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Analysis of cysteine residues in peptides and proteins alkylated with volatile reagents

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Summary. Conditions are described for the reduction and alkylation of cysteines in peptides and proteins with volatile reagents by use of triethylphosphine as reductant, bromopropane as alkylating reagent and triethylamine as base. Alkylated samples need only be vacuum dried prior to subsequent analysis steps. Alkylated samples have been acid hydrolyzed and analyzed on an amino acid analyzer with recoveries of cysteine within 10% of the expected value. Alkylated samples have been directly applied to a sequencer membrane, dried on the surface and cysteines identified by sequence analysis without additional wash steps. In addition proteins blotted onto PVDF have been alkylated *in situ* and sequenced with identification of cysteines. On the analyzer and sequencer the S-propylcysteine derivative elutes at a unique position allowing for the unambiguous identification of cysteine. Cysteine residues are quantitativly alkylated under the conditions developed. The ease of this procedure allows the routine analysis of cysteine in peptides and proteins without additional, time consuming repurification or dialysis steps.

Keywords: Cysteine – Protein sequencing – Amino acid analysis – Amino acid derivatives

Abbreviations: dptu, diphenylthiourea; dmptu, dimethylphenylthiourea; prop-cys, S-propylcysteine

Introduction

Cysteine is one of the most difficult amino acids to quantitate by amino acid analysis or to identify by protein sequence analysis. The reason for this is the high reactivity of the sulfhydryl group on the cysteine side chain. This group is subject to a number of reactions including oxidation, elimination and modification by electrophilic compounds. Cysteine is present in nearly all proteins, additionally, many synthetic peptides contain cysteine either as part

of a sequence or because of the usefulness of the sulfhydryl group in immobilization and other post synthetic modification reactions. In order to obtain reliable analysis, the cysteine residues must first be stabilized. Classically, cysteines have been stabilized by alkylation with reagents like iodoacetamide (Henschen, 1986). Recently several new reagents have been reported for alkylation of cysteines which have improved properties for identification of cysteine by sequence (Brune, 1992; Krutzsch and Inmann, 1993; Jue and Hale, 1993) and amino acid analysis (Hale et al., 1994). While all of these new reagents yield cysteine derivatives that are easily identifiable by sequencing or amino acid analysis, it is necessary to remove excess reducing and alkylating reagents by repurification of proteins or peptides or by extensively washing proteins applied to sequencing membranes (Andrews and Dixon, 1987; Hawke and Yuan, 1987; Jue and Hale, 1994). Repurification of protein or peptide samples is undesirable because of the additional labor intensive steps required. Alkylation of peptides using on-line sequencing procedures (Andrews and Dixon, 1987; Hawke and Yuan, 1987; Jue and Hale, 1994) may lead to unacceptable losses in the wash steps used to remove excess reagents.

We report here the use of new reagents for the alkylation of cysteine that are volatile. By combining these reagents with a volatile reducing reagent and base, conditions have been developed for alkylation of cysteines in peptides and proteins following which excess reagents are removed by evaporation or under vacuum. No repurification or sample washing steps are necessary. Peptides or proteins alkylated in this fashion are suitable for amino acid analysis and protein sequencing. The cysteine derivatives formed elute in unique positions on a post-column amino acid analysis system and on an ABI 477A/120A sequencer without modification of the standard analyzer gradients.

Materials

Bromopropane and triethylphosphine were obtained from Aldrich (Milwaukee, WI). Triethylamine, and BSA were from Pierce (Rockford, IL). Amino acid analysis reagents were from Beckman (Palo Alto, CA) Sequencing and peptide synthesis reagents were from ABI (Foster City, CA). Lysozyme was purchased from Sigma (St. Louis, MO).

Methods

Alkylation of cysteines in proteins or peptides in solution

Protein or peptide (1 nmole to 1 μmole) was dissolved in 50 μl 25–75% acetonitrile in a clean glass reaction tube. A 100 μl volume of alkylation cocktail was added. The alkylation cocktail consisted of 100 μl acetonitrile, 2 μl triethylamine, 2 μl triethylphosphine and 10 μl bromopropane. Depending on the water content of the protein or peptide solution, 2 phases may have formed. If this occured a single phase was restored by addition of small amounts (10–20 μl) of acetonitrile. The alkylation mixture was immediately capped and incubated in the dark at 60°C for 30 min.

Following alkylation the alkylation mixture was either spotted directly onto a sequencer membrane or vacuum dried on a Speed-Vac prior to acid hydrolysis and amino acid analysis.

Alkylation of cysteines in proteins electroblotted onto PVDF

Protein samples were separated on SDS-PAGE gels (Laemmli, 1970) and electroblotted onto PVDF membrane (Matsudaira, 1987). Proteins were visualized with Coomassie blue and bands of interest were excised and placed into a small reaction tube or directly into a sequencer blot cartridge. The blot slices were wetted with enough alkylation cocktail to keep the blot slice moist during the incubation (typically 6–10 μl). The blot alkylation cocktail consisted of 100 μl methanol, 2 μl triethylamine, 4 μl triethylphosphine and 10 μl bromopropane. Following addition of the alkylation cocktail, the reaction tube was capped or the blot cartridge was assembled and the blot was incubated for 30 min at 56–60°C. After incubation the blot slice was air dried or the sequencer was started.

Amino acid analysis

The dried, alkylated protein or peptide in the reaction tube was placed in a hydrolysis vial containing 6 N HCl, placed under vacuum and purged with N_2 three times. The peptide was then placed under vacuum and gas phase hydrolyzed at 120°C for 18 h. Following hydrolysis, the sample was vacuum dried and resuspended in sample buffer to a final concentration of approximately 100 nmole/ml based on the initial peptide concentration. Amino acid analysis was performed on an Beckman 6,300 post-column system which utilizes ninhydrin for detection of amino acids. The average of the methionine and valine standards were used to calculate the recovery of alkylated cysteine.

Protein sequence analysis

Immediately following alkylation, the peptide/alkylation mixture was spotted onto a biobrene pretreated sequencer support and dried under a stream of nitrogen. The sequencer cartridge was then assembled and sequencing performed on an ABI 477A/120A sequencer. Standard begin and reaction cycles (supplied with the sequencer) and standard conversion cycles (from ABI) were used. A standard analyzer gradient optimized for the individual sequencer was used.

Peptide synthesis

All peptide synthesis was performed on an ABI 433 synthesizer using preloaded resins and standard FastMoc chemistry.

Results

Amino acid analysis of cysteines alkylated with bromopropane

Lysozyme was reduced and alkylated with bromopropane as described in the Methods. After the sample had been dried under vacuum and hydrolyzed the amino acids were analyzed on a Beckman 6,300 analyzer. The chromatogram

obtained is shown in Fig. 1. The S-propylcysteine derivative that formed eluted between valine and methionine using a standard analyzer gradient. No additional peaks were seen in the chromatogram indicating that vacuum drying effectively removed the excess alkylation reagents. The amino acid quantitation from this chromatogram is listed in Table 1. The recovery of cysteine was within 3% of its expected value based on the quantitation of the S-propylcysteine derivative. All of the other amino acids were recovered within 10% of the expected value indicating that the alkylation procedure did not cause appreciable modification of any other amino acid.

The alkylated lysozyme sample was analyzed by reverse phase HPLC and compared to unalkylated lysozyme. Alkylation resulted in a significant increase in the retention of the lysozyme sample (Fig. 2A,B) which is consistent with the increased hydrophobicity of the S-propylcysteine residues. The alkylated protein is a discrete peak suggesting uniform alkylation. Electrospray mass spectrometric analysis of this peak determined a mass of 14,647.9 which is essentially identical to the mass calculated for lysozyme with 8 S-propylcysteine residues (14,649).

BSA was also alkylated and analyzed and the results tabulated in Table 2. Cysteine recovery was within 7% of the expected value. All of the other amino acids were recovered within 10% of the expected value with the exception of lysine. Some evidence of alkylation of lysine was seen in BSA (around 10%). This modification could be reduce by shortening the incubation time to 15 min or reducing the TEA concentration.

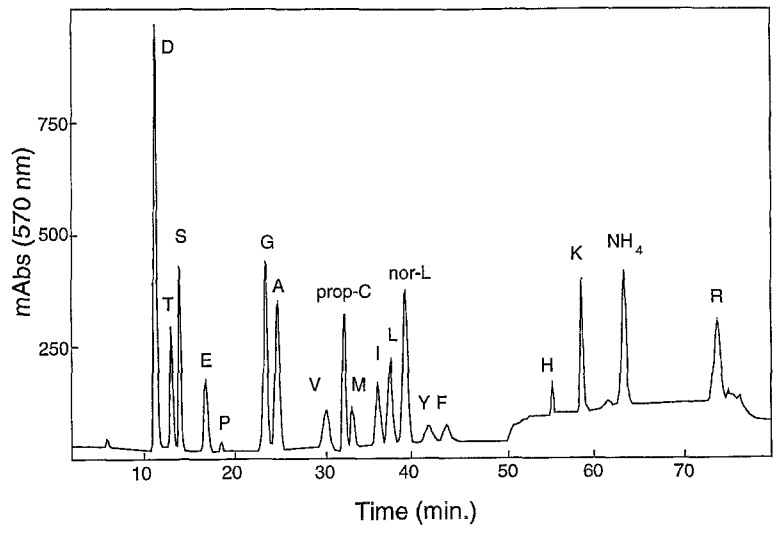

Fig. 1. Ninhydrin chromatogram of the acid hydrolysate of lysozyme alkylated with bromopropane. Lysozyme was alkylated and dried as described in Methods. The protein sample was acid hydrolyzed in the gas phase for 18 h at 120°C utilizing 6 N HCl containing phenol. Following hydrolysis, the sample was dried on a speedvac and the hydrolyzed amino acids resuspended in NaS buffer (Beckman). Amino acids were separated and detected on a Beckman 6,300 amino acid analyzer using an unmodified gradient. Amino acids are labelled with the single letter code

Table 1. Amino acid composition of lysozyme alkylated with bromopropane determined on a Beckman amino acid analyzer

AA	nM AA	AA Ratio	Expec.
D	131.5	21.0	21
T	42.4	6.8	7
S	57.5	9.3	10
E	29.3	4.7	5
P	12.1	2.0	2
G	76.8	12.4	12
A	76.6	12.4	12
V	33.4	5.4	6
Prop-C	48.3	7.8	8
M	13.2	2.1	2
I	32.5	5.2	6
L	50.5	8.1	8
Y	18.7	3.0	3
F	18.0	2.9	3
H	6.5	1.0	1
K	34.7	5.6	6
R	65.4	10.6	11

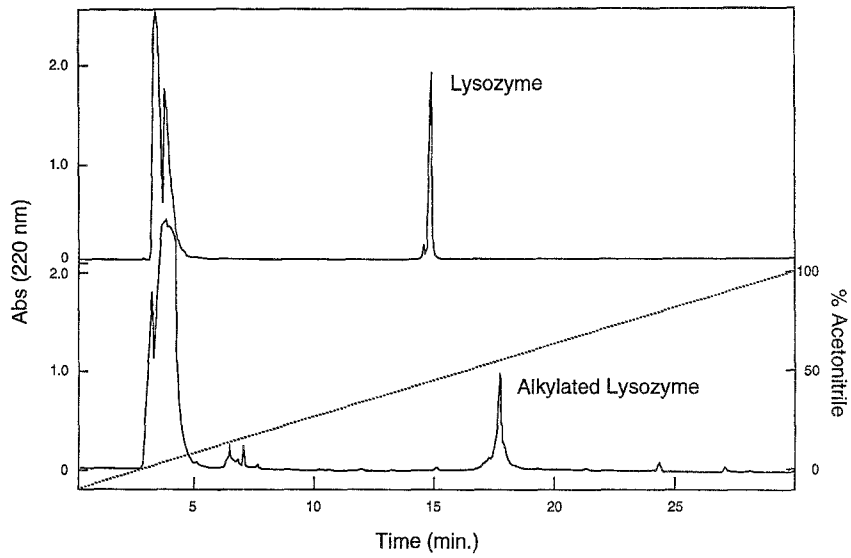

Fig. 2. C-18 reverse phase HPLC monitoring of lysozyme and lysozyme alkylated with bromopropane. Lysozyme and lysozyme alkylated as described in Methods were separated on a 4.5 × 250 mm C-18 column (Vydac). Lysozyme samples were loaded onto the column in 0.1% TFA. Bound protein was eluted with a gradient from 0% to 100% acetonitrile developed over 30 min

A 56 amino acid synthetic peptide containing 7 cysteines was reduced and alkylated with bromopropane and analyzed. The results summarized in Table 3 indicate that cysteine was recovered within 3% of its expected value. The other amino acids were generally within 10% of the expected value with a few

Table 2. Amino acid composition of *BSA* alkylated with bromopropane determined on a Beckman amino acid analyzer

AA	nM AA	AA Ratio	Expec.
D	70.6	54.3	54
T	41.1	31.6	34
S	32.8	25.2	28
E	102.7	79.0	79
P	36.0	27.7	28
G	22.8	17.6	16
A	58.5	45.0	46
V	44.4	34.2	36
Prop-C	42.2	32.3	35
M	5.8	4.5	4
I	17.1	13.2	14
L	78.4	60.3	61
Y	25.3	19.4	19
F	34.2	26.3	27
H	20.6	15.9	17
K	64.6	49.7	59
R	35.2	27.1	23

Table 3. Amino acid composition of a synthetic peptide alkylated with bromopropane determined on Beckman amino acid analyzer

AA	nM AA	AA Ratio	Expec.
D	62.3	4.3	4
T	28.9	2.0	2
S	38.4	2.6	3
E	86.4	5.9	6
P	63.0	4.3	4
G	17.0	1.2	1
A	12.8	0.9	1
V	11.8	0.8	1
Prop-C	99.3	6.8	7
M	30.1	2.1	2
I	64.6	4.4	5
L	107.8	7.4	7
Y	0.1	0.0	0
F	29.7	2.0	2
H	13.8	1.0	1
K	121.5	8.4	9
R	14.9	1.0	1

exceptions. The greatest variation was with the valine recovery and may indicate poor hydrolysis since branched amino acids are known to hydrolyze slowly. The recovery of lysine was about 93% of expected indicating little modification.

Sequence analysis of cysteines alkylated with bromopropane

Lysozyme alkylated with bromopropane in solution was spotted directly onto a biobrene treated glass fiber filter in the alkylation cocktail. The sample was dried under a stream of nitrogen, the sequencer cartridge assembled and the sequencer started. Figure 3A shows step 1 in the sequence of lysozyme. No background peaks due to excess alkylating reagents are seen and only the usual artifact peaks from the Edman chemistry (dptu[2] & dmptu) and lysine (N-terminal amino acid of lysozyme) are seen. Steps 5–7 in the sequence of lysozyme are shown in Fig. 3B. The S-propylcysteine residue is readily identified in step 6 and elutes as the last amino acid (following leucine) using a standard analyzer gradient. The charged residues arginine (step 5) and glutamic acid (step 7) are poorly extracted from the biobrene treated membrane as evidenced by the low yield of these amino acids. The S-propyl cysteine derivative is stable to the sequencing chemistry as is shown in Fig. 4. The S-propylcysteine residue is easily identified in step 30 from the increase over step 29.

Lysozyme was electrophoresed on a 4–20% SDS-polyacrylamide gel under non-reducing conditions and transferred to PVDF. After staining the blot with Coomassie blue the band corresponding to lysozyme was excised and

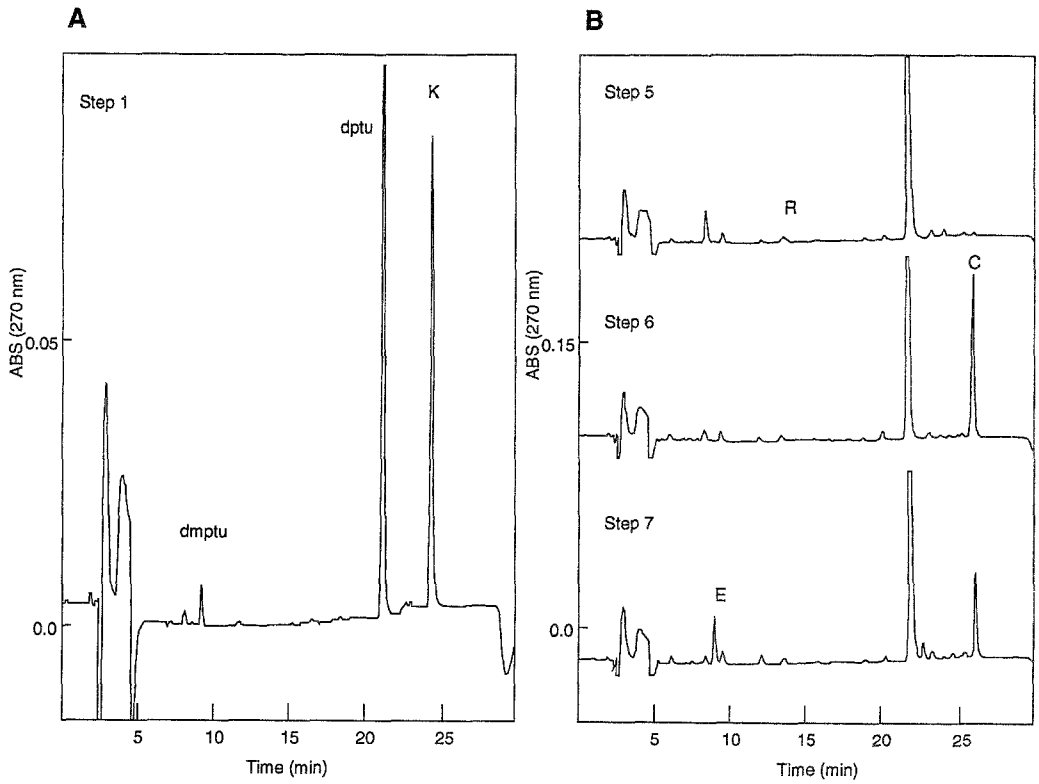

Fig. 3. Protein sequence analysis of lysozyme immobilized on a glass fiber filter. Lysozyme (25 μg) was derivatized with bromopropane and spotted onto a glass fiber filter as described in Methods and loaded into the ABI 477A Protein Sequencer. **A** Cycle 1; **B** Cycles 5, 6 and 7

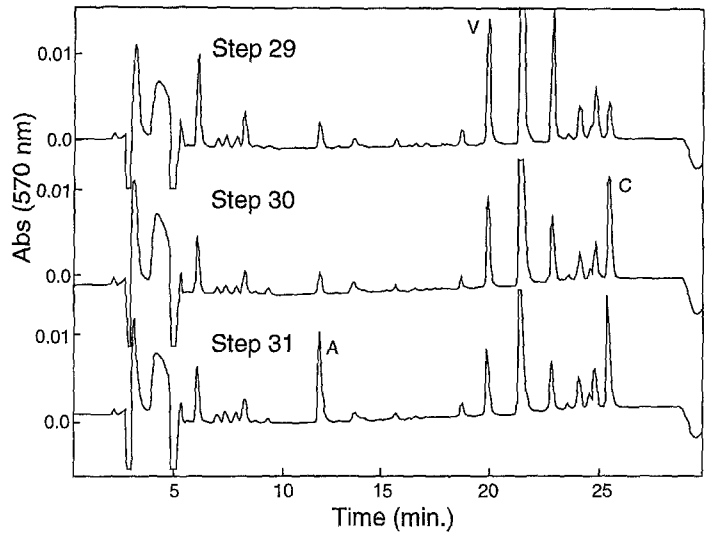

Fig. 4. Cycles 29–30 of the protein sequence analysis of lysozyme immobilized on a glass fiber filter. The derivatized lysozyme sample described in Fig. 3 was sequenced for 31 cycles. Cycles 29–31 are shown

alkylated as described in Methods. Figure 5 shows steps 5–7 of lysozyme alkylated on PVDF in the sequencer blot cartridge. The S-propyl cysteine derivative is easily identified at step 6. The charged residues arginine and glutamic acid are much more efficiently extracted from the PVDF membrane.

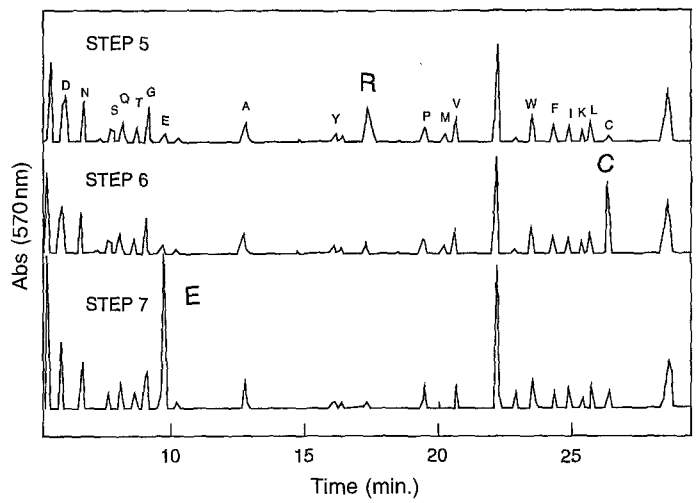

Fig. 5. Protein sequence analysis of lysozyme electroblotted onto a PVDF membrane and alkylated in situ. Lysozyme (10μg) was separated on a 4–20% SDS-PAGE gel, electroblotted onto PVDF, stained with Coomassie blue, the lysozyme band excised and alkylated as described in Methods in the blot cartridge. The ABI 477A Protein Sequencer was started after a 30 min. incubation at 56°C. Cycles 5–7 are shown (baseline corrected data shown)

Discussion

We have developed a method for the analysis of cysteines in proteins and peptides that is rapid, simple and does not require repurification of the alkylated peptide prior to sequencing or amino acid analysis. Use of all volatile reagents for the reduction and alkylation allows the removal of excess reagents by evaporation. The efficiency of removal of the reagents is illustrated in the chromatogram from the amino acid analysis and in step 1 from the sequence analysis of lysozyme alkylated with bromopropane. Both the amino acid analysis chromatogram and step 1 of the sequence showed virtually no background peaks indicating that excess reagent was removed by evaporation during vacuum drying or the nitrogen dry down step. This procedure is thus preferable to existing procedures for the online alkylation of proteins for sequence analysis which require extensive wash procedures for the removal of excess alkylating reagents (Andrews and Dixon, 1987; Hawke and Yuan, 1987; Jue and Hale, 1994). These wash procedures are incompatible for sequence analysis of peptides since unacceptable amounts of peptide will be washed out.

The S-propylcysteine derivative that forms elutes in a favorable position on the Beckman 6300 amino acid analysis system (between valine and methionine) and on the ABI 477/120 sequencer (as the last amino acid following leucine) making identification of cysteine easy. Furthermore the amino acid quantitation indicates that the derivatization of cysteine is complete. This was confirmed by electrospray mass spectrometry of alkylated lysozyme which yielded a mass consistent with complete modification of cysteine residues with propyl groups. No higher (or lower) molecular weight species were detected suggesting that no other amino acids were modified. Step 1 in the sequence analysis of lysozyme revealed no preview of the 2nd amino acid (which is valine) indicating that no alkylation of the N-terminal amino acid occurred (Chang, 1978). The amino acid analysis of the 56 amino acid synthetic peptide yielded values close to those expected for all of the amino acids (with the exception of valine) indicating that no other amino acids were modified by the alkylation procedure. In the BSA sample a small amount of modification of lysine appeared to occur. This could be reduced by shortening the incubation time or by adding less base.

The procedure described herein is compatable with high throughput analysis of peptides. Since the purification step consists only of vacuum drying, many peptides may be alkylated and prepared for acid hydrolysis at once. The time consuming steps of column purification or dialysis have been completely eliminated.

The amino acid analysis reported herein was done on an Beckman 6,300 amino acid analyzer. This analyzer utilizes ninhydrin for detection of amino acids. Bromopropane may not be the optimal reagent (based on the separation of the alkylated cysteine derivative) for other amino acid analysis systems. However, other volatile alkylating reagents can be used such as bromoethane or bromobutane which yield derivatives that elute in different

positions (data not shown). Therefore one could tailor the alkylating reagent used to individual amino acid analysis or sequencing system.

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