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Spectrophotometric Cysteine Analysis

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Procedures have been developed for estimating cystine content of proteins by reducing disulfide bonds with mercaptoethanol and selectively alkylating the generated sulfhydryl groups with 4-vinylpyridine or 2-vinylquinoline. 4-Vinylpyridine reacts with cysteine to produce *S*- β -(4-pyridylethyl)-cysteine (Pe-cysteine), which can be determined in protein hydrolysates either by ion-exchange chromatography or ultraviolet spectrophotometry. Pe-cysteine cannot be determined spectrophotometrically in the intact protein, since the absorp-

tivity varies with size and composition of the protein. 2-Vinylquinoline reacts with liberated sulfhydryls under conditions similar to those for 4-vinylpyridine approximately half as fast to produce *S*-2-(2-quinolyethyl)-L-cysteine (Qe-cysteine). Qe-cysteine absorbs at 318 nm with a molar absorptivity of 10,000 in 0.1 *N* acetic acid; since it is not affected by composition or size, spectrophotometric analysis can be made on solutions of the intact protein, as well as on its hydrolysate.

Demonstrated cystine and cysteine loss during hydrolysis of some natural products has led to the development of several special hydrolyses involving special handling, derivatization, or modification to protect them. Oxidation with performic acid (Schram *et al.*, 1954) and alkylation with iodoacetate (Crestfield *et al.*, 1963) or acrylonitrile (Weil and Seibles, 1961) produce a derivative stable to acid hydrolysis (6 *N* HCl at 100° for 24 hr). These procedures are acceptable in some specific instances, but incomplete reaction or recovery (Moore, 1963), interfering side reactions (Cavins and Friedman, 1967), and analytical difficulties (Kalan *et al.*, 1965) render them not universally acceptable. At present, performic acid oxidation coupled with chromatographic determination as cysteic acid is the most widely used alternate procedure for sulfur amino acids.

Cystine and cysteine contents of protein can be estimated by reducing the disulfide bonds with an excess of mercaptoethanol, followed by selective alkylation of the generated sulfhydryls with either 4-vinylpyridine or 2-vinylquinoline. Alkylation with 4-vinylpyridine yields *S*- β -(4-pyridylethyl)-L-cysteine (Pe-cysteine) in the protein hydrolysate, whereas 2-vinylquinoline yields *S*-2-(2-quinolyethyl)-L-cysteine (Qe-cysteine). Pe-cysteine can be determined in protein hydrolysates either chromatographically (Friedman *et al.*, 1970) or

spectrophotometrically (Friedman and Krull, 1969; Wu *et al.*, 1971). Qe-cysteine can be determined spectrophotometrically in protein solutions or hydrolysates (Krull *et al.*, 1971).

EXPERIMENTAL SECTION

***S*- β -(4-Pyridylethyl)-L-cysteine.** In 50 ml of nitrogen-saturated deionized water was dissolved 2.00 g (0.0165 mol) of L-cysteine (Nutritional Biochemicals Corp.). Under an atmosphere of nitrogen, 2.3 ml (0.017 mol) of triethylamine and 1.75 ml (0.0165 mol) of 4-vinylpyridine were added. The reaction mixture was magnetically stirred under a nitrogen atmosphere for 24 hr and then clarified by filtration. A white crystalline material precipitated from the solution when it was rotary evaporated at 40°. The Pe-cysteine was recrystallized from 95% ethanol as fluffy needles, mp 210–212°, with decomposition.

***S*-2-(2-Quinolyethyl)-L-cysteine.** This derivative was prepared like Pe-cysteine, except that 2.4 ml (0.017 mol) of 2-vinylquinoline replaced the 4-vinylpyridine. The product was recrystallized from a mixture of 80 ml of water and 60 ml of ethanol, mp 219–220°.

Reduction and Alkylation of Proteins. One gram of protein was dissolved in 100 ml of 8 *M* urea, pH 7.5 Tris buffer (Krull *et al.*, 1971), and saturated with nitrogen to remove dissolved oxygen. Mercaptoethanol (100 mol excess over total disulfide) was added under nitrogen and the mixture was stirred for 16 hr at room temperature. The free sulfhydryl

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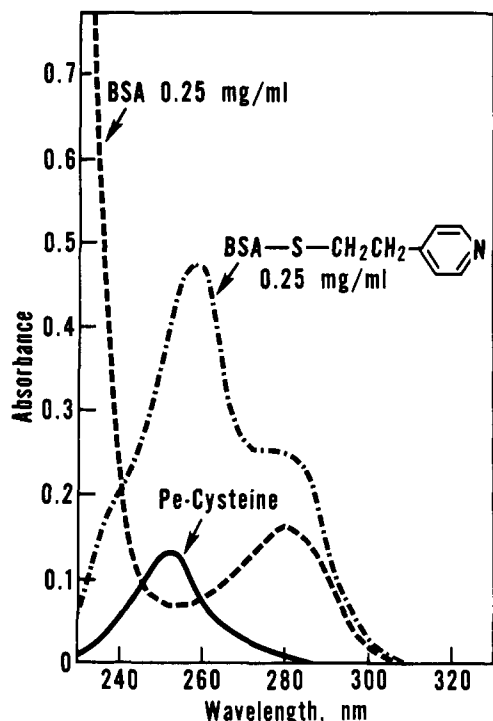


Figure 1. Absorption spectra of *S*- β -(4-pyridylethyl)-L-cysteine (Pe-cysteine) (—), bovine serum albumin (BSA) (---), and 4-vinylpyridine-modified BSA (- · - ·)

groups were then alkylated by addition of the appropriate vinyl compound (1:1 molar ratio with respect to all sulfhydryl groups) and stirred for 90 to 120 min. The solution was adjusted to pH 3 with glacial acetic acid, dialyzed against 0.01 *N* acetic acid, and lyophilized.

Hydrolysis and Amino Acid Analysis. Samples were hydrolyzed in constant-boiling HCl by the sealed tube method. The constant-boiling HCl was saturated with nitrogen before use, and the tubes were sealed under vacuum after being flushed with nitrogen. Sample:acid ratio was approximately 1 mg–2 ml. Hydrolysates were evaporated to dryness under reduced pressure and the residue was dissolved in H₂O. After this operation was repeated several times, the residue was dissolved in pH 2.2 citrate buffer for analysis. Analyses were performed on a Beckman Spinco Model 120 amino acid analyzer by the 3-hr analysis procedure of Benson and Patterson (1965). Peaks were integrated electronically with an Infotronics integrator and results were computer analyzed (Cavins and Friedman, 1968).

Spectrophotometry. Ultraviolet (uv) spectra were determined in 1-cm cells on a Cary Model 14 spectrophotometer, and absorbance was determined on a Beckman Model D.U. spectrophotometer.

RESULTS AND DISCUSSION

Studies on the reaction of vinyl compounds with protein and model compounds (Friedman and Wall, 1964; Friedman *et al.*, 1965) led to the evaluation of 4-vinylpyridine and 2-vinylquinoline as possible reagents to determine cysteine and cystine contents of protein. Since vinyl compounds react 300 times faster with sulfhydryl groups than with amino groups, and since free protein sulfhydryls released by reduction are completely alkylated before all the excess mercaptoethanol is alkylated (Friedman *et al.*, 1965), a 1:1 ratio of free sulfhydryls to vinyl compounds should give the desired deriva-

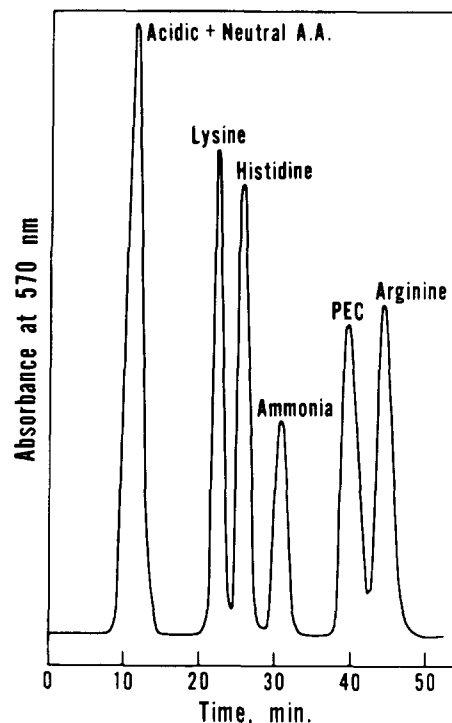


Figure 2. Elution position of *S*- β -(4-pyridylethyl)-L-cysteine (PEC) on a basic column in the Benson and Patterson procedure (1965) for amino acid analysis

tive, which can be determined spectrophotometrically, with no undesirable side reactions.

Uv spectra of Pe-cysteine, bovine serum albumin (BSA), and a solution of BSA alkylated with 4-vinylpyridine are shown in Figure 1. Pe-cysteine has an absorption maximum at 253 nm and an absorptivity of 5000. Unfortunately, the absorptivity of the Pe-cysteine residue in the intact protein is lower than free Pe-cysteine and varies with composition and molecular size of the protein. Pe-cysteine can be determined spectrophotometrically after the protein is hydrolyzed. Determination of cystine as Pe-cysteine spectrophotometrically in hydrolysates requires that alkylation conditions guard against side reactions which produce derivatives that could interfere with absorption. A reaction time of 90 to 120 min produced no undesirable side reactions with 4-vinylpyridine. If conditions are not specific, such as extended reaction time or excess vinyl compound, the Pe-cysteine must be determined chromatographically. The chromatographic procedure is used for all samples that contain starch (Friedman *et al.*, 1970). Pe-cysteine can be separated from other basic amino acids in protein hydrolysates by ion exchange chromatography as illustrated in Figure 2. The final elution position of Pe-cysteine can be readily adjusted because it is more sensitive to pH and ionic strength than is either ammonia or arginine. Quantitation can be based directly on the Pe-cysteine peak or its ratio with lysine or histidine when reaction conditions ensure no modification of these residues. Pe-cysteine can be determined along with the other protein amino acids when alkylation conditions are specific for sulfhydryl residues.

Qe-cysteine has an absorptivity of 10,000 at 318 nm in 0.1 *N* acetic acid, 0.1 *N* HCl, and constant boiling (~6 *N*) HCl; it gives linear Beer's law plots. Uv spectra of wheat gluten, BSA alkylated with 2-vinylquinoline, and Qe-cysteine are compared in Figure 3. No interference was noted between the spectra of normal and alkylated wheat gluten and BSA.

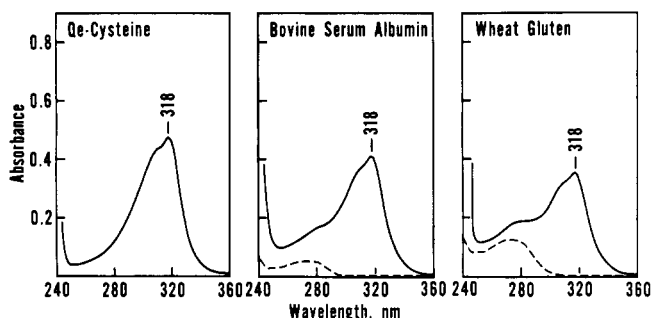


Figure 3. Absorption spectra of S-2-(2-quinolyethyl)-L-cysteine (Qe-cysteine), 2-vinylquinoline-modified whole wheat gluten (0.208 mg/ml), and BSA (0.082 mg/ml) in 0.1 N acetic acid (—) and of unmodified proteins at the same concentration (---)

Table I. Effect of Time of Alkylation in Recovery of S-2-(2-Quinolyethyl)-L-cysteine (Qe-Cysteine) from Reduced Bovine Serum Albumin

Alkylation time, hr	Qe-cysteine, mol/10 ⁵ g
1	38.0
2	42.1
3	47.7
4	49.8
5	49.2
6	51.3

Since the Qe-cysteine spectrum is not influenced by protein size and configuration, this allows quantitative determination of Qe-cysteine residues in solutions of the intact as well as hydrolyzed protein. On the short column in an amino acid analyzer, Qe-cysteine elutes as a broad peak approximately 200 min after arginine; this type peak and slow elution makes chromatographic analysis difficult.

The effect of reaction time on recovery of Qe-cysteine from BSA was examined (Table I). A reaction time of 4 hr was optimum for complete modification. Similar studies with 4-vinylpyridine on several proteins show it to react two times faster than 2-vinylquinoline, and 2 hr was chosen as the optimum reaction time. The optimum time for protein sulfhydryl reaction is not sufficient for complete alkylation of all the excess mercaptoethanol.

Increasing time of reaction over that necessary for complete modification, therefore, increases the chance of the vinyl compound reacting with other sites in the protein, even though the sulfhydryl group is 300 times more reactive than the amino group. An excess of vinyl compound or extended reaction time could result in reaction with the ϵ -amino group of lysine. The possibilities of this side reaction were also investigated (Table II); both pH and concentrates of 4-vinylpyridine were evaluated as to their effect on recovery of Pe-cysteine and lysine residues from alkylated BSA. A pH of 5.0 resulted in slightly lower recovery of half-cysteine residues; however, this decrease was not unexpected since Cavins and Friedman (1968) showed that vinyl compounds react slowly with sulfhydryls at acid pH. At higher mole ratios, a reduction in lysine recovery indicated a reaction of lysine residues with the vinyl compound. The reduction in Pe-cysteine recovery from wheat gluten at higher ratios was unexpected and has not been further investigated.

Half-cysteine content of several model proteins has been determined by alkylation with 4-vinylpyridine or 2-vinylquinoline. These results are shown in Table III. The 4-

Table II. Half-Cysteine^a and Lysine Contents of Protein as Function of Concentration of 4-Vinylpyridine and pH of Buffer for a 2-Hr Reaction Time

		Contents at mole ratio of 4-vinylpyridine-sulfhydryl (mM/100 g)					
		1:1		2:1		3:1	
Protein	pH	Half-cysteine	Lysine	Half-cysteine	Lysine	Half-cysteine	Lysine
Bovine serum albumin	5.0	46.3	103	42.8	104	42.0	85.6
	7.5	48.5	105	45.4	107	40.9	105
Wheat gluten	9.0	47.8	102	42.0	104	42.4	104
	5.0	17.6	8.10	16.7	8.4	17.1	7.46
	7.5	17.5	8.11	17.4	8.26	15.4	8.17
	9.0	18.3	8.33	16.4	8.05	13.4	8.24

^a Determined as S- β -(4-pyridylethyl)-L-cysteine (Pe-cysteine).

Table III. Half-Cysteine Contents of Proteins Determined as S- β -(4-Pyridylethyl)-L-cysteine (Pe-Cysteine) and S-2-(2-Quinolyethyl)-L-cysteine (Qe-Cysteine) (mol/10⁵ g)

Proteins	Pe-cysteine ^a	Qe-cysteine ^b	Cystine/2
Bovine serum albumin	48.5	49.8	49.6 ^c
Wheat gluten	17.4	17.0	17.5 ^d
Lysozyme	55.4	55.2	53.2 ^e
β -Lactoglobulin	29.1	30.0	30.6 ^c
Ovalbumin	13.6	12.9 ^f	13.3 ^g

^a Average of two separate determinations. ^b Average of three separate determinations. ^c Davidson and Hird (1967). ^d Woychik *et al.* (1961). ^e Canfield (1963). ^f Determined on hydrolysate of insoluble product. ^g Gilmore and Fothergill (1967).

vinylpyridine modified proteins were analyzed by quantitating the Pe-cysteine in protein hydrolysates by ion-exchange chromatography and 2-vinylquinoline modified proteins were analyzed spectrophotometrically on solution of the protein or of hydrolysates when the protein was insoluble. Excellent agreement was found between standard literature values and values obtained by reduction and alkylation procedures.

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