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Fluorescent detection of α-aminoadipic and γ-glutamic semialdehydes in oxidized proteins

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ABSTRACT

The oxidative modification of proteins is believed to play a critical role in the etiology and/or progression of several diseases. α-Aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) residues represent major oxidized amino acids generated in oxidized proteins. This paper describes a novel procedure for the specific and sensitive determination of AAS and GGS after their reductive amination with sodium cyanoborohydride and *p*-aminobenzoic acid, a fluorescence reagent, to their corresponding derivatives, followed by a high-performance liquid chromatography (HPLC) analysis. This fluorescent labeling of protein-associated aldehyde moieties is a simple and accurate technique that may be widely used to reveal increased levels of oxidatively modified proteins with reactive oxygen species during aging and disease.
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Introduction

Several lines of evidence indicate that the oxidative modification of proteins and subsequent accumulation of modified proteins have been found in cells during aging and oxidative stress and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis [1–8]. During the oxidative modification of proteins, carbonyl groups are irreversibly introduced into amino acid side chains of proteins through a variety of modification pathways, including the metal-catalyzed oxidation (MCO) of specific amino acid residues [2,3,5] and adduction with carbonyl-containing peroxidized lipids (4-hydroxy-2-nonenal, malondialdehyde, acrolein, etc.) [9–13] and reducing sugars [14]. These oxidized amino acids can be measured by convenient methods using 2,4-dinitrophenylhydrazine (DNPH), which reacts with carbonyl groups of amino acid side chains to generate 2,4-dinitrophenyl (DNP) hydrazones with characteristic absorbance maxima [15–17]. Stable DNP adducts can be determined by a spectrophotometric assay and high-performance liquid chromatography (HPLC). Immunoblotting assays based on the use of anti-DNP antibodies have been also developed to determine oxidatively damaged proteins in tissues. Using these methods, it has been confirmed that carbonyl derivatives accumulate on tissue proteins during aging [18,19] and disease development such as Alzheimer's disease [20,21], rheumatoid arthritis [22], amyotrophic lateral sclerosis [23], diabetes mellitus [24], and Parkinson's disease [21]. Nevertheless, the methods are unfortunately limited to measuring the total carbonyl derivatives formed by various unspecific pathways, and information on the chemical structures and formation mechanisms is barely provided. In addition, chemical difficulties, that is, oxidized amino acids and their reduced products can be destroyed and modified during acid hydrolysis [25–28], have prevented the general quantification of individual protein carbonyls in proteins. Therefore, more specific and reliable methods for the determination of carbonyl derivatives are required in order to understand the chemical nature, oxidation pathway, and distribution level in vivo. For that purpose, we have recently developed a new method for the determination of most predominant carbonyl amino acids, α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) [29]. AAS is the oxidation product of the lysine residue by MCO systems, whereas GGS originated from the oxidation of arginine and proline (Fig. 1). Using this procedure, we have revealed the generation of GGS and AAS on the glycoxidation [30] and Cu^{2+}-catalyzed oxidation of the dopamine-

Abbreviations: AAS, α-aminoadipic semialdehyde; ABA, *p*-aminobenzoic acid; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; DTPA, diethylenetriamine-*N,N,N′,N′′,N′′*-pentaacetic acid; GC/MS, gas chromatography/mass spectrometry; GGS, γ-glutamic semialdehyde; HPLC, high-performance liquid chromatography; HACA, hydroxyaminocaproic acid; HAVA, hydroxyaminovaleric acid; MCO, metal-catalyzed oxidation; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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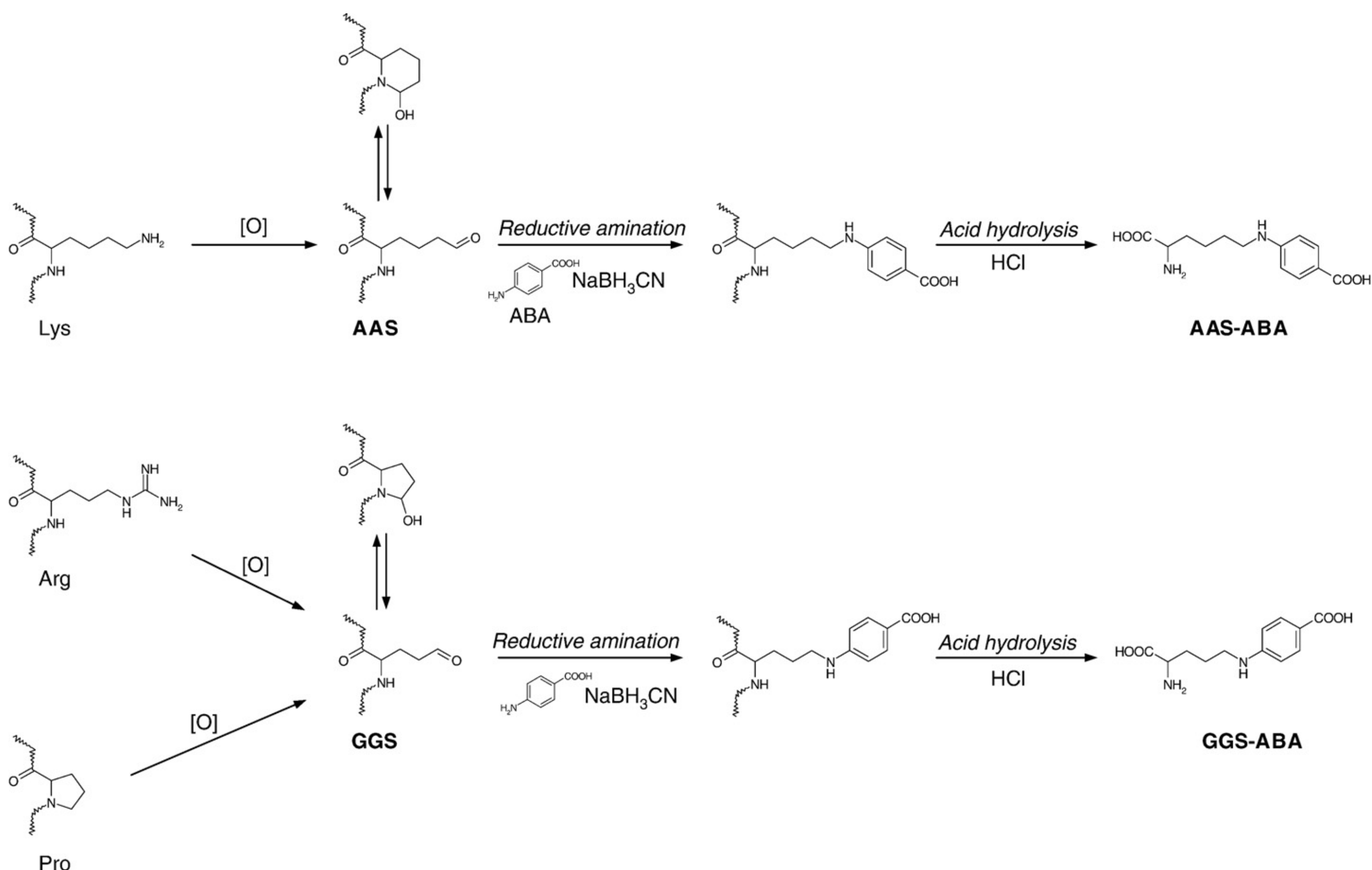

Fig. 1. Reaction scheme for the formation of AAS and GGS, and their derivatization by reductive amination with ABA and $NaBH_3CN$. Reproduced with permission from *Chem. Res. Toxicol*. 19:1059–1065 [29]. Copyright 2006 American Chemical Society.

modified protein [31]. Furthermore, we have also demonstrated their endogenous generation through oxidative stress induced by acute iron overload in vivo [29].

This paper summarizes a novel procedure for the specific and sensitive determination of AAS and GGS after their reductive amination with cyanoborohydride ($NaBH_3CN$) and *p*-aminobenzoic acid (ABA), a fluorescence reagent, to their corresponding derivatives, followed by HPLC analysis. Because of the lack of commercially available standards, we also describe a method for the synthesis and purification of authentic compounds.

Principles

AAS, GGS, and their reduced forms (alcohols) are labile toward acid hydrolysis [25–28] and therefore must be stabilized. Fig. 1 shows a strategy for the derivatization of the AAS and GGS. First, the side chains of these oxidized amino acids are derivatized by reductive amination with $NaBH_3CN$ and *p*-aminobenzoic acid [32,33] to their corresponding derivatives, AAS-ABA and GGS-ABA. These ABA derivatives can be specifically determined by HPLC following acid hydrolysis. The resulting derivatives are highly fluorescent at Ex_{max} 283 nm/Em_{max} 350 nm and stable under the conditions of acid hydrolysis with 6 M HCl.

Materials

*N*α-Acetyl-L-lysine (Cat. No. A2010), *N*α-acetyl-L-ornithine (Cat. No. A3626), and bovine serum albumin (BSA, Cat. No. A2153) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium cyanoborohydride ($NaBH_3CN$, Cat. No. 15,615-9) was from Aldrich (Milwaukee, WI). *p*-Aminobenzoic acid (Cat. No. 01930-32) was obtained from Nacalai Tesque (Kyoto, Japan). Diethylenetriamine-*N,N,N′,N′′,N′′*-pentaacetic acid (DTPA, Cat. No. 347-01141) was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Acetonitrile was of high-performance liquid chromatography (HPLC) grade. All other chemicals were of analytical grade from Nacalai Tesque.

Instrumentation

The quantitative analyses were performed using a Hitachi HPLC system (Hitachi, Tokyo, Japan) that consisted of an L-6020 pump, an L-7300 column oven, and an L-7485 fluorescence detector in a D-2500 data station. A C-18 reversed phase column (COSMOSIL $5C_{18}$-AR-II, 5 μm, 250×4.6 mm, Cat. No. 38145-21, Nacalai Tesque) and a guard column filled with the same material (10×4.6 mm, Cat. No. 38141-61) were used.

Protocol

Synthesis of authentic AAS-ABA and GGS-ABA

The authentic AAS-ABA and GGS-ABA were synthesized from *N*α-acetyl-L-lysine and *N*α-acetyl-L-ornithine, respectively, by lysyl oxidase in an egg shell membrane [29,34]. The egg shell membrane isolated from fresh white leghorn hen eggs was thoroughly washed with distilled water and cut into small pieces (5×5 mm). After removal of the extra moisture on the egg shell membrane using filter paper, the egg shell membrane was weighed. *N*α-Acetyl-L-lysine (10 mM) and *N*α-acetyl-L-ornithine (10 mM) were individually incubated with the egg shell membrane (10 g) in 100 ml of 20 mM sodium phosphate

buffer, pH 9.0, at 37°C for 24 h with continuous shaking. After removal of the egg shell membrane by centrifugation, the reaction mixtures were adjusted to pH 6.0 by the addition of 1 M HCl. The resulting aldehydes were then reductively aminated with *p*-aminobenzoic acid and $NaBH_3CN$. After the addition of ABA (10 g, 73 mmol), $NaBH_3CN$ (5 g, 80 mmol) was slowly added to the reaction mixture with stirring. The mixture was allowed to react at 37°C for 20 h with stirring, followed by evaporation at 40°C in vacuo to dryness. A 6 M HCl (100 ml) was then slowly added to the residues, and the ABA derivatives of *N*α-acetyl-L-AAS and *N*α-acetyl-L-GGS were hydrolyzed for 10 h at 110°C to remove the acetyl group. The hydrolysates were evaporated at 40°C in vacuo to dryness. The resulting AAS-ABA and GGS-ABA were separated by silica gel column chromatography using ethyl acetate/acetic acid/distilled water (20/ 2/1, v/v/v) as the elution solvent. The eluates were fractionated and monitored by thin-layer chromatography (TLC). Silica gel TLC was conducted using an aluminum sheet precoated with Kieselgel 60 (Art. 5553, Merck, Darmstadt, Germany). The chromatogram was developed with ethyl acetate/acetic acid/water (8/1/1, v/v/v) as the solvent, and each spot on the plate visualized by spraying 0.2% ninhydrin in 90% ethanol and then heating at 100°C for 10 min. The R_f values of AAS-ABA and GGS-ABA were 0.53 and 0.50, respectively. As needed, each compound was further purified by preparative HPLC using a C-18 reversed phase column (LiChroprep, 30×240 mm, Lober, Merck, Darmstadt, Germany) with distilled water/acetonitrile/acetic acid (90/10/1) as the eluent at a flow rate of 3.0 ml/min, and monitoring the eluate at 250 nm. The purity and identity of each compound were confirmed using HPLC, MS, and NMR techniques. ^1H NMR spectra of each compound are shown in Fig. 2, and spectral data are as follows.

AAS-ABA: FAB MS 267 $(M+H)^+$; ^1H NMR (500 MHz, D_2O) δ = 1.60 (*m*, 2H, γ-CH_2), 1.85 (*m*, 2H, δ-CH_2) 1.98 (*m*, 2H, β-CH_2), 3.48 (*t*, 2H, *J* = 5.6 Hz, ε-CH), 4.05 (*t*, 1H, *J* = 5.5 Hz, α-CH), 7.70 (*d*, 2H, *J* = 8.5, Ar–H), 8.18 (*d*, 2H, *J* = 8.5, Ar–H).

GGS-ABA: FAB MS 253 $(M+H)^+$; ^1H NMR (500 MHz, D_2O) δ = 1.95 (*m*, 2H, γ-CH_2), 2.04 (*m*, 2H, β-CH_2) 3.52 (*t*, 2H, *J* = 5.6 Hz, δ-CH_2), 4.07 (*t*, 1H, *J* = 5.5 Hz, α-CH), 7.69 (*d*, 2H, *J* = 8.5, Ar–H), 8.11 (*d*, 2H, *J* = 8.5, Ar–H).

Derivatization and acid hydrolysis of proteins

Fig. 3 summarizes the sample preparation scheme for the analysis of AAS and GGS. At least 5 mg of proteins was required for the determination of the AAS and GGS in biological samples. The protein samples (e.g., 100 μl of plasma) were dialyzed at 4°C using a microdialysis cassette (e.g., Slide-A-Lyzer, Pierce, Rockford, IL) or an ultrafiltration device (e.g., Microcon YM, Millipore, Bedford, MA). The dialysis was continued with at least three changes in the phosphate-buffered saline (PBS) containing 1 mM DTPA and a final change in the 0.25 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1% (w/v) sodium dodecyl sulfate (SDS) and 1 mM DTPA. The plasma protein was then diluted with 0.25 M MES buffer (pH 6.0) containing 1% SDS and 1 mM DTPA to 300 μl. After the protein concentration was measured using a commercial kit, 250 μl of the protein solution was transferred to a vial, and 500 μl of 50 mM ABA in 0.25 M MES buffer (pH 6.0) was added. The reaction was started by the addition of 250 μl of freshly prepared 100 mM $NaBH_3CN$ in 0.25 M MES buffer (pH 6.0), and the mixture was allowed to react at 37°C for 90 min with shaking in the dark. After the reaction, the protein was precipitated by the addition of 500 μl of cold 50% (w/v) trichloroacetic acid (TCA). After standing for 10 min in an ice bath, the mixture was centrifuged at 17,000 *g* for 10 min at 4°C, and the pellet of the precipitated protein was separated. The pellet was twice washed with 1.0 ml of cold 10% (w/v) TCA and 1.0 ml of cold ethanol, and then

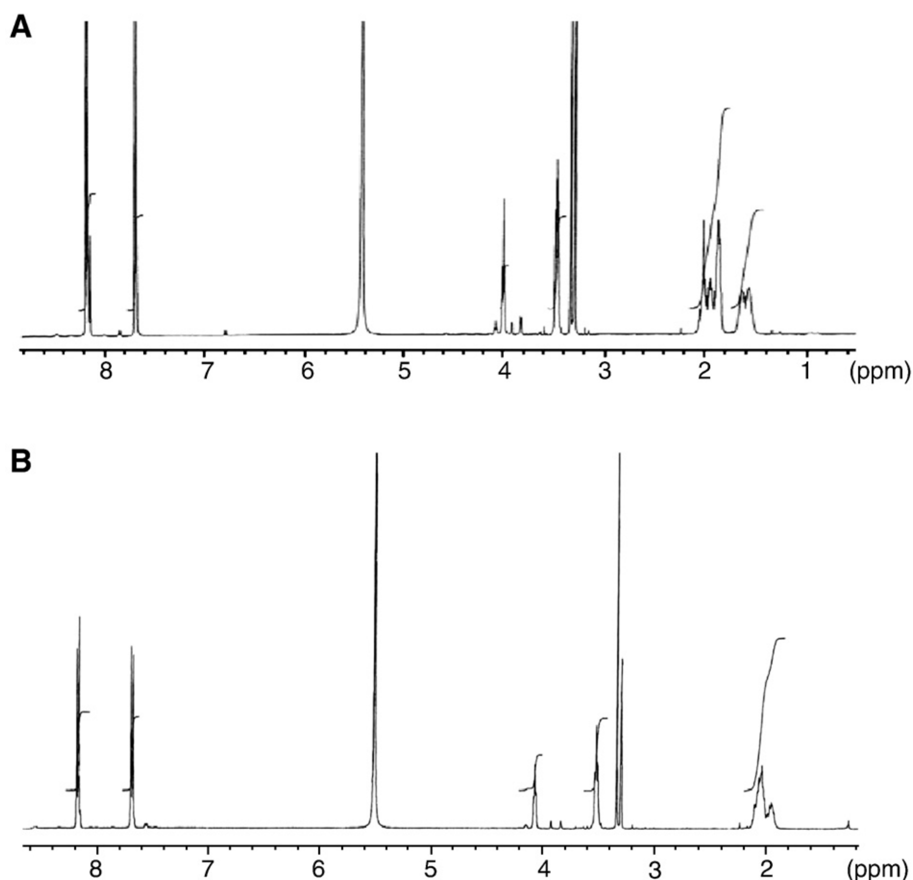

Fig. 2. NMR spectra of synthetic AAS-ABA (A) and GGS-ABA (B).

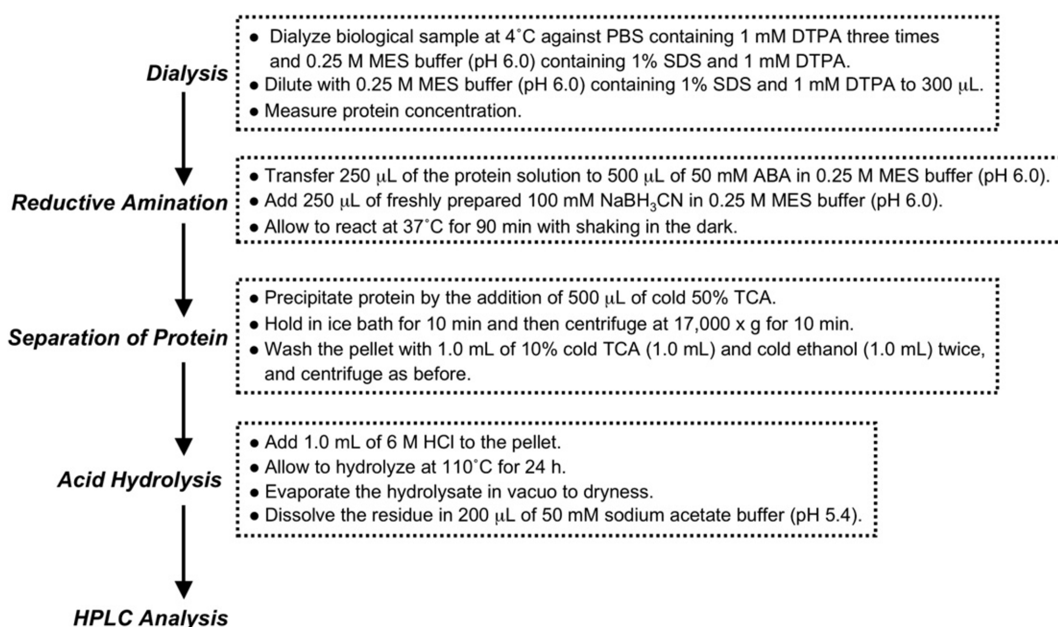

Fig. 3. Scheme for sample preparation in the analysis of AAS and GGS.

centrifuged as before. After the pellet was allowed to air dry, the resulting protein was hydrolyzed for 24 h at 110°C with 1.5 ml of 6 M HCl. The hydrolysate was evaporated to dryness at 40°C in vacuo followed by reconstitution in 200 µl of 50 mM sodium acetate buffer (pH 5.4). The sample was filtered using a PVDF syringe filter (0.45 µm pore size) and then stored at 4°C.

HPLC measurement of AAS-ABA and GGS-ABA

The sample (20 µl) was injected into an HPLC instrument, and eluted with 50 mM sodium acetate buffer (pH 5.4). The column oven was maintained at 40°C, and the flow rate is 1.5 ml/min. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. The components were identified by comparing the elution time to that of the reference compounds. The next sample was injected when the baseline had restabilized.

Calibration procedure

The stock solutions of the reference standards were prepared in methanol, and stored in -20°C. Working standards of the analytes were obtained by dilution with 50 mM sodium acetate buffer (pH 5.4). Calibration curves were obtained for the authentic standards by plotting the peak area versus concentration using a linear regression analysis. Correlation coefficients greater than 0.999 were obtained.

Calculations and expected results

The HPLC analysis of the synthetic GGS-ABA and AAS-ABA showed one single peak at R_t of 7.0 and 20.2 min in each chromatogram (Figs. 4A and B), and the quantification limits of the GGS-ABA and AAS-ABA were approximately 4 and 10 fmol at the signal-to-noise ratio of 10, respectively. Using this method, the peaks of GGS-ABA and AAS-ABA can be detected in human plasma protein (Fig. 4C). We examined the effect of reaction time on derivatization of AAS and GGS in BSA by reductive amination with ABA and $NaBH_3CN$. As shown in Fig. 5, the derivatization was essentially complete after 90 min. We confirmed that the derivatization of AAS and GGS was sufficient for the reaction with 25 mM ABA and 25 mM $NaBH_3CN$ in 0.25 M MES buffer (pH 6.0) containing 0.5% SDS at 37°C for 90 min as described under Protocol. Using this method, the concentrations (mean±SD) of both aldehydic residues in the plasma protein from healthy subjects (n=6) were

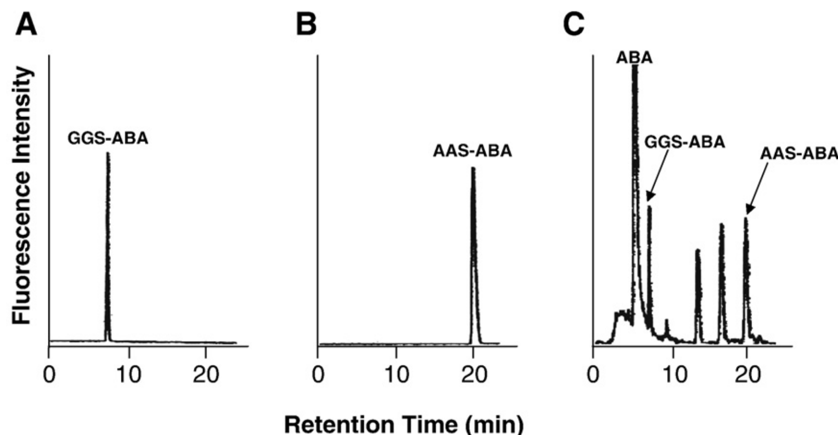

Fig. 4. Determination of AAS and GGS by fluorescent HPLC. Human plasma protein was derivatized by reductive amination with ABA and $NaBH_3CN$. After acid hydrolysis, GGS-ABA and AAS-ABA were analyzed by a fluorometric HPLC with detection at Ex 283 nm/Em 350 nm. (A) GGS-ABA, (B) AAS-ABA, and (C) human plasma protein. Reproduced with permission from *Chem. Res. Toxicol*. 19:1059–1065 [29]. Copyright 2006 American Chemical Society.

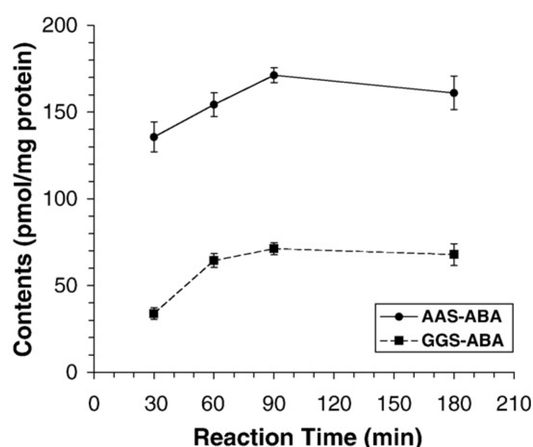

Fig. 5. Effect of reaction time on derivatization of AAS and GGS by reductive amination with ABA and $NaBH_3CN$. BSA was derivatized by reductive amination with ABA and $NaBH_3CN$ for 30–180 min at 37°C. After acid hydrolysis, GGS-ABA and AAS-ABA were analyzed by a fluorometric HPLC. Data are expressed as means ± SD (n = 3).

estimated at 11.0 ± 0.5 pmol/mg protein of GGS and 21.0 ± 2.1 pmol/mg protein of AAS. The concentrations (mean ± SD) of GGS and AAS in commercial BSA were 62.1 ± 2.1 pmol/mg protein (5.21 ± 0.15 mmol/mol) and 172.9 ± 4.0 pmol/mg protein (10.06 ± 0.23 mmol/mol) with a coefficient of variation of 3.4 and 2.3% for five repeated measurements, respectively. The intraassay coefficients of variation for five repeated measurements of GGS and AAS were also established with a rat plasma sample and were of 7.7 and 6.3% at a level of 32.5 and 334.4 pmol/mg protein, respectively.

Discussion

The quantitative and analytical importance of protein carbonyls has prompted the development of methods to analyze specific carbonyl residues because of an understanding of their chemical nature, formation pathway, and distribution level in vivo. Previously, Requena et al. have reported a method based on gas chromatography/mass spectrometry (GC/MS) for the determination of AAS and GGS after their reduction with sodium borohydride ($NaBH_4$) to hydroxyaminocaproic acid (HACA) and hydroxyaminovaleric acid (HAVA), respectively [25]. The use of deuterated internal standards is essential for the quantitative determination by this method because both HACA and HAVA are extensively converted to chloro-derivatives during acid hydrolysis with HCl, and HAVA is partially reverted to proline. After hydrolysis in the presence of deuterated internal standards, hydrolysate is treated to convert to HACA, HAVA, and their deuterated counterparts to volatile trifluoroacetyl-methyl ester derivatives which are then analyzed by GC/MS. A time-consuming and multiple-stage procedure is required for the determination by this method. Furthermore, distinct synthesis of deuterated HACA and HAVA is quite complicated. Another derivatizing method based on reductive amination of AAS and GGS with fluoresceinamine and $NaBH_3CN$ has also been reported [28]. Nevertheless, the resulting fluoresceinamine derivatives are unfortunately degraded to nonfluorescent decarboxylated derivatives by acid hydrolysis. In this paper, we documented a novel specific procedure to quantitate AAS and GGS after their derivatization by reductive amination with ABA and $NaBH_3CN$ under mild conditions. It is noteworthy that the resulting derivatives were entirely stable during acid hydrolysis with HCl. Thus, the mild one-pot derivatizing procedure and tolerance of derivatives to acid hydrolysis allowed the reliable and accurate quantification of both semialdehydes. The contents of AAS and GGS in native BSA determined by this method are in good agreement with the contents reported by Requena et al. [25]. Additionally, the fluorescent derivatives can be detected sensitively and rapidly by a fluorometric HPLC. The femtomole quantification limit of the present method is comparable to that of the GS/MS method [35]. Another merit of the method described here is that the authentic compounds as standards can be easily prepared from commercially available chemicals using lysyl oxidase immobilized in egg shell membrane. Accordingly, the present method is considered to be more reliable, simple, and available than other published methods. This fluorescent labeling of protein-associated aldehyde moieties is a sensitive and specific technique that may be widely used to reveal increased levels of oxidatively modified proteins with reactive oxygen species during aging and disease.

Caveats

$NaBH_3CN$ is highly toxic: Avoid skin contact, do not inhale, and use a chemical fume hood when carrying out reductive amination reaction.

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