A NOVEL SPECTROPHOTOMETRIC PROCEDURE FOR HALF-CYSTINE RESIDUES IN PROTEINS

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ABSTRACT

A spectrophotometric assay for half-cystine residues in proteins was developed based on the following observations: (1) Protein SH groups, generated by reduction of disulfide bonds with mercaptoethanol, are selectively modified by 4-vinylpyridine at pH 7.5 to S-β-(4-pyridylethyl)-L-cysteine residues; (2) the molar extinction coefficient of the pyridine ring in S-pyridylethylproteins is generally lower than the corresponding value in S-β-pyridylethylcysteine (PEC); (3) the S-pyridylethylcysteine in the modified proteins is stable to protein acid-hydrolysis conditions; and (4) the molar extinction coefficient of the pyridine ring in hydrolyzed S-pyridylethylproteins is identical to the value found for PEC.

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Selective modification of sulfhydryl groups in reduced proteins with 4-vinylpyridine transforms half-cystine to S-pyridylethylcysteine residues. The pyridine ring in the resulting S-pyridylethylproteins has an absorption maximum near 255 mμ in the ultraviolet region. The molar extinction coefficient (ε) per pyridine ring in several proteins was lower than the ε-value of the pyridine ring in the model compound S-β-pyridylethylcysteine (PEC). Since PEC residues in the modified proteins are stable to protein acid-hydrolysis conditions, an investigation was carried out on the spectral properties of the pyridine chromophore in intact and hydrolyzed S-pyridylethylproteins. It was discovered that the molar extinction coefficient of the pyridine ring increased on hydrolysis to the corresponding value found in PEC. Based on these observations, a new spectrophotometric procedure was developed for estimating half-cystine residues in proteins.

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METHODS

Materials

Wheat gluten was prepared from Ponca wheat; bovine serum albumin (BSA) four times recrystallized and lysozyme came from Pentex[2] (Kankakee, Illinois); ultrapure urea was obtained from Mann Laboratories (New York); PEC was prepared from L-cysteine and 4-vinylpyridine as previously described (1).

Reduction and Alkylation of Proteins

A typical experimental procedure is as follows: One gram of the protein was dissolved by stirring in 100 ml of 8 M urea, pH 7.5, Tris buffer (2), and nitrogen was bubbled through the solution to remove dissolved oxygen. Mercaptoethanol (1 ml, which is about a 100-molar excess over total disulfides) was then added under nitrogen and the mixture was stirred for 16 hr at room temperature. The free sulfhydryl groups were then alkylated with 1.5 ml of 4-vinylpyridine (1 to 1 mole ratio with respect to all sulfhydryl groups) and stirred for 60 min. The solution was then neutralized to pH 3 with glacial acetic acid, dialyzed against 0.01 N acetic acid, and lyophilized.

Protein Hydrolyses

Proteins (about 10 mg) were hydrolyzed by refluxing for 24 hr in 6 N HCl (constant boiling) in a round-bottomed flask equipped with reflux condenser. Protein-to-acid ratio was 1 mg to 4 ml. Hydrolysates were diluted with 6 N HCl to an appropriate volume (100 ml for BSA and lysozyme; 50 ml for gluten). Ultraviolet spectra were then determined in 1-cm cells on a Cary Model 14 spectrophotometer. The calculations were carried out as follows: The absorbance of a 10-mg hydrolysate of the native protein at 255 mμ was subtracted from the corresponding value for S-pyridylethylprotein hydrolysate. The net absorbance per 10^5 g protein was then calculated.

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Division of the net absorbance by the molar extinction coefficient for PEC (5,000) gives the number of half-cystine residues.

RESULTS AND DISCUSSION

The pyridine ring of PEC has an absorption maximum at 255 mμ and an extinction coefficient which is a function of the pH of the solvent. The pH-dependence is undoubtedly due to protonation of the pyridine ring (5).

If the optical densities of the pyridine ring in the modified proteins were an additive function of the absorbance of the pyridine ring in PEC, the half-cystine content of the proteins could be determined directly via ultraviolet spectroscopy. However, the extinction coefficient of the pyridine ring in the S-pyridylethylproteins is generally lower than the corresponding value of PEC and varies from protein to protein. The ratio of the molar ε-values per PEC residue in unhydrolyzed to hydrolyzed S-pyridylethyl-bovine serum albumin, -gluten, and -lysozyme was 0.73, 0.90, and 0.95, respectively. When the S-pyridylethylproteins were hydrolyzed, the ε value per pyridine ring was identical to the corresponding value in PEC.

The half-cystine content of the three proteins was determined by this

Table I. Half-Cystine Content of Proteins Determined Spectrophotometrically as S-pyridylethylcysteine

Protein	Half-Cystine Content ($M/10^5$ g) Found[a]	Reported
Wheat gluten	17.3; 17.1; 18.0	17.5[b]
Bovine serum albumin	50.0; 52.1; 48.6	49-51[c]
Lysozyme	53.1; 52.9; 55.5	53.2[c]

[a] Each value the average of three analyses.

[b] Reference 3.

[c] Reference 4.

method after hydrolysis. The results in Table I are from three separate determinations on each protein and are in good agreement with literature values. A new technique is therefore available to determine half-cystine residues in proteins. The method involves reduction of protein disulfide bonds, alkylation of the liberated SH groups with 4-vinylpyridine, hydrolysis of the resulting S-pyridylethylproteins, and a spectral examination of the hydrolysate.

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