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Communication

A New Method of Measuring Protein-Methionine-*S*-Oxide Reductase Activity[1]

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ABSTRACT

A new assay for measuring protein-methionine-*S*-oxide reductase is reported. The assay measures the conversion of *N*-(9-fluorenylmethoxycarbonyl)-methionine sulfoxide to *N*-(9-fluorenylmethoxycarbonyl)-methionine using fluorescence detection after high-performance liquid chromatography separation. Enzyme activity is linear over a 60-min period and the assay is sensitive enough to detect the consumption of only 1% of available substrate. Protein-methionine-*S*-oxide reductase activity was demonstrated in pea (*Pisum sativum* L.) chloroplasts, and enzyme levels in leaves of etiolated and light-grown seedlings were compared.

Protein methionyl residues can be oxidized to methionyl sulfoxide residues when exposed to increased intercellular levels of hydrogen peroxide, hydroxyl radicals, or singlet oxygen (4). This oxidation often inactivates the respective proteins. Examples of proteins susceptible to this form of injury include ribosomal protein L12 (5), α-1-PI[2] (8), lipoxygenase (9), and erythrocyte membrane proteins (12). Proteins containing a methionyl sulfoxide can be repaired by PrMSR (EC 1.8.4.6) (4, 5). PrMSR reduces the methionyl sulfoxide residues back to methionyl residues in the presence of reduced thioredoxin, often restoring the protein's biological activity (1, 5).

PrMSR activity was measured first by following the repair of oxidized ribosomal L12 protein containing Met(O); the oxidized L12 protein was not able to be acetylated like the normal L12 protein (5). This difference allowed the PrMSR activity to be measured from its coupled activity with the acetylase. However, this PrMSR assay method is difficult to do and has not been used routinely.

Brot et al. (6) developed an easier PrMSR assay method using tritiated *N*-acetyl-methionine sulfoxide and DTT as substrates. *N*-acetyl-methionine sulfoxide replaced the oxidized protein or peptide, and DTT replaced reduced thioredoxin. This method is sensitive to small amounts of PrMSR activity and can track the enzyme in chromatography fractions; however, the method is not well suited for measuring enzyme activity in tissue extracts. In some assays, 75% or more of the initial substrate was consumed within the assay period (6). Sanchez et al. (11) used this method to study the presence of PrMSR in plants. The relationship between the amount of enzyme per assay and the amount of product formed was found not to be linear. The reported reaction rates did indicate differences in PrMSR activity among tissues and cellular fractions; however, the data could not be used quantitatively. Therefore, this method in its current form has the problem of substrate depletion. Increasing the substrate concentration at the same specific radioactivity would require too much radiolabel, and increasing the substrate concentration by lowering the specific radioactivity would lower the assay's sensitivity.

A known source of the enzyme was needed in order to experiment with the method of measuring PrMSR activity. Sanchez et al. (11) had reported high PrMSR activity using *N*-acetyl-methionine sulfoxide as the substrate in stromal fractions from chloroplasts. We chose to verify that the enzyme contained within the chloroplast stroma (11) was capable of repairing a protein with an oxidized methionyl residue. To accomplish this, the chloroplast enzyme preparation was tested to determine if it could restore oxidized α-1-PI activity. α-1-PI is a proteinase inhibitor that inhibits several serine proteinases, including elastase. In the trap site of α-1-PI, a methionyl residue is easily oxidized with a resulting loss of inhibitory activity (8). Abrams et al. (1) observed that *Escherichia coli* PrMSR could restore the inhibitory activity to previously oxidized α-1-PI. We chose to use this assay to test the chloroplast PrMSR preparation.

With the PrMSR activity verified by the repair of α-1-PI, an alternative quantitative method of measuring PrMSR activity was developed. This method measures the conversion of FMOC-Met(O) to FMOC-Met with fluorescence detection after HPLC separation. This method provides the needed sensitivity previously unavailable and uses less than 1% of the substrate during the assay.

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[2] Abbreviations: α-1-PI, α-1-proteinase inhibitor; FMOC, 9-fluorenylmethyl chloroformate; FMOC-Met, *N*-(9-fluorenylmethoxycarbonyl)-methionine; FMOC-Met(O), *N*-(9-fluorenylmethoxycarbonyl)-methionine sulfoxide; PrMSR, protein-methionine-*S*-oxide reductase.

MATERIALS AND METHODS

Chloroplast PrMSR Activity

The chloroplast PrMSR fraction was isolated by the procedure of Sanchez et al. (11) from young green pea (*Pisum sativum* L.) leaves. Protein concentration in these experiments was determined using the bicinchoninic acid protein assay (13) with BSA as the protein standard.

Repair of Oxidized α-1-PI

An assay to measure the repair of oxidized α-1-PI was used to verify PrMSR activity in chloroplast preparations. Bovine α-1-PI was obtained from Boehringer Mannheim. Porcine pancreatic elastase was purchased from Worthington Biochemical, and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide was purchased from Sigma.

α-1-PI (4.8 nmol) was oxidized with 12 μmol of chloramine-T in 500 μL of buffer (160 mM Tris-HCl [pH 8.0]) at 20°C for 120 min. The α-1-PI solution then was dialyzed overnight against 1500 mL of 20 mM Tris-HCl (pH 7.5) at 4°C to remove the chloramine-T. The recovered α-1-PI solution was used in the following assays.

The chloroplast PrMSR's ability to repair the oxidized α-1-PI was assayed. Details of this assay are given in Table I. Following the PrMSR reaction, the amount of Table I α-1-PI inhibitory activity was measured with an elastase assay. To the 100-μL reaction mixture containing α-1-PI (Table I), elastase (0.003 units) in 400 μL of buffer A (0.2 M Tris-HCl [pH 8.0], 0.1% Triton X-100) was added and mixed. A unit of elastase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide per min at 25°C. This mixture was incubated at 25°C for 5 min to allow α-1-PI to inhibit the elastase. The elastase substrate (500 μL of buffer A containing 1 mg of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide) (3) was then added to the partially inhibited elastase solution. The elastase activity was measured by following the change in A_{410} at 25°C over a period of 4 min.

Synthesis of FMOC-Met(O)

FMOC and the Amino-Tag[3] C-18 HPLC column were purchased from Varian Associates. Methionine sulfoxide was purchased from Sigma, and amino acid Standard H was obtained from Pierce. FMOC-Met(O) was synthesized by derivatizing methionine sulfoxide with FMOC by a modified procedure of Einarsson et al. (7) using a 0.2 M sodium bicarbonate buffer instead of a borate buffer. After pentane extraction, the aqueous phase containing the FMOC-Met(O) was taken to dryness with a centrifugal evaporator. The FMOC-Met(O) pellet was then resolubilized in one-tenth the original volume in 20% (v/v) acetone. This solution was filtered through a 0.2-μm nylon filter, aliquoted, and stored at −20°C.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Table I. *Restoration of Oxidized α-1-PI Activity by Chloroplast PrMSR*

The complete 100-μL assays contained 25 μg of protein from the chloroplast PrMSR preparation, 10 μg of oxidized α-1-PI, and 4 μmol of DTT in buffer (42.75 mM Tris-HCL [pH 7.5], 12 mM $MgCl_2$, and 34 mM KCl). Assays were incubated at 25°C for 120 min. Reactions were stopped by placing tubes on ice. The α-1-PI inhibitory activity was determined with an elastase assay.

Assay Components Present (+) or Absent (−)			Percent Inhibition (mean ± SE)
PrMSR	DTT	α-1-PI	
+	+	+	46.8 ± 1.35
−	+	+	34.9 ± 0.81
+	−	+	32.3 ± 2.26
+	+	−	0

The FMOC-Met(O) concentration after each synthesis was measured by fluorescence detection following separation on a HPLC equipped with a C-18 column. The HPLC separation program (2) was designed for amino acid analysis. A standard curve comparing quantity of FMOC-Met(O) versus peak height was made with oxidized amino acid Standard H. The methionine within this standard was oxidized to methionine sulfoxide by adding 5 μL of 30% H_2O_2 to 200 μL of the amino acid standard and mixing. After incubating for 1 h at 25°C, the amino acids were diluted, derivatized, and analyzed on the HPLC.

Measurement of FMOC-Met

A new HPLC program was developed to separate FMOC-Met(O) from FMOC-Met in a short time period at room temperature with the same C-18 column. The flow rate was 1.0 mL min^{-1}. This program eluted the sample with an isocratic mobile phase (52% [v/v] of 50 mM acetate buffer [pH 4.2] [Solvent A]; 48% [v/v] acetonitrile [Solvent B)]) for the first 6 min. Within this period, FMOC-Met(O) eluted with the solvent front and FMOC-Met eluted with a retention time of 5.6 min. To clear the column of other components, Solvent B was increased from 48 to 60% over a period of 5 min and then was left at 60% for 10 min. The proportion of Solvent B was returned from 60 to 48% over the next 5 min. In the final step of the program, Solvent B was left at 48% for 6 min.

A standard curve was prepared by plotting the concentration of FMOC-Met and peak height using this new program. FMOC-Met was obtained from Advanced ChemTech (Louisville, KY). A 1-mM FMOC-Met solution was made and diluted with acetone. A 20-μL sample loop was used to inject the samples. Using the fluorescence detector, amounts of FMOC-Met as low as 0.25 pmol could be detected.

PrMSR Assay with FMOC-Met(O)

PrMSR assays using FMOC-Met(O) are described below. In these assays, the buffer included 20 mM Tris-HCl (pH 7.5), 40 mM DTT, 12 mM $MgCl_2$, and 34 mM KCl. The concentration of FMOC-Met(O) in assays was 500 μM, with the exception of those noted in Figure 1. The amounts of protein used in

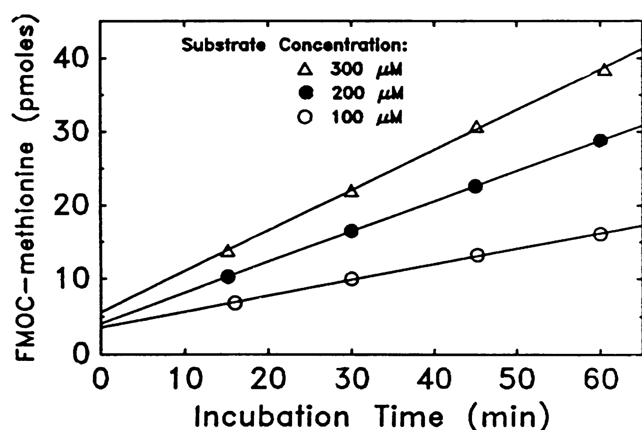

Figure 1. The amount of FMOC-Met produced within a 50-μL aliquot increases linearly with increasing incubation time. Each aliquot contained 1.26 μg of partly purified chloroplast PrMSR. The results are shown of assays containing three different FMOC-Met(O) concentrations.

the assays are reported with the experimental results. The assays were started by the addition of the plant extracts and thoroughly mixed. Immediately after the initiation of the reaction, the assay mixture was subdivided into 50-μL aliquots. These aliquots were incubated in a 25°C water bath, and the reactions stopped at the specified times by adding 450 μL of cold acetone (−20°C) to the 50-μL aliquots. After mixing, the samples were stored overnight at −20°C.

The samples were mixed and centrifuged at 16,000*g* for 5 min to remove the precipitated protein. The supernatant was filtered through a 0.2-μm nylon filter. Twenty microliters of each sample were loaded on the HPLC and separated by the program described above. The results are reported as picomoles of FMOC-Met within the original 50-μL aliquot before the dilution to 500 μL with acetone. The number of picomoles of FMOC-Met within the 20-μL sample loaded on the HPLC was multiplied by 25 to obtain the picomoles in the original 50-μL aliquot. Routinely, each assay consisted of two aliquots that were stopped at approximately 15 and 30 min, respectively. The precise stop time was recorded for each aliquot. The rate of FMOC-Met production was calculated as the slope between these two points (y values= picomoles of FMOC-Met; x values = time).

PrMSR Activity in Etiolated and Green Pea Leaves

Pea seeds were sown in two flats containing moist vermiculite and kept in a dark incubator at 20°C. After 6 d, one flat was placed where the plants received 50 μmol m^{-2} s^{-1} of light at room temperature (20–22°C), whereas the other flat was left in the dark. After 9 d, leaf samples were harvested from the etiolated and green plants. These leaf samples were ground immediately to a fine powder in liquid nitrogen and stored at −80°C.

To extract the soluble proteins from these samples, the powder was transferred to a precooled tube and extraction medium added at a proportion of 1 μL mg^{-1} tissue. The extraction medium contained 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 34 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 10 μM leupeptin, 5.6 μM *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine, and 10% (v/v) glycerol. The resulting slurry was vigorously mixed for 3 min before centrifuging at 16,000*g* for 5 min. The supernatant was immediately assayed for PrMSR activity and protein concentration.

To make relative comparisons, a standard curve was made from the extract obtained from the first sample of the etiolated leaves; this standard curve compared FMOC-Met(O) produced per min with the amount of protein used in the assay. With this standard curve, the relative amounts of PrMSR activity in the other samples were determined.

RESULTS AND DISCUSSION

The ability of chloroplast stromal preparations to reduce oxidized α-1-PI via PrMSR is described in Table I. By definition, the amount of elastase inhibition was zero when α-1-PI was omitted from the assays. When PrMSR or DTT was omitted from the assays, no repair of oxidized α-1-PI should occur; however, there was 34.9 and 32.3% inhibition of elastase in these two respective assays. This result indicates that not all of α-1-PI was inactivated by the oxidative treatment. When PrMSR, DTT, and oxidized α-1-PI were included in the assays, the inhibition by α-1-PI increased to 46.8%. The 11.9% increase in inhibitory activity in the complete assay is attributed to repair of α-1-PI proteins by chloroplast PrMSR.

Having demonstrated the presence of PrMSR activity in the chloroplast stromal samples, efforts to develop a new assay method were begun. Information on the substrate requirements for PrMSR indicated that only the N terminus of the methionine sulfoxide had to blocked (6). We chose to test Met(O) blocked with FMOC at the N terminus as a potential substrate. To test the utility of this substrate, FMOC-Met(O) was reacted with chloroplast PrMSR, and the conversion of FMOC-Met(O) to FMOC-Met was evaluated.

The conversion of FMOC-Met(O) to FMOC-Met was linear for the 60 min tested (Fig. 1), and the rate of FMOC-Met accumulation was dependent on substrate concentration. During these assays, less than 1% of the substrate was depleted. Because the conversion of FMOC-Met(O) to FMOC-Met was dependent upon substrate and enzyme concentrations and because less than 1% of the substrate was depleted, measurements of the K_m for FMOC-Met(O) and V_{max} of this enzymic reaction were attempted. Measurements of K_m and V_{max} were impaired, however, because of insolubility of FMOC-Met(O) at concentrations above 600 μM. The inclusion of acetone within the assay increased FMOC-Met(O) solubility, but also negatively affected the enzyme activity (data not shown). Therefore, PrMSR assays using FMOC-Met(O) cannot be done at substrate concentrations that maintain V_{max}, where a one-to-one relationship exists between reaction rate and amount of enzyme. Subsaturating levels of substrate can be used to determine enzymic activity if the relationship between the reaction rate and amount of enzyme is defined by a standard curve relating the relationship between reaction rate and amount of PrMSR.

The results from assays where the substrate was held constant at 500 μM and the amount of chloroplast enzyme

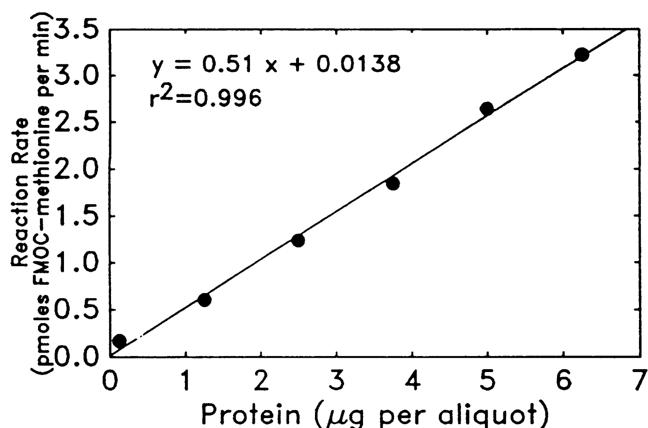

Figure 2. The relationship between the amount of partially purified chloroplast PrMSR in each 50-μL aliquot and the rate of FMOC-Met synthesis. The concentration of FMOC-Met(O) was 500 μM.

varied by 50-fold are shown in Figure 2. A linear relationship between these two variables was found, suggesting that with an appropriate standard curve, the relative amounts of PrMSR could be compared in different tissue extracts.

Using this method, the relative amount of PrMSR activity was 100 ± 3% in the etiolated pea leaves and 156 ± 4% in the green pea leaves. Extracts from etiolated leaves had significant amounts of PrMSR activity in the absence of chloroplasts. This activity may represent the PrMSR from several cellular locations, including etioplasts. Greening and the associated chloroplast biogenesis resulted in a 56% increase in PrMSR activity in the leaves. These results are consistent with the idea that the chloroplast is a prominent cellular location of PrMSR; however, direct measurements remain to be done. The chloroplast is a cellular location with significant potential for oxidative injury (10, 14). Because PrMSR repairs proteins exposed to oxidants, its presence in the chloroplast would be reasonable.

A disadvantage of this method is that PrMSR activities of unknowns are relative to the PrMSR source used to make the standard curve. Assays resulting in a standard curve need to be included whenever measurements are being made. If this is done, the method developed in the present study permits accurate measurement of PrMSR activity in tissue extracts, unlike previously published methods. There was little depletion of the substrate, and activities over a 50-fold range of enzyme concentration can be measured. These characteristics are in contrast with the PrMSR assay method using *N*-acetylmethionine sulfoxide described by Brot et al. (6) and utilized by Sanchez et al. (11). Future plans include the use of FMOC-Met(O) as substrate to measure PrMSR activity in plants exposed to environmental stresses.

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LITERATURE CITED

1. **Abrams WR, Weinbaum G, Weissbach L, Weissbach H, Brot N** (1981) Enzymatic reduction of oxidized alpha-1-proteinase inhibitor restores biological activity. Proc Natl Acad Sci USA **78:** 7483–7486
2. **Betner I, Foldi P** (1988) The FMOC-ADAM approach to amino acid analysis. LC·GC **6:** 832–840
3. **Bieth J, Spiess B, Wermuth CG** (1974) The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem Med **11:** 350–357
4. **Brot N, Weissbach H** (1982) The biochemistry of methionine sulfoxide residues in proteins. Trends Biochem Sci **7:** 137–139
5. **Brot N, Weissbach L, Werth J, Weissbach H** (1981) Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci USA **78:** 2155–2158
6. **Brot N, Werth J, Koster D, Weissbach H** (1982) Reduction of N-acetyl methionine sulfoxide: A simple assay for peptide methionine sulfoxide reductase. Anal Biochem **122:** 291–294
7. **Einarsson S, Josefsson B, Lagerkvist S** (1983) Determination of amino acids with 9-fluorenylmethylchloroformate and reversed-phase high-performance liquid chromatography. J Chromatogr **282:** 609–618
8. **Johnson D, Travis J** (1979) The oxidative inactivation of human alpha-1-proteinase inhibitor. Further evidence for methionine at the reactive center. J Biol Chem **254:** 4022–4026
9. **Rapoport S, Hartel B, Hausdorf G** (1984) Methionine sulfoxide formation: the cause of self-inactivation of reticulocyte lipoxygenase. Eur J Biochem **139:** 573–576
10. **Salin ML** (1987) Toxic oxygen species and protective systems of the chloroplast. Physiol Plant **72:** 681–689
11. **Sanchez J, Nikolau BJ, Stumpf PK** (1983) Reduction of N-acetyl methionine sulfoxide in plants. Plant Physiol **73:** 619–623
12. **Seppi C, Castellana MA, Minetti G, Piccinini G, Balduini C, Brovelli A** (1991) Evidence for membrane protein oxidation during *in vivo* aging of human erythrocytes. Mech Ageing Dev **57:** 247–258
13. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC** (1985) Measurement of protein using bicinchoninic acid. Anal Biochem **150:** 76–85
14. **Takahashi M, Asada K** (1988) Superoxide production in aprotic interior of chloroplast thylakoids. Arch Biochem Biophys **267:** 714–722