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Acid-Stable Fluorescent Advanced Glycation End Products: Vesperlysines A, B, and C Are Formed as Crosslinked Products in the Maillard Reaction between Lysine or Proteins with Glucose

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Vesperlysines A and B, 6-hydroxy-1,4-di{6-(L-norleucyl)}-1*H*-pyrrolo[3,2-*b*]pyridinium and its 5-methyl derivative, respectively, and Vesperlysine C, 5-hydroxymethyl-1,6-di{6-(L-norleucyl)}-1*H*-pyrrolo[3,4-*b*] pyridinium, are isolated as major fluorescent advanced glycation end products (AGEs) from hydrochloric acid hydrolysis of AGE–BSA, bovine serum albumin (BSA) modified by the Maillard reaction with glucose. These fluorophores are glycation products and not artifacts of hydrolysis, since they are also detected in the reaction mixture of lysine and glucose prior to hydrolysis. Vesperlysines are crosslinked products from two lysine sidechains in proteins and are considered to be generated from lysines and the oxidative degradation of glucose, because the six carbon skeleton of glucose in its original form was not incorporated into each structure. These compounds are most likely glycoxidation products like pentosidine. This reasoning is supported by the formation of the same compounds in the Maillard reactions in which ascorbic acid or other sugars with shorter carbon chains are used.

Recently, *in vivo* accumulation of a characteristic-fluorescence substances with Ex_{max} 370/Em_{max} 440 nm has been detected in the proteins from patients (or animals) who developed diabetic complications (1,2). The fluorescence is believed to be derived from "advanced glycation end products" (AGEs) produced by the Maillard reaction between the amino groups (e.g. ϵ-NH_2 of lysines) of proteins and glucose. Such fluorescent AGEs have been thought to be formed by the inter- and intramolecular crosslinking of proteins. However, even though the total fluorescence (Ex 370/Em 440 nm) has been widely used as a marker of AGE levels in proteins, nobody had shown the true chemical nature of such fluorophores before we proposed the crosslinked structure, crossline (3), as the first candidate for AGEs with such fluorescence. The AGE theory indicated (4) that such crosslinking is related to the initiation and/or progression of diabetic complications. Immuno- and immunohistochemical evidence indicated that crossline structures accumulate in tissue proteins of diabetic animals (5-7). We have attempted to isolate crosslines from the acid hydrolysates of proteins or tissues but were as yet successful due to their instability under acid hydrolysis. However we have found another fluorescent materials from the AGE-modified proteins after acid hydrolysis. We now report the structures of such acid stable fluorophores, and their formation in AGE-modified proteins *in vitro.*

MATERIALS AND METHODS

Reagents. L-Lysine hydrochloride, *n*-pentylamine, D-glucose, D-fructose, D-mannose, D-ribose and ascorbic acid were from Kishida chemicals (Japan); bovine serum albumin (BSA) and glyceraldehyde from Sigma (St.Louis,MO); glycolaldehyde dimer from Janssen (Belgium) and D-erythrose from ICN Biochemicals (Cleaveland,OH). 3-Deoxyglucosone was prepared according to Kato *et al.* (8). *Analytical instrument:* A Hitachi 650-40 fluorescence spectrophotometer was used for fluorescence analyses, a Beckman DU 650 spectrophotometer for UV spectra and a JASCO 900 system for HPLC. The SIMS were obtained using a Hitachi M-80B spectrometer with glycerol as matrix, and the NMR spectra were measured with Bruker AM-400 and ARX-500 spectrometers in CD_3OD using 3-(trimethylsilyl)-propanesulfonic acid as internal standard.

AGE-BSA was prepared by incubating BSA (80mg/mL) with D-glucose (0.2M) in 0.25M phosphate buffer (pH 7.4) at 37°C for 6 weeks and dialyzed against deionized water. The Maillard reaction mixtures containing AGE-*n*-pentylamine or AGE-lysine were prepared by incubating equimolar amounts of D-glucose and appropriate

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Abbreviations: AGE(s), advanced glycation end product(s); BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; SIMS, sputtered ion mass spectrum; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; COLOC, correlation *via* long-range coupling; nOe, nuclear Overhauser effect; HMQC, ^1H detected heteronuclear multiple quantum coherence; HMBC, ^1H detected heteronuclear multiple bond connectivity.

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amine (*n*-pentylamine or L-lysine) as before (3) in 0.25M phosphate buffer (pH 7.4) for 6 weeks at 37°C. Similarly, equimolar amounts of appropriate sugar or oxyaldehyde [D-fructose, D-mannose, D-ribose, DL-erythrose, 3-deoxyglucosone, glycolaldehyde dimer, glyceraldehyde and ascorbic acid] and *n*-pentylamine were incubated in 0.25M phosphate buffer (pH 7.4) for 9 days at 37°C. The preceding AGE-BSA or Maillard reaction mixtures (10μL) were dried under reduced pressure and hydrolyzed in 6N HCl (200μL) for 24h under nitrogen in sealed glass tubes.

Purification of the AGE-lysine and various hydrolysates of the Maillard reaction mixtures by HPLC. The fluorescent products were identified (a) on a 0.46 × 25cm Capcell pak C18 SG column using a linear gradient of 0 to 50% MeOH in water during 0 to 30min (flow rate of 0.8mL/min with 0.1% TFA). The eluate were monitored by fluorescent detectioin (Ex 370/Em 440 nm), and (b) on a 0.46 × 25cm Inertsil ODS-3V column with 7mM phosphoric acid (flow rate was 0.8mL/min). The two fluorescent crosslinked products from AGE-lysine (the lysine-glucose mixture) were isolated by treatment with sulfonic acid type ion exchange column (H^+ form) and 3N ammoniacal eluent was purified by reverse phase preparative column chromatography and reverse phase HPLC to give two major fluorophores (yield: **1**, 0.002%; **2**, 0.001%). During purification of **1**, a minor fluorophore **3** was also isolated in 0.001% yield.

After mixing the reaction mixture of BSA (20 g) and glucose with an equal volume of conc. HCl, the solution was heated at 110 °C for 24 h and evaporated to dryness *in vacuo*. The residue was purified as above to give 0.1 and 0.05 mg of fluorophores **1** and **2**, respectively.

The fluorescent material from the AGE-*n*-pentylamine (the *n*-pentylamine-glucose mixture) was purified using a sulfonic acid type ion exchange column (H^+ form) and 3N ammoniacal eluent was further purified on a XAD-2 column and reverse phase HPLC to give fluorophore **4** and **5** in 0.03 and 0.02% yield, respectively.

Structure determination of the fluorophores. Instrumental analyses using proton- carbon nuclear magnetic resonance and mass spectroscopy lead to the structures of these compounds. Each fluorophore was also acetylated in pyridine with acetic anhydride. Vesperlysine A (**1**), 6-hydroxy-1,4-di{6-(L-norleucyl)}-1*H*-pyrrolo[3,2-*b*] pyridinium, had ^{1}H NMR (400MHz, CD_3OD): δ 8.31 (5H, d, *J* 2Hz), 8.07 (7H, brd, *J* 2Hz), 7.96 (2H, d, *J* 3Hz), 6.91 (3H, brd, *J* 3Hz), 4.71 (1''H, t, *J* 7Hz), 4.35 (1'H, t, *J* 7Hz), 3.89 and 3.88(5' and 5''H, t, *J* 6Hz), 1.4-2.2 (2'-4'H and 2''-4''H, m) ppm; ^{13}C NMR (100MHz, CD_3OD): δ 176.6 and 176.5 (6' and 6''C,s), 151.6 (6C,s), 140.6 (2C,d), 136.5 (7aC,s), 136.3 (3aC,s), 129.5 (5C,d), 116.1 (7C,d), 97.6 (3C,d), 59.8 (1''C,t), 56.9 (5''C,d), 56.8 (5'C,d), 49.3 (1'C,t), 32.6 and 32.6 (4' and 4''C,t), 31.6 (2''C,t), 31.5 (2'C,t), 24.2 and 24.2 (3' and 3'',t) ppm; positive SIMS: m/z 393; Ex_{max}/Em_{max}: 366/442 nm (pH 1), 383/442 nm (pH 11). The triacetate of **1** had m/z 519 (positive SIMS).

Vesperlysine B (**2**), 6-hydroxy-5-methyl-1,4-di{6-(L-norleucyl)}-1*H*-pyrrolo[3,2-*b*] pyridinium, had ^{1}H NMR (400MHz, CD_3OD): δ 7.97 (7H, brs), 7.85 (2H, d, *J* 3Hz), 6.86 (3H, brd, *J* 3Hz), 4.74 (1''H, dd *J* 6,7Hz), 4.32 (1'H, t, *J* 7Hz), 3.97(5''H, t, *J* 6Hz), 3.92 (5'H, t, *J* 6Hz), 2.80 (5-Me, s), 1.4-2.2 (2'-4'H and 2''-4''H, m) ppm; ^{13}C NMR (100MHz, CD_3OD): δ 175.7 (5' and 5''C,s), 149.8 (6C,s), 141.5 (5C,d), 138.6 (2C,d), 136.2 (7aC,s), 133.7 (3aC,s), 114.7 (7C,d), 97.8 (3C,d), 55.9 (5' and 5'',d), 55.5 (1''C,t), 48.9 (1'C,t), 32.4 (4'',t), 32.2 (4',t), 31.5 (2''C,t), 30.3 (2'C,t), 24.0 (3''C,t), 23.9 (3'C,t),15.4 (5-Me,q) ppm; positive SIMS: m/z 407; Ex_{max}/Em_{max}: 366/442 nm (pH 1), 383/442 nm (pH 11). The triacetate of **2** had m/z 533 (positive SIMS).

Vesperlysine C (**3**), 5-hydroxymethyl-1,6-di{6-(L-norleucyl)}-1*H*-pyrrolo[3,4-*b*] pyridinium, had ^{1}H NMR (400MHz, CD_3OD): δ 9.45 (7H, s), 8.18 (2H, d, *J* 3Hz), 8.14 (4H, s), 6.93 (3H, d, *J* 3Hz), 4.97 (5-CH_2, s), 4.71 (1''H, t, *J* 7Hz), 4.49 (1'H, t, *J* 7Hz), 4.01(5''H, t, *J* 7Hz), 3.95(5'H, t, *J* 7Hz), 1.4-2.2 (2'-4'H and 2''-4''H, m) ppm; ^{13}C NMR (100MHz, CD_3OD): δ 172.4 (6'C and 6''C,s),143.7 (5aC,d), 143.6 (2C,s), 139.3 (3aC,s), 133.2 (5C,s), 131.7 (7C,d), 119.2 (4C,d), 104.7 (3C,d), 61.5 (5-CH_2,t), 57.4 (1''C,t), 54.2 (5' and 5''C,d), 48.2 (1'C,t), 32.2 (2''C,t), 31.1 (2' and 3''C,t), 31.0 (3'C,t), 23.2 (3''C,t), 23.1 (3'C,t) ppm; positive SIMS: m/z 407; Ex_{max}/Em_{max}: 345/405 nm (pH 1), 345/405 nm (pH 11). The triacetate of **3** had ^{1}H NMR (400MHz, CD_3OD): δ 5.52 (5-CH_2, s) ppm, and m/z 533 (positive SIMS).

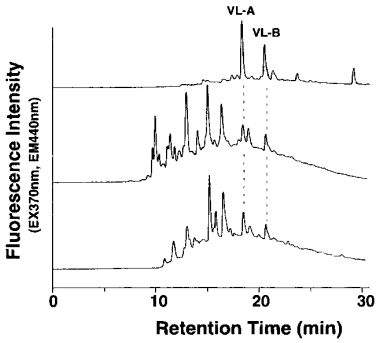

FIG. 1. HPLC analysis of hydrolysate of AGE–BSA (top), reaction mixture of lysine with glucose(middle) and its hydrolysate (bottom). Conditions for the HPLC are described in Experimental section. VL-A: Vesperlysine A; VL-B: Vesperlysine B; VL-C: Vesperlysine C.

Compound **4** had ^{1}H NMR (400MHz, CD_3OD): δ 8.29 (5H, d, *J* 2Hz), 8.02 (7H, dd, *J* 1,2Hz), 7.96 (2H, d, *J* 3Hz), 6.89 (3H, dd, *J* 1,3Hz), 4.68 (1''H, t, *J* 7Hz), 4.31 (1'H, t, *J* 7Hz), 1.4-2.2 (2'-4'H and 2''-4''H, m), 0.92 (5''H, t, *J* 7Hz), 0.90(5'H, t, *J* 7Hz); ^{13}C NMR (100MHz, CD_3OD): δ 151.9 (6C,s), 138.7 (2C,d), 135.2 (7aC,s), 134.3 (3aC,s), 128.2 (5C,d), 113.8 (7C,d), 96.0 (3C,d), 58.8 (1''C,t), 48.3 (1'C,t), 31.6 (2''C,t), 30.8 (2'C,t), 30.0 (3''C,t), 29.6(3'C,t), 23.3 (4' and 4''C,t),14.2 (5' and 5''C) ppm; positive SIMS: m/z 275; Ex_{max}/Em_{max}: 366/442 nm (pH 1), 383/442 nm (pH 11). The monoacetate of **4** had m/z 317 (positive SIMS).

Compound **5** had ^{1}H NMR (400MHz, CD_3OD): δ 7.89 (7H, brs), 7.85 (2H, brd, *J* 3Hz), 6.84 (3H, brd, *J* 3Hz), 4.71 (1''H, t, *J* 7Hz), 4.28 (1'H, t, *J* 7Hz), 2.78 (5-Me, s), 1.4-2.2 (2'-4'H and 2''-4''H, m), 0.95 (5''H, t, *J* 7Hz), 0.90(5'H, t, *J* 7Hz) ppm; ^{13}C NMR (100MHz, CD_3OD): δ 149.9 (6C,s), 139.7 (5C,d), 136.7 (2C,d), 134.2 (7aC,s), 132.5 (3aC,s), 112.0 (7C,d), 96.5 (3C,d), 54.6 (1''C,t), 48.1 (1'C,t), 31.3 (2''C,t), 30.9 (2'C,t), 30.0 (3''C,t),29.8 (3'C,t),23.4 (4' and 4''C,t),14.2 (5' and 5''C),13.0 (5-Me,q) ppm; positive SIMS: m/z 289; Ex_{max}/Em_{max}: 364/442 nm (pH 1), 383/442 nm (pH 11). The monoacetate of **5** had m/z 331 (positive SIMS).

RESULT AND DISCUSSION

AGE-BSA formed *in vitro* had almost the same characteristic fluorescence (Ex_{max} 370/Em_{max} 445 nm at pH 7.3) as that of lens protein or kidney collagen of diabetic patients. After acid-hydrolysis, the fluorescence spectrum remained unchanged (Ex_{max} 371/Em_{max} 444 nm), albeit with an 11% reduction in intensity. This result indicated the possibility that appreciable amounts of acid resistant fluorescent compounds remained after hydrolysis of AGE-BSA produced *in vitro*, although some AGEs, such as crosslines and X1, decomposed on hydrolysis (3,9). HPLC analysis was used to investigate the existence of acid-stable fluorophores (Fig.1). Two major peaks were detected in the hydrolysate of AGE-BSA in HPLC chromatograms when monitoring the fluorescence (Ex/Em) at 370/440nm). We found these peaks in chromatograms not only of the acid hydrolysate of AGE-lysine but also in the non-hydrolyzed reaction mixture. These facts negate the possibility that

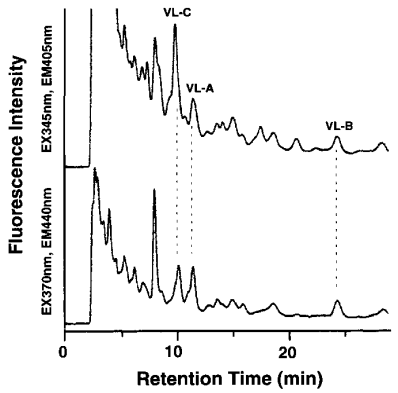

FIG. 2. Reverse Phase HPLC of an acid hydrolysate of reaction mixture of lysine with glucose. Conditions for the HPLC are described in Experimental section. VL-A: Vesperlysine A; VL-B: Vesperlysine B; VL-C: Vesperlysine C.

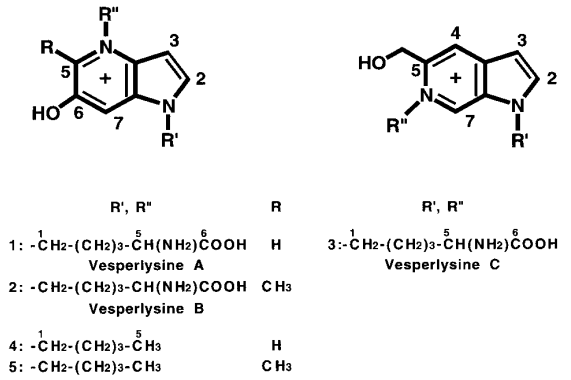

FIG. 3. Structures of Vesperlysine A, B and C and their derivatives.

compounds **1** and **2** might be artifacts, and indicate that both fluorophores were formed during the modification of proteins with glucose *in vitro*. We isolated the pair of fluorophores showing the two peaks first from AGE-lysine and then from the hydrolysate of the BSA-glucose mixture: both experiments gave the same pair of products. We named these Vesperlysines A (**1**) and B (**2**). In the course of the purification, another minor fluorophore was purified from the lysine-glucose mixture. This compound was also detected from the hydrolysate of AGE-BSA (Fig.2) when monitoring the fluorescence (Ex/Em) at 345/405 nm and named it Vesperlysine C (**3**). Similarly a pair of analogues **4** and **5** derived from *n*-pentylamine were isolated from the corresponding Maillard reaction mixture. Structural determination of Vesperlysines A (**1**), B (**2**) and C (**3**) were consistent with the cationic chemical formulae $C_{19}H_{29}N_4O_5$, $C_{20}H_{31}N_4O_5$ and $C_{20}H_{31}N_4O_5$ for **1**, **2** and **3** from the peaks at m/z 393, 407 and 407, respectively. The ^{13}C NMR spectra showed 19, 20 and 20 carbon skeletons, respectively. Vesperlysine B (**2**) was assigned as the mono-methylated derivative of Vesperlysine A (**1**). Since this pair of acid-stable fluorescent 4-azaindole derivatives **1** and **2**, and the 6-azaindole **3** were thought to be formed from two molecules of lysine and some parts of the sugar molecules, seven or eight carbon atoms in total derived from more than two molecules of glucose, *via* dehydration, oxidative fragmentation and condensation of the sugar moieties, which should occur during their formation. In other words, Vesperlysines A (**1**), B (**2**) and C (**3**) are glycoxidation and condensation products as in the case of pentosidine (10). In contrast, crosslines reported earlier (3) were formed from two molecules of amines and two molecules of glucose *via* cyclocondensations without fragmentation and oxidation. Similarly cationic formulae for the pair of fluorophores derived from *n*-pentylamine **4** and **5** were shown to be $C_{17}H_{27}N_2O$ and $C_{18}H_{29}N_2O$, respectively.

We assigned the ^1H and ^{13}C NMR spectra for two pairs of fluorophores (**1** and **4**, **2** and **5**) as follows. Each pair contain the same common pair of aromatic rings($C_7H_5N_2O$ and $C_8H_7N_2O$) in addition to the moieties derived from the corresponding amines. Since the mono-phenolic hydroxyl group was suggested by conversion of **4** to its O-acetyl derivative, and all aromatic protons for **1** and **4** were coupled each other, four kinds of azaindolium rings containing phenolic OH groups were proposed as their structures. One correct structure of the ring was determined as follows. Since the protons of **4** at the 1'-(4.31 ppm) and 1"-(4.68 ppm) positions adjacent to the carbon atom next to the ring nitrogen atom showed nOes with two pairs of aromatic protons, at 7.96 and 8.02 ppm and those at 6.89 and 8.29 ppm, respectively. These data lead to the structures shown in formulae **1** and **4** (Fig.3). The single methyl group of structures **2** and **5** were assigned to position-5 of the rings also from the nOe experiments *viz:* protons of **5** at 1'-(4.28 ppm) and 1"-(4.71 ppm) positions adjacent to the carbon atom next to the ring nitrogen atom, showed nOes with the two pairs of protons, at 7.85 and 7.89 ppm and those at 6.84 and 2.78 ppm,

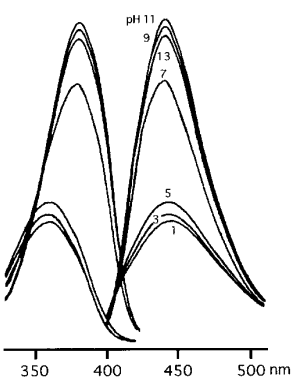

FIG. 4. Fluorescent spectra of Vesperlysine A at various pH values. Excitation and emission spectra were recorded at emission max and excitation max, respectively.

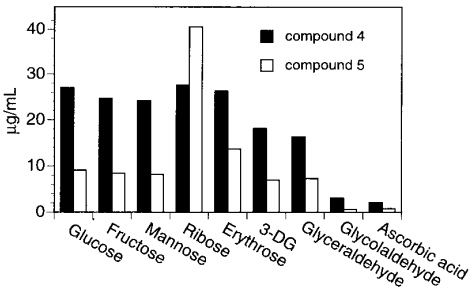

FIG. 5. Formation of Vesperlysines from various sugars.

respectively. These structures and signal assignments were further confirmed by ^{13}C-^{1}H COSY and COLOC spectra. The structure of **3** was also determined as shown in Fig.3 mainly from the results of HMQC, HMBC and nOe experiments in NMR spectroscopy.

The fluorophores, Vesperlysines A (**1**), B (**2**), compounds **4** and **5**, and their analogues, had almost the same fluorescence properties (Fig.4); Ex_{max} 366/Em_{max} 442 nm at pH 1; Ex_{max} 383/Em_{max} 442 nm at pH 11. The bathochromic shift observed in the excitation spectra when the pH was changed from 5 to 7 indicated the presence of a phenolic group in the molecules. The fluorescent intensity at pH 11 was more than twice of that at pH 1, and the spectra at physiological pH were in between. In contrast, the fluorescence of Vesperlysine C (**3**) (Ex_{max} 345/Em_{max} 405 nm) was not affected by pH. The characteristic fluorescence property of Vesperlysines A and B were identical to those observed in cataractous human lens, Ex_{max} and Em_{max} at 370 and 440 nm, respectively. The formation of Vesperlysines analogue **4** and **5** from various sugars of oxaldehydes were investigated using *n*-pentylamine as amine component (Fig.5). After 9 days of incubation, the same fluorophores **4** and **5** were detected in all the reaction mixture examined. Among them, D-ribose was most effective source of these fluorophores. The fact that the fluorophores **1-3** could not be detected in the hydrolysate of BSA alone (data not shown) but detected in that of AGE-BSA might indicate that the azaindole derivatives **1-3** could be the main acid-stable fluorescent AGEs in AGE modified proteins at least produced *in vitro* by Maillard reaction. It must be noted that the fluorescence properties of **1** and/or **2** are closely related to those observed in diabetics. However, we still need more evidence of presence of **1** and/or **2** *in vivo*. Oimomi and coworkers (11) reported the existence of acid stable fluorescent AGE, detected as Peak L1, in hydrolysate of AGE-BSA produced *in vitro* and also from senile cataractous human lens with and without diabetes. More recently, Nagaraj et al. (12) isolated a compound LM-1 from human lens crystallin and characterized its fluorescence. Graham (13) isolated a product Pen K2 as an acid-stable crosslinked product from *in vitro* glycated proteins with ribose and claimed it to be LM-1. Although such fluorescent crosslinked products had not yet been characterized, their fluorescence property and nature are quite similar to Vesperlysine A (**1**) and/or B (**2**). Studies for the detection of Vesperlysines from *in vivo* sources are now in progress.

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