The Chromatographic Determination of Cystine and Cysteine Residues in Proteins as S-β-(4-Pyridylethyl)cysteine*

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SUMMARY

Disulfide bonds in proteins and in seed meals were reduced with β-mercaptoethanol to sulfhydryl groups. The generated sulfhydryl groups were then alkylated by treatment with 4-vinylpyridine. The cysteine residues were thus derivatized to S-(4-pyridylethyl)-L-cysteine residues. The cysteine derivative is stable to acid hydrolysis and on amino acid analysis by ion exchange elutes just before arginine. Complete modification of sulfhydryl groups permits quantitative determination of cysteine and cystine content (half-cystine residues) of proteins and of seed meals. The sulfhydryl groups of cysteine are the only groups modified when the alkylation is performed on solutions of reduced proteins at pH 7.5 for 90 to 120 min with an amount of 4-vinylpyridine equivalent to the mercaptoethanol used for reduction. In order to obtain complete modification of the sulfhydryl groups in seed meals, a longer reaction time, 4.5 hours, and a concentration of 4-vinylpyridine equivalent to 3 times the mercaptoethanol concentration were required.

Ion exchange procedures, coupled with the ninhydrin reaction, are at present widely used to determine the amino acid composition of biological fluids, peptides, proteins, and seed meals (2-10). The procedures are rapid and convenient; the operations and calculations of the amino acid data are largely automated (3). Sulfur-containing acids including cysteine, penicillamine, and glutathione may be chromatographically separated on cation exchange resins by automatic systems for amino acid analysis by ion exchange chromatography. The cysteine derivative is stable to acid hydrolysis and on amino acid analysis chromatogram.

S-β-(4-Pyridylethyl)cysteine residues in proteins and of seed meals. Another disadvantage of the performic acid oxidation is the destruction of other amino acids.

Protein sulfhydryl groups may be alkylated with either iodoacetate or iodoacetamide. Hydrolysis of the modified proteins yields S-carboxymethylcysteine. This derivative elutes as a broad unresolved peak before that of aspartic acid, and may be used to estimate the half-cystine content of proteins (15-22).

Well and Seibles (22) proposed the use of acrylonitrile as a specific reagent for the chemical modification of SH groups in proteins based on the conversion of the thiol groups to cyanoethyl derivatives. Subsequent studies by Friedman (23-30) and his collaborators have shown that other protein functional groups, especially ε-amino groups of lysine side chains, are also modified under the conditions used by Well and Seibles. Protein—SH groups may be selectively modified under specific conditions with three related reagents—methyl acrylate, acrylonitrile, and acrylamide. In each one hydrolysis of the modified proteins yields S-carboxymethylcysteine (30).

Estimating S-carboxymethylcysteine has been suggested as a way to determine the cysteine content of proteins (31), but the method has its limitations. The S-carboxymethylcysteine is partially destroyed during acid hydrolysis of the proteins. On chromatograms of protein hydrolysates that contain a large amount of glutamic acid, the S-carboxymethylcysteine is not well resolved (30).

A reagent was therefore sought that would modify protein sulfhydryl groups to yield a derivative which would be stable to protein acid hydrolysis and which would elute at a convenient concentration of 4-vinylpyridine equivalent to 3 times the mercaptoethanol concentration during hydrolysis. For these reasons a number of attempts have been made chemically to modify cysteine and cysteine residues of proteins to yield derivatives that would be stable to acid hydrolysis (6 x HCl at 100°C for 24 hours) and that would elute as discrete peaks on an amino acid analysis chromatogram.

Schram, Moore, and Bigwood (13) demonstrated that oxidation of proteins with performic acid transforms cysteine and cystine residues to cysteic acid. The oxidized protein is then hydrolyzed and analyzed for its amino acid composition. Cysteic acid, which elutes as a discrete peak on the Moore and Stein amino acid analysis system, can be analyzed quantitatively for the determination of half-cystine residues in the proteins (4). Moore (14) has also shown that the addition of a reducing agent, such as HBr, to destroy excess performic acid results in a 94% recovery of cysteic acid during the oxidation step. However, in our experience, the cited recovery is seldom achieved with cereal proteins and with seed meals. Another disadvantage of the performic acid oxidation is the destruction of other sulfur-containing amino acids.

Protein sulfhydryl groups may be alkylated with either iodoacetate or iodoacetamide. Hydrolysis of the modified proteins yields S-carboxymethylcysteine. This derivative elutes as a well resolved peak before that of aspartic acid, and may be used to estimate the half-cystine content of proteins (15-22).

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position on an amino acid analyzer. The results of this study indicate that 4-vinylpyridine meets all of these requirements; the derivative is eluted just preceding arginine (32). A new procedure is described which can simultaneously assay for $S$-(4-pyrildylethyl) cysteine and other amino acid residues in proteins.

**EXPERIMENTAL PROCEDURE**

**Materials**

Whole wheat gluten was prepared from Ponca wheat; bovine serum albumin, four times recrystallized, and lysozyme came from Pentex; ribonuclease (crystalline) was from Mann; ovalbumin (two times crystallized) was from Worthington; $\beta$-lactoglobulin was a gift of the late Dr. L. Weil; wheat and soy flours were milled at the Northern Laboratory; 4-vinylpyridine came from Fishel Tar Company; mercaptooethanol was from Eastman; Tris and EDTA were from Fisher. $S$-$\beta$-(4-Pyridylethyl)-L-cysteine was prepared from $L$-cysteine and 4-vinylpyridine as previously described (32).

All liquid reagents were redistilled before use. Deionized, distilled, nitrogen-saturated H$_2$O was used throughout the studies.

**Methods**

**Reduction and Alkylation of Proteins**—A typical experimental procedure is as follows. 1 g of the protein was dissolved by stirring in 100 ml of 8 M urea, pH 7.5, Tris buffer (30). (The buffer was prepared by dissolving 16.11 g of Tris, 7.12 g of HNO$_3$, 0.75 g of KCl, 484.8 g of urea, and 1 mg of EDTA disodium salt in deionized water and adjusting to a final volume of 1 liter.) Mercaptooethanol, 1 ml (about 100 molar excess over total thiols), was then added under nitrogen and the mixture was stirred for 16 hours at room temperature. The free sulfhydryl groups were then exposed to 1.5 ml of 4-vinylpyridine (1:1 mole ratio with respect to all sulfhydryl groups) and the solution was stirred for 90 to 120 min. The solution was neutralized to pH 3 with glacial acetic acid, dialyzed against 0.01 N acetic acid, and lyophilized.

**Reduction and Alkylation of Seed Meals**—The reduction and alkylation of defatted soy flour and of wheat flour were carried out as described for proteins, except that for each gram of meal 4.5 ml of 4-vinylpyridine were used (a 3:1 mole ratio with respect to all sulfhydryl groups) and the suspension was stirred for 4.5 hours.

**Amino Acid Analysis** Ten milligrams of the reduced-alkylated proteins were hydrolyzed by constant boiling hydrochloric acid in a sealed tube at 110° for 24 hours utilizing the techniques described by Crestfield, Moore, and Stein (21) to exclude oxygen. The acid was then removed on a rotary evaporator at 40° under reduced pressure and the residue was dissolved in 10 ml of pH 2.0 citrate buffer.

Samples of the reduced-alkylated seed meals, 100 mg, were hydrolyzed by refluxing the sample for 24 hours in 250 ml of constant boiling hydrochloric acid. Nitrogen was bubbled through the solution during the hydrolysis. The acid was removed on a rotary evaporator and the residue was stirred with 10 ml of pH 2.0 citrate buffer. The insoluble material was removed by filtration.

Portions of these samples were then analyzed on either a Beckman amino acid analyzer model 120 or a Phoenix amino acid analyzer model 8000. The results were electronically integrated and area ratios for lysine and $S$-(4-pyridylethyl)cysteine with respect to histidine were computed. The half-cystine (or lysine) content was then determined by multiplying the $S$-(4-pyridylethyl)cysteine-histidine (or lysine-histidine) ratio by the molar concentration of histidine in the protein (Equation 1, where Pe-cysteine is $S$-(4-pyridylethyl)cysteine).

Concentrated Pe-cysteine (mm per 100 g)

$$\frac{\text{area Pe-cysteine}}{\text{area histidine}} \times \frac{\text{mm histidine per 100 g}}{\text{color yield of histidine}} \times \text{color yield of Pe-cysteine}$$

The color yield from $S$-(4-pyridylethyl)cysteine is about 1.02 times that from leucine (32). The imidazole nitrogen of histidine remains unaffected under conditions used to modify the sulfhydryl groups (28, 30). All results are averages of two to four determinations.

Similar results can be obtained with calculations utilizing the arginine content of the proteins instead of the histidine content.

**RESULTS AND DISCUSSION**

A series of proteins and seed meals has been examined by this procedure to determine the optimum conditions for the alkylation of the total sulfhydryl content. Table I shows the influence of time of reaction on the $S$-(4-pyridylethyl)cysteine recovery from several proteins and seed meals. The reduced proteins were alkylated with 4-vinylpyridine for up to 6 hours. An equimolar concentration of 4-vinylpyridine to all sulfhydryl groups (including mercaptooethanol) in the reaction was used with the soluble proteins. Between 30 and 120 min the $S$-(4-pyridylethyl)-cysteine content of the protein changed little. The lysine content remained constant through 4.5 hours, although there was some decrease at more extended reaction times. The total cysteine and cystine contents of the proteins determined as $S$-(4-pyridylethyl)cysteine at the recommended reaction time of 90 to 120 min are in good agreement with standard literature values (Table I).

An excess of 4-vinylpyridine and a longer reaction time were required for the complete modification of seed meals. When reduced wheat flour was reacted with an equimolar concentration of 4-vinylpyridine, low results were obtained (Table I). Increasing the 4-vinylpyridine concentration to a 3-fold excess over the mercaptooethanol concentration and extending the time to 4.5 hours led to results for wheat and soy flour consistent with the literature (Table I). Other functional groups in the flour may be modified under these conditions; however, such modifications would not interfere with the analytical procedure for the quantitative estimation of the half-cystine content of seed meals.

The effects of pH and concentration of 4-vinylpyridine on the recovery of $S$-(4-pyridylethyl)cysteine and lysine residues are summarized in Table II. At a 1:1 mole ratio of 4-vinylpyridine to all sulfhydryl groups, only the alkylation at pH 5 effected the recovery of $S$-(4-pyridylethyl)cysteine. This was expected, as it has been shown that sulfhydryls react slowly with vinyl compounds at acid pH values and the rate of addition increases rapidly with increasing pH (24). At the higher molar ratios the recoveries of $S$-(4-pyridylethyl)cysteine were low at all pH values. This was an unexpected result.
Determinaton of Half-cystine Residues

Table I

Half-cystine contents of proteins and seed meals determined as s-(d-pyridylethyl)cysteine

| Protein or meal            | Half-cystine contents after alkylation time of | Literature values |
|----------------------------|-----------------------------------------------|-------------------|
|                            | 0.5 hrs | 2.0 hrs | 4.5 hrs | 6.0 hrs | ma/100 g | ma/100 g |
| Bovine serum albumin       | 45.8    | 48.5    | (40.7)  | 46.5    | 49.6    |
| Wheat gluten               | 17.6    | 17.4    | (17.5)  | 17.6    | 17.5    |
| α-Lactoglobulin            | 29.4    | 29.1    | (29.3)  | 25.8    | 30.6    |
| Lysozyme                   | 00.4    | (60.8)  |         |         | 54.4    |
| Ribonuclease               | 59.9    |         |         |         | 58.4    |
| Ovalbumin                  | 13.6    | (13.3)  |         |         | 13.3    |
| Defatted soy flour         | 13.2    |         | 13.7    |         | 13.0    |
| Wheat flour                | 12.2    |         | 12.2    | (11.7)  | 12.1    |
| Wheat flour                | 0.4     |         | 10.9    |         | 10.5    |

*Replicate analysis under identical conditions.
*b Reference 33.
*c Reference 34.
*d Reference 33 and 36.
*e Reference 21.
*f Reference 31.
& Reference 35.
+h Wheat flour modified with equimolar concentration of 4-vinylpyridine.

Table II

Half-cysteine and lysine contents of protein as function of concentration of 4-vinylpyridine and pH of buffer

| Protein                    | pH  | Half-cysteine | Lysine | Half-cysteine | Lysine | Half-cysteine | Lysine | Half-cysteine | Lysine |
|----------------------------|-----|---------------|--------|---------------|--------|---------------|--------|---------------|--------|
| Bovine serum albumin       | 5.0 | 46.3          | 103    | 42.8          | 104    | 42.0          | 104    | 85.6          |        |
|                            | 7.5 | 48.5          | 105    | 45.4          | 107    | 40.9          | 105    |               |        |
|                            | 9.0 | 47.8          | 102    | 42.0          | 104    | 42.4          | 104    |               |        |
| Wheat gluten               | 5.0 | 17.6          | 8.10   | 16.7          | 8.40   | 17.1          | 8.46   | 7.46          |        |
|                            | 7.5 | 17.5          | 8.11   | 17.4          | 8.20   | 15.4          | 8.17   |               |        |
|                            | 9.0 | 18.3          | 8.33   | 16.4          | 8.05   | 13.4          | 8.24   |               |        |

Weil and Seibles (22) treated reduced proteins with large excesses of acrylonitrile and found complete reaction of the cysteine residues. However, the excess acrylonitrile was found to react with the ε-amino group of lysine and decrease the lysine recovery (23). With 4-vinylpyridine at pH 7.5 the recovery of lysine in proteins was unaffected by reaction time or concentration of 4-vinylpyridine. Therefore, to obtain complete modification of the protein sulfhydryls, the alkylation must be carried out at neutral pH with a 1:1 mole ratio of 4-vinylpyridine to protein and mercaptoethanol —SH groups for 90 to 120 min. Insoluble materials, such as seed meals, require an increased concentration of 4-vinylpyridine (3:1 mole ratio) and an extended reaction time (4.5 hours) for complete modification.

Since S-(4-pyridylethyl)cysteine is stable to acid-protein hydrolysis up to 120 hours and since S-(4-pyridylethyl)cysteine elutes in the Moore and Stein amino acid analysis system before, and well resolved from, arginine (32), the cited observations suggest that 4-vinylpyridine can be used as a reagent for the quantitative determination of half-cystine (cysteine and cystine) content of proteins and of seed meals.

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