Identification of *N*^ω-carboxymethylarginine as a novel acid-labile advanced glycation end product in collagen

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Collagen undergoes continuous non-enzymatic glycation during its long life period. The products resulting from the glycation reaction, so-called advanced glycation end products (AGEs), were regarded as potential pathogens of various diseases such as diabetic complications. Although several AGEs were identified from acid hydrolysates of glycated collagen, the major AGE(s) responsible for the diseases have not yet been fully characterized. Moreover, acid-labile constituents were decomposed during acid hydrolysis. To investigate these AGEs, we used the enzymatic hydrolysis method [Bensusan, Dixit and McKnight (1971) *Biochim. Biophys. Acta* **251**, 100–108]. As a result, an acid-labile

unknown compound was discovered from the digested glycated collagen. We identified this compound as *N*^ω-carboxymethylarginine (CMA) by matrix-assisted laser-desorption/ionization-MS and NMR. CMA gradually increased in collagen during incubation with glucose and the yield reached about 8 mol/mol of collagen, which is 100 times higher than that of pentosidine. This result suggests that CMA is a major AGE in collagen.

Key words: carboxymethylation, enzymatic hydrolysis, glucose, Maillard reaction.

INTRODUCTION

Glucose and other reducing sugars react with an amino group non-enzymatically to form unrevealed complex compounds. This process, so-called glycation, involves post-translation protein modification and possibly causes various diseases. In particular, the accumulation of advanced glycation end products (AGEs) in long-lived tissue is thought to be implicated in diabetic complications and aging [1–3].

Collagen, which is one of the typical long-life proteins, loses flexibility and enzymatic digestivity in parallel with aging [4]. Similar changes of collagen are known to happen in the patients with diabetes [5]. These phenomena are attributable in part to accumulation of AGEs [6,7]. Structures of several AGEs have been elucidated, including pentosidine [8], pyrraline [9], carboxymethyl-lysine (CML) [10], crossline [11], and non-fluorescent compounds (NFCs) [12]; some of these were confirmed to accumulate in tissues during aging and diabetic complications. However, the major AGEs in collagen remain unknown. Pentosidine is a well-known AGE, which was first identified from human tissue collagen [8], but its amount in the tissue is too small to account for the changes of physical properties of collagen. Carboxyalkyl-lysine are thought to be major AGEs in lens protein but not abundant in collagen [13].

Since most of the known AGEs have been isolated from tissues after acid hydrolysis, acid-labile compounds that decompose under these conditions remain to be unidentified until present. Bensusan et al. proposed the method of enzymatic hydrolysis of collagen [14]. Utilizing their method, we digested glycated collagen with aminopeptidase that led to the detection of an unknown acid-labile amino acid. In this study, we report the

isolation and structure of the new glycation product from glycated collagen.

EXPERIMENTAL

Materials

[1-¹³C]Glucose was purchased from Aldrich (Milwaukee, WI, U.S.A.). Collagenase N-2 was from Nitta Gelatin Inc., Osaka, Japan. Pronase E (Protease type XIV) was from Sigma. Heptafluorobutyric acid (HFBA) was from Nacalai Tesque Inc., Kyoto, Japan. All other reagents were from Wako Chemicals, Osaka, Japan. Dowex 50W cation-exchange resin was from Dow Chemical Co., Michigan, MI, U.S.A. Shodex C18-5A column (4.6 mm × 150 mm; Showa Denko, Tokyo, Japan) was used for reversed-phase HPLC.

Preparation of pig kidney aminopeptidases mixture

Crude aminopeptidases mixture was prepared from the porcine kidney following the method of Bensusan et al. [14]. The porcine kidney (500 g) was subjected to ammonium-sulphate precipitation. The resultant precipitate at 40–80 % saturation concentration of ammonium sulphate was collected and dialysed against 5 mM Tris buffer (pH 8.0), containing 2 mM MgCl₂. The solution was divided into 0.2 ml aliquots and stored at –25 °C until use.

Preparation of acid-soluble collagen (ASC)

ASC was prepared from the skin of a newborn bovine as described in a previous report [15]. The skin was homogenized by

Abbreviations used: AGEs, advanced glycation end products; CMA, *N*^ω-carboxymethylarginine; CML, *N*^ε-carboxymethyl-lysine; NFCs, non-fluorescent compounds; MALDI, matrix-assisted laser-desorption/ionization; HFBA, heptafluorobutyric acid; ASC, acid-soluble collagen; DHB, 2,5-dihydroxybenzoic acid; α-CHCA, α-cyano-4-hydroxycinnamic acid; HMBC, heteronuclear multiple bond connectivity; HSQC, heteronuclear single quantum coherence.

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Figure 1 Amino acid analysis of enzymatic digestion of ASC

ASC samples were incubated at 37 °C for 40 days with or without glucose. (A) Digested ASC incubated with 200 mM glucose, (B) digested ASC incubated without glucose (control). In (A), the unknown peak (*) appeared while the arginine (R) peak decreased. Standard one-letter symbols for the relevant amino acids are shown.

a Polytron homogenizer. The homogenate was washed with an excess volume of 10 mM disodium phosphate in the presence of protease inhibitors (0.1 mM *N*-ethylmaleimide, 0.01 mM PMSF, and 1 mM EDTA). Then the homogenate was defatted with excess volume of diethyl ether/ethanol [1:1 (v/v)]. Collagen was extracted from the defatted tissues with 50 mM acetic acid and precipitated by centrifugation (10000 *g* for 0.5 h) from the extract by adding NaCl at the final concentration of 2 M. The precipitate was dissolved in 50 mM acetic acid. After centrifugation (10000 *g* for 0.5 h) to remove insoluble materials, collagen was precipitated again by the addition of NaCl at the final concentration of 1 M. The precipitate was dissolved in 50 mM acetic acid and dialysed against 5 mM Tris/HCl buffer (pH 7.5). Because of its poor solubility at neutral pH in the absence of salt, collagen precipitated during dialysis and was collected as ASC by centrifugation (10000 *g* for 0.5 h). ASC dissolved in 1 mM HCl was used for experiments. This procedure was carried out below 4 °C. Purity of ASC was checked by SDS/PAGE and amino acid analysis.

In vitro glycation of collagen

ASC (1.25 mg/ml) was dissolved in 50 mM phosphate buffer at pH 7.5, placed in microtubes (0.8 ml/tube) and incubated at 37 °C in the presence of 3 mM Na₂S₂O₃. ASC fibre was reconstituted to form a gel within 1 h; then 0.2 ml of 1 M glucose was added to the gel. The mixture was incubated again at 37 °C for about 30 days. For the preparation of isotope-labelled glycation product, [1-¹³C]glucose was used. ASC incubated without glucose was used as control.

Preparation and fractionations of glycated compounds

Enzymatic digestion of glycated ASC

ASC or glycated ASC gel (1 ml) was centrifuged at 8000 *g* for 10 min to precipitate ASC. The pellet of ASC was washed with 50 mM Tris buffer to remove excess glucose and was subjected to the following digestion sequence. First, to the collagen pellet was added 0.5 ml of 50 mM Tris buffer (pH 7.5) containing 0.04 mg/ml collagenase and 2 mM CaCl₂. Then, 20 µg of Pronase was added to the mixture. Finally, after the solution was adjusted to pH 8 with 1.5 M Tris buffer, the porcine kidney aminopeptidase mixture was added. This procedure was carried out at 37 °C.

Preparative cation-exchange chromatography

The enzymatic digests of ASC were loaded onto a Dowex 50W (H⁺-form) column (90 mm × 50 mm). After washing with 20 ml of water, retained basic compounds were eluted with 10 ml of aqueous 10% pyridine. The eluate was evaporated by a rotary evaporator and dissolved in 1 ml of water. Then, the fraction was loaded onto a column packed with Hitachi Custom Ion Exchange Resin equipped on an L-8500 amino acid analyser (Hitachi Instruments Service Co., Tokyo, Japan). The fraction corresponding to an unknown amino compound at retention time of approx. 64 min was collected.

Preparative C18 HPLC

The fraction containing the unknown amino compound was further chromatographed on a Shodex C18-5A column (4.6 mm × 150 mm). The column was eluted isocratically with 100% H₂O for 10 min at 1 ml/min followed by a linear gradient from 0% to 20% aqueous acetonitrile for 40 min. All mobile phases contained 50 mM HFBA as an ion-pairing agent. After freeze-drying, the fractions were checked by amino acid analysis. The fraction containing the unknown amino compound was subjected to MS and NMR measurements.

Instrumental analyses

Except for enzymatic hydrolysis, samples were heated at 110 °C with 6 M HCl for 24 h for amino acid analysis. The reaction mixture was then evaporated by a rotary evaporator and dissolved in 0.02 M HCl. Amino acid analysis was performed on an L-8500 amino acid analyser using citrate buffers and lithium chloride gradient. Amino acids in the eluate were monitored by post-column reaction with ninhydrin.

Mass spectrometric analyses were performed by Sawady Technology (Tokyo, Japan). Matrix-assisted laser-desorption ionization (MALDI)-MS was recorded on a Voyager Elite (PE-Biosystems, Norwalk, MA, USA). A sample solution (0.01 ml, 10% MeCN) was analysed using matrices of 2,5-dihydroxybenzoic acid (DHB). α -Cyano-4-hydroxycinnamic acid (α -CHCA) dimer and angiotensin I were used for external calibration of the instrument.

NMR spectra were recorded on a 500 MHz JNM A-500 spectrometer (JEOL, Tokyo, Japan) and a 500 MHz DRX-500 spectrometer (Bruker, Karlsruhe, Germany). Standard COSY spectra were measured in ²H₂O solution with the data matrix of 1 K (*F2*) by 256 (*F1*). Residual acetic acid was taken as an internal standard of ¹H chemical shift at 1.84 ppm. Heteronuclear multiple bond connectivity (HMBC) was measured using

synthetic *N*^ω-carboxymethylarginine (CMA) in ²H₂O solution with the data matrix of 1 K (F2) by 256 (F1). The interval corresponding to $1/2\alpha^{2,3}J_{C,H}$ was set at 65 ms. For both COSY and HMBC experiments, 2-fold zero-filling was effected for the F1 data.

Preparation of (*N*^ω-)Carboxymethylarginine

Authentic CMA was prepared from *N*^α-acetylarginine and iodoacetic acid. *N*^α-Acetyl-L-arginine (25 mg) and monoiodoacetic

Figure 2 Time-dependent formation of the unknown compound in collagen

Concentration of the unknown compound was estimated by using leucine as a standard for ninhydrin colourization.

Figure 3 Purification of the unknown compound by column chromatography

Purity of the unknown compound (*) was checked by amino acid analysis at every isolation step by column chromatography as described in the Experimental section. (A) Original enzymatic digest of glycated ASC, (B) aqueous pyridine fraction of (A) from Dowex column, (C) 64-min fraction of (B) from ion-exchange column, and (D) desalted and purified fraction of (C) from C18 column.

Figure 4 MALDI-MS spectra

The unknown compound samples purified by C18 column were subjected to MALDI-MS measurement using DHB matrix. (A) Sample prepared with normal glucose. (B) Sample prepared with [¹³C]glucose. (*) Denotes ion peaks due to the matrix.

acid (19 mg) were dissolved in 1 ml of 1 M NaOH and incubated at 37 °C for 24 h. Equal volumes of 2 M HCl were added and heated at 110 °C for 1 h to deprotect the *N*-acetyl group. The solution was chromatographed as described above. The containing CMA-fraction was loaded again onto a Dowex 50W column and eluted with aqueous pyridine to remove residual organic salts. The eluate was freeze-dried and subjected to MS and NMR measurements.

RESULTS

Formation of CMA from collagen

Amino acid analysis of the enzyme-digested collagen is shown in Figure 1. As shown in the chromatogram from the glycated collagen, arginine decreased significantly upon comparison with that of the control. On the other hand, the unknown peak appeared between phenylalanine and NH₃ (Figure 1, asterisk). The unknown amino compound gradually increased with incubation time and the peak area reached 15 % of that of leucine at 34 days. When we used leucine as a standard for ninhydrin colorization, the yield of the unknown amino compound was estimated to be 8 mol per 1 mol of collagen (Figure 2).

Isolation and structure elucidation of CMA from glycated collagen

After each step of column chromatography, the purity of the unknown compound was checked by amino acid analysis (Figure 3). The unknown compound, which purified by C18 column chromatography (Figure 3D) was subjected to MS and NMR measurements. In the MALDI-MS spectrum, several peaks including those due to a DHB matrix were observed (Figure 4). Among these, the peak at *m/z* 233 was observed only in the spectrum of the sample (Figure 4A). When glycation was carried out in the presence of [¹³C]glucose, the peak at *m/z* 233 in the original spectrum was shifted to *m/z* 234 (Figure 4B). These results show that the unknown amino acid has the molecular mass of *m/z* 232 and possesses a carbon atom derived from C-1 position of glucose.

Figure 5 500 MHz ¹H-NMR spectra of CMA in ²H₂O solution

The spectra were measured with a JMR A-500. (A) Synthetic CMA, (B) the unknown compound obtained from glycated ASC, (C) A (1:1) mixture of (A) and (B). Since the chemical shifts of CMA are highly dependent on pH, (A) and (B) NMR spectra showed a difference in the chemical shifts (*) of the protons.

The NMR data revealed that the amino acid has a proton spin network corresponding to arginine; ¹H-¹H connectivity of H-2/H₂-3/H₂-4/H₂-5 were clearly shown in the COSY spectra. The presence of two proton signal at δ 3.83 in Figure 5(B) (¹³C NMR, δ 46.5 from HSQC) suggested the presence of an N-linked methylene group. The connecting manner of this methylene was determined by HMBC experiment using the synthetic sample, in which a clear ³J_{C,H} correlation was observed between H₂-7 and C-6 while no interaction was obtained between H₂-7 and C-5. The facts unambiguously revealed that the acetic acid residue was substituted at the terminal nitrogen (not at the middle one between C-5 and C-6). Further structural confirmation was carried out by comparing the NMR of the natural and synthetic CMA (Figure 5).

DISCUSSION

AGEs interest us because they are not only diagnostic markers but also potential pathogens of various diseases represented by diabetic complications [16]. Several AGEs have already been identified in the acid hydrolysate of glycated proteins, but major AGE has not been clarified yet. We supposed that glycation of proteins produced acid-labile AGEs which remained as unknown products. To detect acid-labile AGEs we tried the enzymatic digestion of collagen, instead of acid hydrolysis by HCl. As a result of amino acid analysis, a novel glycation product was found from *in vitro* glycated collagen, which was identified as CMA by MALDI-MS and NMR. The amount of CMA increased

in parallel with incubation time. This is further supported by the observation that the generation of CMA was inhibited by aminoguanidine, which is a known inhibitor of glycation (results not shown). Heating with 6 M HCl for 24 h resulted in the disappearance of the CMA peak in the amino acid analysis, which indicates that CMA is acid-unstable, and thus had not been detected in the acid hydrolysate. The acid-lability of CMA is markedly distinct from its lysine equivalent, CML, which accumulates in various tissues with diabetic complications and is thought to be one of the possible pathogens.

The pathway generation of CMA is not clear but active dicarbonyl compounds are probably involved in its formation. Dicarbonyl compounds, such as glyoxal, methylglyoxal and 3-deoxyglucosone, are generated by autoxidation of glucose or degradation of Amadori products, and their concentrations are increased in the blood of diabetic patients [17]. These compounds react rapidly with protein to form cross-links and fluorescent products. New AGEs derived from dicarbonyl compounds, such as carboxyethyl-lysine [18], imidazolone compounds [19] and imidazolium cross-links [13] have been discovered in recent years. One of the biosynthetic routes of CML is also the reaction of glyoxal with a lysine residue of protein [20]. Recently, Paul et al. [12] reported NFCs as novel AGE compounds from glycated collagen. Although NFCs were isolated by acid hydrolysis, their structures were destroyed by further acid hydrolysis, and the expected structure of α -NFC was similar to CMA. NFCs can possibly be derived from CMA by dehydration to form a lactam ring.

The yield of CMA was high in collagen glycated *in vitro*. After incubation for 30 days with 200 mM glucose, the production of CMA was estimated to be approx. 8 mol/mol of collagen on the basis of the ninhydrin colourization of leucine taken as standard. On the other hand, pentosidine was generated at only less than 0.1 mol/mol of collagen under the same conditions. According to Paul et al., NFCs were produced at about the same amount as that of CMA [12]. These results suggest that acid-labile arginine compounds might be major AGEs in collagen.

CMA may be implicated in illnesses associated with aging and the development of diabetes, as speculated for CML. In preliminary studies, CMA could not be detected from *in vitro* glycation products of BSA or lysozyme, which suggested that CMA is a specific product for glycated collagen. Collagen is one of the typical long-lived proteins, and deterioration of collagen-rich tissues generally increases with aging. Serious diabetic complications also appear in collagen-rich tissues, such as blood vessels and renal basement membranes. Glycated collagen not only has altered mechanical properties; physiological functions such as comfortable substratum for cells may be affected [21,22] that may be implicated in progress of senescence and diabetic complications. Currently, we are trying to detect CMA *in vivo* which could be used as a new marker of glycation in collagen-rich tissues and help the study of aging and diabetic complications on the molecular level.

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