide (II) Ac-Lys([1-^{2}H]Fru)-Ala-Ala-Phe-OH

The reaction conditions were the same as reported previously (Stefanowicz et al. 2007). The reaction product after cleavage from the resin was analyzed by HPLC and ESI-MS. The ESI-MS analysis confirmed that one deuterium atom was incorporated into the reaction product. The purtity of crude product was 67% according to HPLC. The peptide was purified by preparative HPLC (yield of purified compound 17 mg from 100 mg of peptydyl resin) and analyzed by NMR, HR-MS and MS/MS techniques. The molecular mass of obtained compounds well to that calculated on the basis of molecular formula. The NMR spectra demonstrate that the structure of the synthesised compound is consistent with the expected one, and that deuterium atom is attached to the carbon C1 in hexose moiety.

The obtained product, regioselectively labeled with deuterium atom, may be useful in mechanistic studies on Amadori products' fragmentation, but is not the derivative of choice for isotopic dilution method, because of small mass shift after labeling (1 Da only).

Labeling by $H_2^{18}O$ and D_2O treatment

The third possible approach to the isotopic labeling of peptide-derived Amadori products is based on reaction of synthetic Amadori products with $H_2^{18}O$ and $^{2}H_2O$. According to the previous NMR studies, the Amadori products in solution exist as equilibrium of furanose, pyranose and open form. The open chain form in water solution exists mainly as a hydrate. The reversible formation of hydrate should result in isotopic exchange of 1 oxygen atom in the hexose moiety. This reaction was observed previously for monosaccharides, such as glucose and fructose (Mega et al. 1990). To check if this reaction could be also observed for Amadori products, we incubated the sample of H-Lys(Fru)-Ala-Ala-Phe-NH_2 with $H_2^{18}O$. The peptide was dissolved in $H_2^{18}O$ and incubated for 2 weeks at room temperature. After 7 days, we found that the exchange level was approximately 50%. After 14 days, the majority of product was isotopically labeled. ESI-MS spectrum indicates that Amadori products in $H_2^{18}O$ undergo isotopic exchange. However, to reach high exchange level, a long incubation time is required, which makes this method inconvenient.

To accelerate the isotopic exchange procedure, we applied the microwave activation. The experimental details were given in "Materials and methods". The samples of peptide VI (H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH) solution were incubated at 75°C for 30 and 90 min, at 100°C for 30 min and at 150°C for 5 min. The results given in Fig. 3 demonstrate that in all cases, the majority of peptide underwent the isotopic labeling. The highest temperature applied (150°) resulted in decomposition of the Amadori product. The predominant product of hydrolysis turned out to be peptide without hexose moiety. Interestingly, the formed peptide did not show any traces of

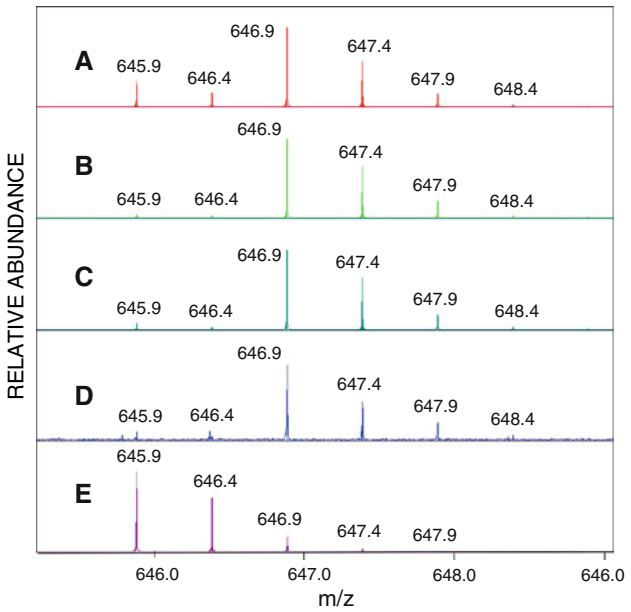

Fig. 3 The isotopic patterns for peptide VI (H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH) subjected to microwave-activated isotopic exchange at specified conditions (temperature; time): **a** 75°C, 30 min, **b** 100°C, 30 min, **c** 75°C, 90 min, **d** 150°C, 5 min, **e** untreated peptide

isotopic labeling. This fact strongly suggests that only aminofructose moiety is susceptible to isotopic labeling. The reaction conducted at 75°C for 30 min did not show decomposition of peptide-derived Amadori product, but the labeling was not complete. The reaction performed at 75°C for 90 min as well as at 100°C for 30 min resulted in almost full isotopic exchange. At 100°C, the abundance of decomposition product was significantly higher than at 75°C/90 min. The similar process was studied by Mega (Mega et al. 1990) on simple sugars. The ^{18}O isotope shift in ^{13}C NMR spectra revealed the exchange of oxygen atom attached to the anomeric carbon of D-glucose, D-mannose and D-fructose. According to the results of our MS experiments, the aminofructose moiety of Amadori products also undergoes the $^{16}O/^{18}O$ isotopic exchange.

To confirm the location of ^{18}O atom the isotopically labeled product was subjected to MS/MS experiment. The fragmentation of the labeled peptide-derived Amadori product indicated that 100% of ^{18}O is eliminated along with the first water molecule. According to all proposed mechanisms of Amadori products fragmentation (Jeric et al. 2002; Frolov et al. 2006a; Stefanowicz et al. 2009), the first step of this process consists of loss of hydroxyl group attached to the anomeric carbon atom. Therefore, the observed fragmentation pattern indicates that the labeling is regioselective and involves only the oxygen atom attached to the anomeric carbon. In addition, the fragment at *m/z* 1,128.7 corresponding to peptide without the hexose moiety

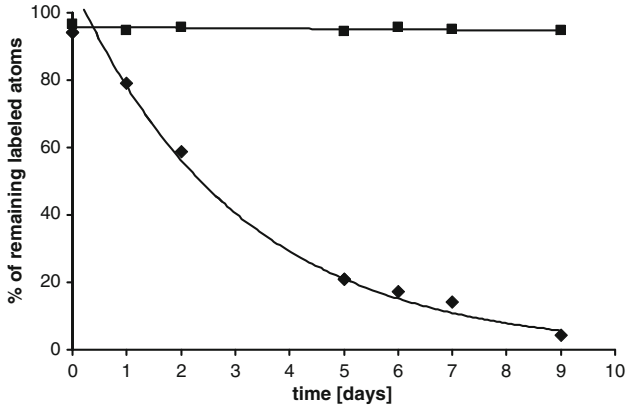

Fig. 4 The kinetics of back exchange process ($^{18}O/^{16}O$ *triangles*, $^{2}H/^{1}H$ *squares*) observed for peptide VI (H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH) at room temperature, water solution, pH approx 4.5

presents isotopic pattern indicating the absence of ^{18}O in peptide chain, thus further confirming the selectivity of isotopic labeling. The isotopically labeled peptide was incubated in water solution and the slow back exchange process was observed with half-life time approximately 60 h (Fig. 4).

Alternatively, the sample of peptide VI was incubated with D_2O. Also in this case, the isotopic exchange was detected. This reaction was previously detected by Roper

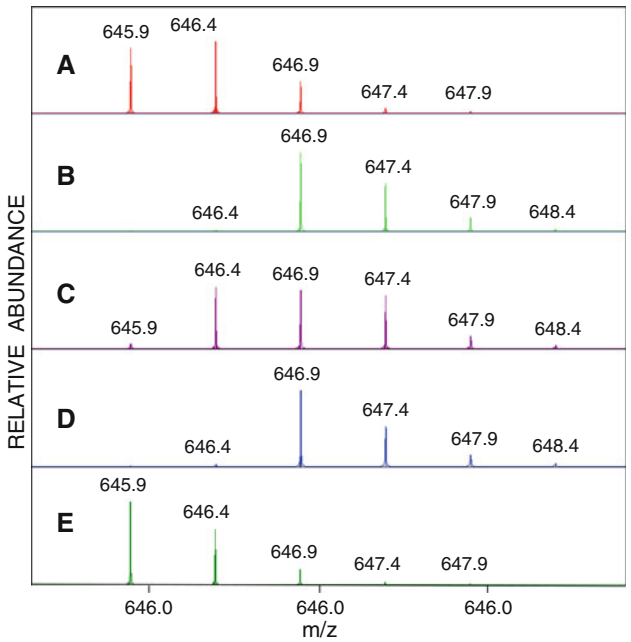

Fig. 5 The isotopic patterns for peptide VI (H-Lys(Fru)-Gln-Thr-Ala-Leu-Val-Glu-Leu-Val-Lys-OH) subjected to microwave-activated $^{1}H/^{2}H$ isotopic exchange at specified conditions (temperature; time): **a** 75°C, 30 min, **b** 100°C; 30 min, **c** 75°C, 90 min, **d** 150°C, 5 min, **e** untreated peptide

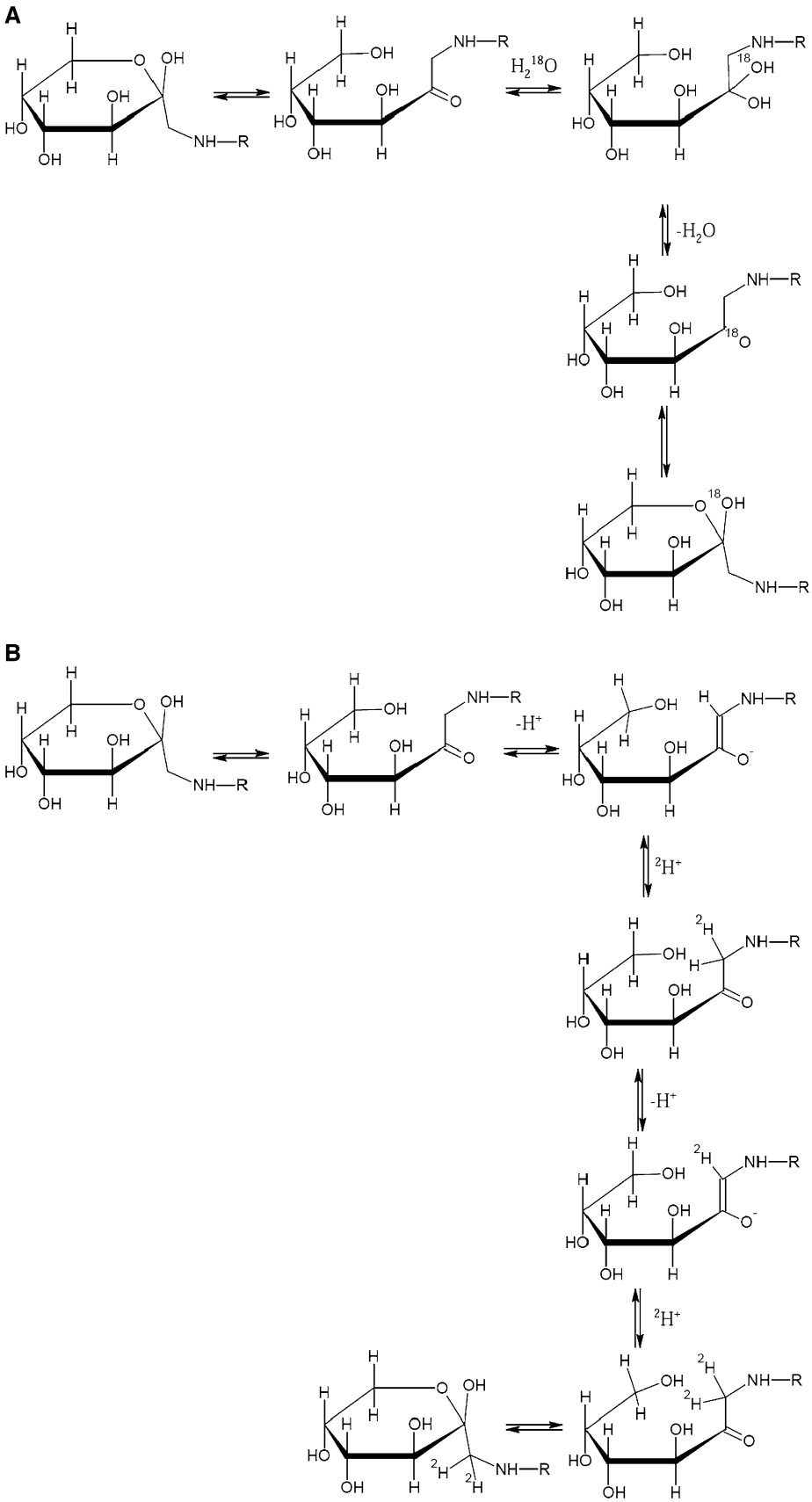

Fig. 6 **a** The scheme of proposed mechanisms of $^{16}O/^{18}O$ isotopic exchange of Amadori products. **b** The scheme of proposed mechanisms of $^{1}H/^{2}H$ isotopic exchange of Amadori products

based on NMR data (Roper et al. 1983). In D_2O solution, the H-1 atom in fructose moiety of Amadori compounds undergoes the slow H/D exchange which is significantly accelerated at more basic pH values.

Because at room temperature, this reaction requires long time (months) to complete, also in this case the microwave activation was applied. The experimental conditions used for H/D exchange were the same as that used for $^{16}O/^{18}O$ exchange. After microwave-assisted isotopic exchange, the sample was freeze-dried to remove D_2O, then dissolved in water and after 1 h (time sufficient for back exchange of amide deuterium and deuterium in side chains of amino acids) the sample was analyzed using ESI-MS procedure. The isotope envelopes showing the isotopic exchange in microwave reactor at various temperatures are shown in Fig. 5. The reaction of deuteration seems slower when compared with $^{16}O/^{18}O$ exchange. After the incubation at 75°C, the isotopic pattern is distinct from unmodified peptide, but deuteration level is low. In addition, deuteration at 75°C for 90 min did not result in full isotopic exchange. The incubation of peptide solution in D_2O at 100°C over 30 min resulted in almost complete isotopic exchange accompanied by partial hydrolysis of Amadori product (<10%), while the application of 150°C over 5 min resulted in almost complete hydrolysis of Amadori product.

Both deuteration and $^{16}O/^{18}O$ exchange are initiated by forming the open chain structure of Amadori product containing the free carbonyl group. Existing at low concentration, the carbonyl containing linear form of Amadori product may form hydrate (which result in oxygen exchange) or form enolate ion—which is an intermediate in H/D exchange. The scheme of proposed mechanisms of isotopic exchange of Amadori products is presented in Fig. 6.

The isotopic exchange was also studied on longer sequences. The solution of glycated ubiquitin incubated with $H_2^{18}O$ under conditions described in experimental part showed nearly a complete exchange of all anomeric OH groups in the hexose moieties. During NMR experiments, we observed that various HSA fragments incubated in D_2O for 3 months exhibited approximately 50% isotopic exchange of protons attached to the carbon C1. These examples demonstrate that the $^{16}O/^{18}O$ and $^{1}H/^{2}H$ isotopic exchange in a hexose moiety is not restricted to short, model peptides with *N*-terminal lysine, but is a general feature of peptide-derived Amadori products. The influence of sequence of the polypeptide on the kinetics of isotopic exchange is currently the object of our studies.

The microwave-assisted isotopic labeling of peptide-derived Amadori products in the presence of $H_2^{18}O$ is a simple and fast procedure. The 2 Da increase in molecular mass enables the use of this method in isotopic dilution method. The obtained product is susceptible to the back exchange of oxygen; however, this process is sufficiently slow to use labeled peptide-derived Amadori product as isotopomers in LC–MS isotopic dilution method. The advantage of the last isotopic labeling method is based on the fact that the isotopic label may be incorporated into the already existing compound, for example, glycated peptides in enzymatic digest. This feature makes the regioselective ^{18}O labeling an attractive approach for quantification of peptide-derived Amadori product in proteomic research and for isotopic labeling based procedures of selective detection of Amadori products. The deuteration, in theory, may be also used in quantitative determination of peptide-derived Amadori products, but the definitely slower labeling process and the possibility of separation of deuterated and undeuterated products makes this approach less attractive when compared with the oxygen labeling. On the other hand, the labeling of the Amadori products with tritium may be potentially a convenient way for radioactive labeling (because of relative simplicity and possibility of preparing the labeled compounds in very small quantities).

Conclusion

The developed methods allow fast and convenient synthesis of isotopically labeled peptide-derived Amadori products. The products were obtained in relatively large scale (10–20 mg of purified compound). Their purity and identity were proven by HPLC, HR-MS and NMR methods. The Amadori products labeled by stable isotopes can be applied as standards for isotopic dilution method in quantification of glycated proteins and as models for mechanistic studies on gas-phase fragmentation of Amadori products and their transformation to AGEs.

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