# Identification of the Carboxymethyllysine Residue in the Advanced Stage of Glycated Human Serum Albumin

Masaya TAKANASHI, Tamiko SAKURAI* and Seishi TSUCHIYA

*Tokyo College of Pharmacy, 1432–1 Horinouchi, Hachioji, Tokyo 192–03, Japan.* *Received July 25, 1991*

**As an advanced stage of glycation, glycated human serum albumin (G-HSA; glucose content, 2 mol of 5-hydroxymethylfurfural equivalent/mol of HSA) was incubated at 37 °C up to 30 d in 0.2 M phosphate buffer, pH 7.4, with 100 μM $Fe^{3+}$. G-HSA incubated for 30 d (G-HSA-30(Fe)) was subsequently hydrolyzed at 110 °C for 24 h in 6 N HCl. In the hydrolysate, $N^{\varepsilon}$-carboxymethyllysine (CML) was identified by cochromatography with synthesized CML on an amino acid analyzer. pI of HSA (4.8) shifted to 4.5 in G-HSA. A more acidic fraction, pI 4.3, appeared in G-HSA-30(Fe). CML content (mol of CML/mol of HSA) of HSA and G-HSA was as follows; 0 in HSA, 0.2 in HSA-30(Fe), 0.4 in G-HSA and 1.5 in G-HSA-30(Fe) pI 4.3. The amino acid compositions also changed in lysine, arginine and tyrosine at the advanced stage of the reaction.**

**Keywords** nonenzymatic glycation; carboxymethyllysine; glycated human serum albumin; autoxidation; amino acid analysis

Glucose and other reducing sugars react nonenzymatically with proteins both *in vivo* and *in vitro* to form ketoamine adducts[1] covalently. This process is called the early stage of the Maillard reaction. Such an adduct produces brown fluorescent chromophores with further incubation under certain physiological conditions[2]; the socalled advanced stage. The reaction products produced in the advanced stage have been studied in relation to diabetic complications and aging.[3] The occurrence of superoxide generation from glycated polypeptides such as glycated polylysine or glycated human serum albumin *in vitro* has been noticed.[4] The superoxide generation through autoxidation of glycated peptide was accelerated in the presence of $Fe^{3+}$.[5]

Ahmed *et al.*[6] found $N^{\alpha}$-formyl fructoselysine, a model compound of ketoamine adduct, to decompose into carboxymethyllysine (CML) and erythronic acid (EA) during incubation for 15 d under physiological conditions. Dunn *et al.* detected the accumulation of CML in lens proteins and skin collagen.[7] Enediol chelated with $Fe^{3+}$ may possibly be oxidized to 2,3-dicarbonyl, which decomposes into CML and EA,[5] as shown in Chart 1. In this study, attention was directed to the formation of the CML residue in glycated human serum albumin during incubation for as long as 30 d at 37 °C in a phosphate buffer containing $Fe^{3+}$.

## Materials and Methods

**Glycation of Serum Albumin** Human serum albumin (Sigma, essentially fatty acid free) was applied to a Sephacryl 200 column to obtain a monomer fraction (HSA). HSA was dissolved in 0.067 M sodium phosphate buffer (pH 7.4) at the concentration of 4% in the presence of 1 M glucose and then incubated at 37 °C for 7 d in the dark and sterilized conditions under the air, with gentle shaking. Following dialysis against water, the glycated HSA (G-HSA) was lyophilized. The glucose content in G-HSA, which was estimated by using thiobarbituric acid[8] and expressed as 5-hydroxymethylfurfural (5-HMF) equivalent, was 2 (mol of 5-HMF/mol of HSA).

**Advanced Reaction of G-HSA** G-HSA (4%) was incubated in 0.2 M sodium phosphate buffer (pH 7.4) in the presence of 100 μM $Fe^{3+}$–adenosine 5′-diphosphate (ADP) at 37 °C for 15 or 30 d under the same conditions as the glycation. To prevent precipitation of ferric phosphate,

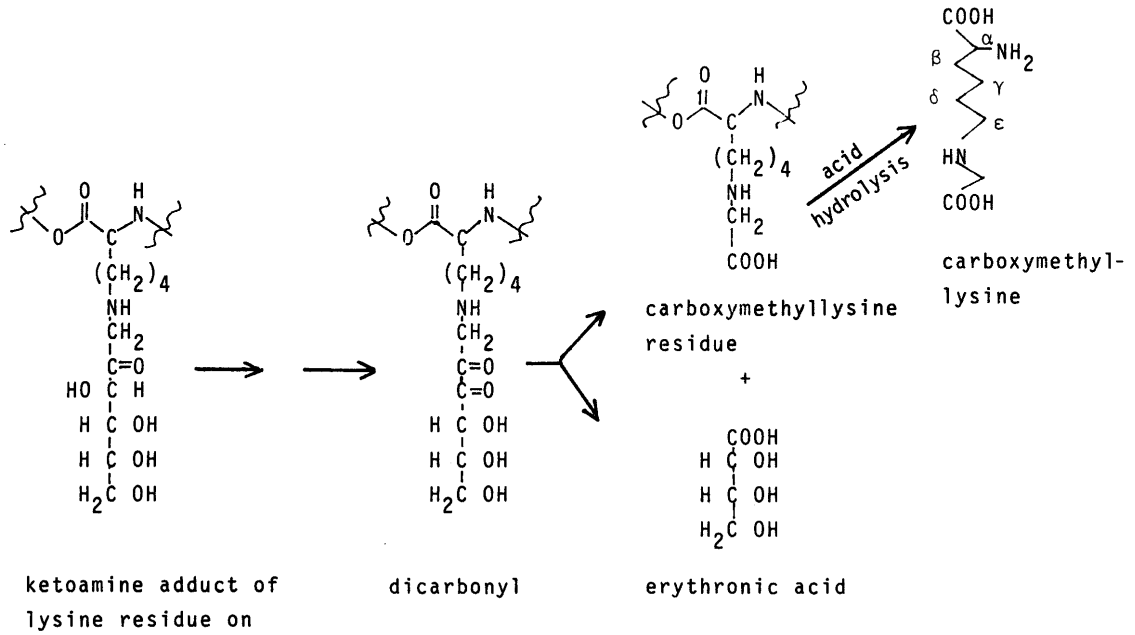


Chart 1

$Fe^{3+}$–ADP (1 : 16.7, mol/mol) solution was used.[9] After the incubation, the incubation mixtures were applied to a column of Sephadex G-25 (2.5 × 80 cm). Void fractions were lyophilized. As the control, HSA was incubated with or without $Fe^{3+}$–ADP under the same conditions as above. The G-HSA and HSA preparations incubated at 37 °C for 15 d and 30 d, respectively, with $Fe^{3+}$–ADP, were referred to respectively as G-HSA-15(Fe), G-HSA-30(Fe), HSA-15(Fe) and HSA-30(Fe).

**Chromatofocusing** Preparations of G-HSA and HSA dissolved in 25 mM of an imidazole–HCl buffer (pH 7.4) were applied to a PBE94 (Pharmacia) column (1 × 44 cm) equilibrated with the same buffer, then eluted with 75 μmol/pH unit/ml polybuffer 74–HCl (pH 4.0). Rechromatofocusing was initiated by 25 mM of an imidazole–HCl buffer (pH 6.2). Absorbance at 280 nm and the pH of each fraction were monitored.

**Amino Acid Analysis** Preparations of G-HSA and HSA were hydrolyzed by 6 N HCl (1 mg/ml) *in vacuo* at 110 °C for 24 h. After evaporation of HCl, the residues were dissolved in 1 ml of 0.02 N HCl and aliquots (10 μl) were analyzed by a Hitachi L-8500 amino acid analyzer.

**Synthesis of CML** CML was prepared by the carboxymethylation of $N^{\alpha}$-formyllysine, previously synthesized by the method of Hofmann *et al.*[10] In brief, L-lysine-hydrochloride (5 g) dissolved in 10 ml of $H_2O$ was applied to an Amberlite-IRA 94 (formate form) and eluted with 1 M formic acid followed by lyophilization. L-Lysine-formate dissolved in 65 ml of 99% formic acid was reacted with 25 ml of acetic anhydride and, following the addition of 85 ml of ice-cold water, the mixture was concentrated. The obtained oil was dissolved in EtOH and crystallized (mp 185—186 °C). Carboxymethylation and deformylation were conducted as follows[11]: $N^{\alpha}$-formyllysine (80 mg) dissolved in 4 ml of 1 N NaOH was reacted with iodoacetate (74 mg) for 40 h at room temperature, followed by the addition of 0.8 ml of ammonia water, and the mixture was then allowed to stand overnight. The reaction mixture was applied onto a Dowex 1-X8 column (2 × 40 cm, acetate) and eluted with 2 N acetic acid. Evaporation of the eluate gave pale yellow crystals of $N^{\alpha}$-formyl, $N^{\varepsilon}$-carboxymethyllysine. Deformylation was carried out by the addition of 2 ml of 2 N HCl and heating at 90 °C for 30 min. The reaction mixture was applied onto a Dowex 50W-X8 (hydrogen form; 1.2 × 18 cm) and eluted with 0.5 M pyridinium acetate (pH 3.1). The proton-nuclear magnetic resonance ($^1$H-NMR) spectrum of the lyophilized material was measured in $D_2O$ and recorded on a Varian EM-390. Chemical shifts are reported as $\delta$ values in ppm with tetramethylsilane as the internal standard ($\delta = 0$). Coupling constants ($J$) are given in herz. Signals in the spectrum are characterized as s (singlet), t (triplet) and m (multiplet). $^1$H-NMR ($D_2O$) $\delta$: 3.69 (1H, t, $J$=6.05, –CH), 3.55 (2H, s, $-CH_2COOH$), 3.02 (2H, t, $J$=7.7, $-CH_2NHCH_2COOH$), 1.86—1.55 (6H, m, $-CH_2CH_2CH_2CH$).

Following derivation of the CML to $N^{\alpha}$,$N^{\varepsilon}$-diacetyl,$N^{\varepsilon}$-carboxymethyllysine methylester, the electron impact-mass (EI-MS) spectrum was obtained using a Hitachi M-800 mass spectrometer. Molecular ion ($M^+$) 316 and deacetylate ($M^+ - CH_3CO$) 257 were obtained, and the other fragments showed agreement with the values in previous literature.[6]

## Results

**Characteristics in Chromatofocusing** Preparations of G-HSA and HSA were applied to a chromatofocusing column. The pH gradient and elution profile of HSA are shown in Fig. 1A and the pH-elution profiles of preparations of HSA, G-HSA and their advanced reactions, in Fig. 1B. The pH of the elution peak indicates the pI value of a protein. The pI of HSA was 4.8 and that of G-HSA, 4.5. Incubation of G-HSA with $Fe^{3+}$ caused a shift in pI to an acidic region. G-HSA-15(Fe) showed a small peak at pI 4.3 as well as at pI 4.5. G-HSA-30(Fe) showed pIs of 4.4 and 4.2. On the other hand, HSA-15(Fe) and HSA-30(Fe) showed a pI of 4.8, though HSA-30(Fe) also gave a peak at pI 4.6. Fractions of pH 4.80—4.30 and pH 4.31—3.90 in G-HSA-15(Fe) and G-HSA-30(Fe) were rechromatofocused, and fractions of pH 4.70—4.45 in the former and pH 4.35—4.00 in the latter were referred to as pI 4.5 and pI 4.3, respectively. Fractions of pH 4.90—4.70 and pH 4.67—4.30 in HSA-30(Fe) were referred to as pI 4.8 and pI 4.6.

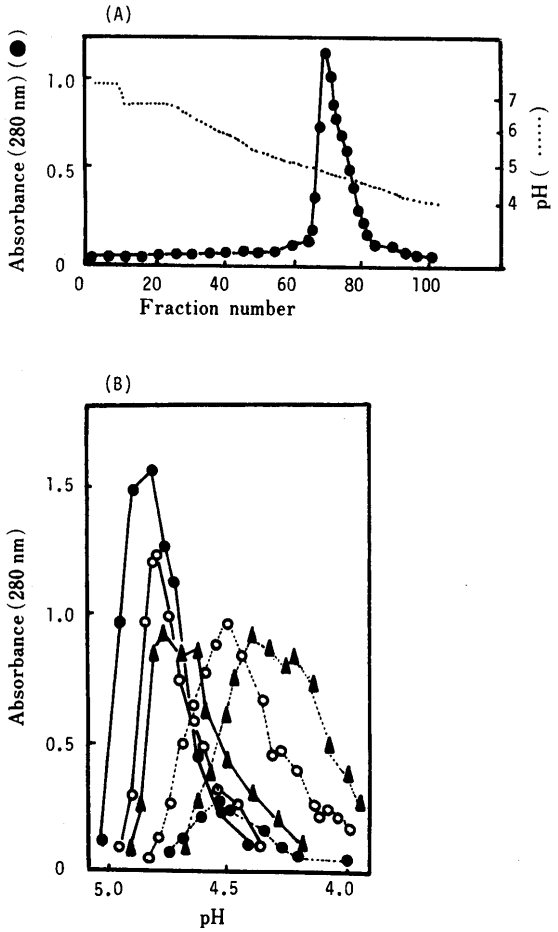


Fig. 1. Typical Profile of Chromatofocusing of HSA (A) and Elution Profiles of HSAs and G-HSAs *vs.* pH (B)

(A) HSA (100 mg) was charged on PBE 94 column (1 × 44 cm) equilibrated with 25 mM imidazole–HCl (pH 7.4) and column was eluted by polybuffer 74–HCl (pH 4.0). Flow rate: 9 ml/h, fraction: 4 ml/each. (B) Elution profiles were replotted *vs.* pH. —●—, HSA (200 mg); --●--, G-HSA (30 mg); —○—, HSA-15 (Fe) (150 mg); --○--, G-HSA-15 (Fe) (150 mg); —▲—, HSA-30 (Fe) (120 mg); --▲--, G-HSA-30 (Fe) (120 mg).

**Detection of CML** When the CML standard was subjected to an amino acid analyzer by the usual method, the retention time of CML overlapped with that of methionine. CML was separated between methionine and valine by reducing the column temperature from 60 °C to 57 °C during a retention time of 25 to 49 min. Elution profiles of the hydrolysates of G-HSA-30(Fe) pI 4.3, CML standard and their mixture are shown in Fig. 2 (A, B and C). When equal volumes of the hydrolysate of G-HSA-30(Fe) pI 4.3 and CML were cochromatographed (Fig. 2C), the retention time of the unknown peak in G-HSA-30(Fe) pI 4.3 coincided with CML. The CML content in typical preparations of HSA and G-HSA was estimated by amino acid analysis (Table I). No CML could be detected in HSA, though HSA-30(Fe) was found to contain 0.2 mol of CML/mol of HSA. The CML content of G-HSA-30 was 2.5 times that of G-HSA, indicating that the incubation of G-HSA accelerated the formation of CML. G-HSA-30(Fe) pI 4.3 showed an increase in CML content compared to G-HSA-30, indicating acceleration by $Fe^{3+}$.

**Amino Acid Composition** The amino acid compositions of preparations of HSA and G-HSA with a theoretical

value of HSA[12] are indicated in Table II. Those of the hydrolysates of HSA-15(Fe), HSA-30(Fe) pI 4.8 and pI 4.6 were the same as that of HSA, though aspartic acid showed a lower value. On the other hand, G-HSA-15(Fe) pI 4.5 and pI 4.3 showed decreases in lysine, arginine and tyrosine compared to HSA-15(Fe). G-HSA-15(Fe) pI 4.3 showed less lysine and arginine than G-HSA-15(Fe) pI 4.5. The pI 4.5 fractions in both G-HSA-15(Fe) and G-HSA-30(Fe) had essentially the same composition. The pI 4.3 fraction of G-HSA-30(Fe) indicated less tyrosine compared to G-HSA-15(Fe).

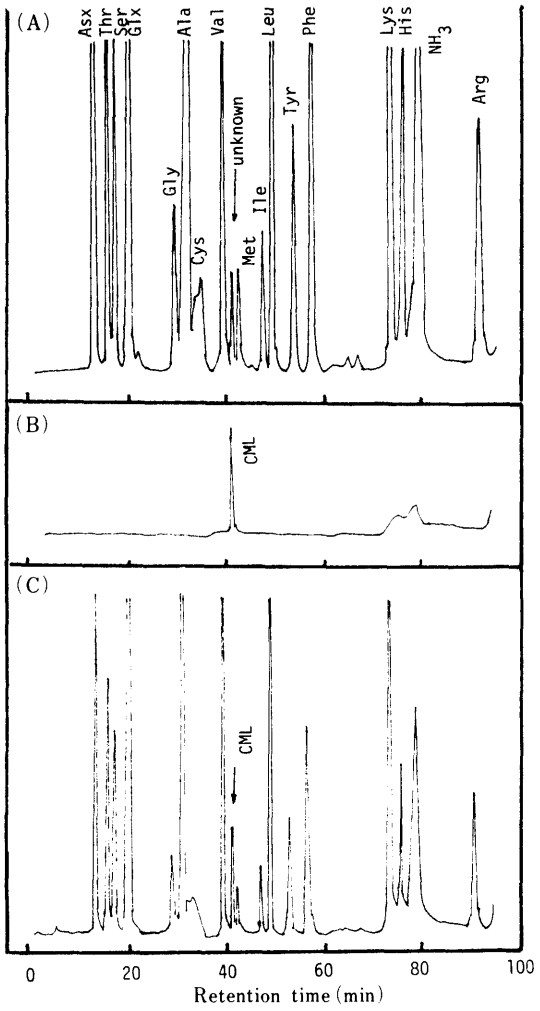


Fig. 2. Elutions of CML by Cochromatography on Amino Acid Analysis Column

(A) Hydrolysate of G-HSA-30 (Fe) pI 4.3 was analyzed; (B) 2 nmol of CML standard was analyzed; (C) mixture of equal volume of hydrolysate of G-HSA-30(Fe) pI 4.3 and CML standard was analyzed.

## Discussion

The long incubation period of glycated proteins corresponds to their long lifespan in the human body. The half-life of HSA in the blood is 14—20 d.[13] In this study, G-HSA was incubated up to 30 d at 37 °C to investigate the effects of advanced reaction by acceleration with $Fe^{3+}$. We could herein identify CML in advanced reaction products of G-HSA by amino acid analysis (Fig. 2). The pI 4.3 fraction of G-HSA-30(Fe), which formed during incubation, showed the highest accumulation of CML (Table I). The appearance of fractions both of pI 4.3 and 4.5, these being less than pI 4.8 of HSA, indicated the appearance of acidic groups and/or the disappearance of basic amino acid residues in G-HSA molecules. CML was partially responsible for the increase in negative charge of G-HSA. CML could not be detected in HSA, while HSA-30(Fe) was found to contain 0.2 mol of CML/mol of HSA. The appearance of the pI 4.6 fraction in HSA-30(Fe) may possibly have been due to the production of CML during incubation from glycated molecules originally present in HSA. That the amino acid compositions of

Table I. Carboxymethyllysine Content

| | CML/HSA (mol/mol) |
|---|---|
| HSA | 0 |
| HSA-30(Fe) | 0.2 |
| G-HSA | 0.4 |
| G-HSA-30 | 1.0 |
| G-HSA-30(Fe) pI 4.3 | 1.5 |

Table II. Amino Acid Composition

| Amino acid residue | Theoretical value[a] | HSA | HSA-15(Fe) | G-HSA-15(Fe) | | HSA-30(Fe) | | G-HSA-30(Fe) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | pI 4.5 | pI 4.3 | pI 4.8 | pI 4.6 | pI 4.5 | pI 4.3 |
| Asp, Asn[b] | 37, 16 | 54.70 | 42.97 | 42.55 | 42.50 | 42.68 | 42.85 | 42.49 | 42.59 |
| Thr | 27 | 26.50 | 28.95 | 28.45 | 27.28 | 28.16 | 28.52 | 28.01 | 27.79 |
| Ser | 24 | 21.90 | 22.50 | 22.64 | 22.23 | 22.85 | 22.46 | 22.61 | 22.15 |
| Glu, Gln[b] | 62, 22 | 85.90 | 86.28 | 79.68 | 79.33 | 86.31 | 86.07 | 80.61 | 79.50 |
| Gly | 12 | 13.10 | 12.45 | 12.29 | 12.08 | 12.68 | 12.49 | 12.31 | 11.69 |
| Ala | 63 | 63.70 | 60.46 | 60.32 | 59.54 | 60.57 | 60.38 | 60.07 | 59.35 |
| Val | 39 | 39.00 | 38.53 | 38.58 | 37.42 | 38.60 | 37.62 | 38.72 | 38.16 |
| Cys | 35 | —[b] | — | — | — | — | — | — | — |
| Met | 6 | —[b] | — | — | — | — | — | — | — |
| Ile | 8 | 8.08 | 8.23 | 7.97 | 7.92 | 8.18 | 8.20 | 8.01 | 7.98 |
| Leu | 61 | 61.00 | 61.00 | 61.00 | 61.00 | 61.00 | 61.00 | 61.00 | 61.00 |
| Tyr | 18 | 17.80 | 17.94 | 13.22 | 13.12 | 17.95 | 18.31 | 14.80 | 11.08 |
| Phe | 31 | 31.40 | 32.17 | 32.26 | 31.85 | 31.65 | 31.69 | 31.95 | 31.50 |
| Lys | 59 | 57.80 | 60.12 | 54.42 | 52.19 | 58.98 | 58.02 | 54.26 | 52.75 |
| His | 16 | 16.40 | 15.83 | 16.15 | 15.83 | 15.92 | 15.50 | 15.90 | 15.76 |
| Arg | 24 | 24.70 | 24.15 | 21.28 | 20.39 | 24.54 | 24.20 | 21.78 | 19.31 |
| Pro | 24 | 26.70 | 25.18 | 24.85 | 24.43 | 24.63 | 26.20 | 24.47 | 24.54 |

*a*) Reference 12). *b*) Present but not quantified.

both fractions of pI 4.8 and pI 4.6 in HSA-30(Fe) (Table II) were essentially the same would be the reason for this. The incubation of HSA caused no decrease in basic amino acid residues, lysine or arginine, as evident in Table II. The pI 4.5 fraction in G-HSA-15(Fe) and G-HSA-30(Fe), however, decreased in about 6 lysine and 3 arginine residues in contrast to HSA-15(Fe). The pI 4.3 fraction in G-HSA-30(Fe) decreased in one more lysine and two more arginine residues than did the pI 4.5 fraction. This may possibly have been due to a reaction such as that with 3-deoxyglucosone.[14] Fluorescence chromophores developed in G-HSA-30(Fe), whose emission maximum at 430 nm during excitation at 350 nm,[15] increased to 5 times that of G-HSA (data not shown). When the fluorescence of the tryptophane residue of HSA and G-HSA was measured during excitation at 290 nm, a maximum emission of 340 nm decreased to 72% in HSA-30(Fe), 51% in G-HSA, and 28% in G-HSA-30(Fe) compared to HSA. Incubation with $Fe^{3+}$ would thus appear to accelerate the degradation of the tryptophane residue. Proteins under conditions such as active oxygen generation underwent attack on their tyrosine, cysteine and tryptophane residues.[16] Decrease in the tyrosine residue of both G-HSA-15(Fe) and G-HSA-30(Fe) was noted (Table II). From these results, it thus follows that accumulation of the CML residue in proteins is an indication of exposure of the tissues to active oxygens, assuming the presence of a trace amount of metal. Nonenzymatic glycation may thus possibly be related to oxidative stress.

An attempt was made to confirm the production of a low molecular substance EA (Chart 1) also, using U-$^{14}$C-glucose (data not shown). To remove the impurities of $^{14}$C-glucose nonspecifically bound to proteins,[17] the incubation of $^{14}$C-glucose with HSA for a short period (5 h) was carried out, followed by the column chromatography of carboxymethylcellulose (Avicel) to obtain free $^{14}$C-glucose. $^{14}$C-G-HSA was obtained by incubating 4% HSA with $^{14}$C-glucose at 37 °C for 14 d (0.5 mol of glucose incorporated/mol of HSA). After the advanced reaction of $^{14}$C-G-HSA with 100 $\mu$M $Fe^{3+}$ for 30 d, gel filtration (Sephadex G-25) of the reaction mixture was carried out. $^{14}$C-G-HSA showed a single radioactive peak corresponding to protein, while $^{14}$C-G-HSA-30(Fe), another radioactive peak (28% of total radioactivity) in the low molecular weight region. The fragmentation or elimination of $^{14}$C-glucose bound to HSA is thus shown to occur. When radioactive low molecular substances obtained from $^{14}$C-G-HSA-30(Fe) were subjected to gas chromatography following trimethylsililation, five of several peaks were found to coincide with that of crude EA synthesized from D-erythrose by reaction with oxygen at 30 °C for 11 h in 0.1 N NaOH solution.[18] The peak at 150 °C showed a signal of $m/z$ 409 ($M^+$ − methyl of EA) by mass spectrometry. From these results, the presence of EA among low molecular substances is clearly indicated.

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