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Determination of plasma thiol bound to albumin using affinity chromatography and high-performance liquid chromatography with fluorescence detection: Ratio of cysteinyl albumin as a possible biomarker of oxidative stress

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Abstract

We examined the influence of oxidative stress on the relative amounts of various albumin-bound thiols in human plasma. To determine the ratio of thiols existing as mixed disulfides following oxidation, we developed a method combining fast purification of albumin using affinity columns and high-performance liquid chromatography (HPLC) with fluorescence detection for low molecular weight thiols which were labeled after reduction.

When the effect of exposure of plasma to radical oxygen species on binding of thiols to albumin was determined by the present method, significant increases in the ratio of cysteine bound to albumin (Alb-Cys) to total cysteine were clearly demonstrated.

Keywords: Oxidative stress; Albumin mixed disulfide; Reactive oxygen species; Human plasma; Cysteinyl albumin; *tert*-Butyl hydroperoxide

1. Introduction

Serum albumin is the most abundant protein in plasma. Its physiological roles include maintenance of colloid osmotic pressure and transport of various ligands. In addition, human serum albumin (HSA) may function as an extracellular antioxidant by scavenging reactive oxygen species (ROS) [1], associated with oxidation of the free Cys-34 residue of HSA, which comprises approximately 80% of the total free thiols in plasma [2]. Davies et al. have clearly shown oxidative modification of the amino acids of bovine serum albumin [3], with particular susceptibility of tryptophan, tyrosine, histidine, and cysteine residues to oxidative stress following exposure to hydroxyl radicals and hydroxyl radicals containing superoxide anions. Intensive investigation has since revealed oxidation of the cysteine residue of purified serum albumin by various ROS [4–7]. These results indicate that the Cys-34 residue of albumin reacts with peroxides. Alternatively, some researchers have proposed that thiol level might be a marker of oxidative stress [8,9] Thus, we focused on alterations involving Cys-34 of HSA following ROS exposure in the present study, since the oxidized form of Cys-34 of albumin is not well understood.

There is evidence to suggest that freshly isolated albumin is heterogeneous with respect to its thiol content, typically ranging from 0.6 to 0.7 SH/HSA (a number of free thiol residue per molecule of HSA) [10]. Although the distribution and alteration of thiols in albumin mixed disulfide (AMD) in human plasma remains unclear, Era et al. suggest that the ratio of AMDs to total albumin might increase with aging [11].

Abbreviations: ABD-F, 4-fluoro-7-sulfamoyl benzofurazan; Alb-Cys, cysteinyl albumin; Alb-CysGly, cysteinylglycinyl albumin; AMD, albumin mixed disulfide; *t*-BuOOH, *tert*-butyl hydroperoxide; CysGly, cysteinylglycine; DTNB, 5,5′-dithio-bis (2-nitro benzoic acid); EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′- (2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; HSA, human serum albumin; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCEP, tris(carboxyethyl)phosphine

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We thus investigated AMD formation upon exposure to ROS in order to assess whether low-molecular weight thiols bound to albumin might be a marker of oxidative stress in human plasma. To analyze AMD formation in human plasma, we developed a method involving rapid purification of albumin using two affinity columns with simultaneous high-performance liquid chromatography (HPLC) determination of low molecular weight thiols covalently bound to the Cys-34 residue of albumin *via* disulfide bonds. We used this method to identify the formation of low molecular weight thiols bound to albumin in plasma during exposure of human plasma to *tert*-butyl hydroperoxide (*tert*-BuOOH), hydrogen peroxide, and peroxynitrite, using the present method.

2. Experimental

2.1. Chemicals

The blue-Sepharose (HiTrap Blue HP; 1 ml) and heparin-Sepharose columns (HiTrap Heparin HP; 1 ml) used in this experiment were purchased from Amersham Bioscience (NJ, USA). *N*-(2-hydroxyethyl)-piperazine-*N*′- (2-ethanesulfonic acid) (HEPES), ascorbic acid, and *tert*-BuOOH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-Fluoro-7-sulfamoyl benzofurazan (ABD-F), and tris(carboxyethyl) phosphine (TCEP) were obtained from Wako Pure Chemical Co. (Osaka, Japan).

2.2. Oxidation of human plasma exposed to ROS

Fresh human blood was obtained from 19 healthy subjects (aged 22–50) with their informed consent. Blood was drawn into vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) and centrifuged immediately at $1000 \times g$ for 15 min at 4 °C. Plasma was incubated in a water bath at 37 °C under aerobic conditions with various concentrations of *tert*-BuOOH (final concentration: 0.1–2.0 mM), 0.5 mM hydrogen peroxide, or 0.5 mM peroxynitrite for the indicated time periods.

2.3. Differential determination of protein bound and free thiols in plasma

Untreated normal plasma samples were assayed immediately for determination of thiol content. 0.5 mM *tert*-BuOOH-treated plasma was prepared as described above. Samples for determination of total thiol content were reduced and directly labeled with ABD-F. Free thiol concentrations were determined after removing protein by ultrafiltration using a filtration tube (cutoff size; 10,000, BIOMAX-10, Millipore, Japan).

2.4. Rapid purification of albumin from human plasma

HSA was purified from plasma using a fast flow heparin-Sepharose column and a blue-Sepharose column [12]. After various lengths of incubation at 37 °C with different peroxide concentrations, samples of plasma were immediately diluted by equal volumes of cold and N_2-purged 0.1 M HEPES buffer (pH 7.0), containing 1 mM EDTA and 2 mM ascorbic acid (Buffer A). After this, 0.4 ml of diluted plasma was applied to a heparin-Sepharose cartridge column (1 ml) connected to a blue-Sepharose cartridge column (1 ml). After 1.0 ml of Buffer A was applied to the connected affinity columns, the heparin-Sepharose column was detached. Following stepwise elution of 4.0 ml of Buffer A from the blue-Sepharose column (fraction 1–8; 0.5 ml/tube), 3.5 ml of 1.5 M NaCl containing Buffer A from same column was obtained (fraction 9–15; 0.5 ml/tube). Each fraction was assayed for the presence of low molecular weight thiols by the method presented below.

2.5. Development of an assay of low molecular weight thiols in plasma

Fifty microliters of plasma or fractionated solution was added to 150 μl of 0.1 M borate buffer (pH 10.5) containing 2.5 mM TCEP and 5.5 mM ABD-F, followed by incubation for 5 min at 60 °C. Twenty-five microliters of 5 M $HClO_4$ was added to the mixture in order to remove protein and the mixture was incubated for 10 min at room temperature. After centrifugation at $12{,}000 \times g$ for 10 min at 4 °C, 10 μl of supernatant from the incubation mixture was injected into a reverse phase column (4.6 mm × 250 mm, COSMOSIL 5C18-MS, Nacalai Tesque, Kyoto, Japan) pre-equilibrated with mobile phase solution containing acetonitrile-sodium acetate (0.1 M) at pH 3.8 (5:95). The chromatographic system consisted of L-6000 pump (Hitachi, Tokyo, Japan), an AS 8000 autosampler (Tosoh, Tokyo, Japan), a L-2500 integrator (Hitachi) and L-7480 fluorescence detector (Hitachi). Standard curves were produced from stock solutions of low molecular weight thiols using concentrations ranging from 1 to 500 μM. A flow rate of 1.0 ml/min was used with a running time of 17 min. Retention times and peak areas were monitored at excitation and emission frequencies of 380 and 510 nm, respectively. Thiol concentrations were measured by extrapolation of the area values from each run on the calibration curves.

2.6. Determination of the ratio of low molecular weight thiols bound to albumin via disulfide bonds

The thiol concentrations of each fraction eluted during blue-Sepharose column chromatography were determined by HPLC with fluorescence detection as described above. Incubation of 0.1 ml of fractionated solution with TCEP to reduce disulfide bonds was followed by labeling of the thiol residues with ABD-F. The ratio of each thiol existing as AMD was calculated by dividing the amount of each thiol recovered from the albumin fractions (4.0–5.5 ml) to that recovered from all the fractions (0–7.5 ml).

2.7. Other method

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli [13].

2.8. Statistics

Each experiment was performed at least four times, and the results expressed as means ± S.D. Data were analyzed by an analysis of variance (ANOVA). Post-hoc comparisons of means between groups were performed using Bonferroni's correction with a significance level of 0.05.

3. Results

3.1. Calibration curves and precision

To study the distribution of AMDs, we devised a new HPLC method for determination of low molecular weight thiols in plasma capable of forming AMDs using ABD-F [14] as the pre-labeling agent and TCEP as the reducing agent.

An isocratic solvent system enabled sufficient separation of four thiols on a C_{18} column. Retention times for cysteine (6.7 min), CysGly (7.9 min), homocysteine (9.8 min), and GSH (14.8 min) are shown in Fig. 1.

The calibration curves for cysteine, CysGly, homocysteine and GSH were linear over the range of 1.0–100 μM, with a correlation coefficients of 0.999, 0.998, 0.999 and 0.999, respectively. The minimum detectable levels were 2 pmol for CysGly, homocysteine, GSH and 10 pmol for cysteine with signal to noise ratio of 3, per 10 μl injection volume. The within-run precision (C.V., $n = 5$) of the assay of cysteine, CysGly, homocysteine and GSH in human plasma was 2.3%, 3.4%, 1.9% and 2.3%, respectively. The between day precision were C.V. < 3.0% for each thiol with analysis of human plasma, repeated for three different days. Recovery experiments were carried out by adding known amounts of cysteine ($n = 5$, at 20 μM), CysGly, homocysteine, and GSH ($n = 5$, 5 μM each) from human plasma were 97.4 ± 3.6, 103.5 ± 3.8, 96.7 ± 2.5, and 100.2 ± 2.2, respectively. Under these conditions, the ABD derivatives in the final mixture were stable for at least 24 h at room temperature.

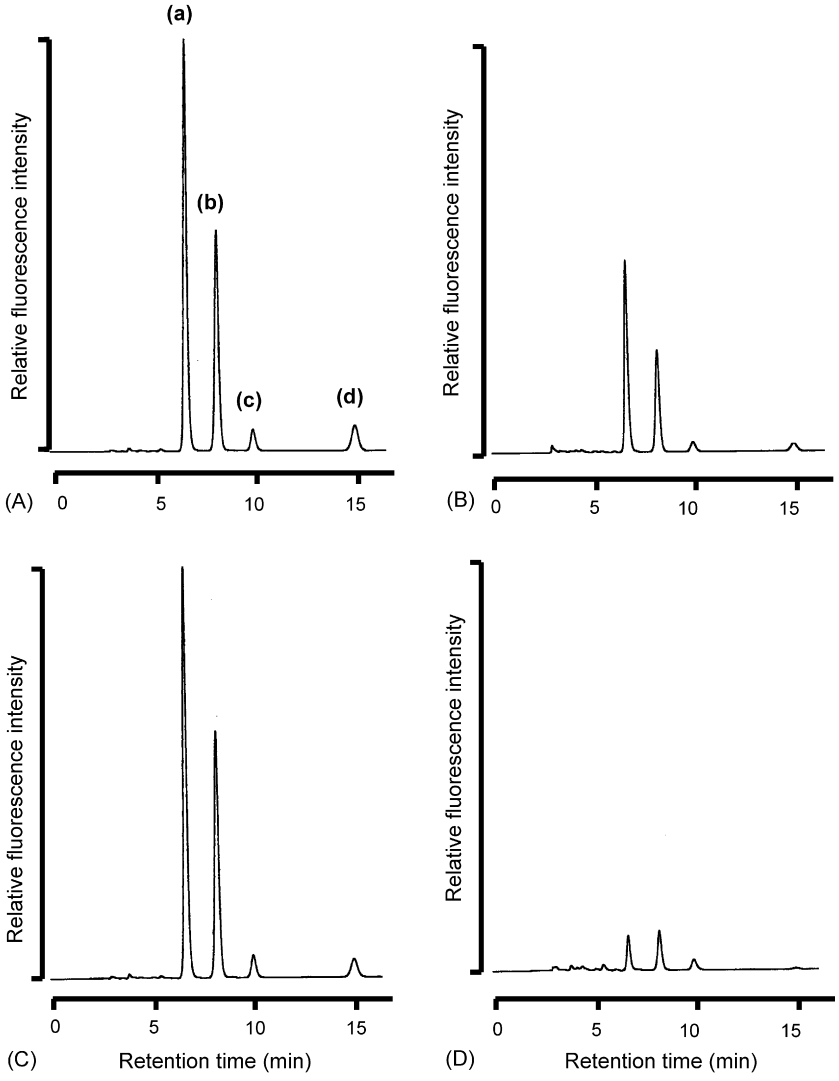

Fig. 1. Chromatograms of thiols isolated from untreated normal plasma or plasma treated with *tert*-BuOOH. (A) Total thiol concentrations in the absence of 0.5 mM *tert*-BuOOH treatment; (B) free thiol concentrations in the absence of 0.5 mM *tert*-BuOOH treatment; (C) total thiol concentrations in plasma treated with 0.5 mM *tert*-BuOOH; (D) free thiol concentrations in plasma treated with 0.5 mM *tert*-BuOOH. Total thiol and free thiol concentrations were determined as described in Section 2. Peaks: cysteine (a), cysteinylglycine (b), homocysteine (c), and glutathione (d).

Table 1
Total concentration of thiol compounds in human plasma

	Concentration[a] (μM)	Range
Cysteine	205.7 ± 18.0	170.3–243.0
Cysteinylglycine	28.5 ± 6.7	19.5–45.1
Homocysteine	9.2 ± 1.9	6.2–12.0
Glutathione	5.8 ± 1.5	3.6–8.6

[a] $n = 12$; values are expressed as means ± S.D.

As shown in Table 1, under optimal conditions, the plasma thiol concentrations determined using our method approached those of previous experiments using other methods [15,16].

3.2. Determination of protein-bound and free thiols in plasma

When plasma thiol concentrations were measured after (Fig. 1B), or in the absence of (Fig. 1A), ultracentrifugation to remove protein, there were obvious differences in the recovery of each plasma thiol. A comparison of Fig. 1A and 1B illustrates that about 60% of plasma thiols were bound to protein with the plasma of normal subjects. Reduced concentrations of free cysteine and CysGly were detected in plasma after *tert*-BuOOH exposure and ultracentrifugation (Fig. 1D), compared with untreated normal plasma (Fig. 1B). Thus, it was shown that the ratios of protein-bound versus total thiols increased in plasma following *tert*-BuOOH exposure. In the absence of ultracentrifugation following *tert*-BuOOH exposure, marked differences in the thiol recovery were not observed (Fig. 1C). These results indicate that the oxidized low molecular weight thiols are reducible by TCEP after exposure of 0.5 mM *tert*-BuOOH.

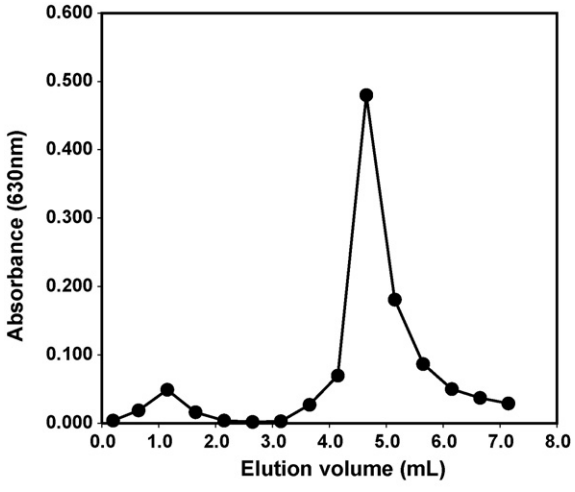

Fig. 2. Elution pattern of albumin during affinity column chromatography. Plasma samples were diluted with equal volumes of Buffer A and 0.4 ml of each sample solution was applied to the heparin-Sepharose column connected with the blue-Sepharose column, as described in Section 2. Albumin was detected by the colorimetric assay with BCG at 630 nm. The results of one representative experiment ($n = 3$) are shown.

3.3. Determination of albumin-bound plasma thiols

To identify the low molecular weight thiols bound to albumin, we examined the conditions to purify HSA from plasma using affinity column chromatography. Each plasma sample was then subjected to heparin-Sepharose and blue-Sepharose column chromatography in order to separate albumin from the other plasma components. A peak representing albumin was observed in the fractions eluted from the blue-Sepharose column (Fig. 2). Albumin was eluted in fractions from 4.0 to 5.5 ml (the main albumin fraction was 4.5–5.0 ml) during this stepwise separation. It was confirmed by SDS-PAGE that a small peak fraction (1.0–1.5 ml) did not contain albumin. Fig. 3 shows the results of SDS-PAGE demonstrating the purity of albumin in the fractions obtained following blue-Sepharose and heparin-Sepharose column chromatography. It is likely that the free low molecular weight thiols and disulfides, as well as most of the protein bound

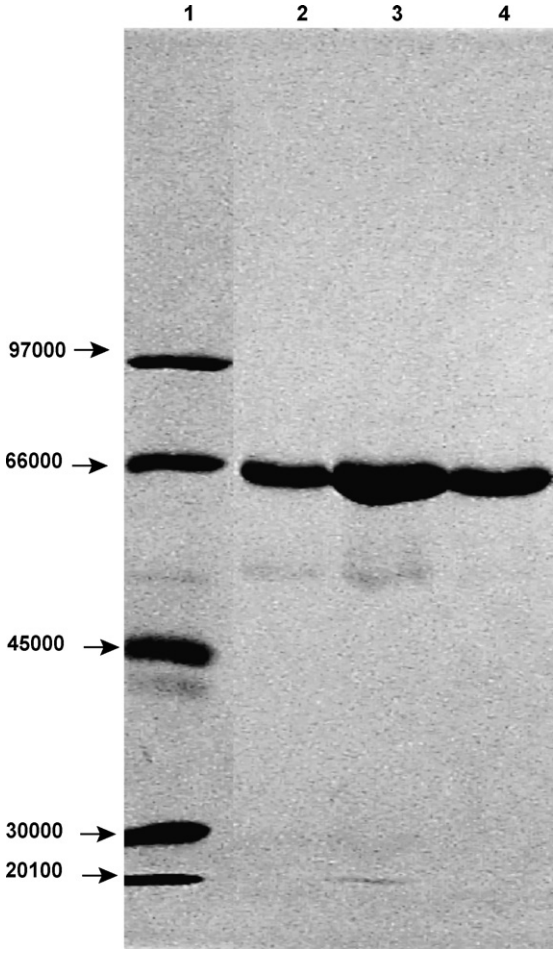

Fig. 3. Albumin fractions from blue-Sepharose column chromatography. Plasma samples were diluted with equal volumes of Buffer A and 0.4 ml of each sample solution was applied to the heparin-Sepharose column connected with the blue-Sepharose column, as described in Section 2. Each fraction was diluted four-fold with Buffer A, and 2 μl of the sample solution was applied to SDS-PAGE. The electrophoretic patterns of the albumin-containing fractions (4.0–5.5 ml; 0.5 ml/tube) are shown in lanes 2–4. Molecular masses in kDa (lane 1) are shown on the left as indicated. Protein was separated using a 12% (w/v) gel and visualized by Coomassie Blue R-250 staining.

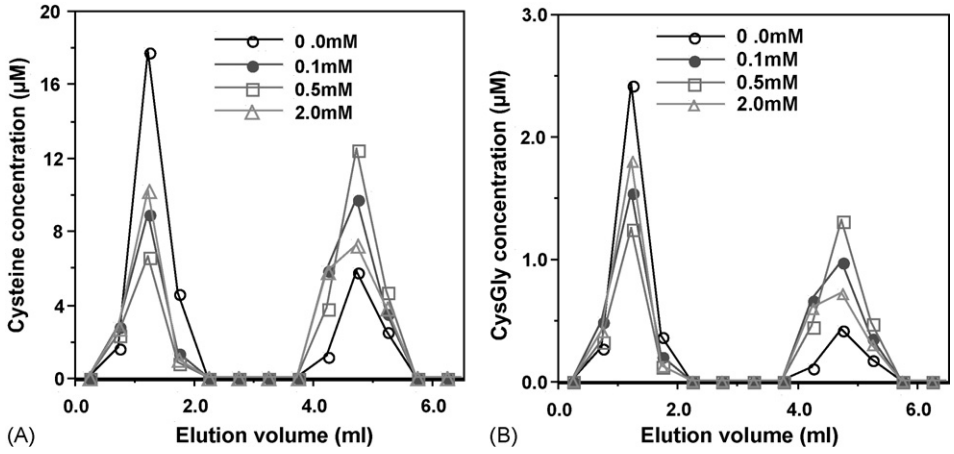

Fig. 4. Effects of various *tert*-BuOOH concentrations on the elution patterns of cysteine (A) and CysGly (B) during blue-Sepharose column chromatography. Plasma incubated with 0, 0.1, 0.5, and 2 mM *tert*-BuOOH for 120 min, was diluted with equal volumes of Buffer A. Then, 0.4 ml of each solution was subjected to affinity column chromatography, after which thiol concentrations were determined in all eluted fractions from the blue-Sepharose column. The results of one representative experiment ($n = 3$) are shown.

thiols, apart from the AMDs, were eluted in the flow-through fractions (0.5–2.0 ml).

3.4. AMD formation following exposure of plasma to tert-BuOOH

We next examined the dose–response of AMD formation. Upon analysis of the albumin fractions eluted from the blue-Sepharose column, cysteine and CysGly, but not homocysteine and GSH, could be quantified since homocysteine and GSH were present at concentrations below the quantification limits.

As shown in Fig. 4, two peaks representative of cysteine and cysteinylglycine were observed in fractions obtained by affinity column chromatography. As shown in Fig. 2, the albumin-containing fractions were from 4.0 to 5.5 ml. Free low molecular weight thiols, as well as disulfides and mixed disulfides, were all eluted in the flow-through fractions (0.5–2.0 ml). The amounts of cysteine (Fig. 4A) and CysGly (Fig. 4B) eluted in the flow-through fractions decreased following incubation with *tert*-BuOOH, while amounts of cysteine and CysGly within the albumin fractions increased. Maximum Alb-Cys and Alb-CysGly formation was observed following treatment with 0.5 mM *tert*-BuOOH, as opposed to 2 mM *tert*-BuOOH. Further, an increase in Alb-Cys was also observed following treatment with 0.1 mM *tert*-BuOOH, compared to untreated normal plasma.

3.5. Alteration of the ratio of albumin-bound thiols in plasma following exposure to tert-BuOOH

Fig. 5 demonstrates changes in the ratios of AMD to total cysteine or CysGly following exposure of human plasma to *tert*-BuOOH. Ratios of Alb-Cys and Alb-CysGly to total cys-

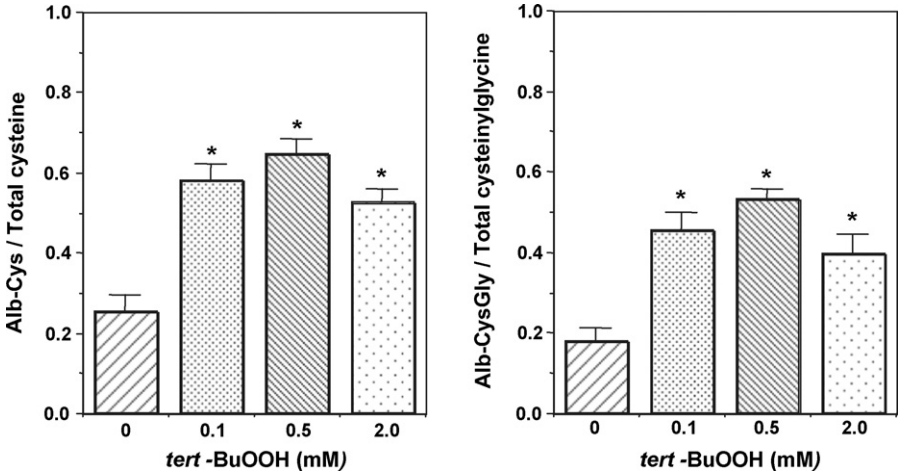

Fig. 5. Alterations in the ratio of Alb-Cys (A) and Alb-CysGly (B) to total amount of each thiol following *tert*-BuOOH exposure. Plasma was oxidized by exposure to various concentration of *tert*-BuOOH for 120 min at 37 °C. Total amounts of cysteine and CysGly were calculated by summing the amounts detected within all fractions eluted from the blue-Sepharose column. Total amounts of cysteine or CysGly bound to albumin were then determined by summing the amounts of each detected within the albumin fractions (4.0–5.5 ml). The ratios of Alb-Cys and Alb-CysGly to total cysteine or CysGly were calculated by dividing the amount of cysteine or CysGly detected within the albumin fractions (4.0–5.5 ml) by the total amount of cysteine and CysGly recovered from the blue-Sepharose column. All data are expressed as means ± S.D. ($n = 4$).

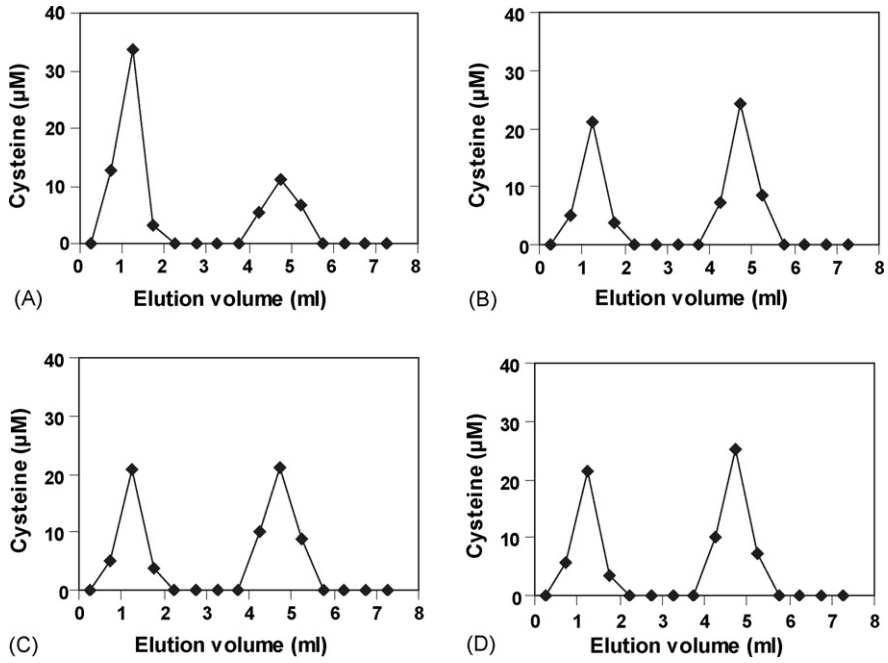

Fig. 6. Elution patterns of plasma cysteine during affinity column chromatography after exposure to various ROS. Cysteine concentrations in all eluted fractions of plasma incubated for 60 min at 37 °C (A), or plasma treated for 60 min at 37 °C with 0.5 mM *tert*-BuOOH (B), peroxynitrite (C), or hydrogen peroxide (D), from the blue-Sepharose column are shown. Results from one representative experiment ($n = 3$) are shown.

teine or CysGly were calculated by dividing the amount of cysteine or CysGly detected in the albumin fractions by the total amounts recovered from the affinity column. Recovery yields of 91% ($n = 3$) and 89% ($n = 3$) were obtained for cysteine and CysGly, respectively, in this separation procedure. Significantly increased ratios of Alb-cysteine (Fig. 5A) and Alb-CysGly (Fig. 5B) to total cysteine and total CysGly were observed following exposure to 0.1-2.0 mM *tert*-BuOOH, compared to control samples lacking *tert*-BuOOH exposure ($p < 0.05$). Ratios of Alb-Cys to total cysteine following exposure of albumin fractions to 0, 0.1, 0.5, and 2.0 mM *tert*-BuOOH were 0.255 ± 0.039, 0.584 ± 0.039, 0.650 ± 0.035, and 0.528 ± 0.031, respectively. Ratios of Alb-CysGly to total CysGly following exposure of albumin fractions to 0, 0.1, 0.5, and 2.0 mM *tert*-BuOOH were 0.183 ± 0.028, 0.456 ± 0.045, 0.533 ± 0.024, 0.397 ± 0.047, respectively.

3.6. Effect of hydrogen peroxide and peroxynitrite exposure on the ratio of Alb-Cys in plasma

Alterations in the ratio of Alb-Cys to total cysteine were also observed following exposure of plasma to peroxynitrite and hydrogen peroxide, as shown in Fig. 6. Elution patterns clearly shift in the elution of cysteine from the flow-through to the albumin-containing fractions, following exposure to 0.5 mM peroxynitrite (Fig. 6C) and 0.5 mM hydrogen peroxide (Fig. 6D), as well as 0.5 mM *tert*-BuOOH (Fig. 6B).

4. Discussion

The results of a previous study suggest that protein sulfhydryl groups contribute significantly to the antioxidant capacity of plasma [17]. Frei et al. demonstrated that exposure of plasma to aqueous peroxyl radicals generated at a constant rate led immediately to oxidation of endogenous ascorbate and sulfhydryl groups [18]. These results suggest that oxidation of the Cys-34 of albumin by ROS might also serve an antioxidant function, since the free cysteine residue of albumin constitutes approximately 80% of the sulfhydryl groups in plasma.

Although there is some suggestion that AMDs are endogenous in plasma [11,19], normal background free and albumin-bound thiol concentrations remain to be established. Further, binding and redox status of thiols with the loading of oxidative stress on AMD formation is unclear so far. Carballal et al. demonstrated formation of albumin sulfenic acid upon exposure of purified HSA to hydrogen peroxide and peroxynitrite, and proposed that albumin sulfenic acid serves as an intermediate in the formation of low molecular weight disulfides and mixed HSA disulfides [2].

Sogami and co-workers developed a HPLC method by which to separate reduced and oxidized HSA at the Cys-34 residue [11,20–22]. This method separates Cys-34 free albumin from other oxidized forms of albumin, including AMDs, using a gel filtration column chromatography system monitored at 280 nm, in which plasma is directly applied to the column. This allows alterations in albumin to be roughly detected by differences in the elution patterns of Cys-34 free and oxidized albumin. However, details regarding the composition of eluted AMDs were not provided in their research. In addition, Mansoor et al. suggested that cysteine in plasma from patients with homocystiuria was moderately increased, whereas protein-bound cysteine and cystine were below normal subjects [23]. Thus, analysis of thiols bound to albuminin in plasma has been required in various clinical studies.

To investigate the nature of AMDs formed upon exposure to ROS in this study, we developed a method by which to combine the separation of albumin using fast cartridge columns with a HPLC method using fluorescence detection to determine the composition of low molecular weight thiols covalently bound to albumin. We also used this method to estimate the possibility that a particular AMD might be a marker of oxidative stress in human plasma.

Although γ-glutamylcysteine was not detected, cysteine, CysGly, homocysteine, and GSH, were all detected in plasma. Quantification of minimal thiol concentrations (μM) is possible using water-soluble phosphine as a reducing agent in relatively brief procedures. Low molecular weight thiols in the fractions obtained from affinity column chromatography were able to be stably stored at −85 °C for at least one month in this experiment. After centrifugation to obtain plasma, it was important to separate albumin from low molecular weight thiols and disulfides as soon as possible. Because auto-oxidation of the free Cys-34 of albumin is achieved more easily *in vitro*, oxidation is more of a problem during procedures requiring more than 30 min, such as dialysis, freezing and column chromatography. In this experiment, we used fast cartridge columns and N_2-purged eluent containing ascorbate, resulting in more than 89% recovery of thiol residues within 20 min during the purification procedure of albumin from plasma.

Using the results indicating dose-dependent changes in Alb-Cys and Alb-CysGly following exposure to *tert*-BuOOH, we calculated the ratios of Alb-Cys to total cysteine and Alb-CysGly to total CysGly. Both ratios significantly increased upon exposure to 0.1, 0.5 and 2.0 mM *tert*-BuOOH for 120 min. Notably, on the mild oxidation (0.1 mM *tert*-BuOOH), Alb-Cys and Alb-CysGly ratios were approximately 2.3-fold and 2.5-fold the levels observed in untreated control samples. In the present study, we observed relatively close ratios of Alb-Cys to total cysteine in normal human plasma ($n = 19$, 0.23–0.29). Alternatively, Beck et al. used mass spectrometry to show that 25% of HSA exists as Alb-Cys in freshly collected normal plasma [24]. These findings suggest that Alb-Cys is a suitable constituent to show the oxidative status because of its homeostasis in human plasma.

Furthermore, we also demonstrated an increased ratio of Alb-Cys to total cysteine on exposure of plasma to peroxynitrite and hydrogen peroxide. These results suggest that Alb-Cys formation is induced by exposure of normal human plasma to various ROS, and that it is possible to estimate oxidative stress by observing the elution pattern of cysteine using affinity column chromatography.

Interestingly, Alb-Cys cannot be reduced using a glutaredoxin (thiol transferase) system [25]. Therefore, Alb-Cys formed in plasma may circulate in the blood stream as a secondary product of oxidation for an extended period of time.

5. Conclusions

The developed chromatographic method is useful to investigate AMDs formation upon exposure to ROS. This method combining fast purification of albumin using affinity columns and HPLC with fluorescence detection for low molecular weight thiols is relatively simple to perform and yields accurate results. The results obtained in the present study clearly demonstrate an increase in Alb-Cys and Alb-CysGly following exposure of plasma to relatively low concentrations of ROS. In particular, the oxidized product Alb-Cys might be used to monitor oxidative stress in humans in the future. To estimate the oxidative status, the value of cysteine existing as AMD should always be expressed as a ratio to the total amount of cysteine detected, since cysteine and other thiol levels differ among individuals, as shown in Table 1. Further research is in progress find the alterations of the Alb-Cys ratio under oxidative stress *in vivo*.

References

[1] B. Halliwell, Biochem. Pharmacol. 37 (1988) 569.
[2] S. Carballal, R. Radi, M.C. Kirk, S. Barnes, B.A. Freeman, B. Alvarez, Biochemistry 42 (2003) 9906.
[3] K.J. Davies, M.E. Delsignore, S.W. Lin, J. Biol. Chem. 262 (1987) 9902.
[4] P. Di Simplicio, K.H. Cheeseman, T.F. Slater, Free Rad. Res. Commun. 14 (1991) 253.
[5] J.W. Finch, R.K. Crouch, D.R. Knapp, K.L. Schey, Arch. Biochem. Biophys. 305 (1993) 595.
[6] B.L. Alvarez, G. Ferrer-Sueta, B.A. Freeman, R. Radi, J. Biol. Chem. 274 (1999) 842.
[7] E. Bourdon, N. Loreau, L. Lagrost, D. Blache, Free Rad. Res. 39 (2005) 15.
[8] K. Kadota, Y. Yui, R. Hattori, C. Kawai, Jpn. Circul. J. 55 (1991) 937.
[9] G.J. Quinlan, T.W. Evans, J.M.C. Gutterridge, Free Rad. Res. 20 (1994) 289.
[10] J. Janatova, J.K. Fuller, M.J. Hunter, J. Biol. Chem. 243 (1968) 3612.
[11] S. Era, K. Kuwata, H. Imai, K. Nakamura, T. Hayashi, M. Sogami, Biophys. Biochim. Acta 1247 (1995) 12.
[12] I. Harrison, D. Littlejohn, G.S. Fell, Analyst 121 (1996) 189.
[13] U.K. Laemmliem, Nature 227 (1970) 680.
[14] K. Imai, T. Toyo'oka, Methods Enzymol. 143 (1987) 67.
[15] A. Andersson, A. Isaksson, L. Brattstrom, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[16] J.-F. Salazar, H. Schorr, B. Herbeth, G. Siest, P. Leroy, J. Chromatogr. Sci. 37 (1999) 469.
[17] D.D.M. Wayner, G.W. Burton, K.U. Ingold, L.R.C. Barclay, S.J. Locke, Biochim. Biophys. Acta 924 (1987) 408.
[18] B. Frei, R. Stocker, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9748.
[19] S. Sengupta, H. Chen, T. Togawa, P.M. DiBello, A.K. Majors, B. Budy, M.E. Ketterer, D.W. Jacobsen, J. Biol. Chem. 276 (2001) 30111.
[20] E. Suzuki, K. Yasuda, N. Takeda, S. Sakata, S. Era, K. Kuwata, M. Sogami, Diab. Res. Clin. Pract. 18 (1992) 153.
[21] A. Hayakawa, K. Kuwata, S. Era, M. Sogami, H. Shimonaka, M. Yamamoto, S. Dohi, H. Hirose, J. Chromatogr. B Biomed. Sci. Appl. 698 (1997) 27.
[22] T. Hayashi, H. Imai, K. Kuwata, M. Sogami, S. Era, Clin. Chim. Acta 316 (2002) 175.
[23] M.A. Mansoor, P.M. Ueland, A. Aarsland, A.M. Svaldal, Metabolism 42 (1993) 1481.
[24] J.L. Beck, A. Ambahera, S.R. Young, M.M. Sheil, J. de Jersey, S.F. Ralph, Anal. Biochem. 325 (2004) 326.
[25] S.A. Gravina, J.J. Mieyal, Biochemistry 32 (1993) 3368.