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Experimental design-guided development of a stereospecific capillary electrophoresis assay for methionine sulfoxide reductase enzymes using a diastereomeric pentapeptide substrate☆

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ABSTRACT

A capillary electrophoresis method has been developed and validated to evaluate the stereospecific activity of recombinant human methionine sulfoxide reductase enzymes employing the C-terminally dinitrophenyl-labeled *N*-acetylated pentapeptide ac-KIFM(O)K-Dnp as substrate (M(O) = methionine sulfoxide). The separation of the ac-KIFM(O)K-Dnp diastereomers and the reduced peptide ac-KIFMK-Dnp was optimized using experimental design with regard to the buffer pH, buffer concentration, sulfated β-cyclodextrin and 15-crown-5 concentration as well as capillary temperature and separation voltage. A fractional factorial response IV design was employed for the identification of the significant factors and a five-level circumscribed central composite design for the final method optimization. Resolution of the peptide diastereomers as well as analyte migration time served as responses in both designs. The resulting optimized conditions included 50 mM Tris buffer, pH 7.85, containing 5 mM 15-crown-5 and 14.3 mg/mL sulfated β-cyclodextrin, at an applied voltage of 25 kV and a capillary temperature of 21.5 °C. The assay was subsequently applied to the determination of the stereospecificity of recombinant human methionine sulfoxide reductases A and B2. The Michaelis–Menten kinetic data were determined. The pentapeptide proved to be a good substrate for both enzymes. Furthermore, the first separation of methionine sulfoxide peptide diastereomers is reported.

1. Introduction

L-Methionine (Met), either bound in proteins or in its free form, is easily oxidized to L-methionine sulfoxide [Met(O)] by reactive oxygen species which are generated by physiological and chemical reactions [1]. The oxidation may result in the loss of protein function, and accumulation of oxidized proteins has been associated with aging [2] and may play a role in degenerative diseases such as morbus Alzheimer [3]. Protein-bound as well as free Met(O) can be reduced by a group of thiol oxidoreductases called methionine sulfoxide reductase (Msr) enzymes, protecting cells and tissues against oxidative damage [4]. Furthermore, recent studies suggest that Msr enzymes may play a role in the regulation of the metabolism of Met [5] and the regulation of enzyme activity [6,7].

Upon oxidation to the sulfoxide, the sulfur atom becomes a chiral center. Thus, Met(O) exists as a pair of diastereomers, i.e. L-methionine-(*S*)-sulfoxide [Met-*S*-(O)] and L-methionine-(*R*)-sulfoxide [Met-*R*-(O)]. For the reduction of the individual diastereomers, two types of Msr enzymes, MsrA and MsrB, exist. MsrA enzymes reduce Met-*S*-(O) bound in proteins or as free amino acid [6,8–10]. MsrB proteins reduce protein-bound Met-*R*-(O) but display only low activity for the reduction of free Met-*R*-(O) [10]. In mammals including humans, there is one gene encoding MsrA while three separate genes encode MsrB proteins, MsrB1, MsrB2 and MsrB3 [4,11]. In addition, an enzyme named free methionine-(*R*)-sulfoxide reductase (fRMsr) has been described in bacteria [12] and fungi [13], which specifically reduces free Met-*R*-(O).

Several in vitro assays have been developed to determine Msr activity with various substrates. The first group of assays is based on the reduction of tritiated *N*-acetyl-[^{3}H]-Met(O) [14,15]. Fluorescent amino acid derivatives substrates such as fluorenylmethyloxycarbonyl-Met(O) [Fmoc-Met(O)] [16] or 4-*N*,*N*-dimethylaminoazobenzene-4-sulfonyl-Met(O)

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[dabsyl-Met(O)] [8,15,17] were utilized in combination with HPLC analysis. Photometric assays are based on the reduction of dabsyl-Met(O) [18] or monitoring the decrease of the UV absorbance of NADPH in combination with various substrates [19–21]. However, only few methods addressed the stereospecificity of Msr enzymes incubating either the individual diastereomers dabsyl-Met-*S*-(O) or dabsyl-Met-*R*-(O) followed by HPLC [8,17] or CE analysis [22]. Incubations with the substrate Met(O) were analyzed following derivatization with o-phthalaldehyde/2-mercaptoethanol and subsequent separation of the diastereomers by HPLC [9] or derivatization with dabsyl chloride and CE diastereomer separation [13]. Finally, a stereospecific electrophoretically mediated microanalysis assay using Fmoc-Met(O) as substrate has been described [23]. However, none of these assays were conducted with peptides which would better reflect the natural substrates. This is particularly relevant for MsrB enzymes which only reduce peptide or protein bound Met(O). Therefore, the aim of the present study was the development of a CE method based on the separation of the diastereomers of a Met(O)-containing peptide. The *N*-acetylated pentapeptide labeled with dinitrophenyl (Dnp) at the C-terminus, ac-KIFM(O)K-Dnp, was selected because it has been shown to be a substrate for Msr enzymes [21].

2. Materials and methods

2.1. Chemicals and enzymes

The pentapeptides ac-KIFMK-Dnp and ac-KIFM(O)K-Dnp (Dnp label at the C-terminus) were synthesized by Peptide-Specialty-Laboratories GmbH (Heidelberg, Germany). Fmoc-β-Ala and 15-crown-5 were purchased from E. Merck (Darmstadt, Germany) and dithiothreitol (DTT) and tris(hydroxymethyl)aminomethane (Tris) were from Sigma-Aldrich (Steinheim, Germany). β-Cyclodextrin (β-CD), γ-CD, carboxymethyl-β-CD, methyl-β-CD and sulfobutyl-β-CD were obtained from Cyclolab Ltd. (Budapest, Hungary) and sulfated β-CD was from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of the highest purity available and used without further purification. Water was purified by a Milli-Q Direct 8 system (Millipore, Schwalbach, Germany).

The construction and cloning of the 6xHis-Tag fusion construct of human Msr enzymes hMsrA and hMsrB2 were described previously [21,24]. The proteins were overexpressed in *Escherichia coli* (strains BL21 or M15) and isolated using Ni-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) as described [25]. The purity of the enzymes was confirmed by SDS-PAGE.

2.2. Instrumentation

CE experiments were performed using a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Krefeld, Germany) equipped with a UV–Vis diode array detector and a sample tray temperature control system set at 10 °C. A 43/53.2 cm, 50 μm I.D., 363 μm O.D. fused-silica capillary (BGB Analytik Vertrieb, Schloßböckelheim, Germany) was used. A new capillary was successively rinsed at a pressure of 138 kPa (20 psi) with 1 M sodium hydroxide for 20 min, water for 10 min, 0.1 M sodium hydroxide for 20 min, water for 10 min, and the background electrolyte (BGE) for 10 min. At the end of each day, the capillary was rinsed with 0.1 M sodium hydroxide for 10 min, water for 10 min, the BGE for 10 min and left in the BGE overnight. Between analyses, the capillary was flushed with 0.1 M sodium hydroxide for 3 min, water for 2 min and the BGE for 3 min. The final BGE consisted of 50 mM Tris, adjusted to pH 7.85 by 1 M hydrochloric acid, containing 14.3 mg/mL sulfated β-CD and 5 mM 15-crown-5. The applied voltage was 25 kV and the capillary temperature was 21.5 °C. Hydrodynamic sample injection was performed at a pressure of 3.4 kPa for 7 s. UV detection was carried out at the cathodic end at 214 nm. All solutions were filtered through 0.2 μm polyester membrane filters and sonicated for 5 min.

2.3. Enzyme incubations

2 mM stock solutions of ac-KIFM(O)K-Dnp were prepared in water and 2 mM Fmoc-β-Ala was prepared in the 50 mM Tris buffer, pH 8.0, and stored at −20 °C. The pH of the Tris buffer was adjusted at 20 °C. Solutions of the Msr enzymes were prepared at a concentration of 1 mg/mL protein in Tris buffer (pH 8.0)/glycerol (2:1, v/v) and stored at −20 °C. Incubations were carried out in 100 μL of 50 mM Tris buffer, pH 8.0, containing 20 mM DTT and the appropriate amount of enzyme. Prior to the assay, the solutions containing enzyme and substrate were preincubated separately at 37 °C for 2 min. The reaction was started by the addition of an appropriate amount of ac-KIFM(O)K-Dnp stock solution to achieve the desired concentration of the substrate. If not indicated otherwise, incubations were carried out at 37 °C for 9 min and stopped by freezing at −80 °C. Before analysis, Fmoc-β-Ala was spiked into the thawed samples at a concentration of 70 μM. Control samples were obtained by incubation without the addition of enzyme and blank samples were obtained by incubations of enzymes without the addition of substrate. For screening of the Msr activity, concentrations of 15 μg/mL of Msr enzymes and 160 μM ac-KIFM(O)K-Dnp were used. The time course was studied at a concentration of 160 μM ac-KIFM(O)K-Dnp with 1 μg/mL hMsrA.

The determination of the Michaelis–Menten kinetic parameters was carried out by varying the concentration of ac-KIFM(O)K-Dnp between 40 and 400 μM (corresponding to 20–200 μM of the individual diastereomers) in the presence of 1 μg/mL hMsrA and 7.5 μg/mL hMsrB2. The plots of initial velocities versus substrate concentration were fitted to the Michaelis–Menten equation using Origin 8.5 (OriginLab Corp., Northampton, MA, USA).

2.4. Experimental design

Modde 7.0 (Umetrics, Umea, Sweden) was used for the experimental design and statistical analysis. For screening and optimization models, the investigated factors were fitted using a polynomial function:

$$y = b_i + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + \cdots + \varepsilon$$

where x_i represents the factors to be optimized; y represents the response functions, i.e., peak resolution, and migration time of analytes. For each response y, the intercept b_i, the main coefficients $b_{1,\ldots,\ n}$, the interaction coefficient $b_{12,\ldots,\ (n\text{-}1)n}$, the quadratic coefficients $b_{11,\ldots,\ nn}$ and the residual ε are calculated. The goodness of fitting [R^2 = (total sum of squares − sum of squares for residuals)/total sum of squares] and the goodness of prediction [Q^2 = 1 − (prediction residual sum of squares/total sum of squares)] were subsequently optimized. R^2 and Q^2 close to 1 describe an excellent model, $Q^2 > 0.5$ a good model and $Q^2 > 0.1$ indicates a significant model [26]. A fractional factorial response IV design was used for the identification of significant variables, while a five-level circumscribed central composite design (star distance 1.682) was used for the optimization of the significant variables.

3. Results and discussion

3.1. Initial separation conditions

Ac-KIFM(O)K-Dnp was selected because it has been previously shown to be a substrate for Msr enzymes [21]. Since the substrate