Identification of Methionine-Containing Proteins and Quantitation of Their Methionine Contents

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We developed an in vitro labeling method that allows identification of methionine-containing proteins after separation on gel electrophoresis and quantitation of their methionine contents. Proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions, blotted onto nitrocellulose membrane, and reacted with [1-^{14}C]iodoacetic acid at pH 2. Autoradiography revealed bands of varying intensities depending on their methionine contents. Proteins of known amino acid compositions and soybean storage proteins were used to establish the specificity and the optimum conditions for the reaction. The ratio of the densitometry scan of the autoradiogram to that of nitrocellulose membrane stained for protein gave an accurate index of the methionine content in each protein band. This method has considerable potential in the identification of methionine-rich polypeptides in legumes and other plant crops where methionine is the most limiting essential amino acid. It should also be generally useful for proteins where methionine is of interest.

Molecular biology has opened up the potential for genetic manipulation of plants with improved traits as food sources. For instance, protein quality can be improved by increasing the amount of methionine and cysteine, the limiting essential amino acids in most legumes and other plant proteins. Our approach and that of other workers require the identification of methionine-rich polypeptides (MRPs) in plant proteins. Currently available methods have serious limitations. Labeling of seed proteins with ^{35}S by injection with sodium [^{35}S]sulfate into the pedicel on various days after flowering requires growing plants, detects nonprotein S compounds, results in dilution of the label by incorporation into nonseed proteins, and may not reflect the methionine content of the mature protein (Schroeder, 1984). Pulse labeling with [^{35}S]methionine and other radiolabeled amino acids used to study biosynthesis and processing of proteins requires an actively metabolizing seed and, again, may not reflect the methionine contents of proteins at their final states of synthesis. Isolation of certain storage proteins by breaking up into subunits and determining the amino acid composition and sequence is more suited to studying storage proteins per se (Nielsen, 1984) but is laborious and time consuming when one's goal is to search for MRPs.

The method we developed is based on the specific alkylation of the thioether moiety in methionine with [1-^{14}C]iodoacetic acid at pH 2 (Gundlach et al., 1959; Lundblad and Noyes, 1984) after gel electrophoresis of the proteins and transfer onto nitrocellulose membrane. The method allows identification of methionine-containing proteins and polypeptides in seeds without growing the plants and a reasonably accurate estimation of their methionine contents. This method should also be generally applicable to proteins where methionine is of interest.

MATERIALS AND METHODS

Proteins. Myoglobin (type I) from equine skeletal muscle, ribonuclease A (type VII-A) from bovine pancreas, papain (type III) from papaya latex, Kunitz trypsin inhibitor from soybeans, bovine carbonic anhydrase, and egg white lysozyme were obtained from Sigma Chemical Co. The S-rich protein fraction from Brazil nut (*Bertholletia excelsa*) was a gift from Dr. Samuel Sun of ARCO Plant Cell Research Institute, Dublin, CA. Soybean seed proteins were obtained by extracting defatted soy flour (cv. Corsoy) with 0.1 M phosphate buffer (pH 7.5) containing 0.5 M NaCl, 0.05 M mercaptoethanol, and 0.001 M phenylmethylsulfonyl fluoride (a protease inhibitor). The crude extract was then dialyzed against distilled water at 4 °C to give albumins (water soluble) and globulins (salt soluble).

Chemicals. [1-^{14}C]Iodoacetic acid (specific activity 6.85 μCi/mol) was obtained from ICN Radiochemicals (Irvine, CA) in 50-μCi quantities. Electrophoresis chemicals and nitrocellulose membranes (0.45-μm pore size) were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were analytical reagent grade and were purchased from Sigma Chemical Co.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed at pH 8.8 essentially as described by Laemmli (1970). The major exception was that the upper electrode (cathodic) buffer solution contained 0.0001 M sodium thioglycolate in addition to the normal recipe. Sodium thioglycolate was required to prevent the destruction of methionine side chain by free radicals or oxidants trapped in the gel matrix (Hunkapiller et al., 1983).

Protein Blotting. The gel was equilibrated in transfer buffer (0.025 M Tris (pH 8.3), 0.192 M glycine, 20% (v/v) methanol) for 30 min prior to blotting. Electrophoretic blotting was achieved with a Trans-Blot Cell (Bio-Rad) with a supercooling coil at 200 V for 2 h (Towbin et al., 1979). The transferred proteins were reacted with [1-^{14}C]iodoacetic acid as described below. The nitrocellulose membrane was stained briefly with 0.125% Coomassie Brilliant Blue R-250 dissolved in 50% (v/v) methanol and 10% (v/v) acetic acid. The destaining solution was 50% (v/v) methanol and 10% (v/v) acetic acid.

[1-^{14}C]Iodoacetic Acid Reaction with Methionine. The nitrocellulose membrane was rinsed twice with distilled water and placed in a dish with 25 mL of 5% (v/v) formic acid (pH 2) containing 50 μCi of [1-^{14}C]iodoacetic acid. To prevent photoxidation of the iodoacetic acid during the reaction, the dish was wrapped with aluminum foil and incubated in the dark at 37 °C on a shaker to ensure mixing. At the completion of the reaction (4–8 days), the membrane was washed at least four times with 50 mL of 5% (v/v) formic acid each time to remove excess and nonspecifically bound label and air-dried overnight at room temperature. To prevent air oxidation of methionine, the reaction mix can be flushed with nitrogen and the reaction performed in a heat-sealable plastic bag.

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Table I. Specificity of the Iodoacetic Acid Reaction

protein	M_r	pI[a]	Met index[b]	Met/mol lit.	Met/mol calcd[c]
myoglobin	17500	7.3	1.0	2	2.0
lysozyme	14400	11.0	1.0	2	1.6 ± 0.1
papain	23000	9.6	0.0	0	0
ribonuclease A	13700	9.3	2.8	4	4.4 ± 0.1
soybean trypsin inhibitor	20100	4.5	1.3	2	1.9 ● 0.3

[a] Isoelectric point. [b] Calculated from eq 1 as described under Materials and Methods. The Met indices of all proteins were normalized with respect to myoglobin, used as the reference protein. [c] Calculated from eq 2 as described under Materials and Methods. Mean ± standard deviation, three determinations each.

Autoradiography. Kodak X-Omat AR film was exposed to nitrocellulose membrane for 4 days at −80 °C. The film was developed manually with Kodak GBX developer and fixer as per instruction recommended by the manufacturer.

Densitometry. The nitrocellulose membrane was scanned at 600 nm in the reflectance mode and the autoradiogram at 525 nm in the transmittance mode on a RFT scanning densitometer (General Transidyne Corp.) interphased with a DEC PDP-8 computer. The computer gave print outs of the scans together with designated peak areas.

Quantitation of Methionine. An index was defined to give an approximation of the relative methionine content of each polypeptide band:

$$\text{Met index} = \frac{\text{peak area, band in autoradiogram}}{\text{peak area, band in nitrocellulose}} \quad (1)$$

This ratio can then be converted to the number of moles of methionine per mole of protein using a reference protein and assuming that there is no differential dye-binding and SDS-binding capacities among the polypeptides (eq 2).

$$\text{Met/mol} = \frac{\text{Met index}}{\text{Met index (ref)}} \frac{M_r}{M_r(\text{ref})} \times \text{Met/mol(ref)} \quad (2)$$

The reference (ref) protein was myoglobin, which contains two methionine per 17.5 kDa molecular weight. The molecular weight (M_r) of each polypeptide was determined from their relative mobilities as compared with molecular weight protein standards (Laemmli, 1970).

RESULTS

Specificity. The specificity of the reaction was studied with papain which contains no methionine, the S-rich protein from Brazil nut, and four characterized proteins. The amino acid sequences of myoglobin (Dautreraux et al., 1969), ribonuclease A (Smyth et al., 1963), papain (Mitchel et al., 1970), soybean trypsin inhibitor (Hoffman et al., 1984), and lysozyme (Canfield, 1963) are known, and their contents of methionine are given in Table I. The gel after electrophoretic blotting, Coomassie blue stained nitrocellulose membrane, and the autoradiogram of four of the proteins are shown in Figure 1. Papain, which has no methionine, showed no band on the autoradiogram although there was a corresponding protein band on the membrane. Even after an extended reaction time of 10 days, papain did not react with iodoacetic acid at pH 2 even though it contains the other potential nucleophiles: cysteine, histidine, and lysine. Furthermore, the iodoacetic acid reaction with papain was also performed in the presence of 0.1 M Tris (pH 8.2), 8 M urea, and 2 mM dithiothreitol, where cysteine would also be carboxymethylated (Anfinsen and Haber, 1961; Lumsden and Coggins, 1978). A distinct band was seen in the autoradiogram when the reaction was performed at pH 8.2 but not at pH 2, indicating that the sulfhydryl groups were present at pH 2 but unreactive. The S-rich protein containing 18% methionine (Sun et al., 1987) showed an intense band in the autoradiogram, but we were unable to quantitate it because it was poorly resolved in that gel. Densitometric scans of the membrane and autoradiogram were quantitated as described in Materials and Methods, and the results are presented in Table I. Good agreement was obtained between literature and calculated values. For quantitation, a reference protein had to be run together with the samples.

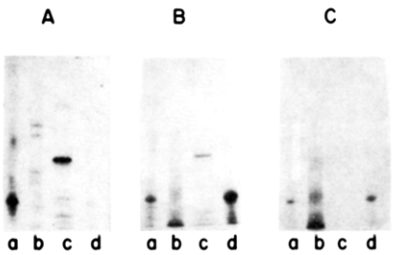

Figure 1. Reactivity of proteins with [1-^{14}C]iodoacetic acid. Proteins were denatured, electrophoresced on a 15% SDS–polyacrylamide gel, electroblotted onto nitrocellulose membrane, reacted with iodoacetic acid for 6 days, and autoradiographed as described under Materials and Methods. Contents of the wells: lane a, ribonuclease A; lane b, sulfur-rich protein fraction from Brazil nut; lane c, papain; lane d, myoglobin. (A) Coomassie blue stained protein bands in gel after electrophoretic blotting. (B) Coomassie blue stained bands on nitrocellulose membrane. (C) Autoradiogram. There is no band corresponding to papain since it contains no methionine.

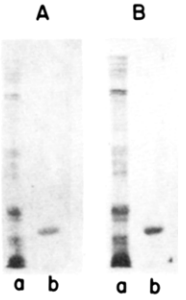

Figure 2. Effect of Coomassie blue staining. Soybean seed albumins (75 μg) and myoglobin (10 μg) were electrophoresced in duplicate on a 17.5% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membrane. After blotting, one set was stained with Coomassie blue and both were reacted with [1-^{14}C]iodoacetic acid for 8 days as described under Materials and Methods. (A) Autoradiogram from stained nitrocellulose membrane. Key: lane a, soybean albumin; lane b, myoglobin. (B) Autoradiogram from unstained nitrocellulose membrane.

Effect of Coomassie Blue Staining. Coomassie Blue, being a bulky molecule, may hinder the reaction of iodoacetic acid with methionine. To study this effect, an autoradiogram resulting from a stained nitrocellulose membrane (Figure 2A) was compared with that from an unstained membrane (Figure 2B). More and better defined bands could be seen in the unstained nitrocellulose. Densitometric scans showed that bands in the unstained nitrocellulose were about 20% more intense than the corresponding bands in the stained membrane.

Sensitivity. The sensitivity of the method was studied with varying amounts of myoglobin (0.5–10 μg). A distinct band could still be seen in the autoradiogram corresponding to 0.5 μg of myoglobin. This was equivalent to 7.5 ng or 57 pmol of methionine.

Effect of Reaction Time. To determine the effect of reaction time, we incubated [1-^{14}C]iodoacetic acid with

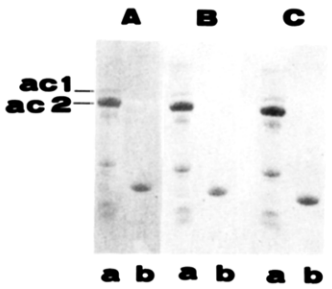

Figure 3. Effect of reaction time. Soybean seed proteins (75 μg) and myoglobin (10 μg) were electrophoresced in triplicate on a 20% SDS–polyacrylamide gel, blotted, and reacted with [1-^{14}C]iodoacetic acid for varying time periods under identical conditions. (A) Autoradiogram resulting from 4-day reaction. Key: lane a, soybean proteins; lane b, myoglobin. (B) Autoradiogram resulting from 6-day reaction. (C) Autoradiogram resulting from 8-day reaction. Ac1 and Ac2 refer to the acidic polypeptides of the glycinin subunits of soybean seed proteins.

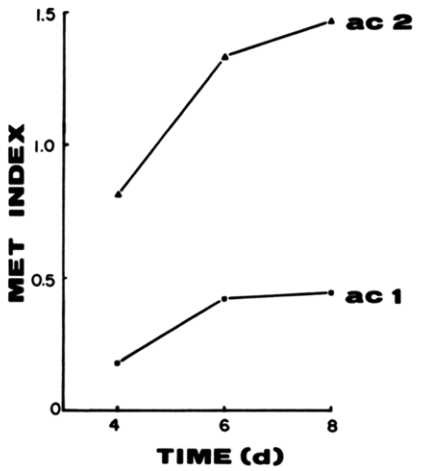

Figure 4. Variation of reaction time with methionine index for two soybean protein bands, designated as Ac1 and Ac2 in Figure 3.

soybean seed proteins and myoglobin, immobilized on nitrocellulose membrane, for varying periods of time (Figure 3). Four-day reaction was insufficient for detecting methionine proteins in soybean as some high molecular weight bands were not observed compared with 6- or 8-day reaction. The Met index of two soybean seed proteins (bands ac1 and ac2 in Figure 3) were quantitated as a function of reaction time (Figure 4). The Met index increased significantly from 4 to 6 days of reaction time but showed no significant differences from 6 to 8 days for both the polypeptide bands.

Application to Soybean Seed Proteins. The method was applied to the quantitation of methionine in the 7S β-conglycinin and 11S glycinin of soybean storage proteins. The amino acid compositions of the major subunits of β-conglycinin (Thanh and Shibasaki, 1977) and the acidic and basic polypeptides of glycinin had been determined (Moreira et al., 1979). The iodacetic reaction was applied to the study of these polypeptides, and the gel and autoradiogram are shown in Figure 5. Their methionine contents quantitated by this method were compared with those in the literature in Table II. Good agreement was obtained taking into consideration that a different variety of soybean was studied.

DISCUSSION

The in vitro labeling method described here is based on the technique of protein blotting and carboxymethylation of methionine by [1-^{14}C]iodoacetic acid. It makes possible

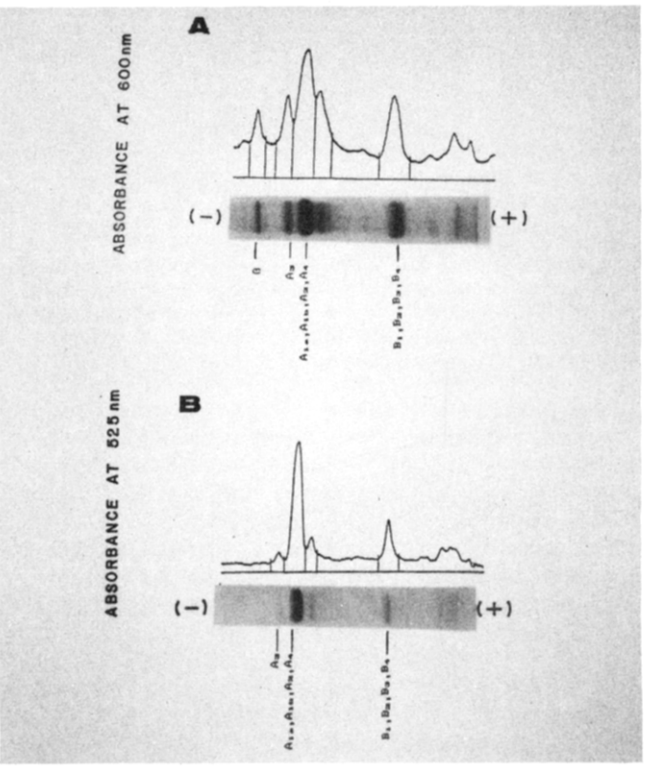

Figure 5. Densitometric scans of soybean seed globulins on (A) Coomassie blue stained nitrocellulose membrane and (B) autoradiogram after reaction with [1-^{14}C]iodoacetic acid. The corresponding gel and autoradiogram are shown below each scan. β refers to the β-subunit of conglycinin, and the acidic and basic polypeptides of glycinin are designated A_1–A_2 and B_1–B_4, respectively.

Table II. Quantitation of Methionine Contents in Soybean Storage Proteins

polypeptide band	M_r	Met/mol lit.[a]	Met/mol calcd[b]
β-subunit (β-conglycinin)	48000	0	0
A_3 polypeptide (glycinin)	42000	2.0	2.0 ± 0.3
A_{1a}, A_{1b}, A_2, A_4 polypeptides (glycinin)	36000	4.5	5.5 ● 0.6
B_1, B_2, B_3, B_4 polypeptides (glycinin)	19500	1.2	1.6 ± 0.2

[a] Obtained from amino acid compositions of isolated subunits and polypeptides. β-Subunit of β-conglycinin has 0 Met/mol (Thanh and Shibasaki, 1977); A_{1a}, A_{1b}, A_2, A_3, and A_4 polypeptides of glycinin have 4, 4, 6, 2, and 1 Met/mol, respectively; B_1, B_2, B_3, and B_4 polypeptides have 2, 3, 0, and 1 Met/mol, respectively (Moreira et al., 1979). Stoichiometry of the acidic and basic polypeptides in glycinin are 1 A_{1a}:1 A_2:$^1/_4$ A_4:$^1/_8$ A_{1b} and 1 B_1:1 B_2:2 B_3:2 B_4, respectively (Moreira et al., 1981; Kitamura et al., 1976). [b] Calculated from eq 1 and 2 as described under Materials and Methods. Mean ± standard deviation, three determinations each.

the rapid identification of methionine-containing polypeptides in proteins. Although the same information may be obtained by excising gel bands followed by electroelution and amino acid analysis, this is a long and tedious process if one's objective is to identify specific methionine-containing proteins since each band would have to be analyzed. The present method represents a simple and rapid screening method to identify methionine-rich polypeptides whose amino acid composition can be subsequently verified once sufficient samples are obtained. Thus, the number of polypeptides for amino acid analysis can be reduced. In cases where protein bands are very close to one another making recovery of single bands very difficult, our method would be the choice for identification.

It is also possible to react the proteins with iodoacetic acid before electrophoresis (Lumsden and Coggins, 1978).

However, there are many advantages to labeling the proteins after gel electrophoresis and protein blotting. First, it permits reaction of several protein samples, including the reference, under one set of conditions, thus allowing direct comparison. Second, since the charge on the protein is changed after reaction, i.e. the thioether group is converted to a sulfonium ion, the labeled protein bands can be compared directly with the unlabeled proteins. This is not possible when the proteins are reacted before electrophoresis since the charge change can alter the mobility of the proteins. However, this is important only when the separation is done without SDS. Third, the transfer efficiency gives an idea of the p*I* of the proteins (Figure 1; Table I) (Gershoni and Palade, 1983). Last, we found that the [1-^{14}C]iodoacetic acid can be reused up to three times if stored in the dark at −20 °C after each use, which is not possible if the reaction is carried out before electrophoresis.

The lack of reactivity of iodoacetic acid at pH 2 with papain demonstrates that the method will not give false-positive results. The fact that papain reacted with iodoacetic acid at pH 8.2 in the same sample further suggests that the cysteine residues were not oxidized to cysteic acid. Besides, the conversion of sulfhydryl groups in reduced proteins to disulfide bonds is extremely slow at acid pH. At pH 2, the other potential nucleophiles in papain, the sulfhydryl group of cysteine (p*K* 8.3), the imidazole group of histidine (p*K* 6.0), ϵ-amino group of lysine (p*K* 10.5), and the terminal amino group (p*K* 9.8) are all protonated and would not react. The specificity of iodoacetate to carboxymethylate methionine residues at acid pH has also been demonstrated by the inactivation of ribonuclease where methionine participates in the active site (Gundlach et al., 1959).

The ability of our method to quantitate methionine is likely due to the fact that the proteins were absorbed onto the surface of the nitrocellulose membrane and remained denatured in the very low pH of the reaction, thus exposing all the thioether groups in methionine for complete and quantitative reaction. Obviously, the reference protein has to be run with the samples always. We also showed that the method was extremely sensitive, being able to detect as little as 8 ng of methionine. The detection limit was most likely lower than this since we did not try applying less than 0.5 g of myoglobin. The reaction time was also an important factor as incomplete alkylation may lead to inaccurate quantitation. Conversely, prolonged incubation may lead to the decomposition of the sulfonium salt. It has been reported that when sulfonium salt is heated at low pH, cleavage products like methionine, homoserine, and *S*-(carboxymethyl)homocysteine are formed (Gundlach et al., 1959). Under our conditions, we found 6–8 days of reaction to be optimum. The accuracy and precision of this method were illustrated by its ability to quantitate methionine not only in single protein but also in peptide fractions.

Our method also overcomes the limitations of current methods for identification of methionine-containing proteins mentioned in the introduction. It allows identification of separated methionine proteins and polypeptides and quantitation of their methionine content in the mature proteins without growing plants. We have also found the method to be very reproducible. The maximum deviation for all the proteins we have studied so far is only 1 Met/mol.

This method is obviously limited by how well the bands are separated by gel electrophoresis and transferred onto the nitrocellulose membrane. This can be overcome by establishing optimum conditions for gel separation or resorting to 2-dimensional electrophoresis and isoelectric focusing. For proteins with extreme p*I*, blotting would have to be optimized too. Strongly basic proteins can be blotted by using very alkaline transfer buffers and the omission of an equilibration step (Szewczyk and Kozloff, 1985). However, complete transfer of proteins is not necessary since the quantitation is based on the amount of protein on the nitrocellulose. This method should have considerable potential in the study of legume and other plant proteins since methionine and cysteine are nutritionally limiting essential amino acids in these proteins. It has been realized in plant-breeding experiments that, to improve the nutritional quality of legume seeds, it would be necessary to improve a protein fraction not only quantitatively but also qualitatively by searching for specific polypeptides with a higher methionine content (Gepts and Bliss, 1984). We have begun to apply our method to legumes and have identified methionine-rich polypeptides in soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), and tamarind (*Tamarindus indica*).

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Registry No. Met, 63-68-3; ribonuclease A, 9001-99-4; lysozyme, 9001-63-2; soybean trypsin inhibitor, 9078-38-0.

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