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Characteristics of Early Maillard Reaction Products by Electrospray Ionization Mass Spectrometry

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It is crucial to characterize early Maillard reaction products and the important compounds formed in the early stage of Maillard reactionas Amadori rearrangement products are the most important modifications in food science. We report here that using electrospray ionization-mass spectrometry (ESI-MS) to directly characterize fragmentation behaviour of Amadori rearrangement products in a reaction model system using six selected amino acids (arginine, asparagine, glutamine, histidine, lysine and tryptophan) and their N-terminal acetylated forms with two reducing disaccharides, lactose and maltose. The fragmentation behaviour of Amadori rearrangement products was illustrated by Tandem MS (MS^2) with collision-induced dissociation (CID). Results showed that the sugar moiety was preferentially fragmented, where by the neutral loss of small molecules, such as 18 Da, 36 Da, 216 Da, 246 Da and 324 Da from disaccharide moieties. Among the fragmented ions, $[M-246 + H]^+$ of disaccharides were relatively stable and they were further studied for fragmentation mechanisms based on representatives of lysine and N^{α}-Ac-lysine. The study is useful to understand the fundamentals of glycation in complex protein systems based on ESI-MS related techniques.

Keywords: Amadori rearrangement products, Maillard reaction, ESI-MS, Collision-induced dissociation, Fragmentation.

INTRODUCTION

Amino acid-sugar systems have been popular as a simple platform for studying the mechanism of the Maillard reaction[1-5]. Fundamental knowledge on derivatives of amino acids with sugars such as Amadoric rearrangement products (ARPs) could potentially improve the production in food industry, predict nutritional status and assess protein quality[6-8]. These compounds were the important targets for characterization and quantification. Due to the complexity of the reaction, it is still a great challenge to control the reaction for food quality, nutritional value and medicinal aspects. In order to simplify the complex processing of Maillard reaction, it was subdivided roughly into three stages:early stage, intermediate stage and final stage. Early stage includes sugar-amine condensation and Amadori rearrangement and the products are colourless. The processing is the nucleophilic of an amine group reacting with the carbonyl group of a reducing sugar to yield a glycosylamine. Glycosylamines are unstable and form N-substituted 1-amino-1-deoxyketoses *via* Amadori rearrangement (Fig. 1)[9-11].

Currently, mass spectrometry (MS) related techniques offer an alternative, highly sensitive method and become powerful for the analysis of glycated products in complex mixture. Electrospray ionization mass spectrometry (ESI-MS) has been used to successfully analyze some Amadori rearrangement products derived from sugars with proteins in complex system[12,13] and other products in complex foods and biological systems[14-16]. MS provided important information for understanding the reaction process and allowed the determination of Maillard reaction products through specific fragmentation patterns[17-21]. Structural identification provided by precursor ions was greatly augmented by product ions evidence. Information obtained from fragmentation species was directly linked to the chemical structure, thereby allowing a higher degree of confidence for identification. Therefore, understanding the fragmentation behavior of Amadori rearrangement products was critical toward establishing strategies for sequencing of proteins[1,22].

In our previous work,we studied the fragmentation behaviour of Amadori rearrangement products in lysine containing peptide/amino acid -glucose model and identified the glycation sites of peptides through the fragments of $[M-84 + H]^+$ in pseudo-MS^3 experiments under low-energy collision induced dissociation (CID) conditions and computational results[23,24].

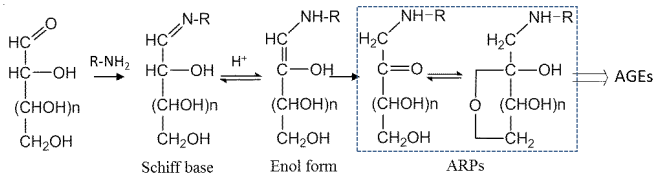

Fig. 1. Production of Amadori rearrangement products (ARPs)

The goal of the present study was to investigate the gas-phase CID fragmentation behaviour of sugars and amino acid moieties of Amadori rearrangement products in anamino acid-disaccharide model and to identifydiagnostic fragment ions for study and understanding of fundamentals of fragmentation behaviours of Amadori rearrangement products in MS^2 and MS^3.

EXPERIMENTAL

The amino acids used are lysine (K), arginine (R), asparagine (N), glutamine (Q), histidine (H) and tryptophan (W). The sugars used are β-lactose. All materials and solvents were obtained commercially (Aldrich and Sigma, St Louis, MO, USA).

Sample preparation: The reaction model was set as following: 0.1 M of amino acids and acetylated amino acids were dissolved in 1 M d-glucose solution and got the ratio of 1:10 by molecular weight. Freezed-dry by SC250DDA Speedvac Plus (Thermo Electron Corporation, Waltham, MA) the samples to get the whiter power mixture and dry-heating the samples in sealed vials for 1 h at 50, 70 and 90 °C, respectively and cooling down the samples in -20 °C immediately after reaction finished. All samples were dissolved with ACN:H_2O:FA (40/60/0.5:v/v/v) just before submitting to MS.

Acetylation of amino acids and purification by HPLC: A solution of the acetylation reagent was prepared through the addition of acetic anhydride (250 µL) to methanol (750 µL). The acetylation reagent (1 mL) was added to a mixture of the amino acid (10 mg) and 50 mM ammonium bicarbonate (100 µL; pH 7.8). The reaction mixture was stirred for 3 h at room temperature. The resulting product was dried using a SC250DDA Speedvac Plus (Thermo Electron Corporation, Waltham, MA) to obtain a solid product, which was further purified through HPLC. HPLC purification of acetylated amino acids (10-20 mg/mL each injection) was carried out in a Waters Delta 4000 system (Milford, MA 01757) with 2487 UV detector (λ = 215 nm), using a reverse phase column: Xterra® C-18 column 19 × 150 mm. The separation was performed using a mobile phase of double-deionized water with 0.1 % (v/v) trifluoroacetic acid (isocratic) and the flow rate was maintained at 12 mL/min. The LC effluent was dried by SC250DDA Speedvac Plus (Thermo Electron Corporation, Waltham, MA).

Mass spectrometry: All MS experiments were conducted in the positive mode of a quadrupole ion trap mass spectrometer, LCQ Deca XP Plus (Finnigan LCQ, Thermo Finnigan, San Jose, CA, USA) equipped with a nanospray ion source. Ion spray tips were flame-pulled from 150 µm (OD) × 50 mm (ID) capillaries. The electrospray voltage was typically kept between 2.8 and 3.0 kV; the inlet capillary was maintained at 180 °C. To obtain MS^2 and MS^n (n > 2) spectra, the normalized collision energy was varied while maintaining the other ion tuning conditions constant. Samples typically comprised 1 µM amino acid in water/methanol (50:50) solutions containing 1 % formic acid. The physical parameters of the interface *i.e.*, the distance between the needle and the hole in the spray shield (1.2-1.5 cm), the voltage (2.8-3 kV) applied to the stainless-steel unit and the temperature of the heated capillary (180 °C)-were optimized at a flow rate of 25 mL/h, using helium as the collision gas. The injection and activation times for MS^2 experiments in the ion trap were 200 ms and 30 ms, respectively.

RESULTS AND DISCUSSION

Amadori rearrangement products in amino acid-disaccharide model: Amino acids and their N-terminal acetylated derivatives were allowed to react with reducing sugars at different temperatures (50, 70 and 90 °C) and reaction times (0.5, 1, 2 and 4 h). Nano-ESI-MS was used to monitor and characterize the Amadori rearrangement products . In MS spectra of glycated amino acids with sugars, the main peaks are protonated amino acids, $[M + H]^+$, with additional peaks of protonated Amadori rearrangement products of amino acid derivatives. The peaks of $[M + 324 + H]^+$ are protonated Amadori rearrangement products of amino acids with disaccharides (lactose and maltose). For example, in the MS spectra, N^{α}-acetyl-lysine reacted with lactose and maltose, *m/z* 189 is the peak of N^{α}-acetyl-lysine and *m/z* 513 is the peak of Amadori rearrangement products of N^{α}-acetyl-lysine reacted with sugars (data not show). ESI-MS results showed that all selected amino acids could react with sugars noticeably at 90 °C and the peaks of Amadori rearrangement products were dominant in the ESI-MS spectra. However, only Amadori rearrangement products of lysine and N^{α}-acetyl-lysine with sugars could be detected at 50 °C for 1 h. It displayed that the ε-NH_2 group was more reactive with sugars than other side-chain amino groups (Table-1).

MS^2 of Amadori rearrangement products of sugar moieties: MS^2 spectra of Amadori rearrangement products in amino acid-disaccharide model showed the main fragmentation ions of Amadori rearrangement products by the loss of molecule of water and even whole sugar from the sugar moieties as shown in Fig. 2. The cleavage of sugar moiety occurred with a mass loss of *m/z* 162 and *m/z* 324. The main fragmentation ions of Amadori rearrangement products with two disaccharides of lactose and maltose were shown in Tables 2 and 3, respectively and the fragmentations were similar to be differentiated from MS^2 data. Among the fragmentation ions, the *m/z*

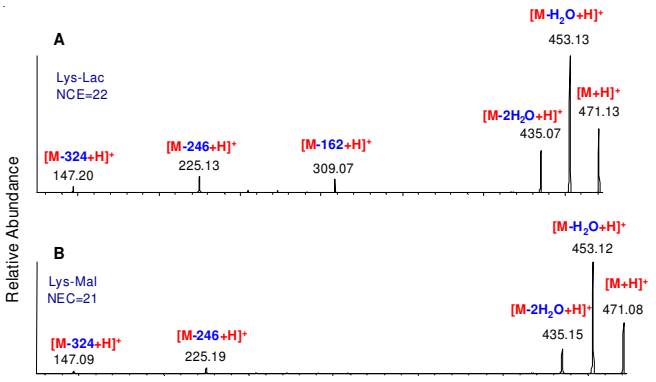

Fig. 2. MS^2 spectra of Amadori rearrangement products(ARPs) of lysine reacting with: (A) lactose and (B) maltose

TABLE-1
GLYCATION RESULTS OF SIX SELECTED AMINO ACIDS AND THEIR N-TERMINAL ACETYLATED FORMS REACTING WITH LACTOSE AND MALTOSE SUCROSE UNDER DIFFERENT TEMPERATURE FOR 1 h*

AA	Lactose			Maltose		
	50 °C	70 °C	90 °C	50 °C	70 °C	90 °C
Arginine	×	×	√	×	√	√
Asparagine	×	×	√	×	×	√
Glutamine	×	×	√	×	×	√
Histidine	×	√	√	×	√	√
Lysine	√	√	√	√	√	√
Tryptophan	×	×	√	×	×	√
Ac-arginine	×	×	×	×	×	×
Ac-asparagine	×	×	×	×	×	×
Ac-glutamine	×	×	×	×	×	×
Ac-histidine	×	×	×	×	×	×
Ac-lysine	√	√	√	√	√	√
Ac-tryptophan	×	×	×	×	×	×

*: AA: Amino acids; Ac-AA: N-terminal acetylated AA; ×: non-detectable; √: detectable

TABLE-2
MAIN FRAGMENTATION IONS AND RELATIVE INTENSITIES IN MS^2 SPECTRA OF ARPS OF AMINO ACIDS REACTING WITH LACTOSE (LAC REPRESENTS LACTOSE)

AA	Compound	$[M + H]^+$ *m/z* (Int. Rel, %)	MS^2 ions *m/z* (Int. Rel, %)	Loss	Fragment lost
Lysine	Lys-Lac (NCE = 22)	471.1 (37)	453.1 (100)	-18	- H_2O
			435.1 (18)	-36	- $2H_2O$
			225.2 (12)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
			147.1 (4)	-324	- $2C_6H_{10}O_5$
Asparagine	Asn-Lac (NCE = 19)	457.1 (23)	439.0 (100)	-18	- H_2O
			421.0 (14)	-36	- $2H_2O$
			337.0 (70)	-120	- $C_4H_8O_4$
			295.1 (1)	-162	- $C_6H_{10}O_5$
Histidine	His-Lac (NCE = 20)	480.1 (45)	462.1 (100)	-18	- H_2O
			444.0 (23)	-36	- $2H_2O$
			156.1 (3)	-324	- $2C_6H_{10}O_5$
Glutamine	Gln-Lac (NCE = 19)	471.0 (40)	453.0 (100)	-18	- H_2O
			435.0 (15)	-36	- $2H_2O$
			309.2 (1)	-162	- $C_6H_{10}O_5$
			225.1 (2)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
Tryptophan	Trp-Lac (NCE = 20)	529.1 (85)	511.1 (100)	-18	- H_2O
			493.1 (6)	-36	- $2H_2O$
			367.2 (9)	-162	- $C_6H_{10}O_5$
			205.1 (1)	-324	- $2C_6H_{10}O_5$
Arginine	Arg-Lac (NCE = 26)	499.2 (58)	481.2 (100)	-18	- H_2O
			463.1 (6)	-36	- $2H_2O$
			301.0 (8)	-198	- $3H_2O$
			175.2 (24)	-324	- $C_6H_{10}O_5$
Ac-Lysine	Ac-Lys-Lac (NCE = 22)	513.1 (36)	495.2 (100)	-18	- H_2O
			477.2 (7)	-36	- $2H_2O$
			267.2 (10)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
			189.2 (1)	-324	- $2C_6H_{10}O_5$

225 was observed, suggesting that the basic skeleton of the disaccharides was similar to glucose in previous study[24].

Fragmentation behaviour of lysine and N^{α}-acetyl-lysine: The ε-NH_2 group of lysine has been shown to be the target group in peptides and proteins that reacts with reducing sugars. Hence, the study of lysine and N^{α}-acetyl-lysine fragmentation pattern should help to understand the cleavage behaviour of glycated peptides and proteins. In the MS^2 spectrum of lysine (Fig. 3a), *m/z* 130 was the dominant ion by loss of N-terminal NH_3. The immoniun (Im) ion of lysine *m/z* 129 could be observed and its structure corresponds to the lysine nominal acylium ion. Furthermore, *m/z* 101 (Im-28 Da) and *m/z* 84 (loss of NH_3 from *m/z* 101) appear in MS^3 spectrum of lysine. In the MS^2 spectrum of N^{α}-acetyl-lysine (Fig. 3d), *m/z* 171 was by loss of H_2O and *m/z* 129 was Im ion of lysine. It implied that the α-NH_2 group was relatively fragile than the

TABLE-3
MAIN FRAGMENTATION IONS AND RELATIVE INTENSITIES IN MS^2SPECTRA
OF ARPS OFAMINO ACIDS REACTING WITH MALTOSE (MAL REPRESENTS MALTOSE)

AA	Compound	$[M + H]^+$ *m/z* (Int. Rel, %)	MS^2 ions *m/z* (Int. Rel, %)	Loss	Fragment lost
Lysine	Lys-Mal (NCE = 21)	471.1 (45)	453.1 (100)	-18	- H_2O
			435.1 (23)	-36	- $2H_2O$
			309.1 (1)	-162	- $C_6H_{10}O_5$
			225.2 (5)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
			147.1 (2)	-324	- $2C_6H_{10}O_5$
Asparagine	Asn-Mal (NCE = 19)	457.1 (23)	439.0 (100)	-60	- H_2O
			421.0 (14)	-60	- $2H_2O$
			337.0 (70)	-120	- $C_4H_8O_4$
			295.1 (1)	-162	- $C_6H_{10}O_5$
Histidine	His-Mal (NCE = 20)	480.1 (45)	462.0 (100)	-18	- H_2O
			444.0 (23)	-36	- $2H_2O$
			318.1 (1)	-162	- $C_6H_{10}O_5$
			156.1 (1)	-324	- $2C_6H_{10}O_5$
Glutamine	Gln-Mal (NCE = 19)	471.0 (40)	453.0 (100)	-18	- H_2O
			435.0 (15)	-36	- $2H_2O$
			309.2 (1%)	-162	- $C_6H_{10}O_5$
			225.1 (2%)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
Tryptophan	Trp-Mal (NCE = 21)	529.1 (85)	511.1 (100%)	-18	- H_2O
			493.1 (6%)	-36	- $2H_2O$
			367.2 (9%)	-162	- $C_6H_{10}O_5$
			205.1 (1%)	-324	- $2C_6H_{10}O_5$
Arginine	Arg-Mal (NCE = 26)	499.1 (58)	481.2 (100%)	-18	- H_2O
			463.1 (7%)	-36	- $2H_2O$
			337.1 (6%)	-162	- $C_6H_{10}O_5$
			175.2 (4%)	-324	- $2C_6H_{10}O_5$
Ac-Lysine	Ac-Lys-Mal (NCE = 21)	513.1 (48)	495.2 (100%)	-18	- H_2O
			477.2 (6%)	-36	- $2H_2O$
			267.2 (30%)	-246	- $C_6H_{10}O_5$ -$3H_2O$-HCHO
			189.2 (1%)	-324	- $2C_6H_{10}O_5$

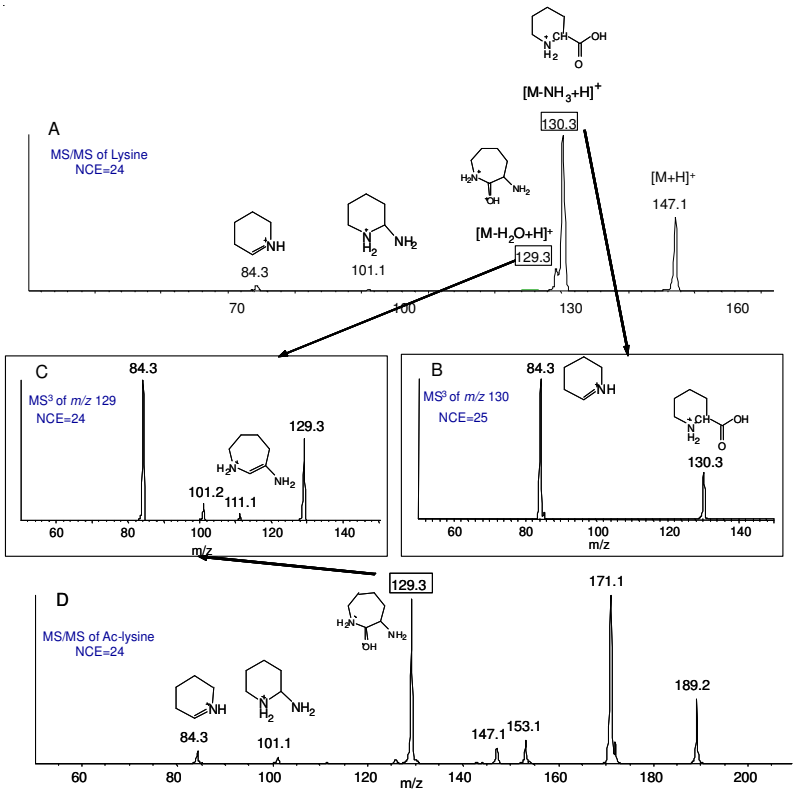

Fig. 3. MS^2 spectra of (A) protonated lysine *m/z* 147 and (D) protonated Na-acetyl-lysine *m/z* 189, and their corresponding MS^3 spectra of (B) *m/z* 130 and (C) *m/z* 129

ε-NH_2 group under the same collision energy. This property might provide additional information for identifying lysine in peptide and protein mapping. In MS^3 spectrum of *m/z* 130 (Fig. 3b), *m/z* 84 was the only fragment, indicating that *m/z* 101 could not be produced from *m/z* 130. The MS^3 spectrum of *m/z* 129 (Fig. 3c) showed not only ions of *m/z* 101 and *m/z* 84, but also *m/z* 111, suggesting that they had similar structures. Based on the MS^2 results, the fragmentation pathway of lysine and N^{α}-acetyl-lysine was postulated as shown in Fig. 4, which was useful to understand the fragmentation behaviour of Amadori rearrangement products.

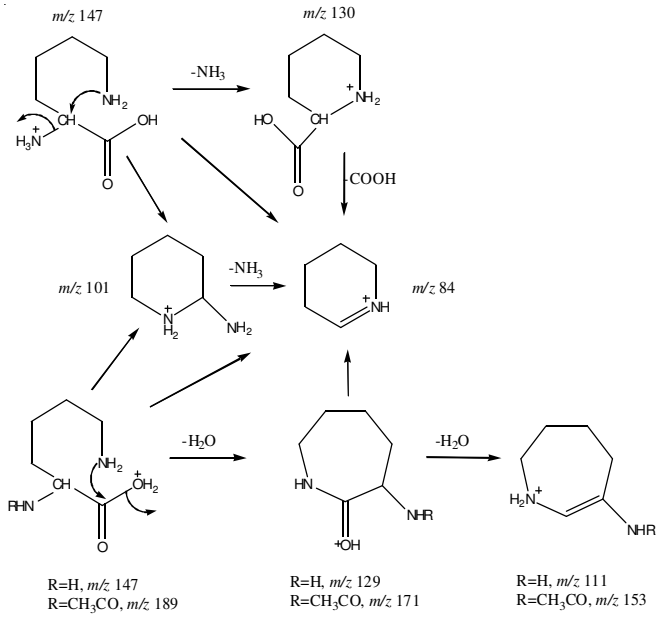

Fig. 4. Proposed pathway of fragmentations of lysine and N^{α}-acetyl-lysine

Conclusion

The amino acid-sugar model used in the present investigation demonstrated positively charged amino acids preferentially reacted with reducing sugars. Besides the primary amino group of the amino acids, the ε-amino group of lysine showed high reactivity. The fragmentation behaviours of the Amadori rearrangement products showed that the sugar moiety tended to be fragmented preferentially by neutral loss of small molecules to form relative oxonium ions $[M-246 + H]^+$. Based on our previous studies[24], the fragmentation behaviours of lysine and N^{α}-acetyl-lysine were studied and the cyclical mechanisms were proposed based on MS^2 data. The fragmentation study in gas phase could serve as the basis for further studies on complex systems, such as the use of lysine-containing peptides[23] and the identification of protein glycation sites, presented in present studies.

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