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A mechanistic study on the fragmentation of peptide-derived Amadori products

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Gas phase fragmentation of peptide-derived Amadori products was investigated using synthetic compounds regioselectively deuterated as well as labeled with ^{18}O at aminofructose moiety. The eliminated molecule CH_2O contains exclusively protons attached to carbon C6 of the aminofructose moiety. The hydrogen atoms connected with the carbon C1 of the aminofructose moiety are partially eliminated as a component of water molecules during the dehydration process and partially dislocated within the fragmented peptide molecule. The labeled oxygen atom attached to the carbon C2 is eliminated in 100% along with the first loss of water. The MS^3 experiments revealed that the product ion formed by triple dehydration of the Amadori product does not eliminate the formaldehyde molecule. On the basis of these observations we proposed a hypothetical mechanism of Amadori products' fragmentation.

Supporting information may be found in the online version of this article.

Keywords: Amadori rearrangement; glycation; neutral losses; mechanism; nonenzymatic protein modifications; microwave synthesis

Introduction

Nonenzymatic glycation is one of the most widely spread nonenzymatic side-chain-specific posttranslational modifications. According to Maillard hypothesis, the formation of protein–sugar conjugates is considered as an important cause of the diabetes associated complications.[1,2]

The first stage of this reaction involves the interaction of glucose with the amino group in the protein molecule. The imines formed in the reversible reaction stage rearrange to Amadori compounds – *N*-substituted (1-deoxy-ketos-1-yl)amines[3,4] – representatives of an important class of Maillard intermediates. (Fig. 1) These compounds undergo oxidation and dehydration giving a rise for a diverse group of heterocyclic compounds, called advanced glycation end products (AGEs). There are numerous pieces of evidence that AGEs mediate diabetes related pathological processes[5] and aging,[6] mainly by changing the physicochemical properties of long-living proteins (like collagen and crystallin) and by the activation of receptors of advanced glycation end products (RAGEs) resulting in inflammatory processes.[7]

On the other hand, recent studies indicate that Amadori-modified proteins of serum may also play a substantive role in the pathogenesis of vascular complications of diabetes, mainly arteriosclerosis. The mechanism of this influence is not clear, however preliminary results suggest that the Amadori-modified human serum albumin may interact with the smooth muscles of the blood vessels increasing blood pressure.[8]

Further understanding of the role of glycation in the diabetic complications development requires the identification of those proteins whose glycation may lead to pathological states as well as the recognition of the amino acid residues that underwent the modification process. This may be solved by the combination of enzymatic hydrolysis and liquid chromatography-mass spectrometry (LC-MS/MS). Earlier studies revealed that the peptide-derived Amadori products in collision induced dissociation (CID) experiments produce a very characteristic pattern of neutral losses (18, 36, 54, 84 and 162 Da).[9–11] This series of neutral losses is attributed to the gradual elimination of 1, 2 and 3 molecules of water, followed by the CH_2O molecule and finally the whole hexose moiety. The given series of neutral losses is characteristic for Amadori products and enables the differentiation of these compounds from the products of enzymatic *O*- or *N*-glycosylation.[9] Although the fragmentation behavior of the glycated peptides is well studied and widely utilized in analysis, there is limited information on the mechanistic aspects of these fragmentations.

In the present paper we reinvestigate the fragmentation of the peptide-derived Amadori products on the basis of the MS^2 and MS^3 measurements. CID experiments were performed on the model peptide–glucose conjugates regioselectively labeled at the sugar moiety with deuterium atoms (attached to the carbon atoms C1 and C6) as well as ^{18}O atom which was attached to the C2 carbon. The isotopic labeling allows us to directly monitor the origin of water and formaldehyde molecules eliminated from the sugar moiety in the CID experiment. The proposed model was supported by the similarity of the fragmentation patterns of the ion produced by the elimination of three water molecules from the glycated peptide with the synthetically obtained compound

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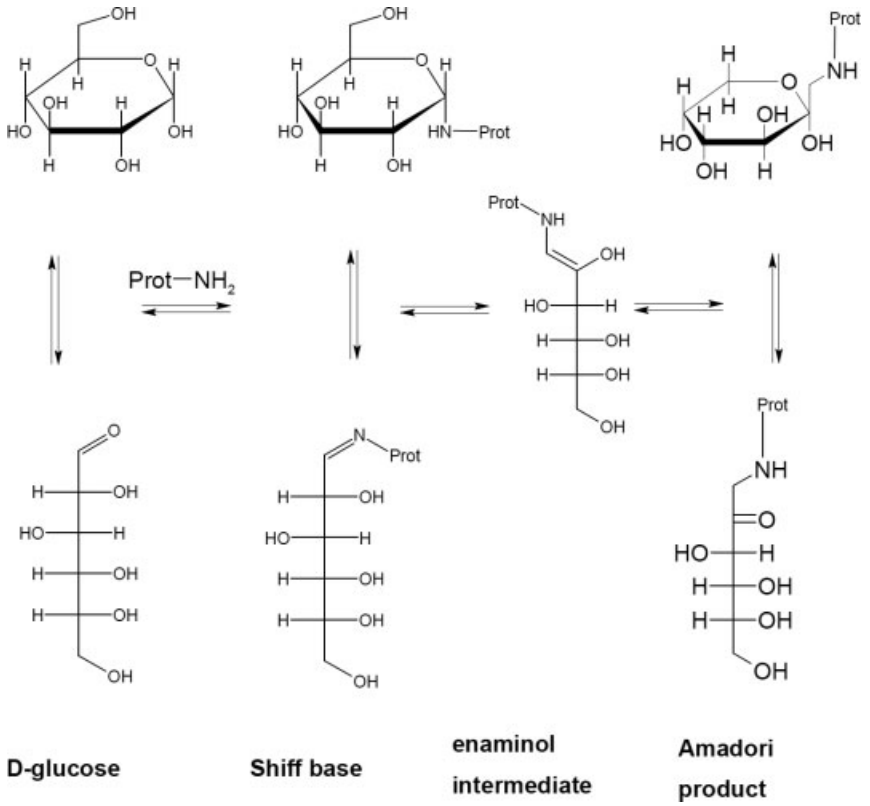

Figure 1. Formation of glucose–protein Schiff base and Amadori rearrangement.

corresponding to the postulated product of the dehydration process.

Material and Methods

Reagents

Deuterated sodium cyanoborohydride ($NaBD_3CN$), D-glucose-6,6-$^2H_2$ and $H_2{}^{18}O$ were purchased from Aldrich. The amino acid derivatives and coupling reagent: *O*-(benzotriazol-1-yl)-*N*, *N*, *N*′, *N*′-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Nova-Biochem.

Peptides

*Ac-Lys(Fru)-Ala-Ala-Phe-OH (**I**) and Ac-Lys([1-^{2}H]Fru)-Ala-Ala-Phe-OH (**II**) (the abbreviation Fru stands for fructose)*

Peptides (**I**) and (**II**) were synthesized according to the solid phase 9-fluorenylmetoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu) protocol. The synthesis has already been presented.[12] Briefly, the lysine side chain designed for modification was protected by 4-methyltrityl group. After completing the peptide synthesis the acid-labile Mtt group was cleaved with 1% triflouroacetic acid (TFA) in dichloromethane (DCM) solution. Peptides **(I)** and **(II)** were obtained on solid support by direct alkylation of the lysine residue's ε-amino group with 2,3 : 4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose, in the presence of $NaBH_3CN$ and $NaBD_3CN$ respectively. All groups protecting amino acids' side chains as well as isopropylidene groups from the sugar moiety were removed during the cleavage from the resin using TFA containing 2.5% of water and 2.5% of triisopropyl silane (TIS) for 24 h at room temperature.

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

Glycoconjugate containing two deuterium atoms in the sugar moiety (**III**) was synthesized using microwave irradiation on CEM Discover system. Hundred milligrams of the resin loaded with peptide was swollen with *N*,*N*-dimethylformamide (DMF). Then 200 mg of D-glucose-6,6-d_2 in 2 ml of DMF was added to the resin. The mixture was irradiated in a microwave oven at 40 W for 20 min. Then the Fmoc group was removed with 20% piperidine in DMF, the solution was drained, the resin was washed with DMF (3×), DCM (3×), MeOH (3×) and dried in vacuum. The resin was treated with the mixture of TFA : TIS : H_2O (95 : 2.5 : 2.5) for 8 h at room temperature in order to cleave the peptide from the support. The resin was washed with TFA and the combined filtrates were evaporated. The final product was purified by preparative high performance liquid chromatography (HPLC).

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

The amide H-Lys(Fru)-Ala-Ala-Phe-NH_2 was synthesized on the solid support according to the protocol given in our recent paper.[12] The purified peptide (1 mg) was dissolved in $H_2{}^{18}O$ (50 µl) and incubated at 25 °C. The MS spectrum of the peptide incubated over 200 h revealed more than 85% of labeling with ^{18}O.

*Ac-Lys(HMF)-Ala-Ala-Phe-OH (**V**)*

Peptide (**V**) was synthesized in solution by the modification of Ac-Lys-Ala-Ala-Phe-OH. Ac-Lys-Ala-Ala-Phe-OH (5 mg) was incubated overnight with 5-(hydroxymethyl)furfural (HMF, 5 mg) in 20 µl of methanol (Fig. 2). The solvent was evaporated and the

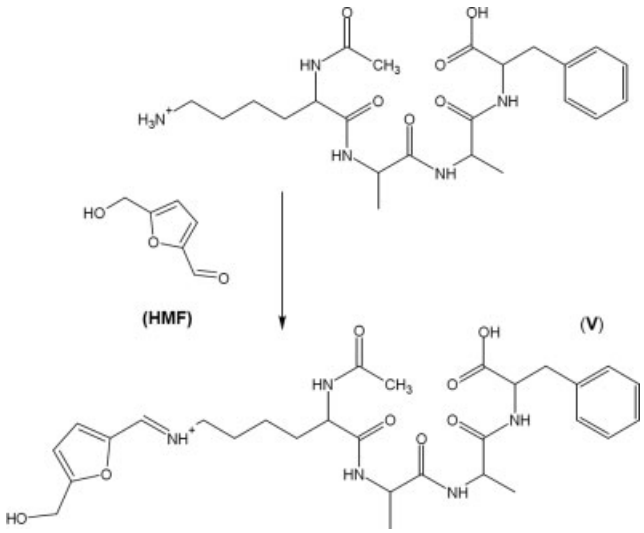

Figure 2. The synthesis of the peptide (**V**) Ac-Lys(HMF)-Ala-Ala-Phe-OH. The abbreviation HMF stands for 5-(hydroxymethyl)furfural.

crude product was used in the MS experiments without further purification.

Mass spectrometry

ESI-CID

The CID experiments were performed using an Apex-Qe 7T instrument (Bruker) equipped with dual electrospray ionization (ESI) source. Spectra were recorded using aqueous solutions of acetonitrile (50%) and formic acid (1%) at the peptide concentration 0.5 μM. The potential between the spray needle and the orifice was set to 4.5 kV. In MS/MS mode, the quadruple was used to select the precursor ions, which were fragmented in the hexapole collision cell applying argon as a collision gas. The product ions were subsequently mass analyzed by the ion cyclotron resonance (ICR) mass analyzer. For CID MS/MS measurements, the voltage 20 V over the hexapole collision cell was applied.

MS^3

The initial fragmentation of the Amadori product molecular ions in the MS^3 experiment was achieved by applying a high potential at the skimmer (70–90 V). The fragments produced by skimmer fragmentation were selected in the first quadruple (Q1) and subjected to CID in the second quadruple (Q2). The secondary product ions were subsequently analyzed in the ICR cell.

Calculation of the extent of deuterium retention

M_{av} – the average molecular masses of the corresponding deuterated fragments of the peptides **(II)** and (**III**) were calculated using the formula:

$$M_{av} = \Sigma(M_i A_i)/\Sigma A_i$$

where M_i are the molecular masses for particular isotopic peaks in the isotopic envelope and A_i are the abundances of corresponding isotopic peaks.

The deuterium content of deuterated product ions was determined by the difference in the average mass between the corresponding fragments of the deuterated and the nondeuterated peptides.

$$D(r) = M_{av}{}^{D} - M_{av}{}^{0}$$

where $D(r)$ is the number of remaining deuterons, $M_{av}{}^{D}$ is the observed average molecular mass of a deuterated peptide fragment; $M_{av}{}^{0}$ is the observed average molecular mass of a nondeuterated peptide fragment.

Results

The undeuterated peptide (**I**) Ac-Lys(Fru)-Ala-Ala-Phe-OH was prepared according to the method described previously.[12] Peptide (**II**) Ac-Lys([1-^{2}H]Fru)-Ala-Ala-Phe-OH was obtained by the same method, using $NaBD_3CN$ instead of $NaBH_3CN$. The molecular mass of the obtained derivative confirmed, that only one deuterium atom was incorporated into the labeled derivative. Peptide (**III**) – H-Lys([6,6′-$^2H_2$] Fru)-Ala-Ala-Phe-OH was obtained by the method presented previously by Frolov *et al.*[13] and Stefanowicz and Szewczuk[14] modified by using microwave activation. The synthesis was performed on solid support, using a solution of D-[6,6-$^2H_2$]glucose in DMF. The obtained peptide showed the expected molecular mass indicating incorporation of two D atoms into the molecule. Peptide (**IV**) labeled selectively on the oxygen atom attached to the carbon C2 was prepared by the incubation of amide H-Lys(Fru)-Ala-Ala-Phe-NH_2 with $H_2{}^{18}O$. After 200 h more than 85% of peptide underwent $^{16}O/^{18}O$ exchange. The literature data show that oxygen exchange takes place at the anomeric carbon atom of some sugars, like glucose, fructose and galactose. The reaction is attributed to the reactivity of linear sugar structure which in a water solution forms reversibly the gem-diol hydrate.[15]

The location of isotopically labeled atoms in the hexose moiety was given in Fig. 3.

Peptide (**V**) was prepared in solution in the reaction of hydroxymethylfurfural with the unmodified peptide Ac-Lys(Fru)-Ala-Ala-Phe-OH according to the procedure given Section on Material and Methods. The molecular mass of the obtained conjugates was consistent with the mass calculated on the basis of the formula.

The identity of obtained peptides including the position of deuterium atoms is confirmed on the basis of NMR (see the supporting information) and HR MS. All compounds were purified on preparative HPLC, and their homogeneity was confirmed by analytical HPLC. Only compound (**V**) was not purified and crude preparation was applied in MS/MS experiments. For this peptide structure was confirmed by HR-MS.

The nondeuterated peptide (**I**) was subjected to the MS/MS experiment. The fragmentation spectrum of the investigated compound is shown in Fig. 4. The analysis of the spectrum reveals that the most abundant peaks are formed by neutral losses of water molecules and the formaldehyde molecule. The elimination of the hexose moiety and the cleavage of the peptide bonds are also visible; however, the abundance of such fragments in relatively low.

The fragments at *m/z* 622.3124, 604.3015 and 586.2906 were subjected to the MS^3 experiment. The ions at *m/z* 622.3124 and 604.3015, generated by the elimination of one and two water molecules respectively, produced in further fragmentation, the fragment ions at *m/z* 568.2796 and 556.279, while the ion at *m/z* 586.2906, arising from the elimination of three molecules of water produced an ion at 568.2796 (elimination of water) but not an ion at *m/z* 556.279 which would be the effect of the elimination of

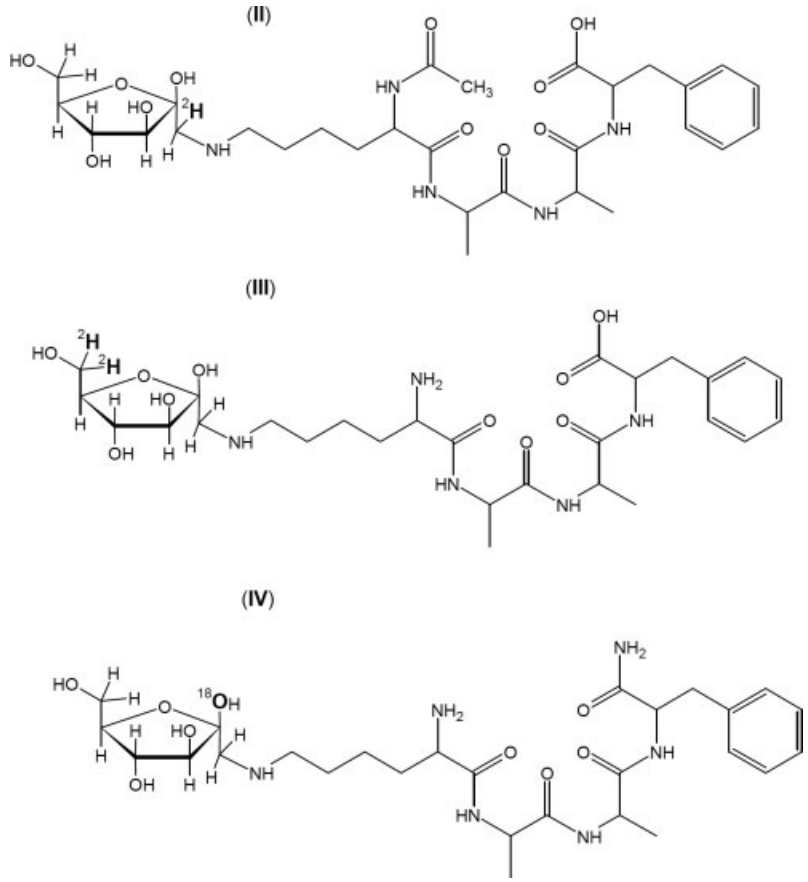

Figure 3. The location of the isotopic labels in the peptides (**II**)–(**IV**).

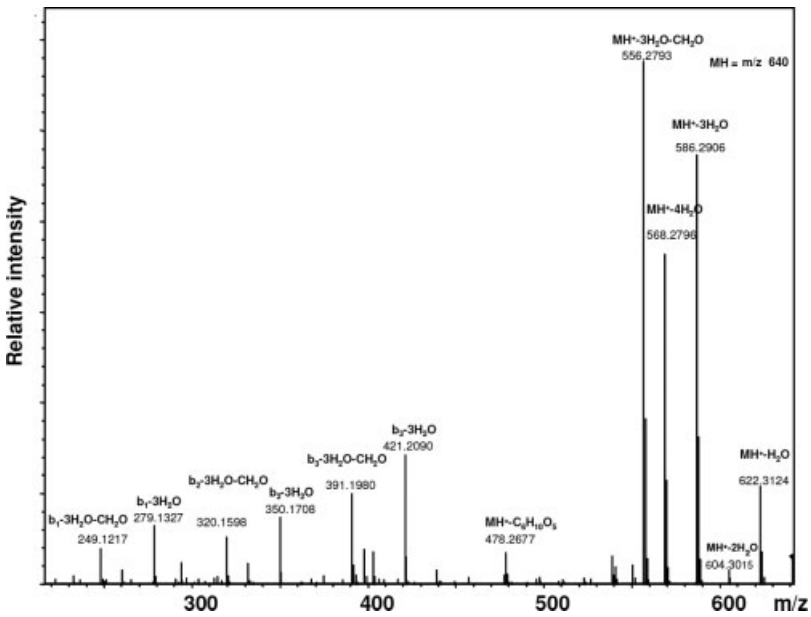

Figure 4. MS/MS spectrum of $[M + H]^+$ ion of glycated peptide **(I)** Ac-Lys(Fru)-Ala-Ala-Phe-OH.

formaldehyde. The summary of MS^3 experiments was given in the Fig. 5

Peptide (**II**) contains only one deuterium atom attached to the carbon 1 of the fructose moiety. The fragmentation of this compound was presented in Fig. 6. The first molecule of water eliminated from this compound did not contain deuterium. The neutral loss of two, three and four molecules of water as well as the loss of three molecules of water along with one molecule of formaldehyde give a rise for the product ions with the deuterium content ($D(r)$) 0.82, 0.75, 0.74 and 0.78, respectively. On the other

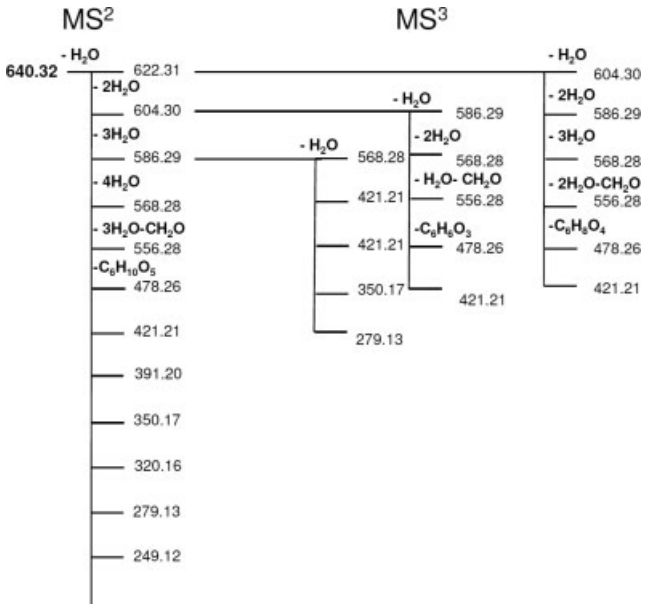

Figure 5. The summary of the products that arise from the MS^3 experiments of each of the first-generation product ions formed from $[M + H]^+$ ion of glycated peptide **(I)** Ac-Lys(Fru)-Ala-Ala-Phe-OH.

hand, the product ion at 478.2677 resulting from the neutral loss of a whole hexose moiety shows an increased intensity of the second isotopic peak (the measured value −39%, while the calculated one −24% of the intensity of the first isotopic peak). The deuterium content in this product ion is low ($D(r) = 0.11$) but in our opinion out of the range of the experimental error.

The deuterium content for ion at *m/z* 392.2044 (b_3-($3H_20, CH_2O$) is estimated as 0.65 what indicates, that the elimination of the Phe moiety reduces the content of D in the peptide. Since Phe is a *C*-terminal amino acid in the sequence, distant from the sugar-baring Lys, the elimination of deuterium with Phe residue may suggest a significant migration of the deuterium atoms attached to carbon 1 in the course of collision induced fragmentation. The difference in the deuterium content resulting from the elimination of the *C*-terminal Phe is close to an experimental error; however, additional pieces of evidence (value of $D(r) = 0.62$, for b_1-$3H_2O$ ion and $D(r) = 0.11$ for the fragment ion produced by the elimination of hexose moiety) seem to support the hydrogen transfer hypothesis during the fragmentation process.

The pattern of the fragmentation of the glycoconjugate labeled on carbon 6 (**III**) is different (Fig. 7). The elimination of water does not remove deuterium atoms from the molecule. However, deuterium is completely removed after the elimination of the formaldehyde molecule. No deuterium migration in the CID of compound (**III**) was observed.

The peptide (**IV**) was obtained by $H_2{}^{18}O$ treatment of amide H-Lys(Fru)-Ala-Ala-Phe-NH_2. The previous studies have proven that only the anomeric hydroxyl group is susceptible to the isotopic exchange.[15] The peptide labeled with ^{18}O at C2 was subjected to the CID experiment. The data presented in Fig. 8 indicate that the loss of the first water molecule is accompanied by the elimination of 100% of ^{18}O. Peptide (**V**) was fragmented (MS^2 experiment) and the fragmentation pattern was compared with the fragmentation of the product ion at *m/z* 586.2906 generated by the elimination of three molecules of water from the parent ion at *m/z* 640.3232. The comparison revealed the similarity of those patterns (Fig. 9). The observed minor differences can be explained by different internal energies of the ions formed in the ion source and obtained as a result of fragmentation. Interestingly, both ions (protonated compound (**V**) and the product ion at *m/z* 586.2906) did not produce product ions arising from the elimination of formaldehyde.

Discussion

The fragmentation of peptide (**II**) suggests that the elimination of water molecules is proton-driven. The first molecule of water is

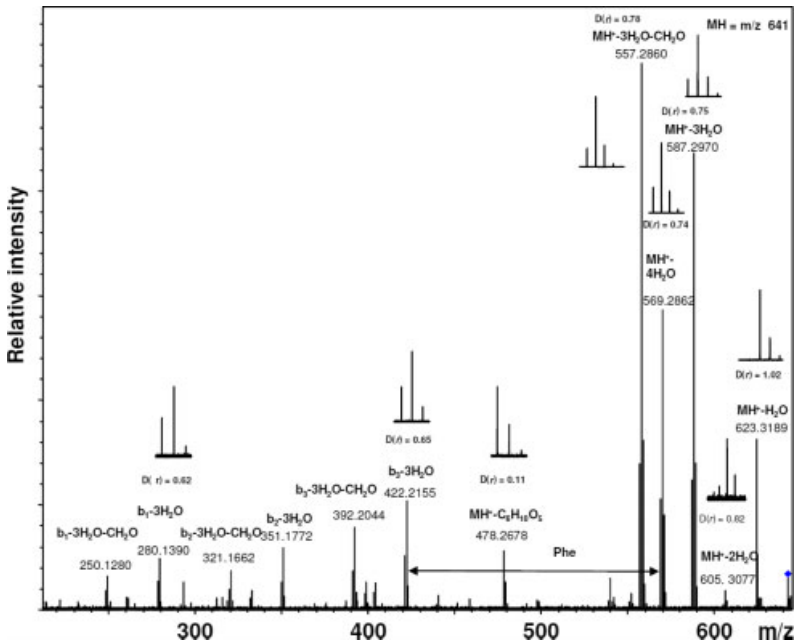

Figure 6. MS/MS spectrum of $[M + H]^+$ ion of regioselectively deuterated glycated peptide (**II**) Ac-Lys([1-^{2}H] Fru)-Ala-Ala-Phe-OH.

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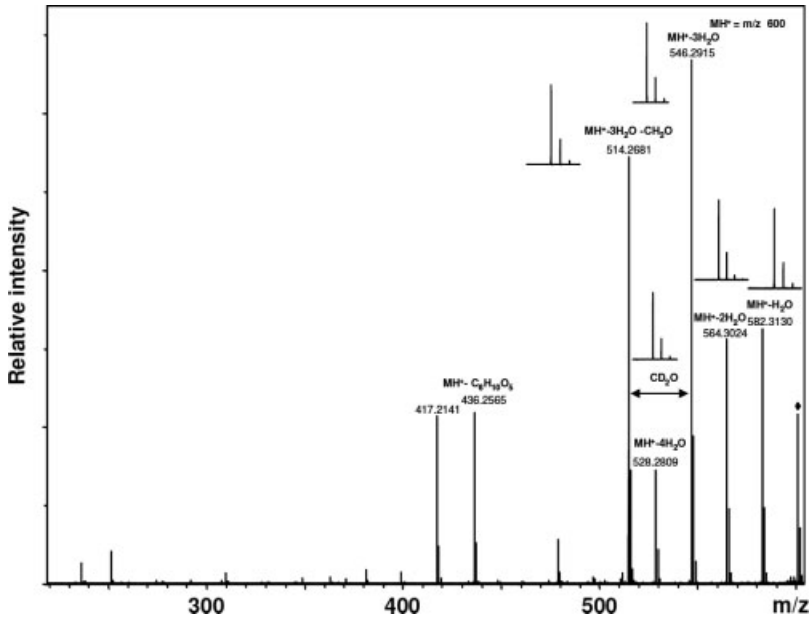

Figure 7. MS/MS spectrum of $[M + H]^+$ ion of regioselectively deuterated glycated peptide (**III**) Ac-Lys([6,6-$^2H_2$] Fru)-Ala-Ala-Phe-OH.

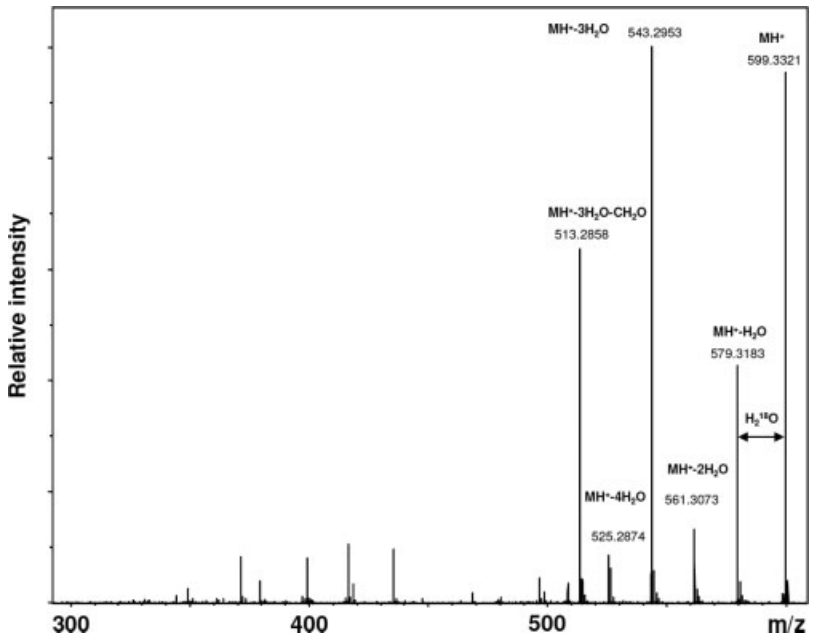

Figure 8. MS/MS spectrum of $[M + H]^+$ ion of regioselectively isotopically labeled with ^{18}O glycated peptide (**IV**) H-Lys([2-^{18}O] Fru)-Ala-Ala-Phe-NH_2.

likely to be eliminated as a result of the migration of the proton attached to the nitrogen atom onto the hydroxyl group attached to carbon 2.

The elimination of water produces an ion with the charge localized on the oxygen (oxonium ion), which is stabilized by the transfer of the hydrogen atom from the carbon 1 onto the nitrogen (the most basic center in the molecule). On the other hand, direct proton transfer in this case would require a three-center reaction what usually requires a high activation energy.[16,17] The alternative mechanism involves the solvation of the proton by the remaining sugar OH group. The hydroxyl group attached to the carbon C2 may serve as an acceptor for the proton eliminated from the C1. This reaction may proceed as a five-center proton transfer. Because the migration of the proton is expected to be faster than the water elimination, proton from the hydroxyl group may be transferred onto the nitrogen in the lysine side chain and scrambled between protonation sites within the hexose moiety. Proton localized on the nitrogen atom can be transferred on the hydroxyl group on carbon 3. The elimination of the second water molecule (which is partially deuterated) removed approximately 18% of deuterium from the fragmented ion. Consecutive elimination of three and four molecules of water caused the removal of 25 and 26% of deuterium respectively. The expected values of $D(r)$ were estimated on the basis of the following assumptions:

- The isotopic effects are neglected.

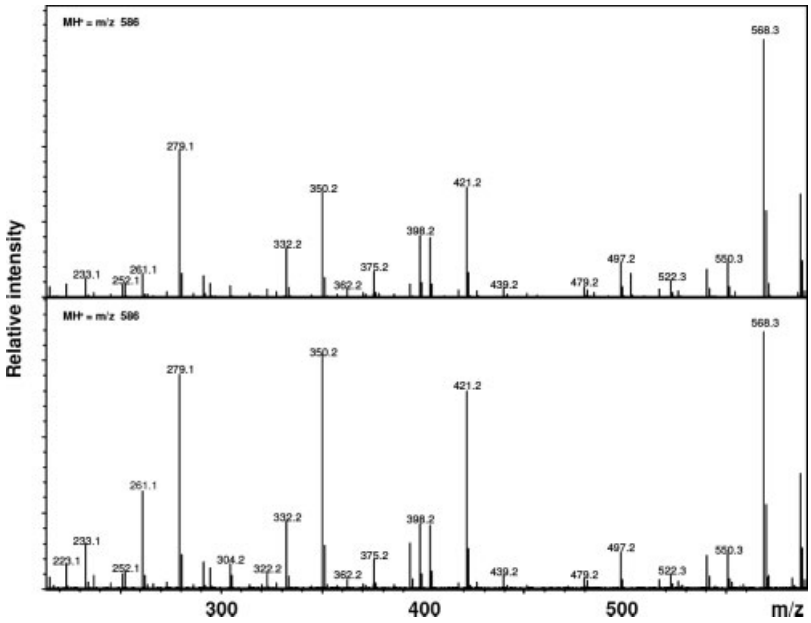

Figure 9. Comparison of MS/MS spectrum for $[M + H]^+$ ion of peptide **(V)** (upper panel) and MS/MS/MS spectrum of product ion obtained by elimination of three water molecules from the protonated ion of peptide **(I)** (lower panel).

- The hydrogen scrambling takes place within the hexose-modified lysine side chain.
- The migration of the protons to the peptide backbone is neglected.
- Deuterium is equally distributed among all protonation sites in the glycated side chain of lysine.

For example, the second water molecule is eliminated from structure E (Fig. 10). The deuterium atom can be localized in five different positions. Among them position 2 results in elimination of D_2O. Because only 0.5 deuterium atoms is distributed in the hexose moiety, the expected value of $D(r)$ is $1 - (2/5 \times 0.5) = 0.8$).

The comparison of $D(r)$ values predicted and measured for the consecutive losses of water (1/1.04; 0.8/0.82; 0.6/0.75 0.5/0.74) reveals a significant difference between the expected and the experimental content of deuterium in the product ions arising from the elimination of three and four water molecules. This discrepancy can be explained by the migration of deuterium, as the hydrogen (deuterium) atom attached to the nitrogen atom in the lysine side chain is mobile and may undergo further dislocation onto the peptide backbone (Fig. 10). The migration of the D atoms is also confirmed by the retention of D in the peptide after the elimination of the whole hexose moiety. The analysis of the fragmentation of (**II**) indicates that D attached to carbon C1 is shifted onto the heteroatom (most likely N in the side chain of lysine) and then initiates the loss of water. This proton can also migrate along the peptide chain. The fragmentation of compound (**III**) gives evidence that the protons eliminated with formaldehyde are attached to carbon C6. In contrast to molecule (**II**) there is no proton migration in the peptide (**III**). Elimination of the protons connected with carbon C6 along with the formaldehyde molecule suggests that the CH_2O molecule contains a carbon C6. The peptide (**IV**) labeled at the anomeric carbon with ^{18}O atom eliminates 100% of ^{18}O with the first eliminated water molecule. The elimination of the hydroxyl group attached to the anomeric carbon C2 at the beginning of the fragmentation process (reaction B–C according to Fig. 10) is in good agreement with the proposed model.

There are a number of papers discussing possible mechanisms of Amadori products fragmentation. Molle *et al*. analyzed the fragmentation of lactolated peptides in gas phase. After the elimination of the galactose moiety, the dehydrated aminofructosyl residue underwent the elimination of a series of water molecules and of formaldehyde. The authors proposed the oxonium structure for the ion formed as a result of the elimination of galactose and two water molecules.[18] The elimination of CH_2O gives a rise for the furylium ion. Jeric and coworkers interpreted the fragmentation of the protonated Amadori products' ions, basing on the pyranose structure of the starting compounds.[11] In this paper, two fragmentation pathways (oxonium and ammonium) were suggested and the elimination of formaldehyde was confirmed.

The model of fragmentation proposed herein (Fig. 10) assumes the furanose form of the aminofructose moiety. Isotopic studies suggest that the fragmentation of peptide-derived Amadori products is initiated by the processes similar to the dehydration of fructose in acid water solution.[19–21] The first step of the reaction involves the elimination of the hydroxyl group attached to carbon 2. This elimination is initiated by the proton transfer from the nitrogen atom onto the hydroxyl group. The elimination of the water molecule gives a rise for the oxonium ion, which potentially can stabilize by the indirect, hydroxyl-mediated transfer of a proton from carbon C1 onto the nitrogen atom.

The proton attached to the glycated ε-amino group is again transferred onto the hydroxyl group, initiating the next dehydration. The loss of 18% of deuterium with the second eliminated water molecule is in good agreement with the proposed model. The deuterium attached to the glycated nitrogen atom undergoes further dislocation, and is transferred onto the other fragments of the peptide, including the *C*-terminal phenylalanine (Fig. 11). The dislocation of the proton in the peptide during CID is in agreement with the mobile proton model.[22] One of the experimental consequences of such a process is a hydrogen scrambling observed in CID of partially deuterated peptides.[23]

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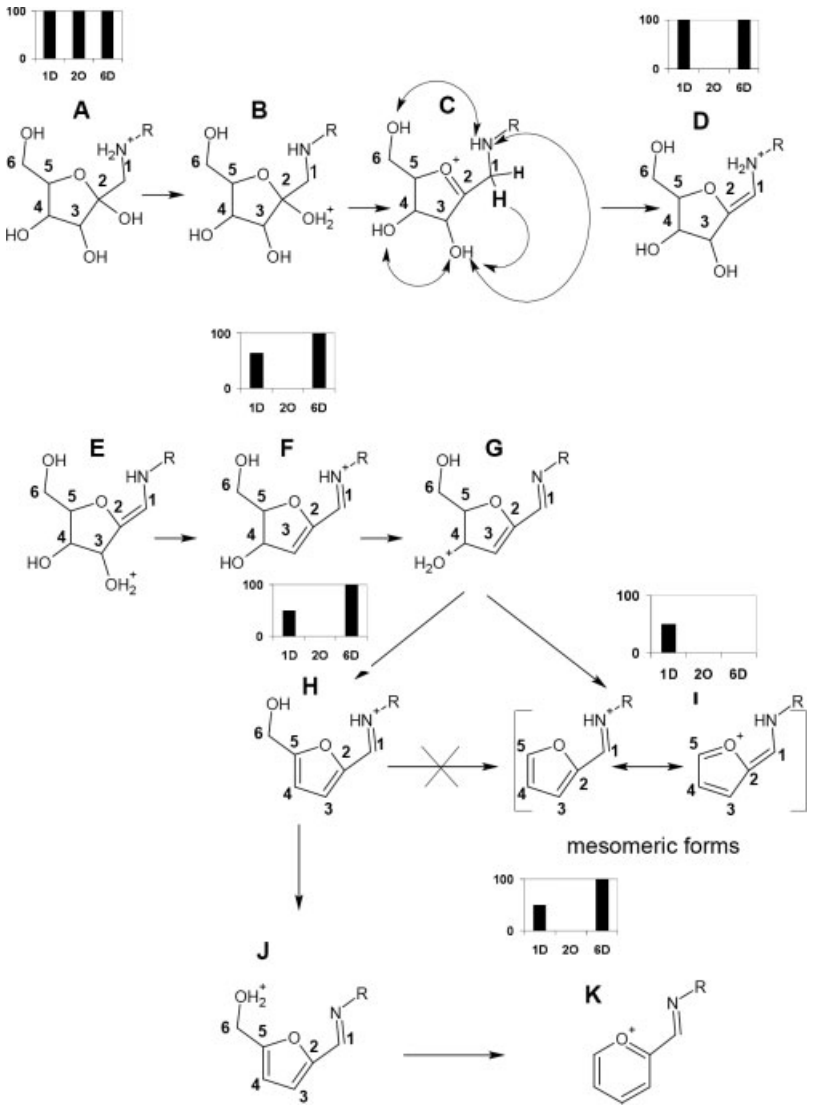

Figure 10. The proposed mechanism of fragmentation of the peptide-derived Amadori products. Bar graphs show the retention of isotopic labels (1-^{2}H; 6-^{2}H; 2-^{18}O) in a consecutive fragmentation steps. The arrows represents the proton transfer within the hexose moiety.

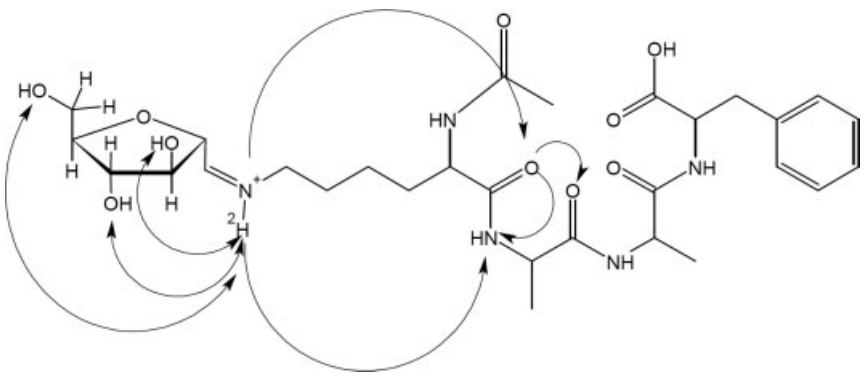

Figure 11. The transfer of protons within the product ion at *m/z* 587.2970 formed by the elimination of three water molecules from the $[M + H]^{+}$ ion of peptide (**II**).

On the other hand, half of the D atoms are permanently bonded to C1, and can be found in all the stable ions containing furylium or pyrylium ions.

The fragmentation of peptide (**III**) indicates that the hydrogens eliminated along with the formaldehyde molecule were attached to carbon 6 only. It is also very likely that the carbon eliminated with CD_2O molecule is the C6 atom of fructose. To support the hypothesis, that the mechanism of gas phase degradation of aminofructose moiety resembles the acid catalyzed dehydration of fructose, the peptide (**V**) (Fig. 2) was synthesized.

The product of the water elimination has the same M and elemental composition as the Schiff base obtained in the reaction of hydroxymethylfurfural with Ac-Lys-Ala-Ala-Phe-OH. The structure of this compound is the mesomeric form of the ion postulated by Molle. We found that this ion does not eliminate the formaldehyde molecule but only H_2O, forming the ion at *m/z* 568.2796. The elimination of CH_2O directly from the ions M-3H_2O obtained by the elimination of three molecules of water from peptide (**I**) at *m/z* 586.2906 is not observed. This phenomenon can be explained assuming that formaldehyde is eliminated simultaneously with the third molecule of water.

According to nuclear magnetic resonance (NMR) studies Amadori compounds exist in solution as an equilibrium of furanose and pyranose forms.[24,25] In water solution, the pyranose form is the predominant one, however in organic solvents [dimethyl sulfoxide (DMSO)] the most populated form of the amino fructose moiety is the furanose. There are also suggestions that similar equilibrium is possible in gas phase.[26] In our model, the furanose form was chosen, because this formula better explains the elimination of CD_2O from the isotopically labeled peptide (**III**). The experiment using model peptide (**V**) also provides support for the furanose structure in gas phase. This model is consistent with the fragmentation mechanism proposed by Molle, and the solution phase mechanism of the fructose dehydration. On the other hand, literature indicates that the cyclic compounds containing the oxonium atom in a six- or five-membered ring undergo fast and reversible interconvertion.[26] It should also be noted that in opinion of some authors, the fragmentation of peptide–sugar conjugates is a complex, multichannel process and most likely each signal represents a mixture of different isomers.[9]

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