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A specific and rapid colorimetric method to monitor the activity of methionine sulfoxide reductase A

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ABSTRACT

Considerable evidence indicates that methionine sulfoxide (MetO) reductase A (MsrA) plays an important role in cytoprotection against oxidative stress and serves as a potential drug target. To screen for MsrA regulators, a rapid and specific assay to monitor MsrA activity is required. Most of current assays for MsrA activity are based on the reduction of radioactive substrates such as [3H]-N-acetyl-MetO or fluorescent derivatives such as dimethylaminoazo-benzenesulfonyl-MetO. However, these assays require extraction procedures and special instruments. Here, we developed a specific colorimetric microplate assay for testing MsrA activity quickly, which was based on the fact that MsrA can catalyze the reduction of methyl sulfoxides and simultaneously oxidize dithiothreitol (DTT), whose color can be produced by reacting with Ellman's reagent (dithio-bis-nitrobenzoic acid, DTNB). The corresponding absorbance change at 412 nm was recorded with a microplate reader as the reaction proceeded. This method to monitor MsrA activity is easy to handle. Our findings may serve as a rapid method for the characterization of recombinant enzyme and for the screening of enzyme inhibitors, pharmacological activators, gene expression regulators and novel substrates.

1. Introduction

Methionine sulfoxide reductase (Msr) system, which eliminates reactive oxygen species (ROS) via participating in the methionine-centered redox cycle and reverses ROS-induced damage to proteins, has been shown to play an important role in cytoprotection against the oxidative insults induced by a variety of causes [1–4]. Methionine sulfoxide (MetO) reductase enzyme A (MsrA) is an important member of the Msr family. Considerable data show that MsrA prevents different cell types from oxidative stress-induced damages, including embryonic stem cells, vascular smooth muscle cells, lens cells and PC12 cells [5–10]. Over-expression of MsrA enzyme also increases lifespan [11–13]. The MsrA system has been shown to decline with aging in many different animal organs, including brain, kidney, liver, tongue and sternohyoid muscle [14–20]. Interestingly, it has been demonstrated that some agents can enhance MsrA system [18–20]. Screening for pharmacological activators and gene expression regulators of MsrA represents a potential approach for the prevention and therapy of age-related diseases. On the other hand, a specific inhibitor of MsrA is also very useful in the research field of oxidative stress.

To screen a pharmacological activator or inhibitor of MsrA, a rapid and specific assay for MsrA activity is required. However, most of current assays for MsrA activity are dependent on the reduction of radioactive substrates such as [3H]-N-acetyl-MetO or fluorescent derivatives such as dimethyl aminoazo-benzenesulfonyl-MetO (DABS-MetO) [21–24]. Using scintillation counter to detect radio-labeling MetO requires radioactive agents and special instruments. Using HPLC to detect fluorescent derivatives of MetO is relatively time-consuming and may also be interfered by inconsistent unexpected derivatives. These assays also involve extraction procedures, which make them impractical

Abbreviations: DABS, dimethyl aminoazo-benzenesulfonyl; DMSO, dimethyl sulphoxide; DTNB, dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; LC-ESI-ITMS, liquid chromatography coupled with electrospray ionization-ion trap mass spectrometry; MetO, methionine sulfoxide; MsrA, methionine sulfoxide reductase A; ROS, reactive oxygen species; RSV, resveratrol; Trx, thioredoxin.

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for rapidly screening regulators of MsrA. Thus, a colorimetric method to monitor the activity of MsrA may help in this field.

Different from other members in the Msr family, MsrA has broad substrate specificity and can reduce a variety of free methyl sulfoxide compounds including dimethyl sulphoxide (DMSO) and L-methionine sulfoxide (L-MetO) [25–28], in the presence of di-thiol substrates, such as reduced thioredoxin (Trx), dithiothreitol (DTT) and dihydrothioctic acid. Recently, a high throughput screening assay to look for inhibitors of MsrA has been developed [29]. This assay is based on the fact that MsrA coupling with Trx reductase can catalyze the reduction of DMSO and simultaneously initiate the oxidation of NADPH. However, this method examines the activity of total MsrA system, which involves MsrA, Trx and Trx reductase. The compounds that affect the activity of any of these components can interfere with the result. We tried to design a more specific method for the rapid detection of MsrA activity. In the MsrA catalytic reaction system, oxidation of di-thiol compound coincides with reduction of methyl sulfoxide to methyl sulfide [29–32]. Thus, monitoring methyl sulfoxides-induced decrease in di-thiol substrates, whose color can be produced by reacting with Ellman's reagent (dithio-bis-nitrobenzoic acid, DTNB), can directly reflect the activity of MsrA. Here, we developed a simple colorimetric assay of MsrA activity via monitoring MetO -dependent oxidation of DTT.

2. Materials and methods

2.1. Materials

Peptone, yeast powder, and imidazole to prepare recombinant rat MsrA (rMsrA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Amresco (cochran solon, OH, USA). PCR kit was purchased from New England Biolabs (Ipswich, MA, USA). BamHI, XhoI and phage T4 DNA ligase was purchased from MBI FERMENTAS (Burlington, ON, CANADA). *Escherichia coli* BL21 (DE3) cells were purchased from TIANGEN BIOTECH (Haidian District, Beijing, China). Thrombin kit was purchased from New England Biolabs (Ipswich, MA, USA). BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Ni-Trap nickel-chelating column was obtained from qiagen GE Healthcare (Hilden, Germany). DTT, DTNB, L-MetO, DMSO were also obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were of chromatographic grade (Tedia Way Fairfield, OH, USA). Amicon Ultra-4 centrifugal filter devices were purchased from Millipore carrigtwohill (Co. Cork, Ireland). Primary antibodies to MsrA were purchased from Upstate-cell Signaling Solutions (NY, USA). Other general agents were available commercially.

2.2. Preparation of recombinant rattus MsrA

Plasmid containing rat MsrA cDNA (pcDNA3.1-rMsrA) was kindly provided by Dr. Bertrand Friguet (Universite' Paris 7-Denis Diderot, France). The cDNA fragment encoding rMsrA was obtained from pcDNA3.1-rMsrA using PCR. A 5′ sense primer containing a BamHI site (5′-AAAGGATCCATGCTCTCCGCCTCCAGAAGGAC-3′), and a 3′ reverse complement primer with an XhoI site (5′-AAACTCGAGTTACTTTTTAATG GCCGTGGGACAGG-3′) were used. Both the amplified product and pET-32a(+) vector were digested with BamHI and XhoI, and the PCR fragment encompassing the complete rattus MsrA coding region was ligated into the restricted pET-32a(+) vector using phage T4 DNA ligase. The nucleotide sequence of the cloned insert was conformed by sequencing at least twice in each direction.

BL21 cells were transformed with the recombinant plasmid and grown in LB medium containing 50 μg/ml ampicillin at 37 °C. When cells reached an absorbance at 600 nm of 0.7, IPTG was added to a final concentration of 0.5 mM and the culture was continued for an additional 4 h. The cells were centrifuged at 5000 g for 10 min at 4 °C and the pellet was suspended in buffer A (20 mM Tris–HCl, pH 7.4; 500 mM NaCl; 0.5 mM imidazole) and sonicated four times for 30 s. The lysate was centrifuged at 40,000 × *g* for 30 min at 4 °C. Supernatant was applied to a 4 ml Ni-Trap nickel-chelating column previously equilibrated with the same buffer A. Column was washed with buffer A containing 60 mM imidazole and the recombinant peptide that contains full sequence of rMsrA was then eluted with buffer A containing 1 M imidazole. 5 ml of collection was treated with centrifugal ultrafiltration and the protein was concentrated. The N-terminal His-tag of recombinant peptide was removed using a Thrombin kit. Protein (500 μg) was digested for 24 h at 20 °C with 1 unit of thrombin. The purity of recombinant peptide was verified by SDS-PAGE stained with Coomassie Brilliant Blue. Recombinant peptide was stored at −80 °C until needed. Antibodies against rMsrA were used for immunological characteristics of recombinant peptide by western blot analysis. Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard. Heat-inactivated MsrA was prepared by boiling recombinant peptide at 95 °C for 30 min and no MetO-reducing activity was detected.

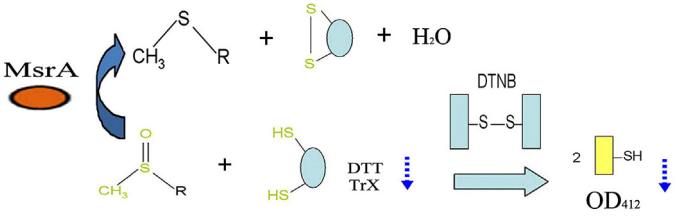

Fig. 1. Principle of colorimetric microplate assay for MsrA activity. MsrA-catalytic methyl sulfoxides-mediated oxidation controls DTT concentration in the reaction system, whose color can be produced by reacting with DTNB.

2.3. Analysis of MetO-reducing activity of recombinant rMsrA

The MsrA activity of recombinant peptide was controlled by a measurement of MetO-reducing activity based on the reduction of the substrate MetO to Met. We monitored the Met content using liquid chromatography coupled with electrospray ionization-ion trap mass spectrometry (LC-ESI-ITMS) as described earlier [33]. Recombinant rMsrA (20 μg) were added into the reaction mixture containing 50 mM sodium phosphate, pH 7.4, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT, 0.5 mM MetO. The mixture was incubated for 0.5 h at 37 °C. The reaction was stopped by addition of diamide. The content of Met were analyzed on a Thermo Finnigan LC–MS system (Thermo Finnigan Corporation, San Jose, CA, USA), including a Surveyor MS pump, LCQ DECA XPPlus MS detector, an auto sampler and Xcalibur TM 1.3 software. A reversed column (Thermo hypersil-Keystone C18, 150 mm × 2.1 mm, 5 μm) was also used. The separation of amino acid was achieved by gradient elution reversed-phase chromatography. The solvent system was composed of 0.2% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Met was eluted with retention time of 6.07 min, matching retention time obtained with the synthetic standards. No interference peak of intrinsic substances was observed. The mass spectra were acquired using electrospray ionization in positive ion mode and MRM. Data were acquired with Xcalibur 1.3. The highest and second highest fragment ions were chosen for Met quantification (m/z = 150.1, 133.0). Equations of the calibration curve for Met was $y = 6E+07x - 871{,}153$, while correlation coefficients (r^2) were 0.9892. MetO-reducing activity of recombinant peptide was expressed as the pmol of the product Met mg protein^{-1} min^{-1}.

2.4. Colorimetric determination of MsrA-catalyzed oxidation of DTT

MsrA-catalyzed oxidation of DTT was performed in the reaction system (pH 8.0) including $MgCl_2$ 10 mM, KCl 30 mM, Tris–HCl 25 mM, indicated concentrations of methyl sulfoxides (DMSO and L-MetO) and DTT 50 μM. Different concentrations of recombinant rMsrA peptide (active or non-active) were added into the reaction buffer and incubated for indicated time. Due to the potential reductive activity of DTT, this reaction was performed in the air-tight and complete darkness. After reaction, 100 μL of the reaction mixture and 100 μL DTNB (4 mM) was added to each well and incubated at 37 °C for 10 min. Absorbance at 412 nm (OD_{412}) was measured with a microplate reader (ELx800; Bio-Tek, Winooski, VT, USA). In each group, OD_{412} in the wells containing a same system incubated for 0 min were monitored and defined as initial values. Compared to initial values, the decreased OD_{412} (ΔA) in the wells after reaction were monitored and calculated. ΔA in the wells containing a reaction system with heat-inactivated MsrA peptide were defined as ΔA_{con}, which reflected a non-enzymatic oxidation of DTT. ΔA in the wells containing a reaction system with active rMsrA peptide incubated for indicated time were defined as ΔA_{Total}, which reflected a total oxidation of DTT. After deduction of non-enzymatic oxidation of DTT, ΔA induced by active MsrA were calculated and defined as: $\Delta A_{MsrA} = \Delta A_{Total} - \Delta A_{con}$.

3. Theory

In addition to detection of the product Met, MsrA activity can be measured by the consumption of another reactant DTT, which can be classically detected by Ellman's spectrophotometric assay. MsrA-catalytic methyl sulfoxides-mediated oxidation controls the concentrations of DTT in the reaction system. Thus, as shown in Fig. 1, the decreased OD_{412} of DTNB, the reduced product by DTNB-DTT reaction, can serve as a rapid indicator for MsrA activity.