Enrichment of cysteinyl adducts of human serum albumin

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We report a method to enrich cysteinyl adducts of human serum albumin (HSA), representing biomarkers of exposure to systemic electrophiles. Because the major site of HSA adduction is the single free sulphydryl group at Cys34, we used thiol-affinity resins to remove mercaptalbumin (i.e., unadducted HSA) from the cysteinyl adducts. Electrospray ionization mass spectrometry was used to detect mercaptalbumin and HSA–Cys34 modifications before and after enrichment of HSA. Differences in adduct content were detected across samples of freshly isolated, archived, and commercial HSA. Cysteinylated and glycosylated adducts were present in all samples, with abundances decreasing in the following order: commercial HSA > archived HSA > fresh HSA. After enrichment of HSA, mercaptalbumin was no longer observed in mass spectra. The ratios of HSA adducts post–preenrichment, quantified via the Bradford assay and gel electrophoresis, were 0.029 mg adducts/mg HSA in fresh HSA and 0.323 mg adducts/mg HSA in archived HSA. The apparent elevation of adduct levels in archived samples could be due to differences in specimen preparation and storage rather than to differences in circulating HSA adducts. We conclude that thiol-affinity resins can efficiently remove mercaptalbumin from HSA samples prior to characterization and quantitation of protein adducts of reactive systemic electrophiles.
plus low abundance has made it difficult to simultaneously measure numerous HSA adducts in samples of human serum or plasma and to identify unknown adducts.

Here we report a method to selectively enrich Cys34 adducts in fresh or archived samples of HSA. The enrichment approach takes advantage of the propensity of HSA containing unmodified Cys34 (mercaptalbumin) to bind with thiol-affinity resin [19]. Because adducted HSA is composed primarily of small mixed disulfides that exhibit reversible binding with HSA–Cys34, disulfide adducts were reduced to mercaptalbumin by dithiothreitol (DTT) prior to treatment with thiol-affinity resin. Using electrospray ionization mass spectrometry (ESI–MS), we characterized intact HSA from various sources and investigated the effects of reduction of mixed disulfides on the enrichment process. Enrichment was assessed both by MS of the intact proteins and by quantification of the amounts of HSA via the Bradford assay and gel electrophoresis before and after enrichment via thiol-affinity resin.

Materials and methods

Chemicals

DTT, tris(2-carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), phosphate-buffered saline, Activated Thiol Sepharose 4B, sequence-grade trypsin, sodium chloride, calcium chloride, ammonium sulfate, nitrogen, Bio-Rad reagent dye, sodium dodecyl sulfate, BioSafe Coomassie G-250 dye (Bio-Rad, Hercules, CA, USA), ammonium sulfate, Tris base, ProteaseMAX, acetonitrile (Fisher Optima grade, 99.9%), and formic acid (Pierce, 1-ml amm. source (Thermo Fisher Scientific, Waltham, MA, USA). Water was purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA, USA).

Isolation of HSA

Whole blood (4 ml) was obtained from a healthy Asian subject (34 years of age) by venipuncture in heparin. The blood was immediately centrifuged for 15 min at 3000g. The plasma layer was transferred to a 15-ml centrifuge tube, and saturated ammonium sulfate was added dropwise to a final concentration of 60% ammonium sulfate. The plasma was vortexed briefly and centrifuged for 30 min at 3000g to pellet the precipitated protein. The supernatant, containing HSA, was transferred to 10-kDa molecular weight cutoff (MWCO) spin columns (Amicon Ultra-4, Millipore), and the protein was desalted using 5 × 15-ml volumes of deionized water. The isolated HSA was immediately aliquoted and frozen at −80 °C. Archived HSA from 40 subjects (pooled and balanced from males/females, smokers/nonsmokers, and black/white subjects with 5 subjects per pool) had previously been isolated according to a similar protocol [20], which included dialysis to remove small molecules, lyophilization to constant weight, and dissolving the purified HSA in deionized water at 50 mg/ml. Archived samples were stored at −80 °C prior to processing in the current study.

MS of HSA

The heterogeneity of HSA with respect to Cys34 adduct status was evaluated in freshly isolated and archived HSA (described above) and commercial HSA from Sigma–Aldrich. Protein samples were analyzed using an Agilent 1200 series liquid chromatograph (Santa Clara, CA, USA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max ESI source (Thermo Fisher Scientific, Waltham, MA, USA). The liquid chromatograph was equipped with C8 guard (Poroshell 300SB-C8, 5 μm, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100-ul sample loop. Solvent A was 0.1% formic acid/99.9% water, and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v/v). For each sample, approximately 50–100 pmol of protein analyte was injected onto the column using an Agilent 1200 autosampler. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 μl/min. The elution program consisted of a linear gradient from 25% to 95% B over 34 min, isotropic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 1 min, and then isotropic conditions at 0.5% B for 14 min at a flow rate of 90 μl/min. The column and sample compartments were maintained at 35 and 10 °C, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection to avoid cross-contamination between samples. The mass spectrometer ESI source parameters were as follows: ion transfer capillary temperature, 275 °C; normalized sheath gas (nitrogen) flow rate, 25%; ESI voltage, 2.0 kV; ion transfer capillary voltage, 33 V; and tube lens voltage, 125 V. Positive ion mass spectra were recorded over the range m/z 500–2000 using the Orbitrap mass analyzer, in profile format, with full MS automatic gain control target settings of 3 × 106 and 5 × 105 charges for the linear ion trap and Orbitrap, respectively, and an Orbitrap resolution setting of 6 × 106 (at m/z 400, full-width at half-maximum [FWHM]). Raw mass spectra were processed using Xcalibur software (version 4.1, Thermo Fisher Scientific), and measured charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novaia, Monmouth Junction, NJ, USA) using the default “large protein” parameters and a background subtraction factor of 1.5. Reproducibility of the measured masses of intact, adducted HSA proteins was within ±3 Da (calculated from the repetitive measurements of the deconvoluted mass spectra of HSA samples over 2 weeks).

Reduction of HSA mixed disulfides

The efficiency of reducing small mixed disulfides bound to HSA—Cys34 was investigated with both DTT and TCEP. Commercial HSA (1-mg portions) was treated with 1 of 11 different concentrations of DTT or TCEP that ranged from equimolar to 270-fold molar excess, with reaction times ranging from 5 to 60 min. All experiments were conducted at room temperature in phosphate buffer (pH 7.4). Following protein reduction, samples were reacted with IAA and the degree of reduction was assessed by monitoring the number of IAA additions (+57 Da per modification) above the measured mass of HSA (66,436 ± 3 Da) [21,22]. Reduction status was assessed before and after reduction using an LTQ Orbitrap XL hybrid mass spectrometer under the same MS conditions described above. Reduction conditions were considered as optimal when they were stringent enough to reduce the Cys34 mixed disulfides while also preserving the intramolecular disulfide linkages. For enrichment experiments, 2-mg portions of fresh HSA (n = 6) and 0.5-mg portions of pooled archived HSA (n = 16) were reduced using a 2.7-fold molar excess of DTT to release Cys34-bound mixed disulfides in 1- and 0.5-mg volumes, respectively. This concentration of DTT had been found to be optimal in preliminary experiments with commercial HSA (described above).

Using IAA-modified HSA as a positive control

IAA was reacted with HSA to create a Cys34 adduct that would serve as a positive control for the enrichment experiments. Commercial HSA was treated with a 2.7-fold molar excess of DTT and was reacted with 150 mM IAA in phosphate buffer (pH 7.4) at 37 °C for 1 h. After removing excess IAA and buffer salts with a
Using thiol-affinity resins to remove mercaptalbumin from HSA

A slurry containing a dry weight of either 250 mg (for 2 mg of fresh HSA) or 75 mg (for 0.5 mg of archived HSA) of Activated Thiol Sepharose 4B hydrated resin in degassed 4B binding buffer (100 mM Tris–HCl and 0.5 M NaCl, pH 7.4) was prepared in a ratio of 75% settled medium to 25% buffer. The slurry was transferred to a 1.5-ml polystyrene spin tube containing cellulose acetate membranes with a 0.22-μm pore size (Pierce Spin Cups, Thermo Fisher Scientific). The reduced HSA starting solution containing either fresh or archived HSA was added to the resin, mixed by vortexing, capped with nitrogen, and sealed with Parafilm. After incubating at room temperature for 16 h on a rotary suspension mixer, the unbound proteins were removed from the thiol-affinity medium by centrifuging at 7000 rpm for 5 min. The flow-through fraction containing the HSA–Cys34 adducts was recovered.

Quantitation of total proteins and HSA

ESI–MS was used to detect mercaptalbumin and HSA–Cys34 modifications before and after treatment of HSA with thiol-affinity resins. The total amounts of proteins in pre- and postenrichment samples were determined in duplicate with the Bradford assay using commercial HSA for the standard curve. The purity of the isolated HSA was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Aliquots containing 10 μg of total protein in 10 μl of deionized water were mixed with 10 μl of 2× Laemmli sample buffer (Bio-Rad) containing 10% SDS and 5% β-mercaptoethanol and were boiled for 10 min. The denatured samples and prestained molecular weight markers (Precision-Plus Protein Standards, Bio-Rad) were loaded on 4–20% polyacrylamide gradient gels (NuSep, Lawrenceville, GA, USA) and separated at 100 V for 1 h. The gels were stained with Bio-Safe Coomassie G–250 dye (Bio-Rad), and the relative concentrations of the various protein bands were determined by densitometry using an AlphaImager HP (Alpha Innotech, San Leandro, CA, USA). The purity of HSA was expressed as the percentage of the sum of all protein bands.

Estimation of HSA–Cys34 adduct levels in HSA samples

The HSA content of samples before and after removal of mercaptalbumin by thiol-affinity resins was quantified by measuring total proteins with the Bradford assay and gel electrophoresis (described above). After removal of mercaptalbumin, the protein content of postenrichment samples reflected the quantity of added HSA–Cys34. Proportions of HSA adducts were, therefore, estimated as ratios of mean quantities of HSA after thiol-affinity treatment to those observed before treatment (mg adducts/mg HSA). For freshly isolated HSA that had been spiked with IAA-modified HSA (positive control), this ratio was adjusted by subtracting the proportion of IAA adducts that had been added (0.047 mg IAA adducts/mg HSA).

Results and discussion

MS of HSA

Using liquid chromatography (LC)–MS, we characterized intact HSA before and after enrichment of HSA–Cys34 adducts. The total ion chromatogram of freshly isolated HSA, given in Fig. 1A, shows a major peak (labeled as HSA) followed by smaller peaks, representing other plasma proteins that were not precipitated by treatment with 60% ammonium sulfate. The raw ESI mass spectrum of HSA (Fig. 1B) exhibits charge states 34+ through 66+.

Heterogeneity of HSA

HSA is heterogeneous with respect to the thiol redox state and exists in both reduced and oxidized forms. The degree of HSA oxidation in vivo has been reported to increase with oxidative stress and various disease conditions, suggesting that HSA is an important scavenger of reactive systemic oxidants [23,24]. However, after collection of blood specimens, Cys34 can be further oxidized during sample storage and processing. In particular, oxidation of Cys34 has been observed to initiate addition reactions with cysteine and mixed disulfides [21,24–26].

In this study, the degree of HSA–Cys34 modification was examined for three different HSA preparations: freshly isolated HSA, archived HSA, and commercial HSA. As shown in Fig. 2, ESI–MS of the intact proteins points to different adduct profiles for these three sources of HSA. The spectrum of freshly isolated HSA (Fig. 2A) was dominated by the mass corresponding to mercaptalbumin (66,436 Da), with a few lower abundance masses also being observed, including one at 66,556 Da representing HSA + Cys (the cysteinylated product) and another at 66,599 Da corresponding to HSA + Gluc (the glycosylated product). (Because the primary site for nonenzymatic glycosylation is Lys [27,28], glycosylation should not preclude modifications of Cys34.) Commercial HSA contained the largest fraction of HSA + Cys, which was twice as abundant as mercaptalbumin (Fig. 2B). The ESI mass spectrum of commercial HSA also showed less abundant masses at 66,473 Da, consistent with nitric oxide (NO) modification, and at 66,512 Da (modification unknown). In the archived HSA spectrum, mercaptalbumin was the most abundant species (Fig. 2C), and prominent peaks corresponding to cysteinylination and glycosylation were observed at higher levels than in the fresh HSA sample.

Reduction of HSA–Cys34 mixed disulfides

As indicated above, the most abundant modification on HSA–Cys34 was cysteinylation. Although cysteinylation was relatively minor in freshly isolated HSA (the 66,556-Da component in Fig. 2A), higher levels of cysteinylated HSA were observed in the archived samples (66,556 Da [Fig. 2C]). As such, it is important to reduce HSA prior to treatment with thiol-affinity resins to achieve maximum enrichment of nondisulfide adducts.

We explored the reduction of mixed thiol disulfides using either DTT or TCEP at room temperature with 1-mg specimens of commercial HSA. A total of 11 different concentrations of DTT or TCEP were tested and ranged from a molar equivalent of reducing agent to 270-fold molar excess, with reaction times ranging from 5 to 60 min. The reaction time did not have a significant effect in any of the experiments using either TCEP or DTT (data not shown). Mass spectra from these experiments provide evidence that between two and five intramolecular disulfides were cleaved, representing 5–11 IAA modifications. In fact TCEP cleaved one intramolecular disulfide at the lowest (equimolar) concentration tested. In contrast, no intramolecular disulfides were broken with
DTT at concentrations between 2.7- and 10.8-fold molar excess, indicating that Cys34 was the only cysteine residue reduced under these conditions.

As illustrated in Fig. 3A, incubation of commercial HSA with a 2.7-fold molar excess of DTT effectively reduced Cys34 mixed disulfides while preserving the intramolecular disulfide linkages. The most intense peak in the deconvoluted ESI mass spectrum was at 66,493 Da, corresponding to HSA with a single IAA modification. The mass in this spectrum at 66,654 Da is likely to be glycosylated HSA–Cys34–IAA, whereas the minor peak at 66,559 Da is probably glycosylated mercaptalbumin [21]. The latter suggests that a small fraction of mercaptalbumin was not modified with IAA. The peak at 66,554 Da was also observed in spectra from HSA that had not been treated with IAA and is, therefore, unlikely to represent an IAA modification of HSA–Cys34. This peak (at 66,554 Da) could represent other covalent modifications of HSA–Cys34 that were not reduced with a 2.7-fold molar excess of DTT.

At a 27-fold molar excess of DTT, commercial HSA was modified with two additional IAA molecules, indicating one broken intramolecular disulfide. When DTT concentrations reached 270-fold excess, as shown in Fig. 3B, a series of peaks was observed with masses close to those expected for addition products representing 1–5 IAA molecules to HSA. The respective observed and expected masses for these peaks, assuming a mass of 66,436 Da for free HSA, were as follows: 66,493 Da (1 IAA: +57 Da observed and expected), 66,554 Da (2 IAA: +118 Da observed vs. 114 Da expected), 66,610 Da (3 IAA: +174 Da observed vs. 171 Da expected), 66,664 (4 IAA: +228 Da observed and expected), and 66,723 Da (5 IAA: +287 Da vs. 285 Da expected). All of these observed masses are reasonable given our mass accuracy of ±3 Da, with the possible exception of the peak at 66,554 Da that corresponds to a mass also observed without IAA treatment and, therefore, could represent a mixture of HSA with 2 IAA additions plus unknown HSA–Cys34 adducts. Although the last major peak in the mass spectrum, at 66,773 Da, could represent HSA with 6 IAA additions, the difference between observed and expected masses (6 IAA: +337 Da observed vs. 342 Da expected) is larger than our nominal mass accuracy.
Based on these results, a 2.7-fold molar excess of DTT was selected for reducing mixed disulfides without disrupting the intramolecular disulfide bridges. Reactions were performed at room temperature for 5 min. These reduction conditions are consistent with previous studies [29].

**Qualitative assessment of HSA–Cys34 adduct enrichment**

**Fig. 4** shows the deconvoluted ESI mass spectra of HSA before and after enrichment of the Cys34 adducts with thiol-affinity resins. Three prominent peaks were observed in the preenrichment spectrum at 66,436, 66,556, and 66,599 Da, corresponding to HCys34–IAA, HCys34–Gluc, and the sum of HCys34–IAA and HCys34–Gluc, respectively. After enrichment, a peak was observed at 66,493 Da corresponding to HCys34–IAA, which was not detected before enrichment. Peaks at 66,545 and 66,705 Da are thought to represent a pool of HCys34 and glycosylated Cys34 adducts, respectively. Note that no peak corresponding to unmodified HSA was observed after treatment with thiol-affinity resins.
A peak at 66,705 (66,545 + 160) Da was also observed in the postenrichment spectrum. This probably represents the corresponding pool of N-linked glycosylated HSA–Cys34 adducts. Because all of the ions resolved in the postenriched spectrum were adducted HSA–Cys34 species, it appears that treatment of HSA with thiol-affinity resins effectively removed mercaptalbumin.

Quantitative assessment of HSA–Cys34 before and after enrichment

The amount of HSA in each sample was calculated using the Bradford assay to measure total protein and SDS–PAGE to determine the purity of HSA. Gel electrophoresis images are shown pre- and postenrichment in Fig. 5. Before treatment with thiol-affinity resins, the fresh HSA sample contained 70.6% HSA and the archived HSA had a mean purity of 46.9% (standard deviation [SD] = 6.8). After treatment, the mean purities were 67.6% (SD = 4.0) and 68.4% (SD = 9.7) for the fresh and archived HSA, respectively. The archived HSA may have been altered during the isolation process (where dialysis and lyophilization were used) or may have degraded over time (11 years at −80 °C), thereby resulting in the observed lower HSA purity. Further work is needed to determine the effects of HSA isolation and storage on levels of HSA–Cys34 adducts and also to confirm adduct levels in fresh HSA from a large sample of subjects. After enrichment, the purity of the fresh and archived HSA specimens were similar, suggesting that the thiol-affinity resin removed the lower molecular weight contaminants from archived specimens of HSA.

One fresh HSA specimen (sample 1.5 in Fig. 5), and two of the pooled archived samples (one of the duplicates of samples 6 and 9 in Fig. 5) were excluded from statistical analysis due to low volumetric and protein recoveries after treatment with thiol-affinity resins. Results for the remaining samples of fresh and archived HSA are summarized in Table 1. The estimated mean adduct level for fresh HSA was 0.076 (SD = 0.010) mg adducts/mg HSA after enrichment. When the amount of HSA–Cys34–IAA positive control (0.047 mg HSA–Cys34–IAA/mg HSA) was subtracted from this value, the mean adduct level was 0.029 mg adducts/mg HSA. The estimated mean adduct level in the archived specimens was
smokers (0.349 vs. 0.289 mg adducts/mg HSA, gender, nonsmokers had significantly higher adduct levels than analysis of variance table shown in Table 2. After adjustment for gender, and smoking status on adduct levels in enriched specimens. P = 0.739) and was dropped from the model. The amounts of HSA measured after enrichment in fresh and archived samples. Table 1

<table>
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<th>Adducts (mg/mg HSA)</th>
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<tr>
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Note. HSA–Cys34 adducts are represented by the amounts of HSA after enrichment via treatment with thiol-affinity resins. M, male; F, female; A, Asian; B, black; W, white.

0.323 (SD = 0.059) mg adducts/mg HSA, which was much higher than that observed in freshly isolated HSA.

We used general linear models to investigate effects of race, gender, and smoking status on adduct levels in enriched specimens of archived HSA. Race (black vs. white) did not significantly affect adduct levels (P = 0.739) and was dropped from the model. The model with gender and smoking status explained 42.5% of the observed variation in adduct levels (R² = 0.425) and produced the analysis of variance table shown in Table 2. After adjustment for gender, nonsmokers had significantly higher adduct levels than smokers (0.349 vs. 0.289 mg adducts/mg HSA, P = 0.0445), and after adjustment for smoking status, males had higher adduct levels than females, although the difference was not statistically significant (0.342 vs. 0.296 mg adducts/mg HSA, P = 0.1121).

Because we expected to observe higher adduct levels in smoking subjects compared with control subjects, interpretation of the apparent increase in adduct levels among nonsmokers must await information about the identities of the particular adducts involved. In any case, these results should be regarded as very preliminary due to the small numbers of HSA samples in our analyses.

Enriched HSA adducts as biomarkers of exposure

As noted in the introductory paragraphs, protein adducts can serve as biomarkers of exposure to a host of xenobiotic toxicants and reactive endogenous species [3,4]. However, because the levels of protein adducts are small compared with the unmodified proteins, they are difficult to detect and identify without prior enrich.

Table 2

<table>
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Acknowledgments

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