A Rapid Method for Detecting Chemical Alteration of Methionine

A chemical procedure is described for the rapid measurement of chemically unmodified methionine of intact proteins as an approach to estimating nutritionally available methionine. In this method, the protein sample is reacted with a dimethyl sulfoxide–hydrochloric acid reagent for 30 min at 50 °C. The methionine of the protein reduces the dimethyl sulfoxide to dimethyl sulfide which is evolved into a confined headspace. Dimethyl sulfide is measured by use of a gas chromatograph equipped with a flame photometric detector and a 394-nm filter for specific detection of sulfur compounds. All residues of methionine in the ribonuclease molecule reacted under the selected conditions. On samples of casein previously oxidized to varying degrees by hydrogen peroxide, the method provided a rapid means of assessing the extent of oxidative damage to methionine since only unaltered methionine responded to this test. Evidence was obtained that this rapid test for methionine damage might be used to empirically evaluate soy protein products.

There is a great need for rapid chemical tests that would be suitable for routine detection of chemical alterations of limiting essential amino acids. Such alterations occur during the processing and preparation of feeds and foods and affect their nutritional value. Compared with feeding trials in an appropriate species, the ultimate measure of a feed or food, chemical tests could have great advantages of speed and economy. The need for rapid chemical tests is especially great for monitoring possible changes in highly processed plant proteins.

Until recently, chemical tests for measuring nutritionally available forms of amino acids were limited to the essential amino acid lysine. The Carpenter procedure and its modifications (Carpenter and Ellinger, 1955a,b; Carpenter, 1960; Friedman, 1975) were mainly used for that purpose. Chemical methods for determining nutritionally available methionine were proposed only recently (Lipton and Bodwell, 1975; 1976a,b; Pieniazek et at., 1975a,b; Ellinger and Duncan, 1976; Finlayson and MacKenzie, 1976), so experience with them is limited. Since methionine is the first limiting essential amino acid for most feeds and foods, except for the cereal grains, there is a particular need for a chemical assay for nutritionally available methionine. Enzymatic, microbiological, and animal methods for determination of available methionine were previously referenced (Lipton and Bodwell, 1975) and all these procedures are more time consuming and difficult than chemical methods.

We now present results of a novel gas chromatographic measurement of methionine (Lipton and Bodwell, 1976a) for the assay of proteins in terms of the dimethyl sulfide released in the reaction with dimethyl sulfoxide. Included are results from studies of crystalline ribonuclease, of casein which had been oxidized to varying degrees with hydrogen peroxide, and of two soy products.

EXPERIMENTAL SECTION

Reagents. Most of the chemicals were from commercial or other sources which were previously specified (Lipton and Bodwell, 1976a). The casein, an ANRC sample from Sheffield Chemical Co., contained 13.83% nitrogen. The 30% hydrogen peroxide (Baker) was diluted with distilled water to the appropriate concentration immediately before use. The catalase was Sigma C-40, a purified product from bovine liver. The soy proteins, one a textured soy product and the other a soy isolate, were from commercial sources. The ribonuclease, a crystalline bovine product from Nutritional Biochemicals, was about 73% pure based on the methionine content determined by ion-exchange chromatography.

Gas Chromatography. The gas chromatograph was a Tracor Model 550 equipped with a flame photometric detector and 394-nm filter which was specific for detection of sulfur compounds. The column was a 6 ft × 0.25 in. glass tube packed with 3% OV-1 on 80–100 mesh Chromosorb W. Rates of gas flow to the detector were adjusted for most of the experiments at the published values and the nitrogen carrier gas rate of 80 mL/min and column temperature of 50 °C were as published (Lipton and Bodwell, 1976a). In a further study of gas flow rate to the detector, response to dimethyl sulfide was also maximal at the following flow rates: H_2, 200 mL/min; air, 100 mL/min; O_2, 35 mL/min. Headspace gas above the reactions was sampled manually using a Pressure Lok Series A gas syringe (Precision Sampling Co., Baton Rouge, La.). A 100 μL volume of gas was ordinarily injected into the gas chromatograph. Our attempts to use a gas sampling valve for automatic injection of the headspace samples into the gas chromatograph were unsuccessful; apparently the dimethyl sulfide was adsorbed to a varying degree by metal surfaces of the valve. Curves for the formation of dimethyl sulfide and for the response of the photometric detector vs. the level of methionine assayed were published (Lipton and Bodwell, 1976a).

Assay of Proteins by Dimethyl Sulfoxide Reagent. Protein samples were assayed in 125-mL Wheaton serum bottles for 30 min at 50 °C, by the procedure used for free amino acids and simple peptides (Lipton and Bodwell, 1976a). The crystalline ribonuclease sample (13.1 mg, 0.96 μmol based on 100% purity) was weighed and transferred to a 125-mL serum bottle and 0.10 mL of 6 N HCl was added. This suspension of ribonuclease was frozen, dimethyl sulfoxide (0.10 mL) was added, and the assay was carried out as previously described.

For the other proteins, the volumes of 6 N HCl and of dimethyl sulfoxide were increased but the volume ratio remained 1:1. Casein samples were oxidized by hydrogen peroxide in the serum bottles, and then were oxidized by dimethyl sulfoxide. By addition of an equal volume of 12 N HCl, the aqueous solution was adjusted to the desired 6 N HCl concentration. For assay of 25 mg of hydrogen peroxide oxidized casein, 2 mL each of aqueous and dimethyl sulfoxide phases were used. In the procedure, which was described in detail (Lipton and Bodwell, 1976a), the aqueous phase was frozen before the dimethyl sulfoxide was added and the bottles were flushed with nitrogen and then sealed with the rubber septum.

The 10–25 mg samples of soy proteins were weighed, transferred to 125-mL Wheaton serum bottles, and suspended in a 1-mL volume of 6 N HCl. This suspension was then frozen before the dimethyl sulfoxide was added and the rest of the procedure was as described.

Oxidation of Casein by Hydrogen Peroxide. Conditions for oxidation of casein by hydrogen peroxide were adapted from those of Cuq et al. (1973). A 15-min oxidation at 50 °C was used for duplicate 25-mg samples of

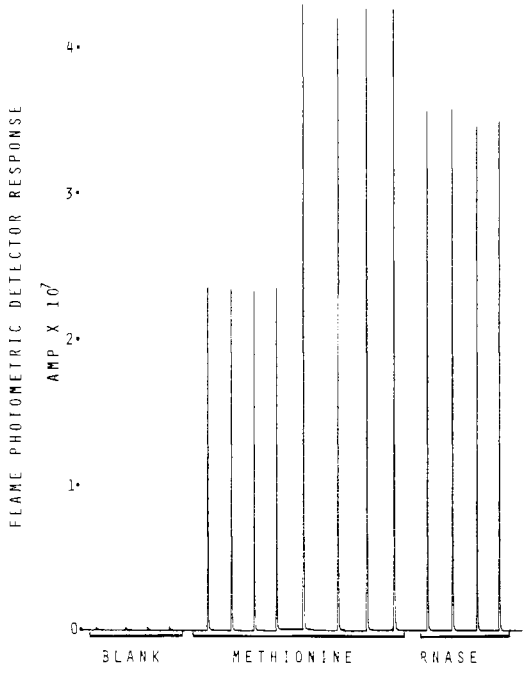

Figure 1. Photograph of recorder chart showing the flame photometric detector response to headspace injections following the dimethyl sulfoxide oxidations of blank (6 N HCl), methionine (2 μmol; 4 μmol) and ribonuclease (13.1 mg) samples. Duplicate 100-μL injections were made from two replicates of each sample. Oxidations were done as described in the text. The electrometer setting was $10^4 \times 512$.

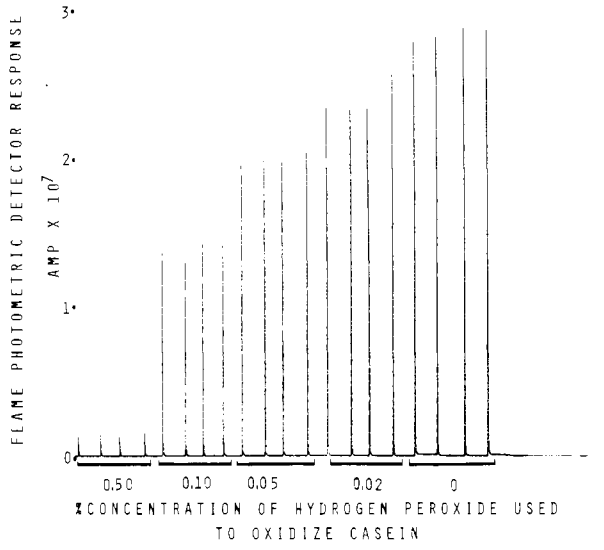

Figure 2. Photograph of recorder chart showing the flame photometric detector response to headspace injections following dimethyl sulfoxide oxidation of casein previously oxidized by hydrogen peroxide. Duplicate 100-mL injections were made from two replicates of each sample. Oxidations were done in 125-mL serum bottles for 30 min at 50 °C, as described in the text. The electrometer setting was $10^4 \times 512$.

casein suspended in 1.0 mL of aqueous hydrogen peroxide of the following percent concentrations: 0, 0.02, 0.05, 0.10, and 0.50. Casein samples were weighed, transferred to 125-mL serum bottles and incubated in a water bath immediately after addition of the hydrogen peroxide. The bottles were swirled repeatedly during the incubation to insure good contact of the protein with the peroxide. After removal to a 25 °C water bath, 5-μL of 2% catalase solution was added to each bottle. With occasional manual mixing, the bottles were incubated at 25 °C and 5 μL of catalase was added after 10 and 30 min. For complete destruction of peroxide, incubation was continued for 1 h at 25 °C and then for 15 min at 50 °C. Samples were then stored at 4 °C overnight prior to assay.

RESULTS AND DISCUSSION

Analysis of Ribonuclease Preparation. Figure 1 illustrates the response of the flame photometric detector to headspace injections after ribonuclease was reacted with the dimethyl sulfoxide reagent and the responses to samples from the blank and from two levels of methionine standard. The ribonuclease sample yielded about 3 μmol of dimethyl sulfide which approximated the 2.8 μmol of methionine found by ion-exchange chromatographic analysis of an identical sample. Apparently all methionine residues of ribonuclease reacted with the dimethyl sulfoxide reagent.

Analyses of Peroxide-Oxidized Casein. Figure 2 illustrates the response of the flame photometric detector to headspace injections after dimethyl sulfoxide oxidation of casein which had been previously oxidized to varying degrees with hydrogen peroxide. The dimethyl sulfide evolution into the headspace, as indicated by the peak heights, was inversely related to the severity of the peroxide treatments. Methionine sulfoxide formation in peroxide-oxidized casein had been shown to be directly related to the severity of the hydrogen peroxide treatments (Cuq et al., 1973). The product of the oxidation of methionine by the dimethyl sulfoxide reagent was previously shown to be methionine sulfoxide (Lipton and Bodwell, 1976a). Thus, the results shown in Figure 2 indicated that dimethyl sulfoxide did not react with residues of methionine which had been oxidized by peroxide and that this gas chromatographic assay was a very rapid method for measuring unaltered methionine.

Cuq et al. (1973) reported that the release of methionine during the in vitro proteolysis of casein by Pronase was lowered by hydrogen peroxide oxidation of the casein. Ellinger and Palmer (1969) reported that hydrogen peroxide treatment of casein depressed net protein utilization (NPU) by rats. This peroxide oxidation of the methionine of casein to methionine sulfoxide resulted in a corresponding loss of methionine as measured by a cyanogen bromide method (Ellinger and Smith, 1971). Those studies support our view that the dimethyl sulfoxide exchange reaction may be a useful indicator of methionine availability. The previously used direct and indirect measurements of methionine sulfoxide (Cuq et al., 1973) were relatively more complicated and laborious than our gas chromatographic measurement of the dimethyl sulfoxide reduction. Direct measurement of methionine sulfoxide after the ordinary hydrolytic procedure with 6 N HCl is not possible because the methionine sulfoxide is reduced back to methionine to varying degrees (see Lipton and Bodwell, 1973).

Evaluation of Soy Proteins. Data on assays of two types of soy protein preparations by the dimethyl sulfoxide–gas chromatographic procedure are summarized in Table I. Ion-exchange chromatographic analyses of the preparations gave methionine contents of 0.040 μmol/mg for the textured soy and 0.0765 μmol/mg for the soy isolate. Protein efficiency ratios (PER) for the preparations were 2.34 for the textured soy and 0.91 for the soy isolate. The peak heights of dimethyl sulfide, following reaction with the dimethyl sulfoxide reagent, indicate that the textured soy preparation was greatly superior to the soy isolate. This assay actually exaggerated the differences in these preparations which were shown by the feeding test.

Chemical Measurements of Nutritionally Available Methionine. In each of the suggested procedures for

Table I. Gas Chromatographic Analyses of Soy Proteins[a] following Their Reaction with a Dimethyl Sulfoxide Reagent

Sample	Dimethyl sulfide peak height,[b] amp × 10^8
Blank	11.4, 11.1
L-Methionine, 1 μmol	103, 101
Textured soy[c,d,e] 25 mg (contains 1 μmol of methionine)	140, 173
Soy isolate[d,e] 13.1 mg (contains 1 μmol of methionine)	33.4, 32.3

[a] Samples were reacted in 125-mL serum bottles with 1 mL of 6 N HCl and 1 mL of dimethyl sulfoxide, as described in the text. [b] Values are averages of duplicate injections of two separate bottles. [c] The textured soy contained about half starch. In a control assay of 12.5 mg of corn starch alone, the response was no higher than the blank. [d] Analyses of the soy protein samples in 0.5 N HCl instead of 6 N indicated the absence of cysteine (Lipton and Bodwell, 1976a). [e] The PER values were 2.34 for the textured soy and 0.91 for the soy isolate.

chemical determination of nutritionally available methionine, chemically unaltered methionine is assumed to be the only available form so the methods focus on determinations of the methylthio group of the methionine side chain. In the procedure of Pieniazek et al. (1976), the nitroprusside reaction responds only to unaltered methionine side chains and not to sulfoxide, sulfone, or to sulfonium derivatives. The method, however, has the disadvantage, which is common to many other colorimetric procedures, of possible interference from other protein and nonprotein food components. Moreover, the necessity for protein digestion prior to the assay is an added disadvantage.

The procedures of Ellinger and Duncan (1976) and of Finlayson and MacKenzie (1976) are most specific in that reaction occurs only with unaltered methylthio side chain groups and not with methionine sulfoxide, sulfone, or other derivatives with altered sidechains. The cyanogen bromide reagent reacts directly on the intact protein, with no need of any preliminary digestion, and the product of its reaction with the methionyl residues is methyl thiocyanate; the gas chromatographic measurement of methyl thiocyanate is the basis of the assay. Disadvantages of the approach are the toxicity of cyanogen bromide and the overnight reaction time required for release of the methyl thiocyanate. Recently MacKenzie (1977) reported a modified procedure with a reaction time of only 2 h. Use of a sulfur-specific detector for measurement of the methyl thiocyanate might further improve the cyanogen bromide method.

Although the proposed dimethyl sulfoxide reagent for available methionine offers a very simple and rapid chemical determination and does not require prior hydrolysis, further work is necessary to establish the usefulness of this approach. Our earlier study (Lipton and Bodwell, 1976a) indicated that other amino acids did not interfere and that only unaltered methionine reacted under our selected experimental conditions. We pointed out that other reducing substances might interfere in the assay of food samples since the assay measured the dimethyl sulfide reduction product. However, for the assay of samples such as the soy proteins, reducing substances would not interfere. This procedure may be of value for empirical use in assaying protein concentrates for available methionine.

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LITERATURE CITED

Carpenter, K. J., Ellinger, G. M., *Biochem. J.* **61**, xi (1955a).
Carpenter, K. J., Ellinger, G. M., *Poultry Sci.* **34**, 1451 (1955b).
Carpenter, K. J., *Biochem. J.* **77**, 604 (1960).
Cuq, J. L., Provansal, M., Guilleux, F., Cheftel, C., *J. Food Sci.* **38**, 112 (1973).
Ellinger, G. M., Palmer, R., *Proc. Nutr. Soc.* **28**, 42A (1969).
Ellinger, G. M., Smith, R. H., *Biochem. J.* **124**, 15P (1971).
Ellinger, G. M., Duncan, A., *Biochem. J.* **155**, 615 (1976).
Finlayson, A. J., MacKenzie, S. L., *Anal. Biochem.* **70**, 397 (1976).
Friedman, M., Ed., "Protein Nutritional Quality of Foods and Feeds. Part I. Assay Methods—Biological, Biochemical and Chemical", Chapters 18, 25, 26, Marcel-Dekker, New York, N.Y., 1975.
Lipton, S. H., Bodwell, C. E., *J. Agric. Food Chem.* **21**, 235 (1973).
Lipton, S. H., Bodwell, C. E., in "Protein Nutritional Quality of Foods and Feeds. Part I. Assay Methods—Biological, Biochemical and Chemical", Chapter 29, Friedman, M., Ed., Marcel-Dekker, New York, N.Y., 1975.
Lipton, S. H., Bodwell, C. E., *J. Agric. Food Chem.* **24**, 26 (1976a).
Lipton, S. H., Bodwell, C. E., *J. Agric. Food Chem.* **24**, 32 (1976b).
MacKenzie, S. L., *J. Chromatogr.* **130**, 399 (1977).
Pieniazek, D., Grabarek, Z., Rakowska, M., *Nutr. Metab.* **18**, 16 (1975a).
Pieniazek, D., Rakowska, M., Szkilladziowa, W., Grabarek, Z., *Br. J. Nutr.* **34**, 175 (1975b).

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A Rapid Bioassay for Pesticide Phytotoxicity

A leaf infusion technique which demonstrates low levels of herbicidal activity in chemical compounds was developed. The test is based on determining the differential accumulation of leaf tissue starch as influenced by the phytotoxicity of the applied chemical. The assay is a half-leaf test that can routinely be completed in 6 h using a minimum of equipment.

Early screening of chemical compounds for phytotoxicity is often hindered by the quantities of chemical required and the number of test plants needed. The purpose of this study was to devise a simple, efficient in vivo bioassay for