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Original article

Identification of acetaldehyde-modified lysine

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Abstract

Intermediate carbonyl compounds produced by abnormalities in glucose and lipid metabolism in vivo induce amino carbonyl reactions that modify proteins in a non-enzymatic manner. This reaction is further accelerated with the progression of lifestyle-related diseases, eventually leading to the formation of advanced glycation end-products (AGEs). Also, alcoholic beverages are consumed as a luxury item. Acetaldehyde (AA), which is produced as an intermediate metabolite of alcohol metabolism, has a highly reactive carbonyl group and may modify proteins in the same manner as AGEs are produced. In this study, we attempted to identify the structure of AA-modified amino acids (AA-A) and measure their amount by immunochemical methods using monoclonal antibodies in order to detect the modification of proteins by AA.

The spleens of mice immunized with AA-keyhole limpet hemocyanin (AA-KLH) were fused with myeloma cells, and antibody-producing cells reactive with AA-bovine serum albumin (AA-BSA) were isolated. The amino acid analysis of AA-BSA showed that lysine (Lys) and histidine were highly modified. Focusing on Lys with amino groups on its side chain, AA-Lys was isolated from the product generated from N^{α}-benzyloxycarbonyl-L-lysine (N^{α}-Cbz-Lys) and AA by HPLC and the structure was analyzed by nuclear magnetic resonance (NMR) and mass spectrometry. As a result, m/z 385 and m/z 411 were detected, and the structure was identified by NMR. The reactivity of these three fractions with antibodies against monoclonal anti-AA-modified proteins was evaluated and the structures were analyzed. In addition, the three fractions in AA-modified BSA incubated in phosphate buffer (pH 7.4) at 37 °C for 7 days were evaluated by ELISA. As a result, the structures of the three fractions were identified, and the highest reactivity with antibodies was observed in fraction 2. These increased in a concentration-dependent manner with AA-modified BSA. These results indicate that three new AA-derived Lys structures have been identified and are formed under physiological conditions, and that this structure will be one of the tools to evaluate protein modification by AA.

***KEY WORDS*:** acetaldehyde, alcohol, post-translational modification

Introduction

Ethyl-alcohol is contained in many foods to enhance flavor and shelf life. Drinking alcohol is an essential part of adult life as a communication tool and recreational activity, and moderate drinking is said to increase appetite and relieve fatigue, making the population of drinkers extremely large [1)]. On the other hand, an increase in blood alcohol concentration causes not only acute alcohol intoxication, but also hangover, alcoholic liver disease, diabetes, and other conditions caused by alcohol metabolites. In recent years, it has also been reported to be involved in the pathogenesis of Alzheimer's disease, ischemic heart disease and so on [2)].

The Ministry of Health, Labor and Welfare reports that the percentage of people who drink alcohol in amounts that increase the risk of lifestyle-related diseases is 11.6%, and the percentage of people who drink five or more days a week is 22.9%. Alcohol from drinking is mainly metabolized and detoxified in the liver. After ingested alcohol (ethanol) is taken up by liver cells, it is metabolized into acetaldehyde

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Glycative Stress Research 2021; 8(4): 201-210

(AA) by alcohol dehydrogenase (ALD), into acetic acid by aldehyde dehydrogenase (ALDH), and after circulating throughout the body by blood, it is broken down into water and carbon dioxide. It is ultimately released from the body through sweat, urine, and exhaled air.

The AA produced in this process is a reactive aldehyde and is thought to proceed in a similar manner to the Maillard reaction. In this reaction, glucose, a reducing sugar, reacts non-enzymatically with the amino groups of proteins to produce advanced glycation end-products (AGEs), which are post-translational modifications [3, 4]. AGEs increase with the onset of diabetes mellitus [5], and their accumulation is enhanced with the complications of diabetes mellitus [6]. In fact, N^{ε}-(carboxymethyl)lysine (CML) [7] and N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) [8], a type of AGEs, have been reported to present in the plasma of patients with renal disease. It has been reported to accumulate in the plasma of patients with renal disease [9]. Therefore, inhibition of AGE formation is expected to prevent or delay the onset and progression of diabetes and its complications.

It is also important to determine and evaluate the specific AGE structure because of the different factors involved, such as oxidative stress in CML [7] and abnormal glucose metabolism in MG-H1 [10]. Hayashi N *et al* [11] reported that AA modifies the amino group of bovine serum albumin (BSA) as well as glucose, AA-BSA decreases hepatocyte viability, and AA-derived AGEs accumulate in the liver of ethanol-treated rats. Currently, alanine transaminase (ALT) is used as a marker of liver dysfunction. If the specific AGE structure produced by AA is clarified, it will be possible to evaluate the accumulation of AA-derived AGEs due to alcohol consumption, and it is expected to become a marker for pathological conditions such as alcoholic liver disease. Therefore, in this study, we evaluated the novel AGE structures produced by AA.

Methods

Preparation of AA-BSA

AA-modified BSA (AA-BSA) was prepared by incubating 600 mM AA (Merck Millipore, Billerica, MA, USA) and 2 mg/mL BSA in sodium phosphate buffer (NaPB; pH 7.2) at 37 °C for 7 days. Subsequently, bicinchoninic acid (BCA; Thermo Scientific, Waltham, MA, USA) was used for protein quantification of AA-BSA.

Reactivity of antibodies to AA-A

Balb/c mice were immunized with AA-BSA (0.1 mg/mouse) three times every 2 weeks and monoclonal antibodies against AA-A were produced according to the conventional method [12], BSA modified with methylglyoxal (MG), glyoxal (GO) were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [13]. Briefly, in the non-competitive ELISA, each well of a 96-well immunoplate (Thermo Fisher Scientific) was coated with 0.1 mL of 1 μg/mL sample dissolved in phosphate-buffered saline (PBS) and blocked with 0.5 % gelatin hydrolysate dissolved in PBS. The wells were blocked with 0.5 % gelatin hydrolysate dissolved in PBS. The wells were incubated for one hour with 0.1 mL of AA-BSA antibody diluted 8 times in triplicate from 1 μg/mL. Antibodies bound to the wells were detected using a horseradish peroxidase-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific). Then, 100 μL of 500 μg/mL of O-phenylenediamine dihydrochloride (Fujifilm Wako Pure Chemicals, Osaka, Japan) in citrate-phosphate buffer (pH 5.0) containing 5.9 mM hydrogen peroxide was added, followed by developing for 3 minutes. The reaction was terminated with 100 μL of 2.0 M sulfuric acid and the absorbance was measured at 492 nm using a Sunrise Rainbow Thermo RC system (Tecan, Männedorf, Switzerland).

Competitive ELISA of monoclonal anti-AA-A antibody against AA-BSA and each fractional component

Prepared AA-BSA samples and each fractional sample prepared by high performance liquid chromatography (HPLC) were measured by competitive ELISA. In the competitive ELISA, each well of a 96-well immunoplate was coated with 0.1 mL of 1 μg/mL sample dissolved in PBS and blocked with 0.5 % gelatin hydrolysate dissolved in PBS. To these wells, 0.05 mL of AA-BSA diluted in triplicate from 1 μg/mL in seven successive step dilutions and BSA diluted in the same manner as the control group and fractions 1, 2, and 3 were added as competitors and well mixed for 10 min with AA-BSA antibody, followed by one-hour incubation. The antibodies bound to the wells were detected using horseradish peroxidase-conjugated anti-mouse IgG antibody. Subsequently, 100 μL of 500 μg/mL O-phenylenediamine dihydrochloride in citrate-phosphate buffer (pH 5.0) containing 5.9 mM hydrogen peroxide was added for 3 min of developing. The reaction was terminated with 100 μL of 2.0 M sulfuric acid and the absorbance was measured at 492 nm using the Sunrise Rainbow Thermo RC system.

Amino acid analysis of AA-BSA

AA-BSA prepared with 100 mM AA was dissolved in 1 mL of 6 M HCl in the presence of 14mM 2-in ercapioethdnol for hydrolysis; after 18 h, the sample was collected and dried in a centrifugal concentrator, then redissolved in 0.02 M HCl and injected into an amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) [14].

Measurement and preparative isolation of AA-Cbz-Lys by HPLC

600 mM AA and 100 mM N^{α}-benzyloxycarbonyl-L-lysine (N^{α}-Cbz-Lys; Sigma, St. Louis, MO, USA) were incubated in 200 mM NaPB at 37 °C for 4 days (AA-Cbz-Lys). The sample (50 μL) was dissolved in H_2O (950 μL) and the entire volume was run through the Sep-pack column. The column was then washed with H_2O (3 mL), followed by 50 % MeOH (2 mL), and the fractions were collected for purification. After complete drying of the generated sample, it was dissolved in 100 μL of 20 % acetonitrile (ACN), filtered through a 0.45 μm polytetrafluoroethylene membrane filter (Merck Millipore) and injected into UV-HPLC. Thereafter, AA-modified N^{α}-Cbz-lysine will be referred to as AA-Cbz-Lys. In preparative HPLC (Shimazdu, Kyoto, Japan: System Controller CBM-20A, Pump LC-20AD, UV Detector SPD-20A, Autosampler SIL-20A, Column Oven CTO-20AC),

30 L of AA-Cbz-Lys was prepared as injected into a Cosmosil Packed Column 5C18-AR-II (20 x 250 mm, Nacalai Tesque Co., Ltd., Kyoto, Japan). The column was maintained at 40 °C. The mobile phase was 0.1 % TFA (Fujifilm Wako Pure Chemicals), and ACN was gradient in two steps. Solution A was 10 % ACN and solution B was 60 % ACN. The gradient was (B: 0-40 min, 45-50 % ACN; B: 41-46 min, 100 % ACN; B: 47-52 min, 45 % ACN) at a flow rate of 5 mL/min. The effluent was monitored by UV at 270 nm and separated into three fractions (Fr. 1: 21-22 min, Fr. 4: 33-34 min, Fr. 5: 35-36.52 min). These fractions were repeated 30 times and each fraction was lyophilized. The lyophilized samples were redissolved in 50 % ACN. Based on the molecular weight determined by mass spectrometry, the concentration of each Fr fraction; concentrations were 17.4 mM, 9 mM, and 20.6 mM, respectively. These fractions were again measured by HPLC and the fractions were collected. Each Fr fraction was diluted to 100 µM with H_2O and 10 µL was injected into the UV-HPLC. The measurement conditions were carried out in the same way.

Structural analysis by LC-ESI-QTOF

Each of the preparative Fr fractions was diluted to 10 µM in 20 % ACN with 0.1 % formic acid (FA) and measured by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF) [15]. For simplicity, the LC was maintained in an oven at 40 °C. The measurements were carried out by flow injection mode. The mobile phase was 0.1 % (FA) with a two-step gradient of ACN (B: 0-2 min, 90 % ACN; B: 2-16 min, 90-10 % ACN; B: 16-19 min, 10 % ACN). The conditions were as follows: flow rate, 0.2 mL/min; injection volume, 5 µL; ionization source temperature, 200 °C; capillary voltage, 4.5 kV. Collision-induced dissociation was performed using nitrogen, with collision energy and pressure set to 20 eV and 1.6 bar, respectively. Data were acquired in the conserved mass range of m/z 50-1000. The compositional formulae of the detected ions were analyzed using Smart Formula.

Nuclear magnetic resonance (NMR)

Compound **1**, **2** and **3** was characterized by NMR using a JNM-ECZ600R/S1 spectrometer equipped with a 5-mm ROYAL probe (JEOL, Tokyo, Japan). The probe temperature was set to 303 K. Each sample was dissolved in 750 µL of acetonitrile-d_3 (99.96 atom % D), and ^{1}H and ^{13}C chemical shifts were reported relative to the internal standard of tetramethylsilane. ^{15}N chemical shifts were indirectly referenced using ^{15}N-^{1}H chemical shift referencing ratio of 0.1013291444 [16]. NMR signals were assigned by 1D ^{1}H, 1D ^{13}C, 2D ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{15}N HSQC, ^{1}H-^{13}C HMBC, and ^{1}H-^{15}N HMBC. NMR data processing, analysis and spectral simulation were performed using Mnova software (version 14.1.2; Mestrelab Research, Santiago de Compostela, Spain).

Statistical analysis

All data were expressed as mean ± standard deviation (± SD). ***Fig. 2*** was examined for statistical significance using one-way analysis of variance (ANOVA) and Bonferroni's post-test. Statistical analysis was performed using the EZR software package (Oxfordshire, United Kingdom) [17].

Results

Reactivity of Monoclonal Anti-AA-A Antibodies

In the present study, monoclonal anti-AA-A antibodies (2F10, 3G4, 4B11), which were produced by immunizing mice with AA-BSA in our laboratory, were used as antibodies against AA modifications. First, a test was conducted to elucidate the reactivity of the antibodies with AA-BSA. The results of ELISA using the prepared AA-BSA and monoclonal anti-AA-A antibodies showed that three antibodies reacted with AA-BSA compared to unmodified BSA in the control group. Among the three antibodies, 2F10 was found to be the most active (***Fig. 1-a***). Therefore, we performed a competitive ELISA with AA-BSA using 2F10. The results showed that 2F10 reacted with AA-BSA in a competitor concentration-dependent manner compared to unmodified BSA (***Fig. 1-b***). Furthermore, the antibody showed no reactivity with various aldehyde-modified BSA, but reacted specifically with AA-BSA (***Fig. 1-c***).

Evaluation of the formation of AA-modified amino acids

AA-BSA prepared at concentrations from 0 to 600 mM of AA was evaluated by ELISA using 2F10, and it was confirmed that AA-modified amino acids were produced in a concentration-dependent manner. In addition, the amount of AA-modified product reached the upper limit when prepared with 30 mM AA (***Fig. 2-a***). Next, AA-BSA prepared with 10 mM AA was evaluated at different temperature retention times. The results showed a time-dependent formation of AA-modified products (***Fig. 2-b***).

Evaluation of AA-modified amino acids

Since it was confirmed that AA reacts with proteins to produce AA-modified products, AA-BSA was prepared to evaluate which amino acids in proteins react with the carbonyl group of AA, and the variation of amino acid content was confirmed by amino acid analysis. As a result, Lys, which is known to be involved in the formation of AGEs, was found to be highly reactive with AA, while arginine (Arg), which is also involved in the formation of AGEs, was found to be less reactive with AA. (***Table 1***)

Preparation of AA-Cbz-Lys generated by AA

From ***Table 1***, it was confirmed that AA has high reactivity with Lys. Therefore, AA-A was generated by mixing AA and Lys, and was preparative. Since some of the AGEs structures are not fluorescent, we used the protecting group Cbz-Lys to identify the structure by HPLC in this study. The prepared AA-Cbz-Lys was detected at a UV wavelength of 270 nm, and a chromatogram containing Fr fractions 1-3 was obtained (***Fig. 3-a***). Fr fractions 1, 2, and 3 were separated from each other and measured again by HPLC under the same conditions, and single peaks were detected (***Fig. 3-b, c, d***).

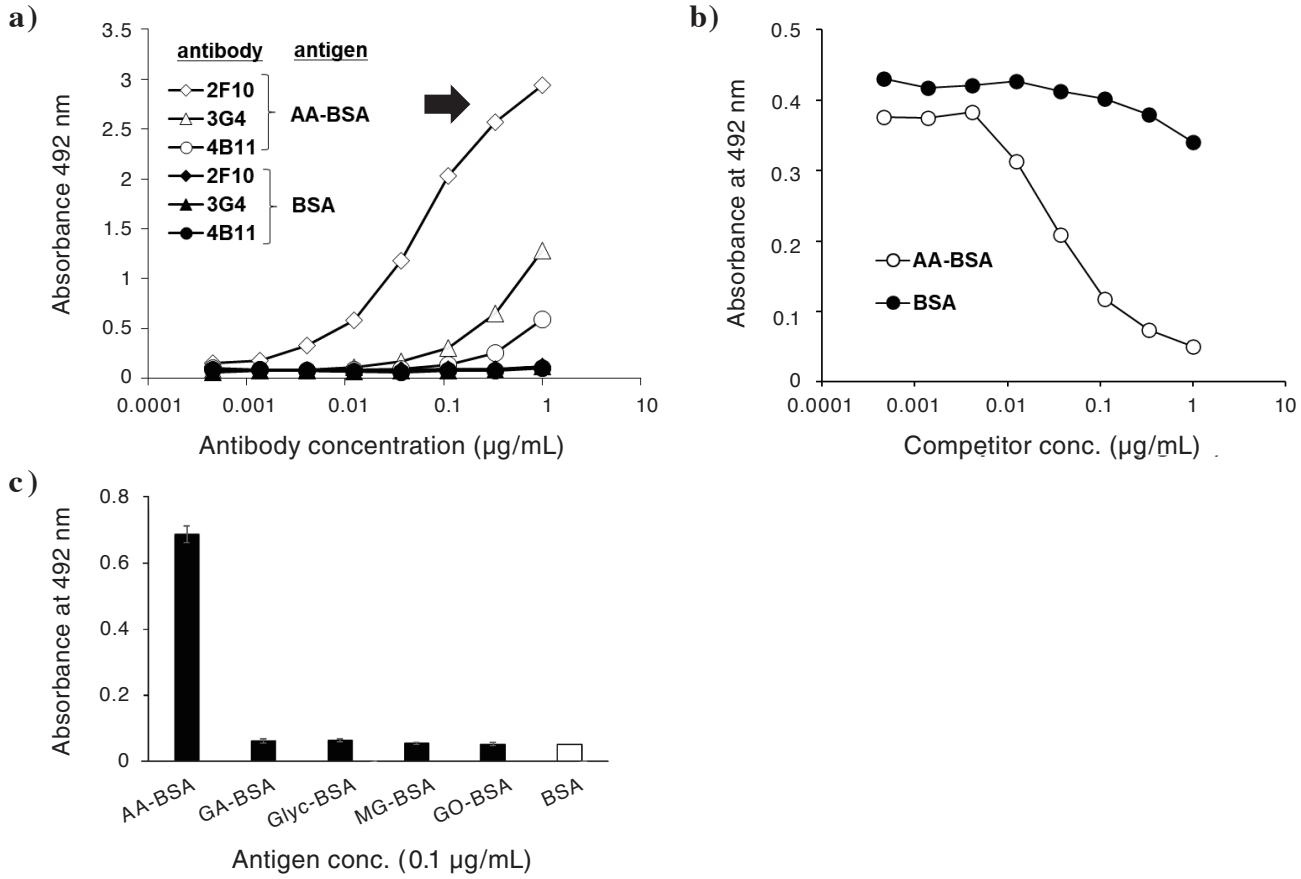


Fig. 1. **Reactivity of monoclonal anti-AA-A antibody.**

The reactivity of the prepared AA-BSA with monoclonal anti-AA-A antibodies (2F10, 3G4, 4B11) was evaluated by ELISA. **a)** Reactivity of AA-BSA with each antibody by non-competitive ELISA. **b)** Reactivity of AA-BSA with 2F10 by competitive ELISA. **c)** Reactivity of BSA modified with AA, GA, Glyc, MG, GO and 2F10 by non-competitive ELISA. Data are presented as mean (± SD). Unmodified BSA was used as control group. AA, acetaldehyde; AA-A, AA-modified amino acids; BSA, bovine serum albumin; ELISA, enzyme-linked immuno sorbent assay; GA, glycolaldehyde; Glyc, glyceraldehyde; MG, methylglyoxal; GO, glyoxal; SD, standard deviation.

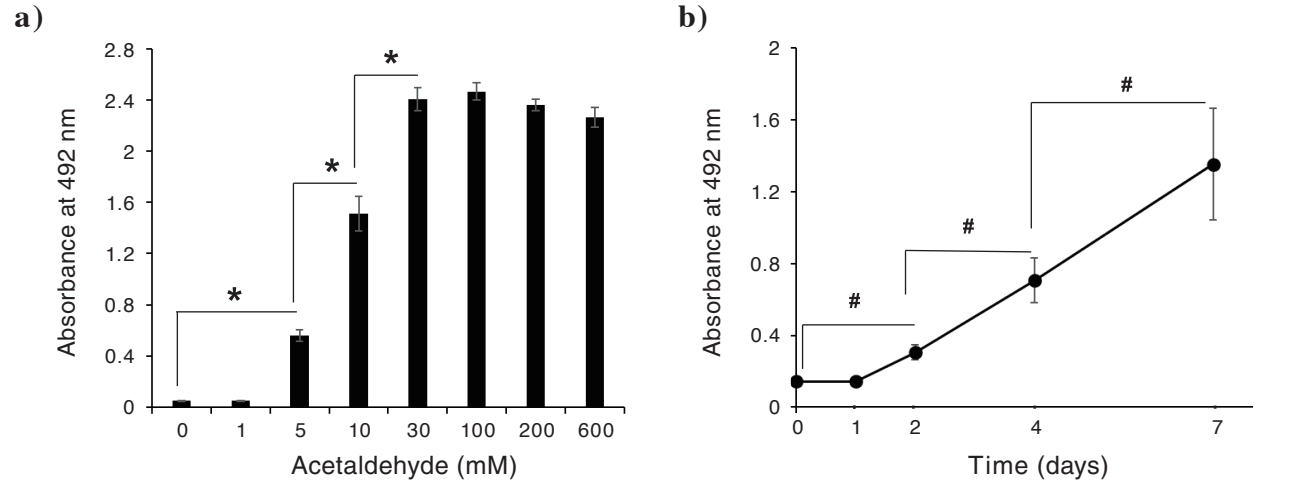


Fig. 2. **Evaluation of AA-A production.**

The production of AA-A was evaluated by non-competitive ELISA at different concentrations of AA and different retention times. **a)** Evaluation of the amount of AA-A produced by changing the concentration of AA. **b)** Evaluation of the amount of AA-A produced by AA-BSA prepared with AA (10 mM) at different retention times. Data are shown as mean (± S.D.), **a)** * $p < 0.01$, 5 vs 0 mM, 10 vs 5 mM, 30 vs 10 mM, by Bonferroni test; **b)** # $p < 0.05$, 2 vs 0 days, 4 vs 2 days, 7 vs 4 days by Bonferroni test. AA, acetaldehyde; AA-A, AA-modified amino acids; ELISA, enzyme-linked immuno sorbent assay; SD, standard deviation.

Table 1.

Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val
2.6 ± 0.7	4.2 ± 0.7	3.7 ± 0.5	3.4 ± 0.6	4.9 ± 0.6	2.2 ± 0.8	0 ± 0	5.2 ± 0.6
Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
10.3 ± 1.0	6.4 ± 0.3	3.5 ± 0.7	8.4 ± 0.6	2.0 ± 0.8	81.3 ± 0.05	70.7 ± 0.4	10.3 ± 0.6

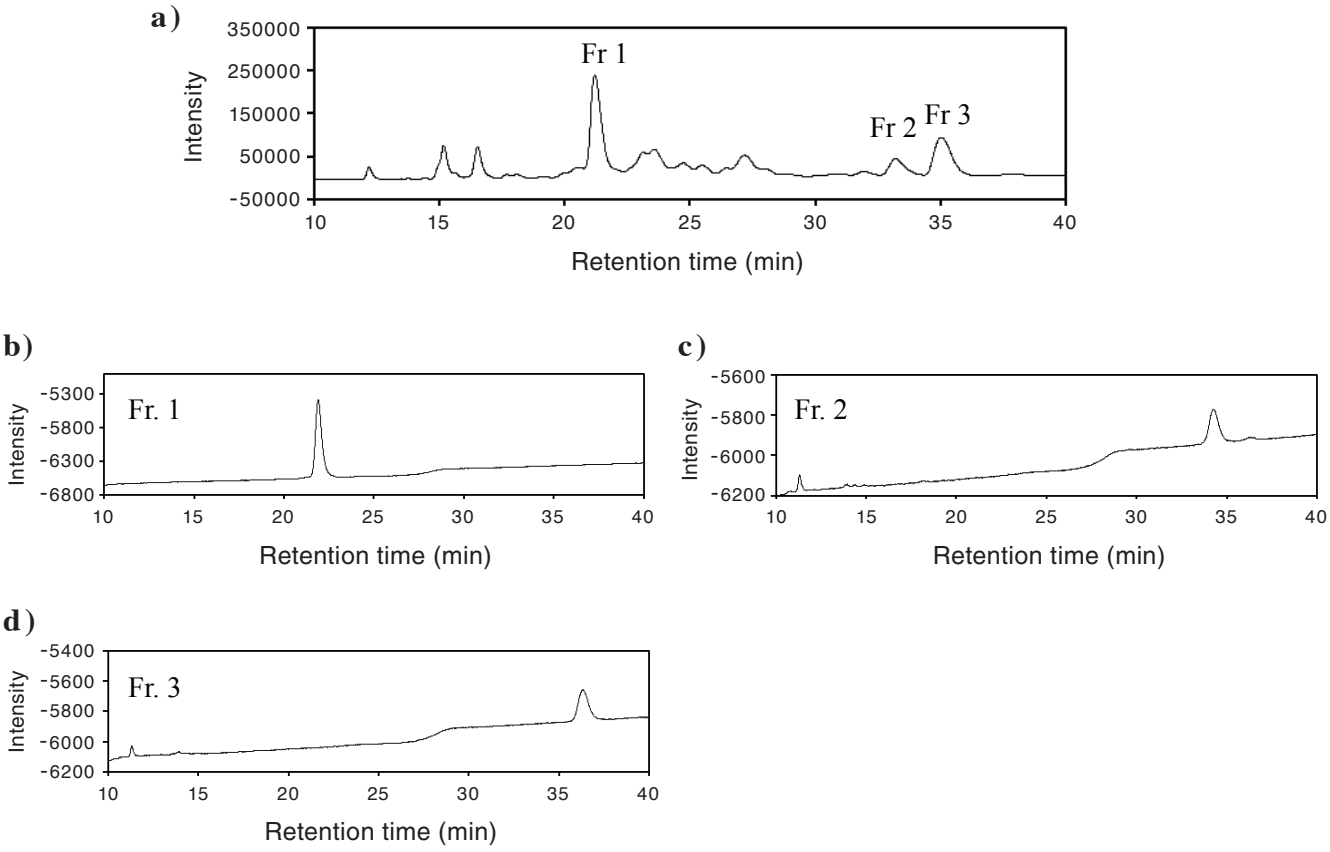


Fig. 3. **Preparation of AA-Cbz-Lys produced by AA.**

a) Chromatogram of AA-Cbz-Lys. **b)** Re-measurement chromatogram of Fr fraction 1. **c)** Re-measurement chromatogram of Fr fraction 2. **d)** Re-assay chromatogram of Fr fraction 3. AA, acetaldehyde; Cbz-Lys; benzyloxycarbonyl-L-lysine.

Structural analysis by LC-ESI-QTOF

LC-ESI-QTOF analysis was performed for each Fr fraction, which were separated as single peaks. The mass-to-charge ratio (m/z) of 385 was detected in Fr fraction 1 (***Fig. 4-a***), and the same m/z of 411 was detected in Fr fractions 2 and 3 (***Fig. 4-b, c***).

Structural analysis by NMR

NMR analyses were performed to determine the structure of **1**, **2**, and **3**. The NMR signals were assigned by a series of one- and two-dimensional experiments and the assignments are shown in ***Table 2***. 1D-^{1}H NMR spectrum of **1** gave three signals at 7.7, 8.2, and 8.5 ppm, which were assigned to pyridine protons (***Fig. 5-a***, left). 2D-^{1}H-^{15}NHMBC spectrum shows the correlation between pyridine nitrogen and neighboring protons (***Fig. 5-a***, right). Furthermore, the pyridine ring is modified with an ethyl group at C-5 and a methyl group at C-2. From the NMR analysis, compound **1** is identified as (*S*)-1-(5-(((benzyloxy)carbonyl)amino)-5-carboxypentyl)-5-ethyl-2-methylpyridin -1-ium (***Table 2***). Compounds **2** and **3** also share a pyridine unit, however the side chains are more extended to have a C = C double bond.

Compounds **2** and **3** are structural isomers, and the isomerism originates from *cis* or *trans* configuration. From the ^{3}J coupling data and spectral simulations, compound **2** is identified as (*S*, *Z*)-1-(5-(((benzyloxy)carbonyl)amino)-5-carboxypentyl)-5-(but-2-en-1-yl)-2-methylpyridin-1-ium with cis configuration, and compound **3** is identified as (*S*,*E*)-1-(5-(((benzyloxy)carbonyl)amino)-5-carboxypentyl)-5-(but-2-en-1-yl)-2-methylpyridin-1-ium with *trans* configuration (***Table 2***, ***Fig. 5-b, c***).

The m/z of each fraction was calculated from the NMR-estimated structure of each fraction using Smart Formula (***Table 3***), and the m/z was consistent with that of the peak detected in ***Fig. 4***.

Reactivity of monoclonal anti-AA-A antibody with each fraction

We elucidated whether the structures detected in each fraction reacted with monoclonal anti-AA-A antibody, which specifically reacts with AA-BSA. As a result, competition with 2F10 was observed in each fraction (***Fig. 6***).

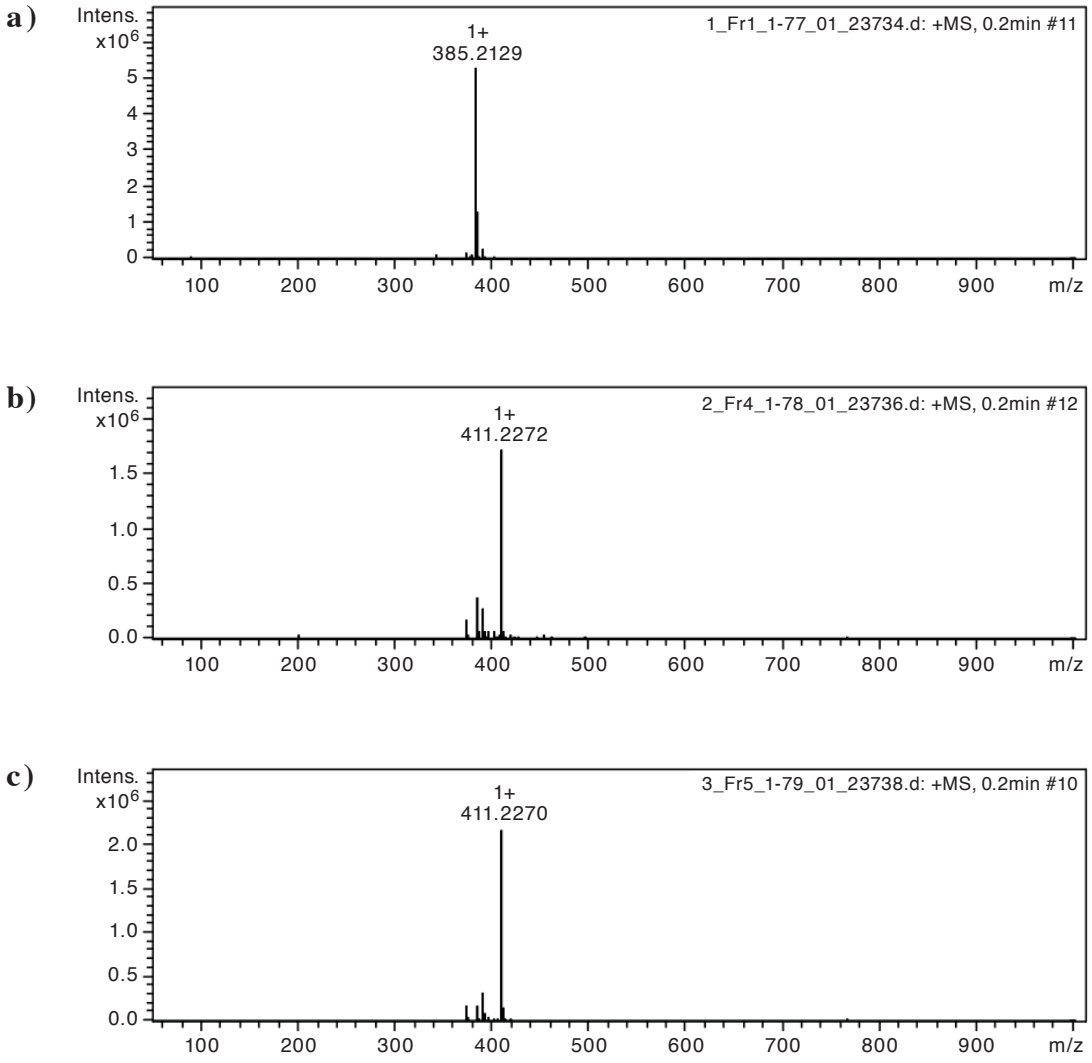


Fig. 4. **Structural analysis by LC-ESI-QTOF.**

Each Fr fraction was measured by LC-ESI-QTOF. **a)** Detection peak from Fr fraction 1. **b)** Detection peak from Fr fraction 2. **c)** Detection peak from Fr fraction 3. LC-ESI-QTOF, liquid chromatography–quadrupole time-of-flight mass spectrometry.

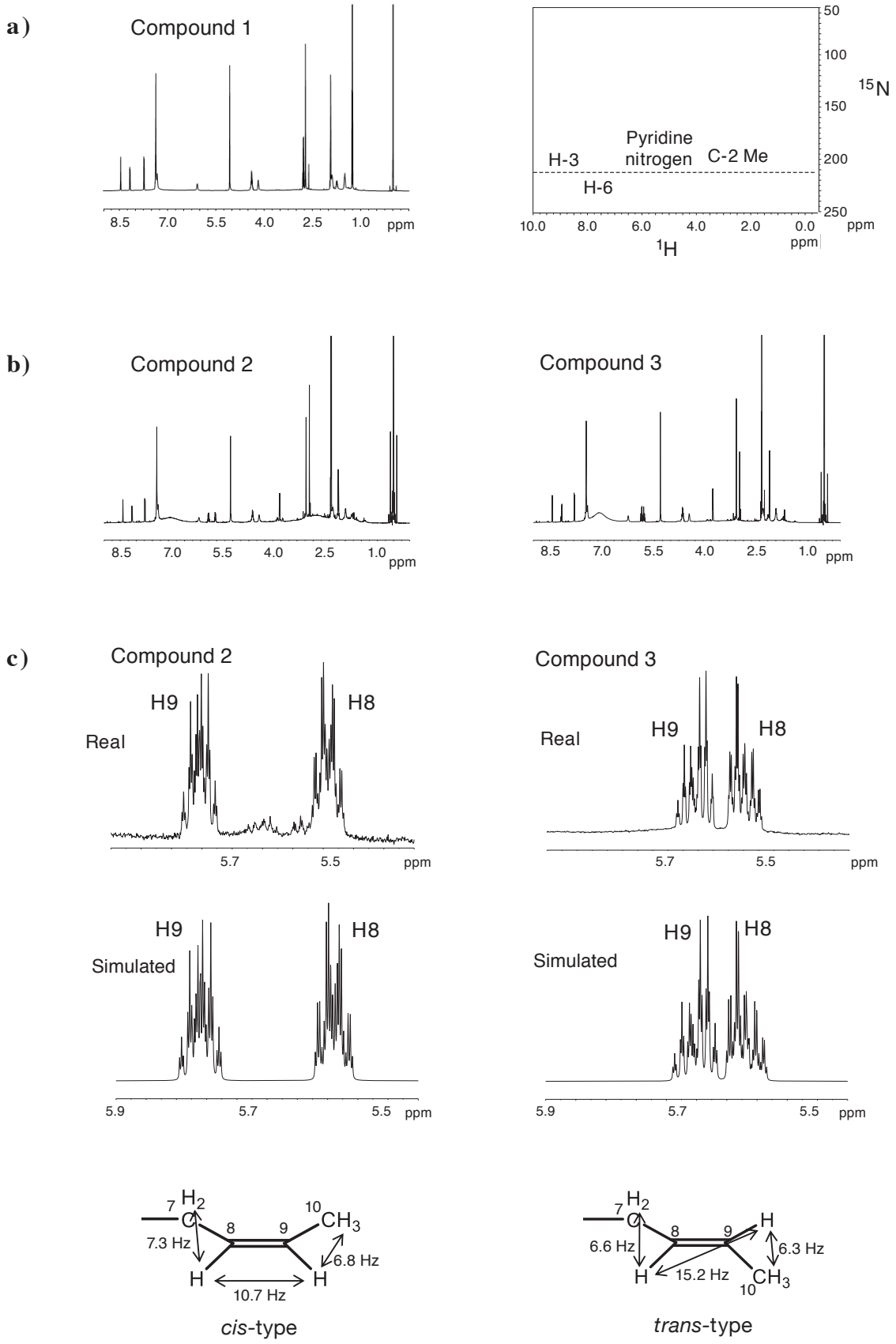
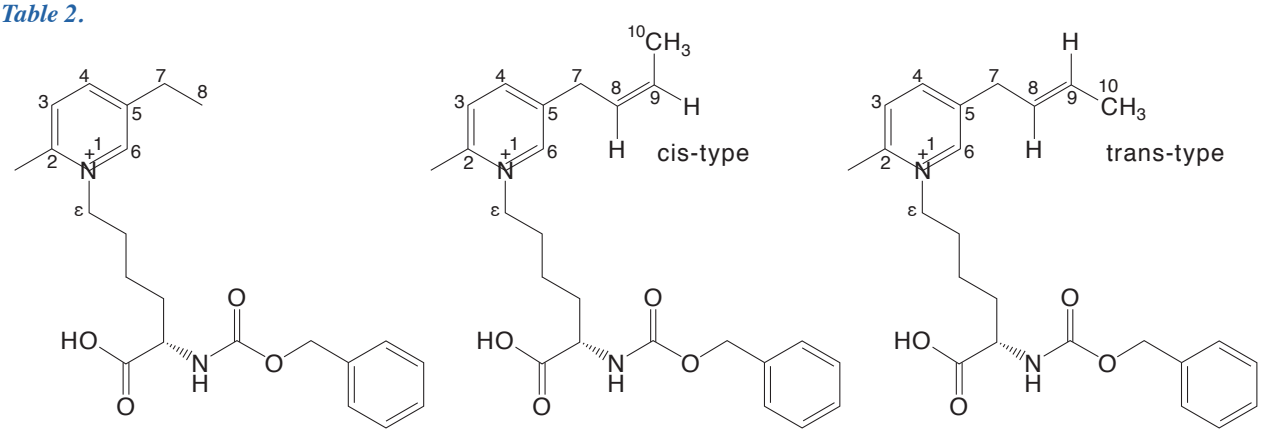


Fig. 5. **NMR analysis of compounds 1, 2, and 3.**

a) 1D ^{1}H and 2D ^{1}H-^{15}N HMBC spectra of compound **1**. **b)** 1D ^{1}H-NMR spectra of compound **2** and **3**. **c)** Expanded ^{1}H-NMR spectra (olefin region) of compound **2** and **3** (upper) and simulated NMR spectra of the corresponding region (lower).

Table 2.



structure	1	2	3
		^{1}H chemical shift [ppm]	
H-3	7.7	7.7	7.7
H-4	8.2	8.1	8.1
H-6	8.5	8.4	8.4
H-7, H-7'	2.8, 2.8	3.5, 3.6	3.4, 3.4
H-8	1.3	5.5 (J = 10.7 Hz)	5.6 (J = 15.2 Hz)
H-9	–	5.8	5.7
H-10	–	1.7	1.7
Lys Hε	4.4	4.4	4.4
		^{13}C chemical shift [ppm]	
C-2	153.7	153.0	154.0
C-3	130.9	131.0	130.9
C-4	145.9	146.2	146.3
C-5	143.8	141.2	141.1
C-6	144.9	145.0	145.2
C-7	26.0	30.0	30.0
C-8	14.7	126.4	127.6
C-9	–	129.1	130.7
C-10	–	18.0	18.0

Table 3.

Fr No.	Compositional formulae	Mass-to-charge ratio (m/z)
1	$C_{22}H_{29}N_2O_4^+$	385.2122
2	$C_{24}H_{31}N_2O_4^+$	411.2278
3	$C_{24}H_{31}N_2O_4^+$	411.2278

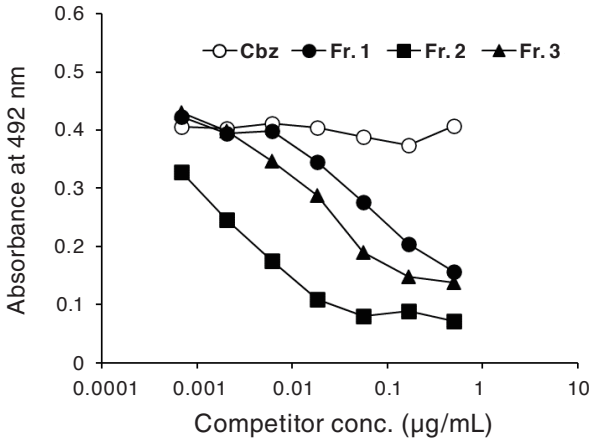


Fig. 6. **Reactivity of each Fr fraction with monoclonal anti-AA-A antibody.**

The reactivity of each Fr fraction with monoclonal anti-AA-A antibody was evaluated by competitive ELISA. AA, acetaldehyde; AA-A, AA-modified amino acids; ELISA, enzyme-linked immuno sorbent assay.

Discussion

The consumption of ethyl alcohol through alcoholic beverages is widely incorporated into dietary habits for a rich diet. However, excessive intake of ethyl alcohol has negative side effects, such as poisoning by its metabolic intermediate, AA, and increased risk of developing Alzheimer's disease with prolonged and excessive intake[18]. While AA-induced tissue damage has been considered, its molecular mechanism remains unclear. As with carbonyl compounds, modified amino acids are thought to be generated from AA, but their structures are not well understood. In this study, in order to elucidate the biotoxicity of AA at the molecular level, we identified the structure of modified amino acids derived from AA and investigated a system that can easily measure the structure using monoclonal antibodies.

The monoclonal anti-AA-A antibody, which was prepared in this study, reacted with AA-BSA in a concentration-dependent manner, indicating that this antibody recognizes AA-modified proteins. In addition, this antibody reacted only with AA-BSA among various aldehyde-modified proteins, indicating that this antibody is specific for the structure formed by the reaction with AA, even though the aldehyde compound has a carbonyl moiety.

Human susceptibility to alcohol fluctuates on a daily basis depending on physical condition, even in the same individual. Currently, only the detection of alcohol in the breath is used to evaluate the amount of alcohol intake, and the concentration of AA, which causes sickness, cannot be evaluated only by the concentration of alcohol in the breath. If the AA-A identified in this study can be easily measured using immunochromatography and other methods, it will be possible to quickly evaluate the "level of intoxication" when drinking. In today's society, traffic accidents caused by drunk drivers are one of social issues. However, when testing the alcohol concentration in the breath, there are cases where accurate measurement is not possible due to excessive water intake, and this has become a social problem. In such cases, AA-A may be used as a new marker that is difficult to cheat.

In the future, the detection of AA-Lys *in vivo* using the obtained antibodies or instrumental analysis will clarify the kinetics of AA production, and it will be possible to analyze the involvement of AA in the development of liver diseases and Alzheimer's disease, in which AA involvement has been reported. Furthermore, the establishment of a quantitative system for AA-A using mass spectrometry may lead to the early detection of various alcohol-related diseases and conditions that have been pointed out to be related to enzymes involved in alcohol metabolism.

Declaration of Conflict of Interest

All authors declare that they have no conflicts of interest.

Acknowledgments

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