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Identification and quantitation of cysteine in proteins separated by gel electrophoresis

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Abstract

A simple technique is introduced to identify and quantitate cysteine (Cys) after acid hydrolysis of protein. The technique involves using 9-fluorenylmethyl chloroformate (Fmoc)-based amino acid analysis that recovers all of the amino acids (asparagine and glutamine are recovered in their acidic forms) except tryptophan. Cys adducts with acrylamide and iodoacetamide have been observed in hydrolysates of gel-separated proteins. To enable quantitation of Cys by amino acid analysis, different conditions of reduction [dithiothreitol (DTT) and tributylphosphine] and alkylation [vinylpyridine, acrylamide and iodoacetamide] were compared. Optimal conditions for *on-blot* reduction (125 m*M* of DTT, pH 8.5, at 80°C) and alkylation (0.25 *M* iodoacetamide, pH 8.5, at 37°C) of proteins which have been separated by gel electrophoresis and blotted onto polyvinylidenedifluoride (PVDF) membrane were established to achieve complete recovery of alkylated Cys. Even with the optimal *on-blot* iodoacetamide alkylation, there may still be some acrylamide adducts present and these were able to be separated by HPLC along with the other 16 amino acids. The Cys content has been successfully determined by Fmoc–amino acid analysis of PVDF-blotted proteins separated by 1D or 2D gel electrophoresis. Lysine alkylation with iodoacetamide and acrylamide has also been characterised. Protein identification using amino acid composition including Cys has been introduced.

Keywords: Cysteine; Proteins; Amino acids

1. Introduction

Cysteine (Cys) is an amino acid with a sulfhydryl group on the β-position, which can undergo a variety of reactions including oxidation and the formation of various oxidation products [1,2]. Cys is oxidised during acid hydrolysis, and as a result, Cys is not recovered during standard amino acid analysis of acid hydrolysed protein. In peptide mass spectrometric analysis, the exact mass of a Cys-containing peptide is difficult to measure because different mass values are generated from Cys oxidation products and adducts. In Edman protein sequencing, Cys cannot be identified because its phenylthiohydantoin (PTH) derivative is unstable, losing H_2S to form PTH-dehydroalanine, which in turn degrades further to a variety of products [3]. It is advantageous for each of these applications to modify Cys to facilitate its correct identification and quantitation.

A number of methods have been published for Cys

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analysis. These include performic acid oxidation of Cys to cysteic acid [4], direct analysis of Cys [5], and reduction/alkylation. Proteins have been reduced by mercaptoethanol [6,7], dithiothreitol (DTT) [8–10] or tributylphosphine (TBP) [10–12], and alkylated under alkaline (pH 8) conditions using iodoacetamide [6,12], vinylpyridine [7,11], bromopropylamine [8,9] or acrylamide [10]. These alkylated products are stable under the conditions of acid hydrolysis used in amino acid analysis [6–8] and Edman degradation used in protein sequence analysis [9–12].

The most commonly used alkylating reagent is vinylpyridine which has several useful analytical properties. It can be applied to proteins bound or blotted to chemically inert membrane supports such as polybrene-treated glass-fiber, PTFE or polyvinylidenedifluoride (PVDF) membranes. The derivative produced from vinylpyridine, *S*-4-pyridylethyl–Cys has a high UV absorbance at 254 nm. *S*-4-Pyridylethyl–Cys containing peptides can be scanned at this wavelength, and this is useful for the selection of Cys-containing peptides in peptide mapping. The pyridylethyl group is labile in mass spectrometry (MS), resulting in the release of a molecular mass of 106. This helps to confirm the presence of Cys in a peptide. Furthermore, in Edman sequencing, the PTH derivative of *S*-4-pyridylethyl–Cys elutes at a unique position in standard HPLC gradients [11]. However, in amino acid analysis with 9-fluorenylmethyl chloroformate (Fmoc) precolumn derivatisation, *S*-4-pyridylethyl–Cys quenches the Fmoc fluorescence, resulting in a low quantitation of Cys (data shown in Section 3.3).

With the advent of semi-preparative high-resolution separation of proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), more and more gel-separated proteins are being analysed. The modification of Cys during gel electrophoresis of proteins has recently been highlighted by the increasing use of peptide mass fingerprinting for protein identification. The conditions for sodium dodecyl sulfate (SDS)–PAGE (reducing environment at pH 8) favour alkylation of Cys during the electrophoresis if free acrylamide exists in the gel, and formation of a Cys–acrylamide adduct has been observed in isoelectric focusing gels [13]. Studies involving NMR [14], MS [15], amino acid analysis [16] and protein sequencing [10] have confirmed the formation of the acrylamide adduct, cysteinyl–*S*-β-propionamide (Cys–pam). Another derivative of Cys has been observed in proteins separated by 2D gel electrophoresis. In the Swiss-2D-PAGE protocol for 2D gels [17], the immobilised pH gradient (IPG) strip is equilibrated between the first-dimension isoelectric focusing and second-dimension SDS–PAGE. This involves a reducing step using an excess of DTT and an alkylation step with iodoacetamide to remove the remaining DTT. Cys in reduced proteins can be alkylated with iodoacetamide to form carboxyamidomethyl–Cys (Cys–cam). Therefore during the 2D separation of proteins there may be several different Cys adducts formed. It is of interest to clarify this situation and hence develop an assay for Cys in gel-separated proteins.

In this study, we have established a technique for the analysis of modified Cys in proteins separated by 2D-PAGE and electro-blotted onto a PVDF membrane. To optimise conditions, proteins were reduced by TBP or DTT, and alkylated using acrylamide or iodoacetamide. After acid hydrolysis, protein hydrolysates were analysed by automated amino acid analysis (AminoMate) using Fmoc precolumn derivatisation [18]. The optimal conditions for complete modification of Cys in proteins separated by 2D-SDS–PAGE and blotted onto a PVDF membrane were established from detailed comparative analyses using the different reagents. Using our approach, a number of proteins have been analysed for their Cys composition and further identified on the basis of amino acid compositional matching using the AACompIDent search program in ExPASy (http://expasy.hcuge.ch) with the new ‘Constellation 5’ that now includes Cys together with 16 other amino acids [19]. The modification of lysine (Lys) by alkylation at the ϵ-amino group has been observed and is discussed.

2. Materials and methods

2.1. Chemicals and apparatus

L-Cysteine (minimum 98% by TLC), 4-vinylpyridine (minimum 95%), *S*-4-pyridylethyl-cysteine,

iodoacetamide, poly-L-lysine and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). *S*-Carboxymethyl-L-cysteine (98%, cysteine-*S*-ethanoic acid) was obtained from Aldrich (Milwaukee, WI, USA). Tributylphosphine (TBP, 85%) was from Fluka (Buchs, Switzerland). Acrylamide and dithiothreitol were supplied by Bio-Rad (Hercules, CA, USA). Triethylamine (TEA) (sequencing grade) was from Pierce (Rockford, IL, USA). All reagents and apparatus used for gel electrophoresis, electroblotting, and amino acid analysis were described in detail by Hochstrasser et al. [20] or Yan et al. [19].

2.2. Preparation of 1D or 2D gel-separated and PVDF electroblotted protein samples

1D-SDS–PAGE was carried out on Mini-protean II (Bio-Rad) apparatus [21]. The procedures for running 2D-SDS–PAGE and electro-transferring protein onto PVDF membrane are outlined in the Swiss-2D-PAGE database (http://expasy.hcuge.ch/ch2d/technical-info.html).

2.3. Synthesis of Cys–pam

Cys–pam was synthesised according to the method of Friedman et al. [22] as follows: a solution of 1.74 g (0.01 mol) of Cys in 20 ml of water was adjusted to pH 8.0 with ammonia. To the Cys solution, 0.78 g (0.011 mol) of acrylamide was added. The reaction mixture was stirred under argon at 45°C. After 30 min, the reaction mixture was cooled in an ice water bath. A white precipitate was formed, which was filtered, and then recrystallised from 50 ml of hot ethanol–water (75:25, v/v). Further product could be obtained by the addition of methanol to the mother liquor. The final product was assayed by ^{1}H NMR spectroscopy. ^{1}H NMR spectra were acquired at 27°C on a Varian XL-400 spectrometer operating at 400 mHz. Deuterium oxide was used as solvent with 3-(trimethylsilyl)propionic acid as internal standard. The spectrum was in accordance with that reported by Chiari et al. [13] and it indicated the presence of only minor impurities. Electrospray ionization (ESI) MS of a solution of the product (2.5 m*M* in 0.1 *M* HCl, stored at 4°C for 3 months) displayed a single ion of molecular mass of 192 (M+H).

2.4. Preparation of Cys–propionic acid

Cys–propionic acid was prepared from standard Cys–pam by acid hydrolysis, which was carried out under the same conditions used for hydrolysis of protein samples (see below).

2.5. On-blot reduction and alkylation of proteins on PVDF membranes

The procedure for reduction and alkylation of proteins on PVDF was as initially reported by Brune [10]. Details of reactions at 37 and 80°C are given in the next section. Reactions at 37°C were carried out on an excised PVDF spot in a 500-μl Eppendorf tube lid to which the reduction and alkylation reagents were added, which was then sealed with parafilm. The enclosed Eppendorf tube lid was placed on a floating tray with the lid in contact with the 37°C water bath. This created a small chamber enabling the solution to fully contact protein on the PVDF membrane whilst maintaining an oxygen-free environment. Reactions at 80°C (heating block) were performed in a 500-μl Eppendorf tube that had been flushed with argon immediately before closure of the lid. All reagents, except borate buffer, were made immediately prior to use.

2.5.1. Using DTT as a reducing reagent

The reducing mixture contained 15 μl of methanol–water (50:50, v/v), 30 μl of 250 m*M* DTT (in 250 m*M* borate buffer, pH 8.5), and 15 μl of 250 m*M* borate buffer (pH 8.5). For reaction at pH> 11.0, 15 μl of methanol–water (50:50, v/v) was combined with 15 μl of TEA–water (11:89, v/v) and 30 μl of 250 m*M* DTT (in water).

After incubation at either 37 or 80°C for 30 min, the reaction solution was discarded. Fifteen μl of methanol–water (50:50, v/v), 15 μl of borate buffer (pH 8.5) and either 30 μl of acrylamide (1 *M*) or iodoacetamide (0.5 *M*) were added immediately; for reaction at pH>11.0, 15 μl TEA–water (11:89, v/v) was added instead of borate buffer. The reaction was incubated at either 37 or 80°C for 30 min. The solution was then discarded and the PVDF spot was

washed three times with methanol–water (50:50, v/v) before drying. The reduced and alkylated protein spot on PVDF was ready for further analysis.

2.5.2. Using TBP as a reducing reagent

2.5.2.1. The combination of TBP and acrylamide

In the case of reduction with TBP and alkylation with acrylamide, the reduction and alkylation reactions can be carried out in one step. The mixture contained 13.5 μl of methanol–water (50:50, v/v) 1.5 μl of TBP (0.1 *M* in *n*-propanol), 30 μl of acrylamide and 15 μl of borate buffer (250 m*M*, pH 8.5). For reaction at pH>11.0, 15 μl TEA–water (11:89, v/v) was added instead of borate buffer. The reaction mixture was incubated at 37 or 80°C for 30 min. The reaction solution was discarded and the PVDF spot was washed three times with methanol–water (50:50, v/v) before drying. The reduced and alkylated protein spot on PVDF was ready for further analysis.

2.5.2.2. The combination of TBP and iodoacetamide

As iodoacetamide reacts with TBP to form a quarternary phosphinium salt, it was necessary to use a two-step procedure involving reaction with TBP followed by alkylation with iodoacetamide. The reducing mixture contained 15 μl of methanol–water (50:50, v/v) 1.5 μl of TBP (0.1 *M* in *n*-propanol), and 43.5 μl of borate buffer (250 m*M*, pH 8.5). For reaction at pH>11.0, 15 μl TEA–water (11:89, v/v) was used instead of borate buffer, and 28.5 μl of water added to make up a total 60 μl.

After incubation at either 37 or 80°C for 30 min, the reaction solution was discarded. Fifteen μl of methanol–water (50:50, v/v) and 15 μl of iodoacetamide (0.5 *M*), and 30 μl of borate buffer (pH 8.5) were added immediately. For reaction at pH> 11.0, 15 μl TEA–water (11:89, v/v) was added instead of borate buffer, and 15 μl of water to make up a total 60 μl. The reaction mixture was incubated at either 37 or 80°C for 30 min. The solution was then discarded and the PVDF spot was washed three times with methanol–water (50:50, v/v) before drying. The reduced and alkylated protein spot on PVDF was ready for further analysis.

2.6. Acid hydrolysis and amino acid analysis

PVDF samples containing protein were hydrolysed with 6 *M* HCl vapour at 155°C for 1 h [19]. The protein hydrolysate was subjected to amino acid analysis using Fmoc precolumn derivatisation as described by Ou et al. [18] and Yan et al. [19].

2.7. Electrospray ionisation mass spectrometry

ESI mass spectra were acquired on a Quattro II mass spectrometer (Micromass, UK). Fractions collected manually from the amino acid analyser were desalted during sample infusion into the ESI-MS spectrometer. A Peptide Trap Cartridge (1×10 mm, Michrom BioResources, USA) containing reversed-phase packing was substituted for the injection loop on a rheodyne injector. The sample was loaded onto the cartridge by a syringe in the normal manner, and then washed with water (500 μl). The samples were desorbed directly into the ionisation source by switching the valve and eluting with acetonitrile–water (60:40, v/v) at a flow-rate of 20 μl/min.

3. Results and discussion

3.1. HPLC separation of the acrylamide adduct of Cys

The acrylamide adduct of Cys (Cys–pam) was synthesised for use as a standard with a similar yield (80%) to that (82%) reported by Friedman et al. [22]. No oxidation products of Cys–pam were detected using ESI-MS.

Since Cys–pam is converted to Cys–propionic acid during the acid hydrolysis conditions used to fully hydrolyse proteins to free amino acids, the standard was subjected to the same conditions and its recovery monitored by HPLC after Fmoc derivatisation. As no starting material (Cys–pam) was detected by HPLC after the acid hydrolysis (retention time indicated in Fig. 1a) we conclude that Cys–pam is quantitatively converted to Cys–propionic acid. The Fmoc derivative of Cys–propionic acid was readily resolved from the other amino acids (Fig. 1a).

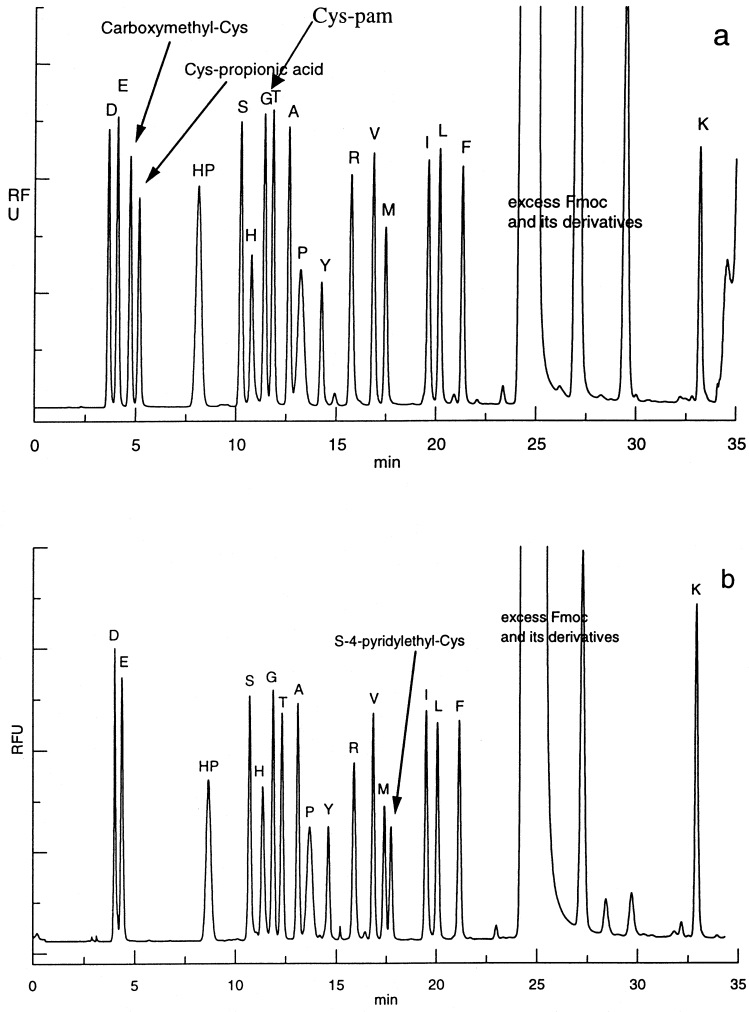

Fig. 1. Chromatograms of separation of Fmoc-derivatised amino acids: (a) 250 pmol each of Cys-propionic acid, carboxymethyl–Cys and other amino acid standards including HP. The retention time of Cys–pam indicated; (b) 1000 pmol of pyridylethyl–Cys and 250 pmol each of other amino acid standard including HP.

3.2. HPLC separation of the iodoacetamide adduct of Cys

Using the standard HPLC gradient for separation of amino acids, Fmoc-derivatised standard carboxymethyl–Cys, which is the product of the acid hydrolysis of the iodoacetamide adduct of Cys (Cys–cam), eluted earlier than Fmoc-derivatised Cys–propionic acid, and was well separated from the other amino acids, including glutamic acid (Fig. 1a).

3.3. HPLC separation of the vinylpyridine adduct of Cys

Alkylation with vinylpyridine produces *S*-4-pyridylethyl–Cys. Using the standard gradient the Fmoc derivative of *S*-4-pyridylethyl–Cys eluted in a unique position shortly after methionine (Fig. 1b). In this chromatogram there is four times (1000 pmol) the amount of *S*-4-pyridylethyl–Cys compared to the other amino acids (250 pmol of each), which clearly

illustrates that the pyridylethyl group quenches the fluorescence of the Fmoc derivative. Thus a small peak is obtained which reduces the accuracy of quantitative estimation. Since estimation of amino acid composition is challenging for 2D gel-separated proteins, which are typically present at levels of 5–50 pmol, it is not practical to use vinylpyridine as the alkylating reagent for the detection of Cys using Fmoc-based amino acid analysis.

3.4. Detection of iodoacetamide and acrylamide adducts of Cys in 2D gel-separated proteins

In a standard procedure of 2D gel electrophoresis [17], after the first-dimension isoelectric focusing separation, the IPG strip is equilibrated with reducing buffer containing (125 m*M*) DTT in order to resolubilise the proteins and reduce disulfide bonds. The strip is subsequently equilibrated with an (135 m*M*) iodoacetamide-containing solution. Iodoacetamide, as an alkylating reagent, serves the purpose of blocking –SH– groups, derived either from Cys, or from the excess of DTT (the DTT must be removed as it causes a dark background in gels when using silver staining). Thus, the Cys in proteins is alkylated by iodoacetamide before the second-dimension SDS–PAGE to form the derivative, Cys–cam. During acid hydrolysis of the protein, Cys–cam is quantitatively converted into its acid form, carboxymethyl–Cys, which separates from the acrylamide adduct Cys–propionic acid.

Human serum albumin (HSA) was separated by 2D gel electrophoresis and blotted onto PVDF in the standard way. Fig. 2a shows a chromatogram from the analysis of hydrolysed HSA where carboxymethyl–Cys is indicated. The detection of carboxymethyl–Cys demonstrated the modification of Cys by iodoacetamide solution during IPG strip equilibration before the second-dimension in 2D-PAGE. However, the modification was incomplete and the yield of iodoacetamide adduct was found to be inconsistent in different samples. On some occasions both acrylamide and iodoacetamide adducts were observed (e.g. Fig. 2b). Obviously complete acrylamide alkylation is not occurring when proteins are focused at their isoelectric point in a thin IPG strip or when the strip is equilibrated in iodoacetamide. Formation of the acrylamide adduct (Cys–pam) was also observed on analysis of PVDF-blotted BSA that had been separated by 1D gel electrophoresis. The recovery of the Cys adducts was variable and sometimes insignificant. We presume that this is caused by differing amounts of free mono-acrylamide present in the gels.

Neither carboxymethyl–Cys nor Cys–propionic acid were detected in the analysis of a control sample of BSA that had been prepared in water and subjected to acid hydrolysis under the standard conditions (data not shown). It is clear that the alkylation of Cys in 2D electrophoresis is variable and incomplete. Post-gel-separation alkylation is required to enable the quantitation of Cys. We have optimised the conditions necessary for the quantitative analysis.

3.5. Optimisation of a post-separation on-blot reduction and alkylation of Cys

BSA contains 35 Cys residues which form 17 intra-disulfide bonds (Swiss Prot: albu_bovin), and it was chosen as a model for exploring recovery of Cys modification. It was separated by 1D-SDS gel electrophoresis, and blotted onto PVDF. Without a reducing environment, Cys residues on PVDF-blotted protein will reform disulfide bonds; whether they form intra- or inter-disulfide bond remains unclear. Therefore, a reducing procedure is essential to break up cystine to Cys in order to undergo Cys alkylation. *On-blot* reduction and alkylation described in Section 2.5 were carried out with DTT or TBP as reducing reagents, and iodoacetamide or acrylamide as alkylating reagents using TEA to provide a high pH ($>$11.0) environment.

3.5.1. Comparison of DTT vs. TBP as reducing agents and iodoacetamide vs. acrylamide as alkylating agents

3.5.1.1. Recovery of Cys

The reducing and alkylating reagents: DTT (125 m*M*), TBP (2.5 m*M*), acrylamide (0.5 *M*) and iodoacetamide (0.135 *M*) commonly used [17,23] in gel electrophoresis were compared in this study. For gel strip equilibration, the Swiss-2D-PAGE protocol uses iodoacetamide solution at pH 6.5, which is not optimal for alkylation. However, we use iodoacetamide (0.25 *M*) at pH$\geq$8.5 for the alkylation re-

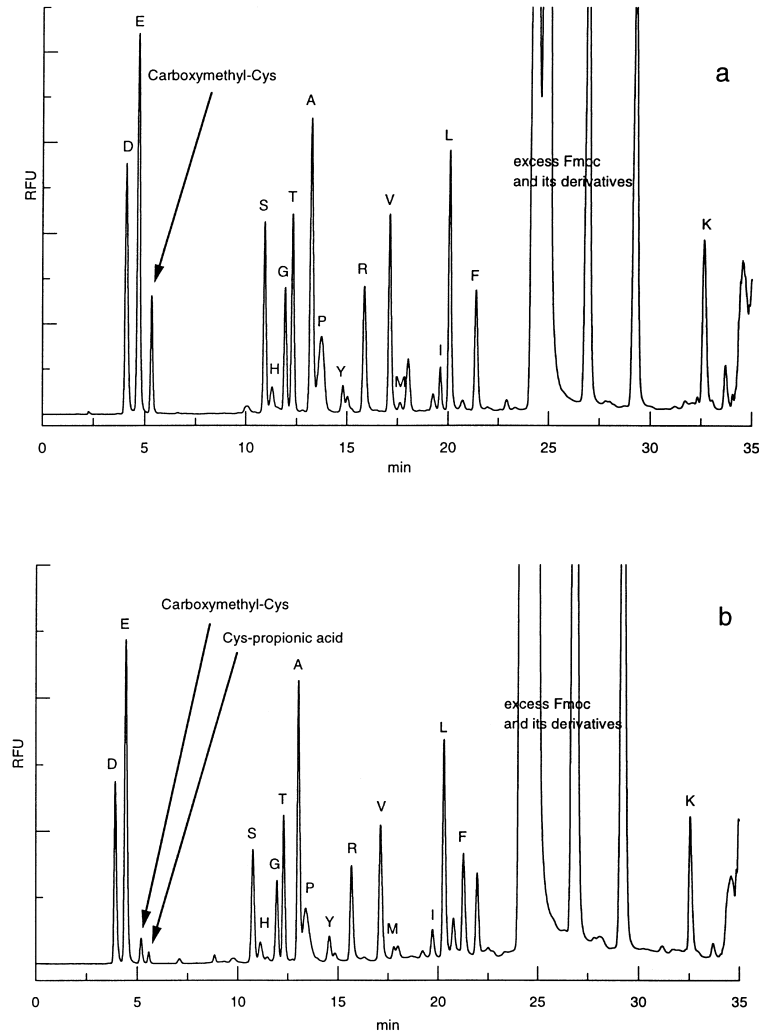

Fig. 2. Chromatograms of separation of hydrolysates from human serum albumin separated by 2D-PAGE and blotted on PVDF membrane where no specific additional reduction and alkylation step was used, where (a) carboxymethyl–Cys detected; (b) both carboxymethyl–Cys and Cys–propionic acid detected.

action. Table 1 shows the average recoveries (triplicates) of Cys from BSA using four different reduction and alkylation treatments: DTT and acrylamide; DTT and iodoacetamide; TBP and acrylamide; and TBP and iodoacetamide, as described in Section 2.5. The reactions were carried out at 37°C. DTT (125 m*M*) as a reducing reagent, and iodoacetamide (0.25 *M*) as an alkylating reagent gave the highest recovery of Cys. Further experimentation using (2.5 m*M*) TBP and (0.25 *M*) iodoacetamide treatment gave lower and non-reproducible recoveries (64–76%, $n=12$). When a final concentration of 50 m*M* TBP was used (the concentration reported by Brune [10]), it gave an average of only 50% recovery with both acrylamide or iodoacetamide as alkylating reagents. This result suggested a lower concentration of TBP is more efficient for alkylation but we did not find completely reproducible conditions. We were unable to increase the recovery of Cys–propionic acid by manipulating either reagent concentration or extending reaction times.

Table 1
The recovery of reduced and alkylated Cys from BSA[a] (triplicate samples) separated by 1D gel electrophoresis and electro-blotted onto PVDF membrane using four different conditions at pH>11.0, 37°C

Reduction and alkylation treatment	Cys–propionic acid (% recovery[b])	Carboxymethyl–Cys (% recovery)
125 m*M* DTT and 0.5 *M* acrylamide	70±3	—
125 m*M* DTT and 0.25 *M* iodoacetamide	—[c]	97±6
2.5 m*M* TBP and 0.5 *M* acrylamide	65±16	—
2.5 m*M* TBP and 0.25 *M* iodoacetamide	—[c]	87±6

[a]Approximately 10 pmol of protein hydrolysed. The theoretical %Cys composition is 8.9% calculated by number of Cys/total number of amino acids (excluding Asx, Glx, Trp and Lys)×100%.
[b]The % recovery is calculated by experimental %Cys composition/theoretical %Cys composition×100%. The %Cys composition=pmol of Cys/pmol of total amino acids (excluding Asx, Glx, Trp and Lys)×100%. In all cases, significant amounts of Lys were alkylated.
[c]The amount of Cys–propionic acid (from gel electrophoresis) was insignificant (<3% of carboxymethyl–Cys).

We conclude that using a combination of DTT and iodoacetamide provides near quantitative recovery of Cys in proteins separated by gel electrophoresis and electro-blotted onto a PVDF membrane.

3.5.1.2. Quantitation of Cys content in various proteins separated by gel electrophoresis

A variety of standard proteins were separated by 1D or 2D gel electrophoresis, blotted onto PVDF membrane and subjected to *on-blot* reduction and alkylation at 37°C with DTT and iodoacetamide. The resultant amino acid analysis is shown in Table 2. The results show that this technique is effective for the analysis of Cys in 13 different proteins with widely differing Cys contents. In comparison with other amino acids (Ala, Arg and Val) which normally show stable results (Table 2), Cys analysis is reliable. However, in all cases, the amount of Lys recovered was low and variable. This was found to be due to the concomitant alkylation of the ϵ-NH_2 group of Lys with iodoacetamide (see Section 3.5.2), the product of which eluted with aspartic acid (Asx, a mixture from both aspartic acid and asparagine). Therefore these amino acids were not used in the calculation of recoveries.

3.5.2. Lys alkylation with iodoacetamide or acrylamide

3.5.2.1. Observation of Lys alkylation

It was observed that during the reduction and alkylation of Cys, Lys recovery decreased and varied. Lys was now the amino acid which was unable to be quantitated. Fig. 3a and Fig. 3b show the typical separation of amino acids from hydrolysed 1D separated BSA, including Cys–propionic acid and carboxymethyl–Cys, modified *on-blot* by acrylamide and iodoacetamide, respectively. It was repeatedly found that the recovery of Lys (K) was very low compared with the total composition as shown in Fig. 3c (BSA contains 10% Lys).

Normally, Lys reacts with Fmoc at both amino groups (α- and ϵ-NH_2) to form a di-substituted Fmoc derivative which is highly hydrophobic and elutes late in the chromatogram [18]. It has been reported that amino groups of Lys can be alkylated under reducing and alkaline conditions [25,26]. When Lys residues are present in a protein, the ϵ-NH_2 can become singly or doubly alkylated.

The possibility that the ϵ-amino group of Lys was being alkylated by iodoacetamide and acrylamide was being investigated using poly-Lys. Alkylation with iodoacetamide led to the formation of the dialkylated (dicarboxymethyl-) Lys (Fig. 4a). The identity of this species which elutes at the position of Asx (Fig. 4a) was confirmed as mono-Fmoc–di-carboxymethyl–Lys (M+H=485) by off-line ESI-MS. Alkylation with acrylamide generates a mixture of products (Fig. 4b). Mono-Fmoc–di-Lys–propionic acid (M+H=513) and di-Fmoc–mono-Lys–propionic acid (M+H=663) were identified by off-line ESI-MS. The mixture of derivatives and the presence of non-alkylated Fmoc–Lys indicated that alkylation by acrylamide is incomplete using these conditions. Fig. 4c shows the chromatogram of Fmoc derivatives

Table 2
Cys analysis of various PVDF-blotted proteins separated by gel electrophoresis

Sample source[a]	Protein	Swiss-Prot	Amount hydrolysed[b] (pmol)	Cys comp.[c]		Ala comp.[c]		Arg comp.[c]		Val comp.[c]		Lys comp.[d]	
				Theor.	Exp.	Theor.	Exp.	Theor.	Exp.	Theor.	Exp.	Theor.	Exp.
2D	Heat shock protein	P60_human	8.4	0.8	**0.7**	14.6	**15.7**	4.5	**4.4**	14.3	**13.1**	12.3	**3.8**
	Glutamate dehydrogenase isomerase	dhe3_human	12.2	1.6	**1.5**	10.2	**12.1**	8.1	**7.7**	8.9	**9.1**	8.1	**1.5**
	Triose phosphate	tpis_human	18.6	2.9	**2.4**	16	**16.3**	4.6	**4.5**	14.3	**12.4**	10.3	**1.7**
	Catalase	cata_human	7.1	1.1	**2.1**	11.3	**12.2**	7.8	**7.7**	10.0	**8.5**	7.5	**1.3**
	Encoyl-CoA hydratase	echm_human	7.4	3.3	**2.2**	18.1	**17.4**	3.3	**4.9**	8.2	**11.1**	11.7	**1.3**
	Hemoglobin β-chain	hbb_human	26.3	1.8	**1.3**	13.8	**15.2**	2.8	**3.2**	16.5	**15.1**	9.2	**1.6**
	Tear prealbumin	vegp_human	24.8	2.7	**2.1**	8.8	**10.5**	6.2	**5.9**	9.7	**9.7**	8.9	**1.8**
	α-1-Antitrypsin	a1at_rat	33.2	0.4	**1.2**	8.5	**9.1**	4.8	**5.1**	8.8	**8.8**	8.7	**0.1**
1D	Carboxypeptidase inhibitor	mcpi_soltu	40	22.2	**16.1**	14.8	**13.6**	3.7	**4.3**	3.7	**4.4**	6.9	**2.6**
	T-cell surface antigen[e]	cd4_rat	29.3	3.7	**3.5**	6.1	**6.4**	6.7	**6.5**	9.4	**9.7**	10.6	**0.3**
	Exoglucanase I	gux1_trire	30	6.4	**5.9**	7.7	**8.6**	2.7	**3.0**	6.1	**6.9**	3.4	**0.2**
	β-Casein	casb_bovin	25	0[f]	**0.7**	3.4	**3.9**	2.7	**3.5**	12.8	**11.4**	6.9	**0.9**
	Serum albumin (BSA)	albu_bovin	8.9	8.9	8.7	11.9	**13.4**	6.6	**7.9**	9.4	**9.0**	13.0	**0.6**

All proteins were *on-blot* alkylated using 125 m*M* dithiothreitol as reducing reagent and 0.25 *M* iodoacetamide as alkylating reagent, at 37°C, pH>11.0. In all cases, the recovery of Lys was lower than the theoretical content.

[a]All analyses is for a single spot analysed. 1D, SDS–PAGE; 2D, 2D gel electrophoresis. In both cases gels were electroblotted to PVDF membrane prior to reduction and alkylation, acid hydrolysis and Fmoc derivatisation. In all cases, two Cys adducts were observed as Cys–propionic acid and carboxymethyl–Cys that were alkylated by acrylamide (in-gel) and iodoacetamide (*on-blot*), respectively. Thus, Cys composition was calculated by a sum of Cys–propionic acid and carboxymethyl–Cys.

[b]Protein amount determined by amino acid analysis (described in Section 2.6).

[c]For Cys, Ala, Arg and Val, the theoretical % amino acid composition=number of amino acid/total number of total amino acids (excluding Asx, Glx, Trp and Lys)×100%; and the experimental % amino acid composition=pmol of amino acid/pmol of total amino acids (excluding Asx, Glx, Trp and Lys)×100%.

[d]The theoretical % Lys composition=number of Lys/total number of total amino acids (excluding Asx, Glx and Trp)×100%. The experimental % Lys composition=pmol of Lys/pmol of total amino acids (excluding Asx, Glx and Trp)×100%.

[e]Recombinant rat T-cell surface antigen (RsCD4) without signal amino acid sequence and lacking the GPI anchor (AA 28–393) [24] was constructed in Dr. Neil Barclay's laboratory in the University of Oxford.

[f]β-Casein contains 1 Cys in its signal sequence region. Thus, there is no Cys in the mature protein chain. This shows that it is difficult to quantitate a single Cys in a protein.

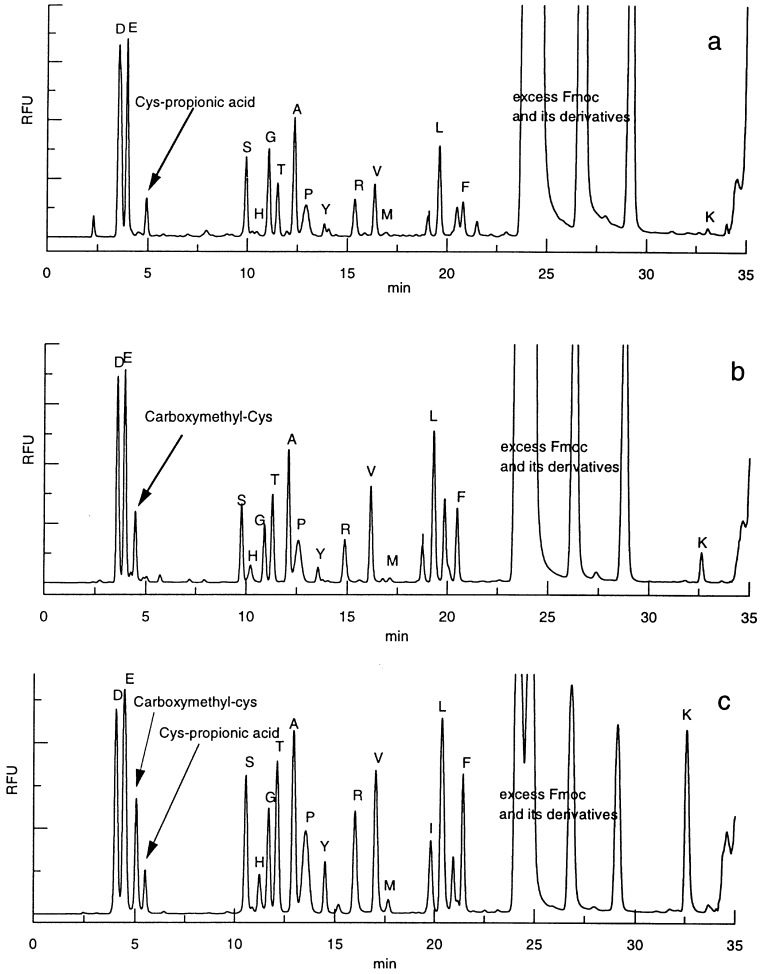

Fig. 3. Chromatograms of separation of hydrolysates from 1D-PAGE separated and PVDF blotted BSA, which was *on-blot* reduced and alkylated prior to acid hydrolysis, where (a) alkylation by acrylamide at pH>11.0, Cys–propionic acid fully recovered and Lys partially alkylated; (b) alkylation by iodoacetamide at pH>11.0, both carboxymethyl–Cys and Cys–propionic acid detected, and Lys fully modified; (c) by iodoacetamide at pH 8.5, both carboxymethyl–Cys and Cys–propionic acid detected, and Lys fully recovered.

of acid hydrolysed, non-alkylated poly-Lys, where a significant Fmoc–Lys peak is observed as expected and no adducts are apparent. The minor amino acid peaks in Fig. 4a–c are contamination of poly-Lys, a synthetic product which has been dot-blotted onto PVDF membrane without any purification in this study.

Using iodoacetamide or acrylamide as alkylating reagent at pH>11.0, there were no apparent effects on quantitation of histidine (His) and tyrosine (Tyr). Since the possibility of His and Tyr alkylation exists, we explored this further using poly-His and poly-Tyr. Only the Fmoc His and Tyr derivatives were obtained after reduction, alkylation and acid hydrolysis (data not shown). This is in agreement with the conclusions of Crestfield et al. [6] that His alkylation requires a pH of 5.5. Cotner and Clagett [27] demonstrated that after acid hydrolysis, O-derivatives of Tyr are hydrolysed back to the free Tyr residue.

3.5.2.2. Comments on Lys alkylation

At high pH (>11.0), using DTT and iodoacetamide for *on-blot* reduction and alkylation, Cys can

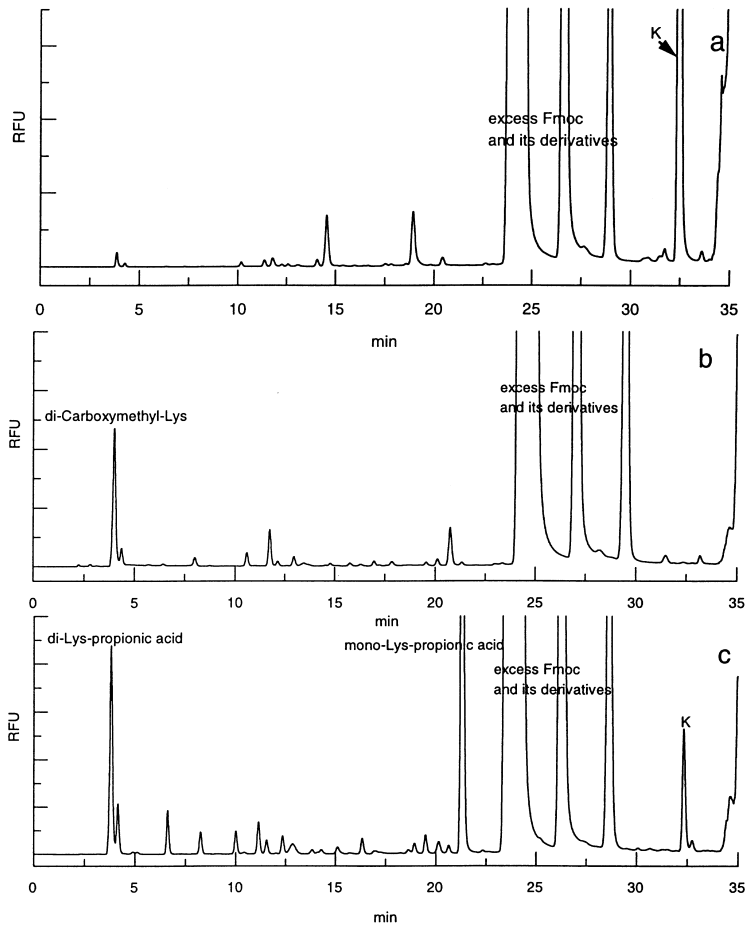

Fig. 4. Chromatograms of separation of acid hydrolysed poly-Lys (spotted onto PVDF membrane), where (a) the Cys was not alkylated; a major peak of Lys detected; (b) Lys was alkylated by iodoacetamide at pH>11.0 followed by acid hydrolysis, almost all Lys modified as di-carboxymethyl-Lys (mono-Fmoc derivative) coeluting with Asp; (c) Lys was alkylated by acrylamide at pH>11.0 followed by acid hydrolysis; Lys partially modified as mono-Lys–propionic acid (di-Fmoc derivative) eluted after Phe, and di-Lys–propionic acid (mono-Fmoc derivative) coeluting with Asx.

be reliably quantitated. This treatment also leads to almost quantitative alkylation of Lys, although its derivatives require further separation from the co-eluted amino acid, Asx. Other amino acids are not affected by the treatments. It has been demonstrated that a high pH environment favors both reduction and alkylation enabling 100% alkylation of Cys, while the de-protonated ϵ-NH_2 group in Lys is also alkylated. Carboxyamidomethylation of the ϵ-NH_2 group in Lys may have some uses: (1) in normal Fmoc amino acid analysis, Lys elutes after excess Fmoc at around 32 min. If Lys were modified quantitatively and was able to be separated from the other amino acids as above, the analysis time may be shortened. (2) In peptide mass spectrometry analysis, the commonly used enzyme, trypsin, cannot cleave Lys sites if the Lys side chain is modified [28]. Using carboxyamidomethylation, it may be possible to selectively obtain arginine-terminated peptides to simplify the peptide spectrum, or to give a better interpretation of the different masses obtained.

3.5.3. Overcoming the complication of Lys alkylation

The pK_a of the ϵ-amino group in Lys is 9.5, and SH-group of is Cys 8.5 [13]. As the rate of alkylation

is dependent on the pH, the level of protonation of these groups can be altered. In an attempt to reduce the alkylation of Lys while maintaining the yield of Cys the reduction and alkylation conditions were varied as a function of pH and temperature.

3.5.3.1. Reduction and alkylation of Cys as a function of pH and temperature

Table 3 shows the recovery of Cys from BSA (1D gel-separated and blotted on PVDF) using four combinations of reducing and alkylation reagents (DTT or TBP; iodoacetamide or acrylamide) at pH 8.5 at different temperatures. When both reactions were carried out at 37°C, all treatments gave between 53 and 60% recovery of Cys with complete absence of Lys alkylation. In an attempt to increase the level of Cys alkylation we increased both reaction temperatures to 80°C. The combination of DTT and iodoacetamide did not lead to Lys alkylation but the percentage of Cys alkylation was not increased significantly, while the other three treatments at this temperature led to alkylation of Lys without dramatically increasing the alkylation of Cys. This may be attributed to oxidation of Cys in the alkylation reaction at 80°C. There was no reducing reagent present during the alkylation step and Cys may have been oxidised at 80°C, even though the reaction mixture in the Eppendorf tube was flushed with argon. When we used DTT and conducted the reduction step at 80°C, followed by alkylation using iodoacetamide at 37°C, we achieved 100% recovery of Cys without concomitant alkylation of Lys (Fig. 3c shows a typical chromatogram of *on-blot* reduced and alkylated BSA using these conditions, note the presence of the acrylamide adduct as well as the iodoacetamide adduct). This was confirmed by replicated analyses ($n=12$). Thus, we conclude that using the condition of reduction (125 m*M* of DTT, pH 8.5, at 80°C) and alkylation (0.25 *M* iodoacetamide, pH 8.5, at 37°C) on proteins blotted to PVDF membrane after gel electrophoresis, all amino acids (except Trp) are able to be quantitated in one HPLC separation.

3.5.3.2. Demonstration of Cys analysis in various proteins separated by gel electrophoresis without loss of Lys

A group of gel-separated proteins were *on-blot* reduced and alkylated using the optimal conditions

Table 3
The recovery of Cys from BSA[a] separated by 1D gel electrophoresis and electro-blotted onto PVDF membrane using different reduction and alkylation reagents at pH 8.5 and different reaction temperatures

Reducing and alkylation treatment (pH 8.5)	% recovery	Lys alkylation
Reduction at 37°C and alkylation at 37°C ($n=3$)		
125 m*M* DTT+0.25 *M* iodoacetamide	60±0[b]	No
125 m*M* DTT+0.5 *M* acrylamide	53±12[b]	No
2.5 m*M* TBP+0.25 *M* iodoacetamide	53±3[b]	No
2.5 m*M* TBP+0.5 *M* acrylamide	57±3[b]	No
Reduction at 80°C and alkylation at 80°C ($n=3$)		
125 m*M* DTT+0.25 *M* iodoacetamide	63±5[b]	No
125 m*M* DTT+0.5 *M* acrylamide	76±3[c]	Yes
2.5 m*M* TBP+0.25 *M* iodoacetamide	48±9[c]	Yes
2.5 m*M* TBP+0.5 *M* acrylamide	54±7[c]	Yes
Reduction at 80°C and alkylation at 37°C ($n=12$)		
125 m*M* DTT+0.25 *M* iodoacetamide	100±12[b]	No

[a]Average amount hydrolysed was 10 pmol. The theoretical % Cys composition is 8.9% calculated by number of Cys/total number of amino acids (excluding Asx, Glx, Trp and Lys)×100%; or 6.0% calculated by number of Cys/total number of amino acids (excluding Trp only)×100%.

[b]The % recovery was calculated by experimental % Cys composition/theoretical % Cys composition×100%. The experimental % Cys composition=pmol of Cys/pmol of total amino acids (excluding Trp)×100%. In these cases, Lys was not alkylated.

[c]The % recovery was calculated by experimental % Cys composition of BSA/theoretical % Cys composition×100%. The experimental % Cys composition=pmol of Cys/pmol of total amino acids (excluding Asx, Glx, Trp and Lys)×100%. In these cases, Lys was alkylated.

Table 4
Demonstration of Cys analysis in various PVDF-blotted proteins separated by gel electrophoresis, using 125 m*M* dithiothreitol as reducing reagent at 80°C and 0.25 *M* iodoacetamide as alkylating reagent at 37°C, at pH 8.5

Sample source	Protein identified	Protein hydrolysed (pmol)	Cys composition[a]		Lys composition[a]	
			Theor.	Exp.	Theor.	Exp.
2D	Heat shock protein (p60_human)	12.9	0.5	0.4	9.7	9.1
	Haptoglobin (hpt_rat)	12.8	2.8	2.7	8.7	8.4
	Fetuin (a1hs_rat)	3.4	3.6	3.6	3.9	3.8
	Alpha-antitrypsin (a1at_rat)	17.2	0.3	0.4	6.8	7.0
	Tear prealbumin (vegp_human)	24.7	1.9	1.6	7.0	7.3
1D	T-cell surface antigen[b] (cd4_rat)	26.1	2.6	2.4	8.7	8.1
	Ca^{2+}ATPase[c] (atcd_human)	4.7	3.0	3.0	5.4	5.4
	GTP-binding protein RAM[c] (RB27_human)	24.0	2.8	3.0	5.5	5.1

The % amino acid composition=pmol of amino acid/pmol of total amino acids (excluding Trp)×100%. In all cases, Lys was not alkylated. All proteins were identified by AACompIDent search program (http://expasy.hcuge.ch).
[a]In all cases, two Cys adducts were observed as Cys–propionic acid and carboxymethyl–Cys that were alkylated by acrylamide (*in-gel*) and iodoacetamide (*on-blot*), respectively. Thus, Cys composition was calculated by a sum of Cys–propionic acid and carboxymethyl–Cys.
[b]Recombinant rat T-cell surface antigen (RsCD4) without signal amino acid sequence and lacking the GPI anchor (AA 28–393) [24] was constructed in Dr. Neil Barclay's laboratory in the University of Oxford.
[c]These two proteins were purified from human red blood cells by Dr. Kailing Wang in the University of Sydney.

present above. Table 4 shows the protein names, amount hydrolysed and comparison of Cys composition with theoretical data. In all cases, alkylated Lys was not detected and was estimated accurately. These proteins were positively identified by their experimental amino acid composition (excluding Trp) using Constellation 5 in AACompIdent search program in ExPASy (http://expasy.hcuge.ch) [19,29]. Cys has been accurately quantitated in this range of proteins which contain between 0.5 and 3.6% Cys. Using Constellation 2 in AAcompIdent (excluding Cys and Trp), similar identification results have been achieved (data not shown here). Also we have observed that for high Cys-containing proteins, such as wool intermediate filament proteins, protein identification using amino acid composition including Cys has improved the confidence of the identification (manuscript in preparation).

4. Conclusions

Conditions for the *on-blot* reduction and alkylation of Cys residues in proteins separated by 2D gel electrophoresis have been established. This has enabled the development of a rapid, reliable and quantitative analysis of these alkylated Cys products using Fmoc-based amino acid analysis. Quantitation of Cys has increased the confidence in identification of high Cys-containing proteins by their amino acid composition.

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