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A fluorescent-based HPLC assay for quantification of cysteine and cysteamine adducts in *Escherichia coli*-derived proteins

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ABSTRACT

Recombinant proteins expressed in *Escherichia coli* are often produced as unfolded, inactive forms accumulated in inclusion bodies. Redox-coupled thiols are typically employed in the refolding process in order to catalyze the formation of correct disulfide bonds at maximal folding efficiency. These thiols and the recombinant proteins can form mixed disulfide bonds to generate thiol-protein adducts. In this work, we apply a fluorescent-based assay for the quantification of cysteine and cysteamine adducts as observed in *E. coli*-derived proteins. The thiols are released by reduction of the adducted protein, collected and labeled with a fluorescent reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The derivatized thiols are separated by reversed-phase HPLC and can be accurately quantified after method optimization. The estimated thiol content represents total amount of adducted forms present in the analyzed samples. The limit of quantification (LOQ) was established; specifically, the lowest amount of quantifiable cysteine adduction is 30 picograms and the lowest amount of quantifiable cysteamine adduction is 60 picograms. The assay is useful for quantification of adducts in final purified products as well as in-process samples from various purification steps. The assay indicates that the purification process accomplishes a decrease in cysteine adduction from 0.19 nmol adduct/nmol protein to 0.03 nmol adduct/nmol protein as well as a decrease in cysteamine adduction from 0.24 nmol adduct/nmol protein to 0.14 nmol adduct/nmol protein.

1. Introduction

The benefit of using a bacterial host to manufacture a recombinant protein is its ability to produce large quantities of the protein at lower cost [1–3]. However, a significant drawback of using a bacterial expression system is that the protein is often produced as an unfolded, inactive form and accumulated in the inclusion bodies [4–6]. Thus, solubilization and proper oxidative refolding are required to regain proper conformation and biological activity in many disulfide-containing molecules as described in a number of reports and reviews [7–11]. Proteins, including those fused with the Fc region of antibodies such as protein-Fc fusion molecules and peptibodies (biologically active peptides fused to the Fc moiety); contain multiple disulfide bonds in their native states. Such proteins can be bacterially expressed and purified following the above mentioned solubilization and refolding process.

During oxidative refolding, thiol redox couples, often cysteine and cystamine, are employed in order to catalyze correct disulfide bond formation at maximal folding efficiency. These thiols and recombinant proteins can spontaneously form mixed disulfide bonds to generate thiol-protein adducts in the refolding process. These protein adducts are usually minimized by optimization of refolding conditions or can subsequently be removed by downstream chromatographic separation. However, residual amounts of thiol-protein adducts can inevitably remain in the refolded solution and can potentially be co-purified in the final purified product. These adducted species are product-related heterogeneities and their presence may potentially have an impact on the safety, activity, and stability of the protein. Therefore, the quantitative measurement of adducted species and/or additional process understanding may be required for product quality assessment and manufacturing process control once complete evaluation of this attribute has been achieved.

Numerous articles address detection and quantification of thiols [12–20], but accurate quantification of total adduct content present in purified preparations has been difficult and not been previously reported. Cysteine adduction was investigated by Yotsu-Yamashita [21], however, this work did not address thiol adducts in purified proteins. In our attempts, ion exchange and RP HPLC (reversed-phase HPLC) have been used to separate and characterize various

Abbreviations: RP HPLC, reversed-phase high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; TCEP, Tris (2-carboxyethyl) phosphine; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; TFA, trifluoroacetic acid; AP, acid precipitation.

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adducts. However, these methods suffer from poor resolution of the adducted species from the desired final product and do not allow for precise and reproducible quantification of the level of adduction (our unpublished data). A method capable of quantifying total thiol content present in adducted molecules is therefore needed for routine assessment of refolding efficiency and product quality. For precise measurement of total adduct content, it is highly feasible to quantify the released cysteine and cysteamine as total thiol content after reduction of the adducted forms. We describe here the application of a fluorescent-based HPLC method for the quantification of cysteine and cysteamine adducts in peptibody products. The method was developed by adapting amino acid analysis methodologies which utilize pre-column derivatization to specifically measure these two thiol compounds [22]. The method includes release of thiols from the samples of interest by TCEP (Tris (2-carboxyethyl) phosphine) reduction, labeling of the released thiols with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ, Waters AccQFluor reagent), and analysis of the labeled thiols by RP HPLC.

2. Materials and methods

2.1. Materials

All peptibodies used in the study were manufactured by Amgen. These molecules are engineered to contain functionally active peptides fused to the Fc portion of an antibody at the N or C termini or inserted into the Fc polypeptide chain. These fusion proteins consist of two identical polypeptide chains, with various number of intramolecular and intermolecular disulfide bonds. Several engineered peptibodies (A, B, C, D, E, and F) were utilized as test samples, including pools and fractions from various purification process steps. A recombinant protein (referred to as protein G) which was not refolded with a thiol redox couple was used as a negative control.

AccQ-Fluor reagent was purchased from Waters (Milford, MA, USA). TCEP was purchased from Pierce (Rockford, IL, USA). Triethylamine, Amicon Ultra 4 10 K filters and GuHCl (guanidine hydrochloride) were purchased from VWR Scientific (Westchester, PA, USA). HPLC grade acetonitrile, phosphoric acid, acetic acid, and anhydrous sodium acetate were purchased from JT Baker (Phillipsburg, NJ, USA). Formic acid, hydroxylamine, and monobasic and dibasic sodium phosphates were all purchased from Sigma–Aldrich (St Louis, MO, USA). L-Cysteine and cystamine dihydrochloride were purchased from Fluka (Milwaukee, WI, USA). Endopeptidase Lys-C was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dithiothreitrol (DTT) was purchased from Invitrogen (Carlsbad, CA, USA). Urea was purchased from Amresco (Solon, OH, USA). Sequencing grade TFA (trifluoroacetic acid) was purchased from Applera (Carlsbad, CA, USA). Glacial acetic acid was purchased from J T Baker (Phillipsburg, NJ, USA). Sodium acetate (3 M) was purchased from Teknova (Hollister, CA, USA).

2.2. Chromatography

Chromatographic separation of intact peptibodies and adducted peptibody species was performed on an Agilent 1100 series LC system, equipped with a Zorbax C18, 5 μm particle size, 2.1 mm × 150 mm column (Agilent, Santa Clara, CA, USA). Separation was achieved using a gradient mobile phase consisting of 0.1% TFA (v/v) in water (solvent A) and 90% acetonitrile, 0.1% TFA, and 9.9% water (v/v, solvent B); UV detection was achieved at 215 nm. The column was equilibrated at 37% mobile phase B for 18 min prior to running samples. Gradient conditions were: 0–10 min, 37% B; 10–48 min, 37–43% B; 48–58 min, 43% B; 58–65 min, 43–90% B; 65–75 min, 90% B and return to 37% B in 1 min. Flow rate was 0.2 mL/min, injection amount was 12 μg and the column temperature was maintained at 60 °C. Total run time was 76 min and the post-delay time for reconditioning the column with 37% B was 18 min.

Chromatographic separation of peptides from the enzymatic digestion of peptibody D was performed on an Agilent 1100 series LC system equipped with a Jupiter C18, 5 μm particle size, 2.1 mm × 250 mm column (Phenomenex, Torrance, CA, USA). Separation was achieved using a gradient mobile phase consisting of 0.1% TFA (v/v) in water (solvent A) and 90% acetonitrile, 0.1% TFA, and 9.9% water (v/v, solvent B); UV detection was achieved at 215 nm. The column was equilibrated at 0% mobile phase B for 20 min prior to running samples. Gradient conditions were: 0–10 min, 0% B; 10–34 min, 0–24% B; 34–76 min, 24–45% B; 76–80 min, 45–98% B; 80–85 min, 98% B and return to 0% B in 1 min. Flow rate was 0.2 mL/min, injection amount was 12 μg and the column temperature was maintained at 60 °C. Total run time was 85 min and the post-delay time for reconditioning the column with 0% B was 20 min.

2.3. Mass spectrometry

Mass analysis of intact peptibodies and adducted peptibody samples was performed in positive ion mode using a Thermo Fisher Scientific LCQ Advantage mass spectrometer equipped with an ESI source. The capillary voltage and capillary temperature were set at 30 V and 300 °C, respectively. The on spray voltage was set at 4 kV, and the tube lens voltage at 10 V. Full mass analysis was performed in the range of 300–2000 m/z. The raw data was processed using Bioworks software (Thermo Fisher Scientific) to obtain deconvoluted parent masses.

Peptide mapping analysis was utilized to investigate the location of cysteine and cysteamine adducts in a peptibody of interest. Reduced and non-reduced maps were compared to identify the peptides involved in disulfide linkage. Two aliquots of 100 μg of a peptibody D purification fraction enriched in adducted species were dried to completion. One of the dried peptibody samples, hitherto referred to as Pb D1, was resuspended in reduction buffer (8 M GuHCl, 0.1 M DTT) at a final concentration of 4 mg/mL, and incubated at 37 °C for 1 hour. The other tube of dried peptibody, hitherto referred to as Pb D2, was resuspended in 8 M GuHCl at a final concentration of 4 mg/mL. Both Pb D1 and Pb D2 were diluted 20X in digestion buffer (4 M Urea, 20 mM hydroxylamine, 0.1 M sodium phosphate, pH 7). Enzymatic digestion with endopeptidase Lys-C was achieved by incubating both reduced (Pb D1) and non-reduced (Pb D2) peptibody samples at 37 °C overnight at a protein/enzyme ratio of 20:1. Mass spectrometric analysis of the resulting Lys-C map was performed in positive ion mode on a ThermoFisher Scientific LCQ Advantage equipped with an ESI source. The capillary voltage and capillary temperature were set at 35 V and 275 °C, respectively. The ion spray voltage was set at 4.5 kV, and the tube lens voltage was set at 10 V. The instrument was operated in data-dependent mode, cycling through full mass scan, zoom scan, and MS/MS scan of the most intense ion.

2.4. Adduct Assay method

Chromatographic separation of cysteine and cysteamine adducts was achieved using an Agilent 1200 series LC system, equipped with a fluorescence detector, quaternary pump, and a column heater. A Zorbax 300SB C18, 5 μm particle size, 2.1 mm × 150 mm column (Agilent, Santa Clara, CA, USA) was used for analyzing cysteine and cysteamine adducts. Fluorescence detection was achieved using an excitation wavelength of 250 nm and emission wavelength of 395 nm, with a photomultiplier gain of 14.

Separation was achieved using a gradient mobile phase consisting of 0.1 M sodium acetate, 0.1% triethylamine (v/v), pH adjusted to 6.78 with phosphoric acid, (solvent A); 100% acetonitrile (solvent B); water (solvent C). The column was equilibrated in solvent A for 12 min. Gradient conditions were: 0–5 min, 100% A; 5–25 min, 0–18% B; 25–30 min, 18% B; 30–38 min, 18% B/82% C; 38–46 min, 90% B/10% C; 46–54 min, 90% B/10% C and return to 100% A in 1 min. Injection volume was 10 μL and the column was held at room temperature. Total run time was 55 min and the post-delay time for reconditioning the column with 100% A was 12 min. The flow rate was at 0.3 mL/min.

2.5. Cysteine and cysteamine standard preparation and derivatization with AccQTag fluorophore

A stock solution of 6 ng/μL L-cysteine and a stock solution of 12 ng/μL cystamine were both prepared using 0.05% acetic acid as diluent. Several 192 μL aliquots were made from each stock solution, and were stored at −20 °C. For the assay, 8 μL 0.5 M TCEP were added to a thawed aliquot of either L-cysteine or cystamine stock solutions, and incubated at 37 °C for 1 h (L-cysteine standard solution A and cysteamine standard solution A). These reduction conditions were determined to be optimal for complete conversion of cystamine to cysteamine (data not shown). Reduction of L-cysteine standard was also done to remove any trace cystine impurity, and also to produce uniform HPLC conditions between samples and standards. L-cysteine standard solution B and cysteamine standard solution B were then prepared by making a 1:5 dilution of standard solution A for both L-cysteine standard and cysteamine standard. A buffer solution consisting of 0.008% acetic acid and 0.084% formic acid in water was also prepared (AF buffer). A series of L-cysteine standards with concentrations of 0.012 ng/μL, 0.024 ng/μL, 0.048 ng/μL, 0.096 ng/μL, 0.144 ng/μL, 0.192 ng/μL, 0.48 ng/μL, 0.72 ng/μL and 0.96 ng/μL in a final volume of 50 μL were prepared by diluting L-cysteine standard solutions A and B, using AF buffer as the diluent. Similarly, a series of cysteamine standards were prepared with concentrations of 0.024 ng/μL, 0.048 ng/μL, 0.096 ng/μL, 0.192 ng/μL, 0.288 ng/μL, 0.384 ng/μL, 0.96 ng/μL, 1.44 ng/μL and 1.92 ng/μL, in a final volume of 50 μL. The standard mixtures were then derivatized with AccQTag fluorescent label by adding 52 μL of Borate buffer and 3 μL AccQTag reagent, and heated at 55 °C for 10 min (both Borate buffer and AccQTag reagent are provided in the kit purchased from Waters, Milford, MA, USA). Derivatized standard mixtures were allowed to cool at room temperature and 95 μL mobile phase A were added to each standard. A volume of 10 μL of each standard was injected on the HPLC. The final amounts of derivatized L-cysteine standard injected were 30 pg, 60 pg, 120 pg, 240 pg, 360 pg, 480 pg, 1200 pg, 1800 pg, and 2400 pg. Final amounts of derivatized cysteamine standard injected were 60 pg, 120 pg, 240 pg, 480 pg, 720 pg, 960 pg, 1200 pg, 1800 pg, and 2400 pg.

2.6. Sample preparation

Prior to HPLC analysis, free cysteine and cystamine from the redox couple used in refolding the protein were removed from the non-reduced protein by filtration. Some common buffers (i.e. TRIS, 2-amino-2-hydroxymethyl-propane-1,3-diol) have also been found to compete for the AccQ-fluor reagent and were similarly removed. The removal was accomplished by spinning the peptibody samples (200 μg) for 30 min at 4.8 K rpm on AmiconUltra4 V-shaped 10K-cutoff filters. The retentate, containing the peptibody, was buffer-exchanged into 0.1% formic acid and reduced with TCEP at 37 °C for two to three hours; the final concentration of TCEP was 20 mM in 100 μL 0.1% formic acid. The reduction released cysteine and cysteamine previously adducted on the protein. Full release of the thiol species through reduction was confirmed by LC/MS analysis of the reduced peptibody. Released cysteine and cysteamine were collected by filtration of the reduced protein on Amicon Ultra4 V-shaped 10K-cutoff filters, and collection of the flow-through. Collection of released thiols was repeated three times, and the combined collections were pooled and dried to 100 μL in a speedvac centrifuge. Collected thiols were derivatized with Waters' AccQTag fluorophore. If mass analysis suggested an abundance of adducted forms, less than 42 μL were labeled with the fluorophore. Otherwise, 42 μL were labeled with the fluorophore. For derivatization with the fluorophore, the volume of thiols to be labeled was adjusted to 42 μL with 0.1% formic acid, and 8 μL of 0.05% acetic acid was added. The thiols were then gently mixed with 52 μL Borate buffer and 3 μL AccQTag fluorophore. The AccQTag fluorophore was prepared according to the instructions in the kit [23,25]. The mixture was heated for ten min at 55 °C in a heat block. After heating, 95 μL mobile phase buffer A were added, and 10 μL were injected and analyzed by RP HPLC.

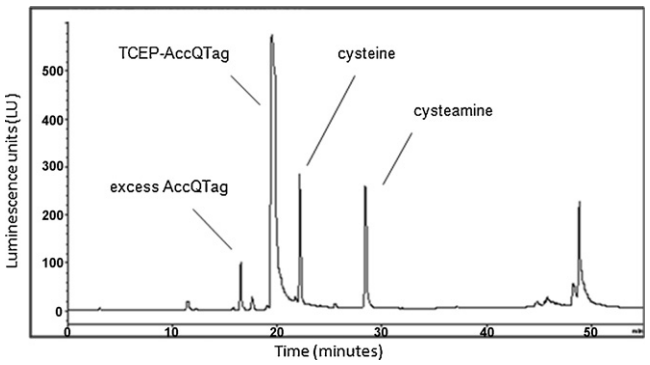

Fig. 1. RP HPLC of derivatized cysteine and cysteamine standards. Cysteine and cysteamine were derivatized with AccQTag fluorescent reagent, and 1200 picograms derivatized samples were injected onto RP HPLC.

2.7. Calculations

The amount of each thiol from adducted species is expressed as nmol adduct/nmol protein. The amount of adduct was calculated utilizing the thiol peak area obtained in the analysis of the sample of interest and the slope and y intercepts of the corresponding standard curve. The limit of quantification (LOQ) was defined as the lowest point on the standard curve. Specifically, the lowest amount of quantifiable cysteine adduction is 30 picograms and the lowest amount of quantifiable cysteamine adduction is 60 picograms.

3. Results and discussion

3.1. RP HPLC method development

Development of the Adduct Assay was initiated by testing the chromatographic separation of fluorescently labeled cysteine and cysteamine standards. ACQ, 6-aminoquinolyl-N-hydroxysuccinimydyl carbamate (commercialized by waters, AccQTag) is a fluorescent reagent commonly used in amino acid analysis and was first described by Strydom and Cohen as a pre-column derivatizing agent [4,6]. The AccQTag reagent reacts with primary and secondary amines, in this case, the amino groups on cysteine and cysteamine, forming stable unsymmetrical ureas that fluoresce strongly at a wavelength of 395 nm [24,26]. Separation of the fluorescently labeled cysteine and cysteamine standards was achieved by RP HPLC. A chromatographic trace resulting from the injection of AccQTag-derivatized cysteine and cysteamine standards is shown in Fig. 1. Two additional peaks were also observed: a

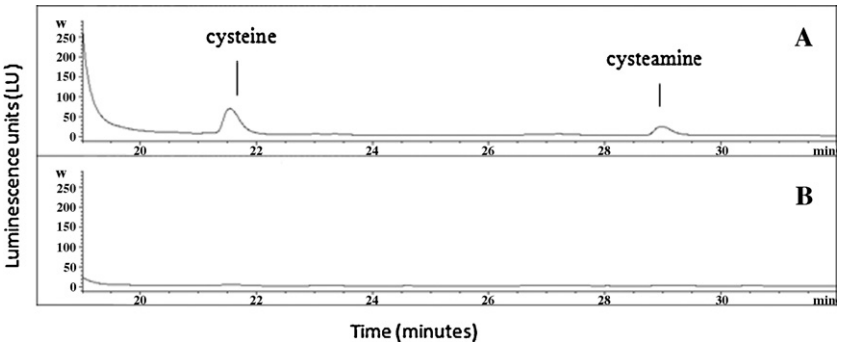

Fig. 2. Cysteine and cysteamine analysis of flow-through fraction (A) and retentate fraction (B) derived from peptibody E sample (see Section 3 for details).

peak resulting from AccQTag reacting with the amine moiety in the reductant, TCEP, and a peak related to excess AccQTag. As the figure shows, AccQTag labeled cysteine and cysteamine are well resolved.

3.2. Linearity

Standard curves for both cysteine and cysteamine standards were generated for quantification of adducted species in the samples of interest by the Adduct Assay. Nine-point curves were generated by plotting thiol peak areas against corresponding known concentrations of derivatized cysteine and cysteamine. The cysteine standard solution was prepared from L-cysteine, and was reduced with TCEP prior to use to ensure full reduction of any potential cystine impurity. The cysteamine standard solution was generated by reduction of cystamine with TCEP. Both cysteine and cysteamine standards were derivatized with AccQTag reagent, and the amounts previously described in the section describing standard curves were injected. A linear relationship between injected amounts of standards and the resulting HPLC peak areas was achieved. For derivatized L-cysteine a linear range was established from 30 pg to 2400 pg; for derivatized cysteamine, linearity was established between 60 pg and 2400 pg.

For both standards, all values for the correlation coefficient (r^2) of the linear regression are 0.99.

3.3. Assay reproducibility

Adduct Assay reproducibility was determined as the variance in the peak areas resulting from injection of derivatized cysteine and cysteamine standards from six analyses performed over a period of two years. Both retention time and peak area showed acceptable reproducibility for the six experiments compared, in which 1200 pg of derivatized standards were injected. The percent coefficients of variation (% CV) for cysteine and cysteamine standard injections were 3.3 and 5.6, respectively. For both cysteine and cysteamine standard injections, peak retention time for standard injections had a % CV value of 0.2. Triplicate experiments were performed with peptibody C to investigate the reproducibility of the release of thiols associated with adducted protein.

The variance in the peak areas corresponding to the release of cysteine and cysteamine was determined, and the calculated percent coefficients of variation (% CV) for cysteine and cysteamine thiol release were 8.9 and 8.1, respectively.

3.4. Recovery of released thiols from adducted protein

As described in the Section 2.6 in Section 2, several filtration steps were necessary to isolate the thiols released from adducted samples. It was thus necessary to investigate the efficiency and specificity of the filtration steps. A peptibody (peptibody E) known to contain both cysteine and cysteamine adducts, was reduced. After reduction of peptibody E, both the retentate (containing reduced protein) and the flow-through (containing released thiols) were derivatized with AccQTag fluorophore and analyzed by RP HPLC. As Fig. 2 shows, released thiols are only present in the flow through fraction. Complete reduction of peptibody E was also confirmed by LC/MS analysis of the retentate; no adducted species were observed (data not shown). Thus, the recovery of released thiols was shown to be specific and efficient.

Table 1
Recovery of thiol standards spiked into peptibodies A and B.

	Cysteine (peak area)	Cysteamine (peak area)
Standard	1938	1932
Peptibody A	329	279
Peptibody A + standard	2145	2393
Peptibody B	680	671
Peptibody B + standard	2406	2557
Percent standard recovery Pb A	95	92
Percent standard recovery Pb B	92	98

Thiol recovery was also investigated through spiking experiments where known quantities of cysteine and cysteamine standards were added to two adducted peptibody samples, A and B, prior to release of the thiol adducts. Peak areas were compared from the analyses of spiked and non-spiked peptibody samples and also from the analyses of thiol standards. The results of these experiments are tabulated in Table 1. Recovery of both cysteine and cysteamine was calculated to be over 90% in both spiking experiments.

3.5. Analysis of oxidized thiol standards, cystine and cystamine

The sources of the standards used to generate a standard curve are the same as those used in protein refolding. It was thus necessary to investigate whether oxidized forms of the standards could be also detected by the Adduct Assay. Moreover, since the cysteamine standard was generated by reducing cystamine, it was necessary to determine what amount, if any, of non-reduced cystamine might be remaining post-reduction. Reduced and non-reduced standards were analyzed by the Adduct Assay. Oxidized species, cystine and cystamine, were identified as peaks observed only in the non-reduced sample (see Fig. 3). The retention times of the oxidized species were determined to be different from those of the reduced forms.

3.6. Analysis of adduct-containing peptibody samples

As proof of concept, adduct levels were quantified by the Adduct Assay in peptibody samples that had been shown to have

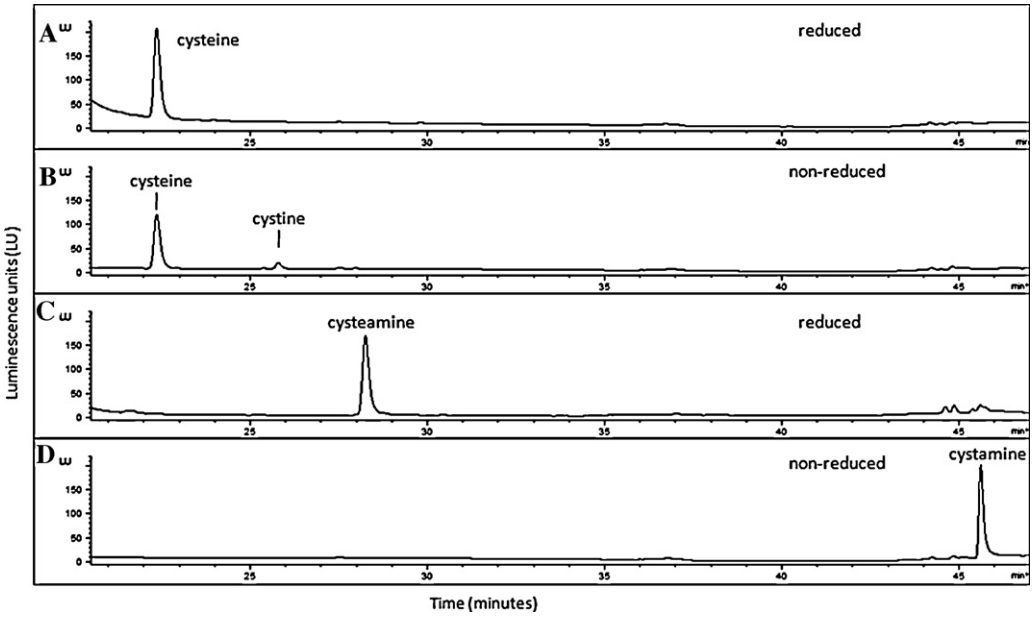

Fig. 3. Fluorescent RP HPLC analysis of cysteine (A), cystine (B), cysteamine (C) and cystamine (D).

significant adduction by several orthogonal methods, including cation exchange chromatography, LC/MS, and peptide mapping. Purification of recombinant therapeutic proteins requires several chromatographic steps. One such step (final polishing), is often applied to separate protein aggregates and other impurities from pure protein product. LC/MS analysis of purified fractions from the final polishing step of peptibody D indicated significant levels of cysteine and cysteamine adduction. However, lack of chromatographic resolution did not allow definitive identification and quantification of the adducted species. Mass spectrometry of a fraction from the final polishing step revealed multiple masses in addition to the theoretical mass (56,708 Da). From the additional masses, it was possible to postulate different combinations of both cysteine (+120 Da) and cysteamine adduction (+76 Da), but not to quantify the level of adduction accurately, as shown in Fig. 4. Peptide mapping was also used to characterize adduction in this sample by subjecting it to enzymatic digestion with endopeptidase Lys-C. All of the peptides involved in disulfide bonds were recovered in the peptide map. Cysteine adduction was observed on all of the recoverable cysteine-containing peptides. Work by D. Ren et al. showed the possibility of accurately quantifying cysteinylation on a single cysteine in an IgG by LC/MS [27]. However, adduction on multiple sites, as was determined by our peptide mapping, would not be amenable to quantification using this approach. No cysteamine adduction was observed on any of the peptides. Fig. 5 shows the chromatographic separation of Lys-C peptides, before and after reduction of the sample. Differences between the observed masses in the analysis of whole peptibody D and the observed adduction in the peptide map emphasize the limited adduct information provided by both methods and the need for a quantitative method.

A purified fraction from the final polishing step of Peptibody D was also subjected to CEX (Cation Exchange-HPLC) analysis. Definitive identity and quantification of adducted species was complicated by co-elution of deamidated peptibody with some of the adducted species in this technique (data not shown). Cysteine and cysteamine adduction in the peptibody D fraction were determined by the Adduct Assay prior to full assay optimization; 0.28 nmol cysteine adduct per nmol of peptibody and 0.16 nmol cysteamine

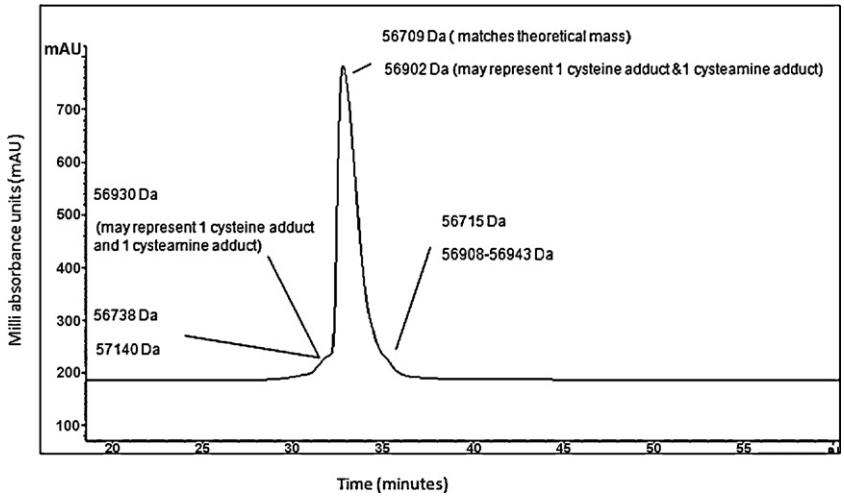

Fig. 4. LC/MS analysis of a purification fraction from the final polishing step of peptibody D. The theoretical mass of peptibody D is 56708 Da. Observed additions of 76 Da (cysteamine) and 120 Da (cysteine), suggesting the presence of adducted peptibody species, are indicated.

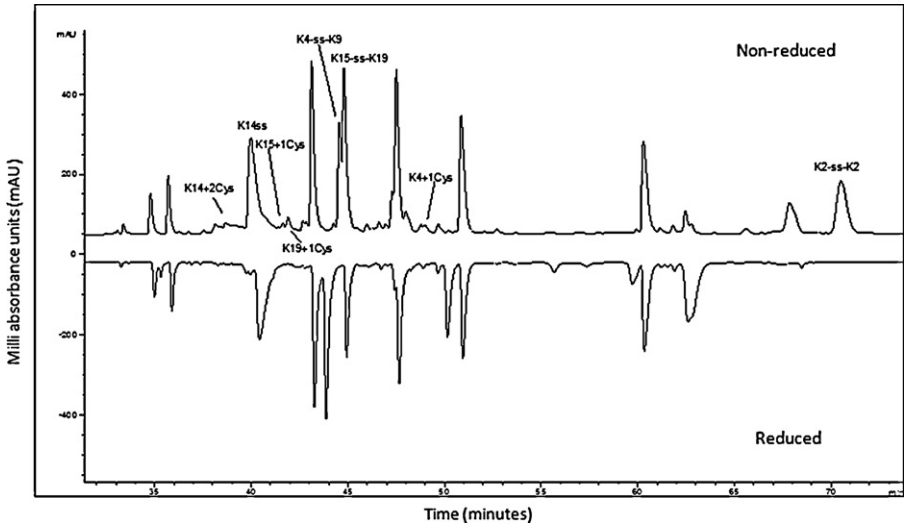

Fig. 5. Non-reduced vs. reduced Lys-C peptide maps of peptibody D. Disulfide bonded peptides are shown as "–ss" and adducted peptides are shown as "–Cys". No cysteamine peptide adduct was found.

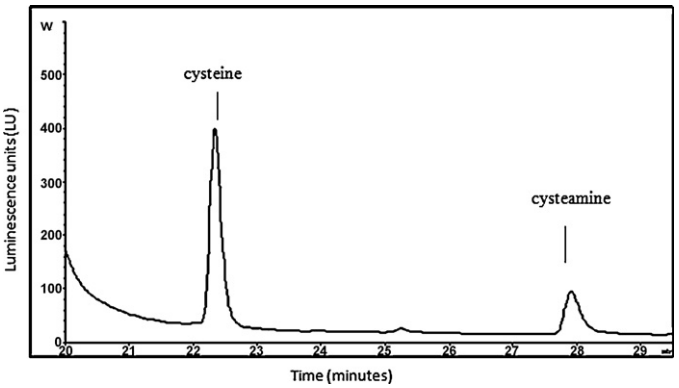

Fig. 6. RP HPLC of recovered cysteine and cysteamine adducts from an enriched purification fraction of peptibody D.

Table 2
Adduct recovery from peptibody C at different purification steps.

	Cysteine (nmol adduct/nmol peptibody)	Cysteamine (nmol adduct/nmol peptibody)
Peptibody C, AP pool[a]	0.19	0.24
Peptibody C, capture pool	0.04	0.24
Peptibody C, intermediate polish pool	0.04	0.24
Peptibody C, final polish pool	0.03	0.14

[a] Pools were collected from successive purification steps, and assayed on the Adduct Assay.

adduct per nmol of peptibody were detected. The chromatographic separation for quantification of cysteine and cysteamine adducts extracted from peptibody D is shown in Fig. 6.

The Adduct Assay was also used to quantify the levels of cysteine and cysteamine adduction in the purification process pools of a peptibody. The purification of peptibody C involved AP (acid precipitation) followed by a chromatoghraphic capture step, intermediate polishing, and final polishing purification steps. These purification steps were designed to accomplish removal of host cell proteins and aggregated forms and were not explicitly designed to remove adducted species. Pools from the individual purification steps were collected and analyzed using the Adduct Assay. The results, as shown in Table 2, indicate that the capture step removed a significant amount of the cysteine-adducted species that were observed in the acid precipitation pool. The pool from the final polishing step showed the least amount of both cysteine and cysteamine adduction. Thus, we demonstrated that the assay could be effectively used to monitor adduct levels in various purification steps.

During the purification of several other peptibodies, purification process pools and final purified bulks were collected and analyzed by the Adduct Assay to quantify cysteine and cysteamine content for degree of adduction. The results of the assay were used to optimize peptibody refolding conditions to minimize generation of various adduct forms, and also to monitor adduct removal at different steps of the peptibody purification process. Table 3 summarizes typical Adduct Assay results for the peptibody purification pools and final pure bulks. Peptibodies A and B in final purification stage were found to have significantly higher level of cysteine and cysteamine adducts, suggesting that oxidative refolding is not very complete and the subsequent chromatographic separations were not able to completely remove these adducts away from the desired product. The efficiency of the purification process for removal of adducted forms can be assessed by the Adduct Assay. The assay can be used to quickly compare the content of cysteine and cysteamine as well as the level of adduction in various fractions or pools, as demonstrated by the analysis of peptibodies E and F. For example, the high cysteamine adduction observed in peptibody E correlates with the observed cysteamine adduction detected by LC/MS. For peptibody F, intermediate polishing step fraction 2

Table 3
Adduct Assay results from the analysis of several purification process pools from the purification of several peptibodies. Protein G, used as a negative control, was refolded without the presence of a cysteine/cystamine redox couple.

	Cysteine (nmol adduct/nmol peptibody)	Cysteamine (nmol adduct/nmol peptibody)
Peptibody A, FPB[a]	0.49	0.32
Peptibody B, FPB	0.54	0.63
Peptibody E, IP fraction	0.22	1.70
Peptibody F, IP fraction 2	0.09	0.49
Peptibody F, IP fraction 48	0.31	0.07
Peptibody F, IP fraction 51	0.06	0.07
Peptibody F, IP pool (fractions 44-50)	0.16	0.07
Protein G, neg. control	Undetected (0)	Undetected (0)

[a] FBP, final purified bulk; IP, intermediate polishing step. Peptibody F, IP fraction 51 was not included in the final IP pool, most likely because of high aggregation levels, or low UV absorbance.

contains higher level of cysteamine adduct, intermediate polishing step fraction 48 contains higher level of cysteine adduct while intermediate polishing step fraction 51 contains the lowest level of both adducted forms. Therefore, adduct analysis of these intermediate polishing step fractions suggests that this purification step provides some degree of separation of the adducted forms. When the intermediate polishing step fractions containing parent peptibody were pooled between fractions 44 and 50, the determined cysteine adduct remains higher than the cysteamine adduct, suggesting that the cysteine adduct is not completely resolved from the parent peptibody form. Our initial focus on pooling of intermediate polishing step fractions is to minimize the amount of aggregation in the peptibody, with low adduction level as a secondary goal.

4. Conclusions

The Adduct Assay was developed to quantify cysteine and cysteamine adduction in *Escherichia coli*-derived proteins from our production process. These proteins require refolding, often utilizing cysteine/cystamine redox couples. Specifically, the Adduct Assay provided adduct quantification for engineered peptibodies. In an effort to control adduct levels, the purification scientists have relied on the Adduct Assay to successfully monitor adduct levels as a function of different redox couple conditions. The assay has also been utilized to monitor adduct levels at different steps in the purification process. The Adduct Assay simplifies comparison of adduct levels between samples. A number of experiments have validated the sensitivity, linearity, and reproducibility of the assay as well as confirmed an acceptable level of recovery of the adducted thiols. The method should also prove useful with other redox agents such as reduced/oxidized glutathione couples.

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