Poster Presentation

The Protein Society
Seventh Symposium
July, 1993

Poster #301-T

Improved Cysteine Analysis Using Disulfide Interchange

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Abstract

Amino acid analysis has become a simple and effective tool for quantitation of protein and peptide amino acids. However, under standard hydrolysis conditions unprotected cysteine is highly susceptible to thermal degradation and is characteristically difficult to quantify. A recently reported method uses disulfide interchange reagents for cysteine protection [1]. Cysteine reacts with each dithiocompound to form a thermally stable mixed disulfide. Recently, we have used this method in conjunction with the novel derivatizing agent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, for sensitive amino acid analysis of proteins [2, 3]. This poster will compare the analyses of several protein samples hydrolyzed in the presence of three dithio-compounds dithiodiglycolic acid (DTDGA), dithiodipropionic acid (DTDPA), and dithiodibutyric acid (DTDBA). As expected, the three cysteine disulfide adducts were detected. When necessary, modifications were made in the normal hydrolysate chromatographic conditions to improve resolution of the cysteine analog. In addition to the cys-derived peak, a smaller previously unidentified peak was detected, and has been tentatively identified for each of the three.

Protein fractions (β lactoglobulin and cytochrome C) were collected on a membrane fraction collector following separation by capillary electrophoresis. These proteins were then hydrolyzed on the membrane in the presence of DTDPA and analyzed using AQC derivatization.

[1] Barkholt, V., and Jenson, A.E., *Anal. Biochem.* 177 (1989) 318.
[2] Strydom, D.J., and Cohen, S.A., (1993) *in* Techniques in Biochemistry IV, (R.H. Angeletti, ed.), Academic Press, San Diego.
[3] Cohen, S.A., and Michaud, D.P., *Anal. Biochem.*, 211 (1993) 279.

Instrumentation

Hydrolysis Instrumentation
Waters Pico•Tag Workstation
Precision Scientific Model 16 hydrolysis oven (Chicago, Illinois)

Chromatographic Instrumentation
Waters 715 autoinjector
Waters 625 pump
3.9 x 150 mm AccQ•Tag™ C18 reverse phase column
Waters Temperature Control Module
Column Temp. 37°C for DTDGA and DTDPA, 39°C for DTDBA
Waters 470 Scanning Fluorescence Detector
λ Excitation 250 nm
λ Emission 395 nm
Waters Expert Ease™ software

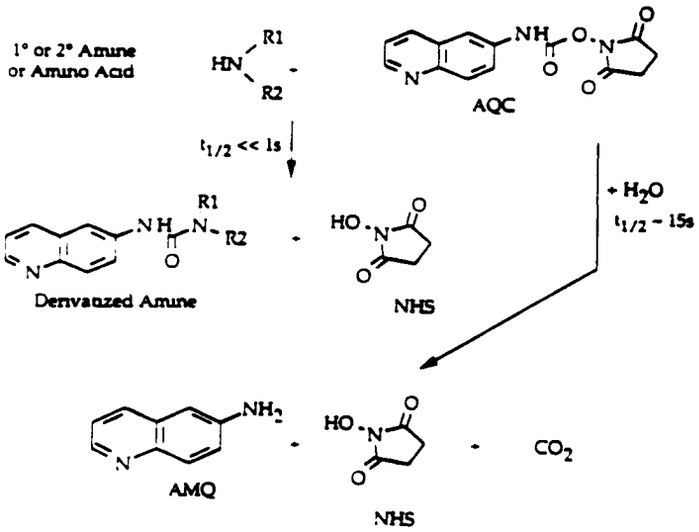
CE Membrane Fraction Collection
Waters Quanta 4000E™
Membrane Fraction Collector

HPLC Analysis
Amino acid analysis was achieved using a ternary gradient.

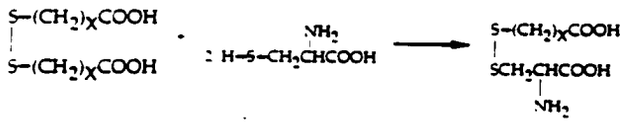
Eluent A	140 mM NaOAc, 17 mM TEA, 3μM EDTA, pH 5.00 for DTDGA or 5.05 for DTDPA and DTDBA
Eluent B	Acetonitrile
Eluent C	Water
Flow Rate	1 mL/min

Different gradients were used for each of the three reagents in order to resolve each of the disulfide interchange products from the other amino acids.

Derivatization Chemistry



Proposed Disulfide Interchange Chemistry*



X = 1 for DTDGA, 2 for DTDPA, and 3 for DTDBA

* Hoogerheide, J.G., and Campbell, C.M., *Anal. Biochem.*, 210 (1992) 146.

Sample Preparation

Hydrolysis

1. Deliver the protein containing section of the membrane (washed first with methanol and water) or an aliquot of amino acid or protein sample to a pyrolyzed 6 x 50 mm tube.
2. Put all tubes in a hydrolysis vial and dry under vacuum.
3. Add an aliquot of the disulfide interchange reagent to each tube.
4. Vortex each tube and dry under vacuum.
5. Add constant boiling HCl and phenol to bottom of hydrolysis vial.
6. Evacuate and purge with nitrogen several times.
7. Evacuate and seal under vacuum.
8. Hydrolyze at 110°C for 22 hours.

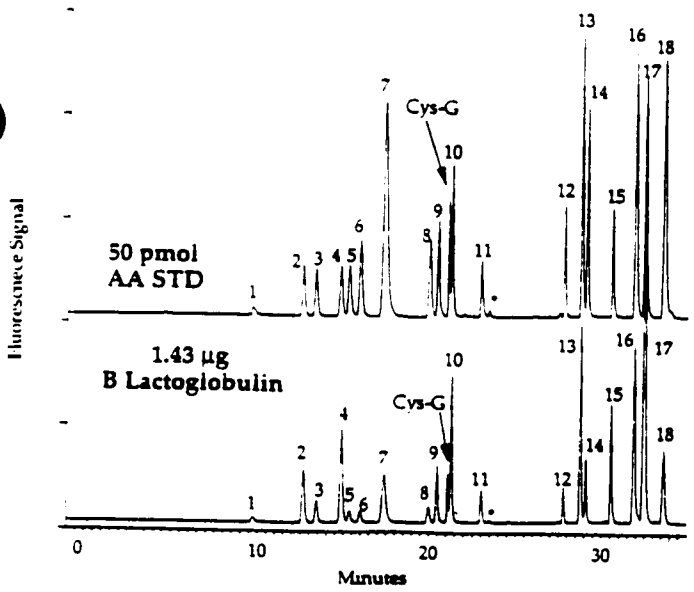
Derivatization

Standards
1. Mix 1 volume of derivatizing reagent to 4 volumes of buffered (pH 8.8) amino acid standard.
2. Vortex.
3. Heat for 10 minutes at 50°C.
4. Inject.

Hydrolyzed Proteins
1. Dry contents of hydrolysis vial.
2. Dissolve hydrolyzed sample in dilute HCl.
3. Buffer solution to pH 8.8.
4. Mix 1 volume of derivatizing reagent to 4 volumes of sample.
5. Vortex.
6. Heat for 10 minutes at 50°C.
7. Inject.

Hydrolyzed Membrane Bound Proteins
1. Extract hydrolyzed amino acids from the membrane using 50:50 Acetonitrile:4% SDS.
2. Remove membrane.
3. Follow steps 1-7 for hydrolyzed proteins.

DTDGA Analysis



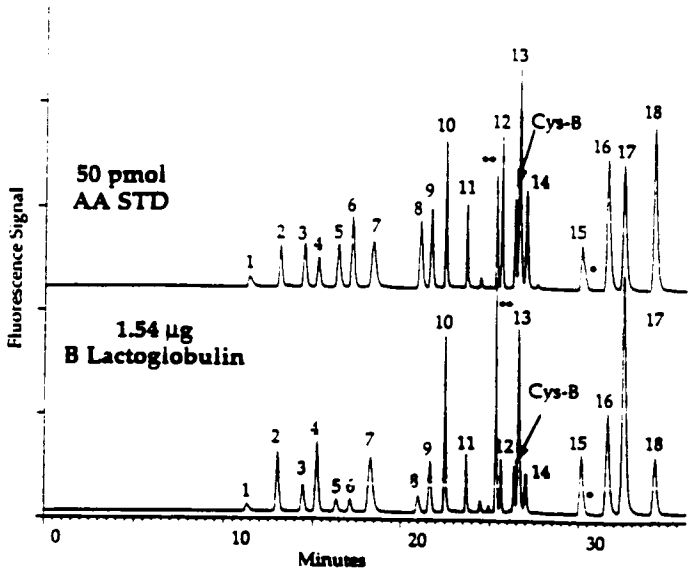
1. AMQ
2. Asp
3. Ser
4. Glu
5. Gly
6. His
7. NH3
8. Arg
9. Thr
10. Ala
11. Pro
12. Tyr
13. Val
14. Met
15. Lys
16. Ile
17. Leu
18. Phe

* Reagent byproduct
** Hydrolysis byproduct

Gradient:

Time	%A	%B	%C	Curve
Inut.	100	0	0	
0.5	99	1	0	L
18	95	5	0	L
19	91	9	0	L
24	90	10	0	L
28	83	17	0	L
30	83	17	0	L
34	0	60	40	S

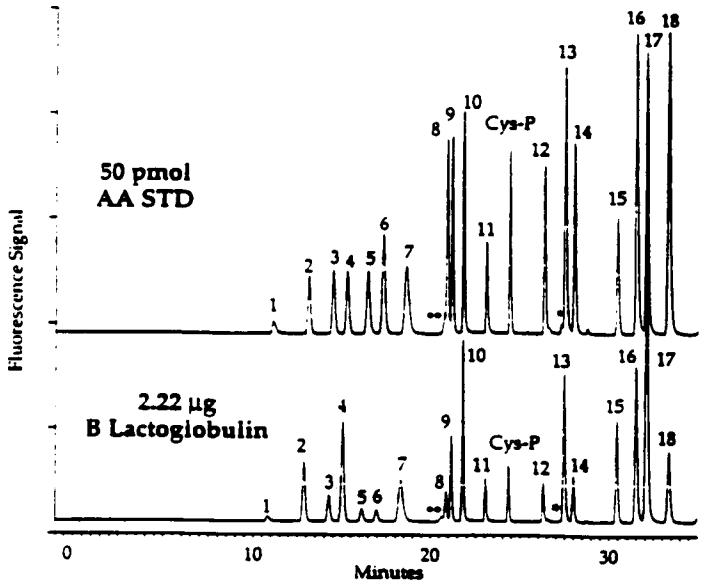
DTDBA Analysis



Gradient:

Time	%A	%B	%C	Curve
Inut.	100	0	0	
0.5	99	1	0	L
18	95	5	0	L
19	91	9	0	L
22	86	14	0	L
26	86	14	0	L
34	83	17	0	L
35	0	60	40	S

DTDPA Analysis



Gradient:

Time	%A	%B	%C	Curve
Inut.	100	0	0	
0.5	99	1	0	L
18	95	5	0	L
19	91	9	0	L
29.5	83	17	0	L
33	0	60	40	S

DTDGA - AAA

AA	B Lactoglobulin Comp.	B Lactoglobulin		Cytochrome C Comp.	Cytochrome C	
Asp	16	15.5	15.6	8	7.8	7.7
Ser	7	6.6	6.6	0	nm	nm
Glu	25	24.9	24.8	12	12.0	11.7
Gly	3	3.1	3.0	12	11.1	10.8
His	2	2.0	2.0	3	3.0	3.1
Arg	3	3.0	3.0	2	2.3	2.5
Thr	8	7.8	7.8	10	10.0	10.4
Ala	14	13.3	13.3	6	5.8	5.7
Pro	8	7.9	7.8	4	3.9	3.9
Cys	5	5.2	5.2	2	2.2	2.2
Tyr	4	4.6	4.4	4	5.1	5.1
Val	10	9.7	9.7	3	3.0	3.0
Met	4	4.3	4.4	2	2.0	2.0
Lys	15	14.9	15.4	19	18.0	17.8
Ile	10	9.4	9.3	6	6.0	6.0
Leu	22	21.7	21.6	6	5.9	5.9
Phe	4	4.0	4.1	4	4.0	4.0
Avg. Error µg Hyd.		3.7	3.8		5.0	5.8
		1.4	1.4		1.5	1.5
		4.0	4.0		7.1	7.7
		1.4	1.4		1.6	1.5

- Good average error values for both proteins.
- Cys value high due to slight coelution with Ala.
- Highest yield of intact Met for the three disulfide interchange reagents.

nm not measured

DTDPA - Membrane Fraction Collector

AA	B Lactoglobulin Comp.					Cytochrome C Comp.			
Asp	16	15.8	16.5	17.0	17.0	8	8.8	8.8	8.5
Ser	7	9.0	10.0	9.2	8.7	0	nm	nm	nm
Glu	25	22.8	23.8	24.1	24.9	12	11.9	11.6	11.9
Gly	3	4.7	6.9	6.4	4.4	12	12.9	12.5	12.8
His	2	1.3	1.7	1.5	1.5	3	2.4	2.2	2.4
Arg	3	4.2	6.0	4.1	4.0	2	1.7	3.0	3.2
Thr	8	8.4	7.8	7.9	7.9	10	9.5	9.4	9.5
Ala	14	15.0	14.3	14.6	15.1	6	6.4	6.1	6.3
Pro	8	7.1	7.2	7.4	7.6	4	3.9	3.7	4.1
Cys	5	11.5	7.3	10.3	7.5	2	4.4	3.4	3.7
Tyr	4	3.8	4.0	4.0	4.0	4	4.0	2.9	3.1
Val	10	9.5	9.5	9.6	10.0	3	3.2	3.2	3.1
Met	4	4.0	3.7	3.6	3.9	2	1.9	1.7	1.8
Lys	15	12.8	12.4	12.2	12.9	19	16.4	14.6	15.0
Ile	10	8.7	8.8	8.8	9.2	6	6.0	5.7	5.7
Leu	22	20.7	20.7	21.0	21.8	6	6.1	6.2	6.0
Phe	4	3.9	4.0	4.1	4.1	4	4.1	3.8	3.8
Avg. Err*		22 (15)	24 (22)	23 (18)	14 (11)		15 (8)	17 (13)	16 (12)
µg Hyd.		0.19	0.31	0.23	0.38		0.63	0.68	0.71
Recovery		32%	52%	40%	64%		76%	83%	86%

- Average error of all amino acids except Cys in parenthesis.
- A hydrolysis or membrane related impurity coelutes with Cys-P and causes high Cys values.
- Good overall error for low level Cytochrome C without Cys-P.

nm not measured

Performic Acid Oxidation

AA	B Lactoglobulin Comp.				Cytochrome C Comp.			
Asp	16	17.1	17.5	17.3	8	8.4	8.3	8.2
Ser	7	5.9	5.9	5.9	0	nm	nm	nm
Glu	25	25.3	26.0	25.9	12	12.1	12.1	12.0
Gly	3	3.5	3.2	3.3	12	12.3	12.3	13.1
His	2	2.0	2.0	2.0	3	3.0	3.0	3.0
Arg	3	3.0	2.9	3.0	2	2.0	1.9	2.1
Thr	8	7.4	7.4	7.2	10	9.0	8.9	8.7
Ala	14	13.4	13.6	13.4	6	5.8	5.7	5.6
Pro	8	8.1	8.1	8.1	4	3.9	4.0	3.9
Cys	5	4.2	4.3	4.3	2	1.0	1.1	1.1
Val	10	9.5	9.6	9.6	3	2.9	3.0	3.0
Met SO2	4	5.6	5.7	5.8	2	2.8	2.8	2.7
Lys	15	21.6	21.8	21.7	19	20.3	21.5	21.1
Ile	10	9.2	9.3	9.2	6	5.7	5.8	5.7
Leu	22	21.5	21.7	21.6	6	5.8	5.9	5.9
Phe	4	4.2	4.2	4.1	4	4.5	4.3	4.1
Avg. Error		10.8	10.7	11.0		9.7	9.4	9.2
µg Hyd.		3.0	3.0	3.0		4.8	4.5	4.7

nm not measured

Comparison with Disulfide Interchange Data (reported as an average, n=3):

	# Cys	Cys	Cys-G	Cys-P	Cys-B
B Lac	5	4.8	5.3	5.0	5.7
Cyt C	2	1.1	2.2	2.0	2.7

DTDPA - AAA

AA	B Lactoglobulin Comp.				Cytochrome C Comp.			
Asp	16	17.9	18.3	18.4	8	9.1	9.1	8.8
Ser	7	7.0	7.0	7.1	0	nm	nm	nm
Glu	25	25.7	26.1	26.0	12	12.6	12.0	12.2
Gly	3	3.4	3.2	2.9	12	11.6	11.5	11.6
His	2	2.0	1.9	1.9	3	3.0	3.0	2.0
Arg	3	2.8	2.7	3.6*	2	1.9	2.3	2.3
Thr	8	7.6	7.7	7.8	10	9.5	9.2	9.2
Ala	14	13.6	14.0	14.0	6	6.1	5.8	5.7
Pro	8	7.9	8.0	7.9	4	4.0	3.9	3.8
Cys	5	5.0	5.1	5.0	2	2.0	2.0	2.0
Tyr	4	4.0	4.0	4.0	4	3.7	3.9	4.0
Val	10	9.4	9.2	9.6	3	2.9	2.9	2.9
Met	4	4.1	4.1	4.0	2	2.0	2.0	2.0
Lys	15	15.0	15.1	15.0	19	18.8	18.2	18.7
Ile	10	8.9	9.0	9.0	6	5.8	5.7	5.8
Leu	22	21.5	22.1	21.8	6	6.1	5.9	6.1
Phe	4	4.1	4.1	4.1	4	4.0	4.2	4.0
Avg. Error		2.2	2.3	2.3		2.3	2.5	2.4
µg Hyd.		4.3	4.1	4.0		3.5	4.6	3.8

- Arg results complicated by coelution with a hydrolysis related impurity.
- Good average error values for both proteins.

nm not measured

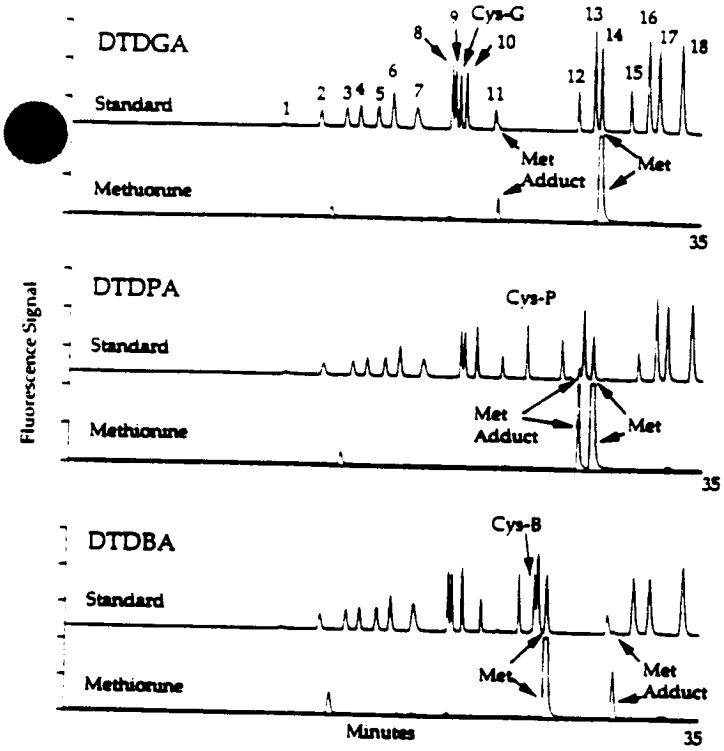
DTDBA - AAA

AA	B Lactoglobulin Comp.				Cytochrome C Comp.			
Asp	16	16.9	16.8	16.8	8	7.7	7.7	7.6
Ser	7	6.8	7.0	7.0	0	nm	nm	nm
Glu	25	26.0	25.3	25.8	12	14.3	13.8	13.8
Gly	3	3.0	3.1	3.1	12	11.8	11.7	11.6
His	2	1.9	1.9	2.0	3	2.9	2.9	2.9
Arg	3	3.0	3.0	3.0	2	2.0	2.0	2.0
Thr	8	8.0	8.2	8.0	10	10.2	9.9	9.7
Ala	14	14.0	14.1	14.0	6	5.8	5.8	5.8
Pro	8	8.0	8.0	8.0	4	3.8	3.9	3.8
Cys	5	6.0	5.5	5.6	2	2.7	2.8	2.7
Tyr	4	4.1	4.1	4.0	4	4.2	4.2	4.1
Val	10	9.8	9.6	9.6	3	3.1	3.1	3.1
Met	4	4.6	4.5	4.4	2	2.3	2.4	2.3
Lys	15	13.2	13.0	13.1	19	15.5	15.4	15.3
Ile	10	8.9	8.8	8.8	6	5.7	5.7	5.7
Leu	22	22.1	21.7	21.5	6	5.8	5.8	5.8
Phe	4	4.0	4.0	4.0	4	3.8	4.0	3.9
Avg. Error		4.9	4.4	4.2		7.87	8.06	7.82
µg Hyd.		1.5	1.6	1.6		1.48	1.56	1.61

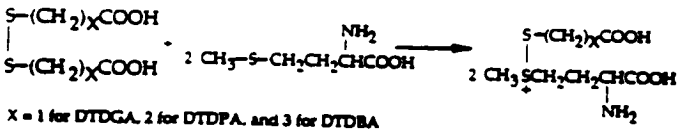
- Cys values are high due to poor resolution between Cys and Val.
- Low Lys values due to disulfide interchange related impurity which coeluted with Lys in the standard.

nm not measured

Methionine Adduct



Proposed Methionine Reaction:



X = 1 for DTDGA, 2 for DTDPA, and 3 for DTDBA

Conclusions

- Modified chromatographic gradients were developed which resolved the disulfide interchange products from the other amino acids.

- Analysis using AQC and disulfide interchange gave good overall error results and good results for cysteine for both β lactoglobulin and cytochrome C at the 1-5 µg level. The overall error results were in the 2 - 8% range.

- The disulfide interchange results for cysteine are as good as the results generated by the performic acid oxidation technique.

- A disulfide interchange reagent byproduct present in each of the standard mixtures appears to be a methionine adduct. When methionine was hydrolyzed in the presence of each of the disulfide interchange reagents, a product was generated which was coeluted with the reagent byproduct.

- Based on the relative size of the peaks generated by the reagent byproducts in the methionine samples, reaction with methionine appears to to be the slowest with DTDGA. In order to minimize methionine loss, DTDGA might be the best disulfide interchange reagent to use.

- Membrane fraction collection was attempted. An impurity from either the membrane or the disulfide interchange reaction was coeluted with the Cys-P thus complicating the result. The overall error ignoring the Cys-P result was good for the low level of protein (100 - 700 ng) extracted from the membrane. Chromatographic resolution of the impurity from the Cys-P peak or the use of DTDGA will enhance this technique for analysis of cysteine from membrane bound proteins.