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Short communication

Cyclic interconversion of methionine containing peptide between oxidized and reduced phases monitored by reversed-phase HPLC and ESI-MS/MS

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ABSTRACT

The cyclic oxidation and reduction of methionine (Met) containing peptides and proteins play important roles in biological system. This work was contributed to analysis the cyclic oxidation and reduction processes of a methionine containing peptide which is very likely to relate in the cell signal transduction pathways. To mimic the biological oxidation condition, hydrogen peroxide was used as the reactive oxygen species to oxidize the peptide. Reversed-phase high-performance liquid chromatography and mass spectrometry were employed to monitor the reactions and characterize the structural changes of the products. A rapid reduction procedure was developed by simply using KI as the reductant, which is green and highly efficient. By investigation of the cyclic oxidation and reduction process, our work provides a new perspective to study the function and mechanism of Met containing peptides and proteins during cell signaling processes as well as diseases.

1. Introduction

The cyclic interconversion of DNA and cell signaling proteins between modified forms and unmodified forms is implicated in the regulation of many important processes *in vivo* [1–3]. Besides the well-known methylation/demethylation, phosphorylation/dephosphorylation and ubiquitination/deubiquitination interchanging processes, the cyclic oxidation and reduction of methionine (Met) containing proteins also play an indispensable role in controlling homeostasis in biological systems and regulating cellular signal transduction [4–6]. The redox status of Met residues in proteins is closely related with disorders in aging as well as in some pathological conditions [7–9]. It has been indicated that the oxidation of Met-35 in the β-amyloid peptide leads to insolubility and stability of β-amyloid peptide and further resulted in Alzheimer's disease [10]. The oxidation of methionine in calmodulin significantly impairs its function in calcium signaling [11]. Voltage-dependent K^+ channel proteins are also modulated by the redox process of Met residue [12]. Therefore, researches on the cyclic interconversion between methionine residues and methionine sulfoxide residues in proteins or peptides are crucial for understanding cell signaling and mechanism of many diseases [13,14].

Many efforts have been devoted to study the cyclic oxidation and reduction of Met in organisms. It has been demonstrated that *in vivo* methionine residues are readily oxidized to methionine sulfoxide residues by reactive oxygen species (ROS), such as H_2O_2, hydroxyl radicals and hypochlorite [7,15]. And by the effect of methionine sulfoxide reductase, methionine sulfoxide can be reduced back to methionine [16]. This reversibility suggests that methionine residues may serve as an important antioxidant species to mitigate oxidative stress in body [17]. To mimic such process, *in vitro* researches on the oxidation of methionine containing proteins and peptides have been carried out mainly based on the ROS pathways [18,19]. Without methionine sulfoxide reductase, chemical protocols primarily relying on organic reagents have been used for the reduction of methionine sulfoxide residue in peptides, such as trimethylsilyl bromide/1,2-ethanedithiol reduction and *N*-methylmercaptoacetamide method [20–22]. However, the general problems with these available methods are time-consuming and poor selectivity leading to the destruction of disulfide bridged cysteine residues. Hence, further investigations into more efficient reduction protocols and the whole cyclic oxidation and reduction process are highly desired.

Mass spectrometry, with its unparalleled high-content information acquisition capability, has recently emerged as a dominant technique in characterizing protein and peptide components of complex biological systems. Compared with traditional analysis

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methods such as gel electrophoresis, isoelectric focusing, Edman sequence analysis, it can provide useful data to elucidate the structural information including the modification sites through fragmentation [18,23–26]. Tandem mass spectrometry (MS/MS) is particularly attractive in the identification of bioactive peptides [27–29] and has been applied to characterize methionine sulfoxide residues in proteins and peptides [30–33]. By combined with reversed-phase high performance liquid chromatography (RP-HPLC), matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) and electrospray MS/MS (ESI-MS/MS) can be effective in analyzing the whole oxidation and reduction process of methionine containing peptides.

Recently, a new cancer biomarker termed lysosomal protein transmembrane 4 beta (LAPTM4B) was found overexpressed in the majority of solid tumors [34]. The importance of LAPTM4B in regulating cell survival, proliferation, migration and carcinogenesis indicates that this protein participates in the cancer cell signaling. And an extracellular fragment (EL2) containing a Met residue may contributes to this close relationship with the signal transduction evidenced by the characteristic of its sequence [34–36]. By using EL2 as the "epitope", the generated antibody and peptide probe has been used for functional study and effective detection of live cancer cells [34,37]. Based on these findings, the modification of EL2 may be critical during tumor progress. In this study, the cyclic methionine oxidation and reduction process of EL2 peptide was analyzed *in vitro*. Different oxidation conditions were evaluated to obtain a mild process. Subsequent characterization of the structural modifications of EL2 was realized by utilizing RP-HPLC, MALDI-TOF MS and ESI MS/MS techniques. In the reduction process, a method employing KI as the reductant was developed, which is green, high efficient and universal. Structural and functional analysis of the EL2 peptide in oxidation and reduction environment may be potential in understanding the mechanism of signal transduction of hepatoma cells and developing new diagnostic strategies.

2. Experimental

2.1. Chemicals

2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 9-fluorenylmethoxycarbonyl (FMOC)-derivatized amino acids and 1,2-ethanedithiol were purchased from Siam (USA). FMOC–amino acid–Wang resin was from Advanced ChemTech (USA). Trifluoroacetic acid (TFA) and ascorbic acid were obtained from Sigma (USA). Acetonitrile (HPLC grade) was from Fisher (USA). *N*-methylmorpholine, piperidine, dichloromethane (DCM), *N,N*-dimethylformamide (DMF) and 30% H_2O_2 were of analytical grade and purchased from Beijing Chemical Factory (China). KI was obtained from Sinopharm (China). Ultra-pure water from a MilliQ water purification system (Millipore, Bedford, MA) was used throughout. All other chemicals were of analytical grade and used without further disposal.

2.2. Solid-phase peptide synthesis

The peptide EL2 (NH_2–Pro–Tyr–Arg–Asp–Asp–Val–Met–Ser–Val–Asn–COOH) was synthesized using FMOC strategy [38] on an automated solid phase peptide synthesizer (PS3, Protein Technologies Inc., USA). FMOC–amino acid–Wang resin was used as the starting material. The elongation cycles for peptide synthesis on the Wang resin were performed in the presence of *N*-methylmorpholine and HBTU. After completion of synthesis cycles, the peptide resin was washed with DCM and methanol respectively and dried under high vacuum for 4 h. The peptide cleavage was carried out by using TFA–ethanedithiol–water (95:2.5:2.5). The mixture was occasionally stirred at room temperature for 4 h and then was filtered. The filtrate was collected and evaporated to 1 mL. The peptide was finally precipitated by adding cold diethyl ether. The white precipitate was dried under high vacuum.

2.3. RP-HPLC analysis of the peptides

The HPLC system consists of a Hitachi L-7610 degasser (Japan), a Hitachi L-7100 pump (Japan), a Hitachi L-7420 UV-Vis detector (Japan), a Hitachi L-7300 column oven (Japan), a Hitachi D-7000 interface (Japan) and a Rheodyne 7725i injection valve (USA). The UV data were monitored at 220 nm.

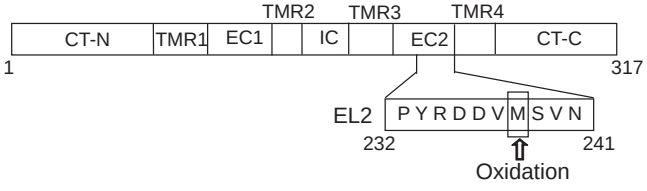
The synthesized peptide EL2 was analyzed and purified by using the Hitachi HPLC system. The purity of the peptide was analyzed on a Diamonsil C18 column (250 mm × 4.6 mm i.d.) at a flow rate of 1.0 mL/min. Mobile phases were acetonitrile and water containing 0.1% TFA. Gradient: 0–25–25.1–30 min, 15–30–80–80% acetonitrile containing 0.1% TFA. The purification was performed on a Kromasil C8 column (250 mm × 10 mm i.d.). Blank chromatogram was obtained by injecting the solvent under the same conditions. After subtracting the blank from the sample chromatogram, the purity was calculated by measuring the peak areas.

2.4. Oxidation of EL2 with H_2O_2

The aqueous solution of EL2 (1 mg/mL) was prepared and mixed with 0.6% H_2O_2 in the volume ratio of 39:1. The final concentrations of EL2 and H_2O_2 were approximate 1 mg/mL and 0.015%, respectively. The resultant solution was incubated at 37 °C for oxidation. The reaction process was monitored by RP-HPLC at different time, *i.e.* 1, 4 and 9 h. For each run, 10 μl of the reaction solution was sampled and injected for analysis. Chromatographic peaks separated during RP-HPLC were collected and lyophilized for further MS characterization.

2.5. Reduction of the methionine sulfoxide with KI

A portion of the oxidized EL2 peptide was dissolved with TFA to obtain a 2 mM solution and cooled to 0 °C. KI powder (20 eq.) was then added to the tube. Brown iodine precipitate was observed which subsequently disappeared with the addition of a saturated solution of ascorbic acid. After being shaken at room temperature for 3 min, the mixture was moved to ice bath and reacted for 1 h. The mixture was filtrated and the filtrate was collected and evaporated to about 2 mL. Ultrapure water was added to dilute the resultant solution to a volume of 10 mL. The obtained solution was further analyzed by RP-HPLC. The chromatographic peaks were collected and lyophilized for MS characterization.



Scheme 1. Schematic representation of LAPTAM4B protein and amino acid sequence of the decapeptide EL2. (CT-N, N-terminal cytoplasmic tail; TMR, transmembrane regions; EC, extracellular loops; IC, intracellular loop; CT-C, C-terminal cytoplasmic region.)

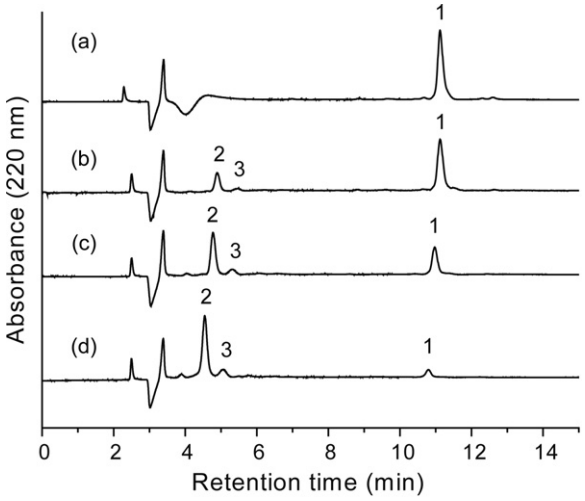


Fig. 1. RP-HPLC analysis of EL2 peptide samples. The synthesized EL2 (a); EL2 solution after oxidation in 0.015% H_2O_2 for 1 h (b), 4 h (c), 9 h (d), respectively. Peak 1: EL2; Peak 2 and Peak 3: two oxidation products of EL2.

2.6. Characterization of the peptides by MALDI-TOF-MS and ESI-MS/MS

The originally synthesized EL2 peptide was identified by MALDI-TOF-MS (Bruker Daltonics, USA) after purification. The oxidized EL2 and the subsequently reduced product were also characterized by MALDI-TOF-MS before tandem MS analysis.

Positive-ion electrospray ionization mass spectra were obtained on a Micromass Q-TOF mass spectrometer (Waters, USA). The ESI-MS/MS characterization was carried out off-line. The chromatographic fractions of peptide samples were manually collected from HPLC and lyophilized. After re-dissolved by a mixture of 50% CH_3CN and 50% H_2O added with 0.1% formic acid, the collected samples were injected into the mass spectrometer through the ESI probe needles, respectively. The spray voltage of the mass spectrometer was 3 kV and the cone voltage was 90 V. The desolvation temperature was 373 K and the source temperature was 373 K. Nitrogen was used as both cone gas and desolvation gas with a flow rate of 50 L/h and 500 L/h, respectively. The collision energy was set up to 10 V. Collision-induced dissociation MS/MS spectra were obtained in the range of 100–1200 *m/z*. The collision energy was set to 30–40 eV and argon was used as the collision gas. Mass Lynx (ver. 4.0) software was used for analysis and post processing.

3. Results and discussion

3.1. Peptide synthesis

The decapeptide EL2 locating at the second extracellular loop of LAPTM4B protein with the sequence of NH_2–P–Y–R–D–D–V–M–S–V–N–COOH (1194.53 Da) was chosen as the model to investigate its redox status (Scheme 1). By

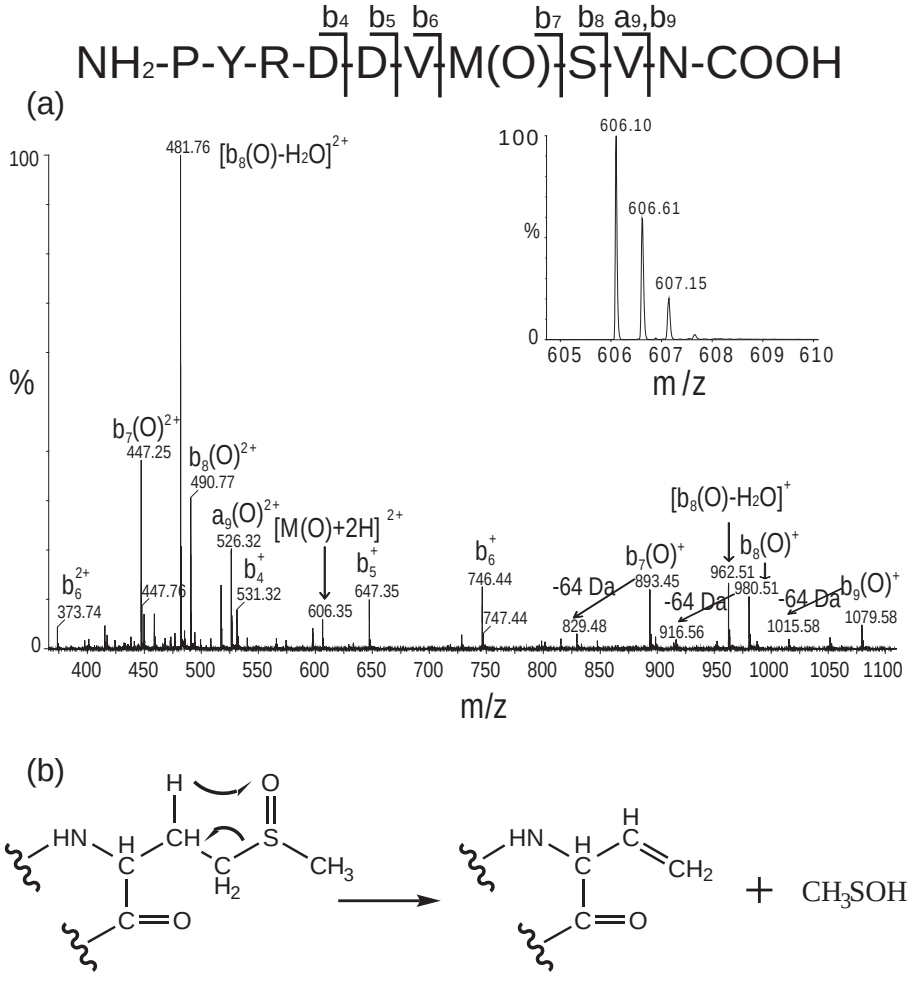


Fig. 2. Characterization of the structural changes of the species in chromatographic peak 2. (a) ESI-MS/MS spectra of peak 2; (b) schematic diagram of fragmentation pathway of the neutral loss of 64 Da.

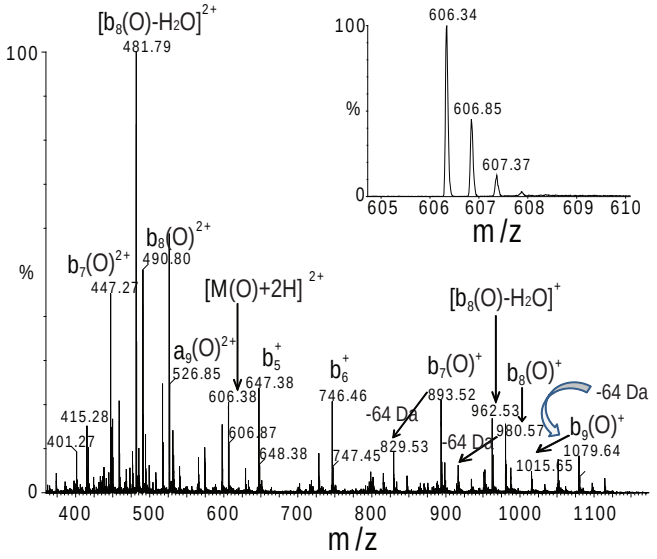


Fig. 3. ESI-MS/MS characterization of the structural changes of the species in chromatographic peak 3.

using FMOC solid phase peptide synthesis strategy, EL2 was firstly synthesized as the starting material. After purification, the obtain EL2 peptide was analyzed by RP-HPLC. A chromatographic peak was detected with the retention time of 11.09 min, which is subsequently identified by MALDI-TOF-MS as EL2 (*m*/*z* 1195.6). The relative content of EL2 in the purified product was calculated by measuring the peak areas in the chromatogram (Fig. 1a). With blank subtracted, the chromatographic peak of EL2 showed an area percentage of 96.5%, denoting that the peptide product had a high purity.

3.2. Monitoring the oxidation of peptide by RP-HPLC

ROS induced oxidation of proteins and peptides is a type of post-translational modification and crucial for cellular signal transduction [4,5]. To investigate such biological phenomenon, many *in vitro* methods have been developed for the oxidation of Met in proteins and peptides. Herein, the oxidation of EL2 under different conditions was carried out to examine its susceptibility to oxidant modification, which would provide useful information on the biological response of this fragment to cell signals. Firstly, Fenton's reaction [39] and UV/H_2O_2 system generating hydroxyl radicals were respectively employed to oxidize EL2. Unfortunately, the results from RP-HPLC separation indicated that EL2 peptide was totally degraded within 30 min treatments. This is quite in accordance with the previous finding that Met residues are often further oxidized to methionine sulfone by these two methods [31]. Such oxidation conditions were too harsh to treat EL2 and also cannot happen *in vivo* [19]. With this consideration, H_2O_2 was added as the only source for ROS which was moderate, easy to operate as well as more closely resembling *in vivo* oxidative environment. In the experiments, incubation at 37 °C was also chosen for mimicking the body temperature.

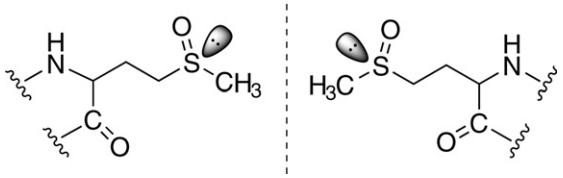


Fig. 4. Methionine sulfoxide diastereoisomers formed during oxidation of EL2.

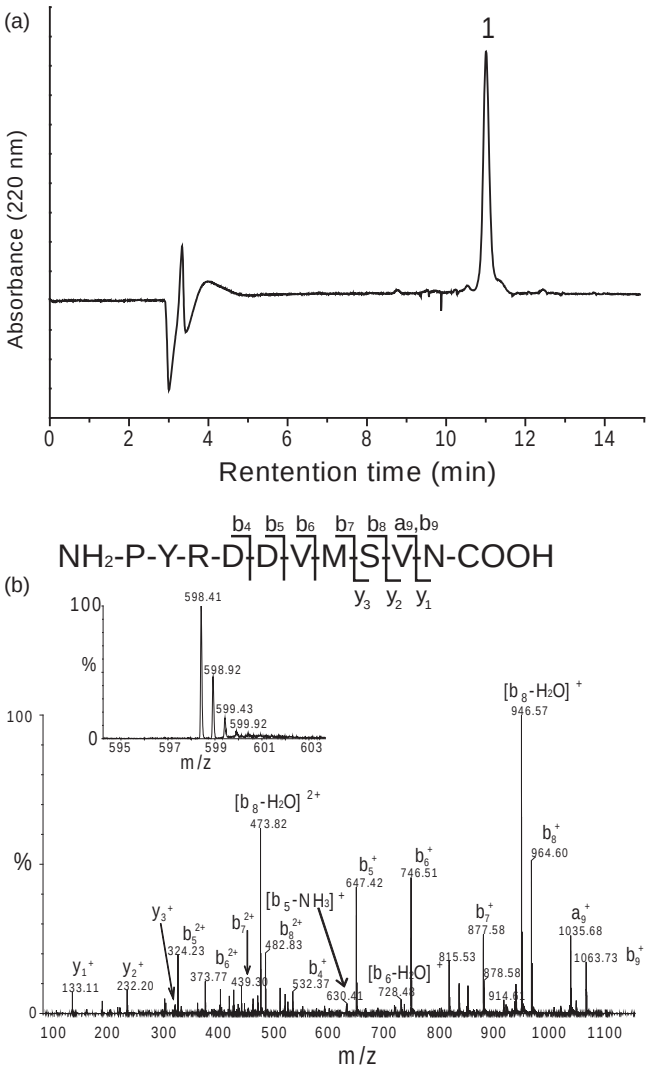
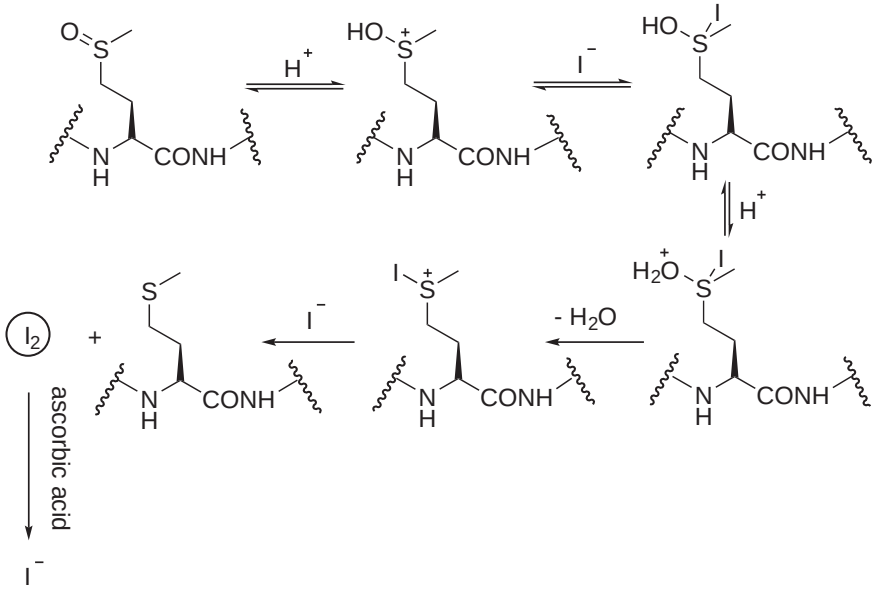


Fig. 5. Analysis of the reduced peptide sample. (a) RP-HPLC separation of the reduced peptide solution, Peak 1: EL2; (b) ESI-MS/MS spectra of the chromatographic peak 1.

RP-HPLC was used to monitor the oxidation process by analyzing reaction solutions at different time points, 1 h, 4 h and 9 h. As shown in Fig. 1b–d, peak 1 identified as EL2 peptide decreased as the reaction time increased, suggesting the consumption of EL2. Meanwhile, two new peaks (peak 2 and peak 3) with retention time of 4.78 and 5.31 min were detected with enhanced intensity, implying that these new species were most probably the oxidation products of EL2. Further characterization by MALDI-TOF MS demonstrated that both products have an increase of 16 Da (*m*/*z*: 1211.6, 1211.5) over EL2. And each may have insertion of one oxygen atom in the peptide. Since Met residue shows the highest tendency to oxidation among the whole amino acid sequence of EL2, the oxidation of Met to methionine sulfoxide can be conferred. As calculated from the chromatograms, over 91% of EL2 peptide was oxidized after 9 h reaction, which was faster than some reported data [31]. Together with the fact that EL2 was unstable in Fenton's and UV/H_2O_2 systems, the high susceptible of this peptide to oxidation can be conferred.

3.3. ESI-MS/MS analysis of oxidation process of EL2

In order to further characterize the structural change of EL2 peptide during oxidization, ESI-MS/MS was employed. Fig. 2a shows



Scheme 2. Mechanism of the reduction reaction of methionine sulfoxide residue in EL2 peptide.

ESI-MS/MS spectra of the species in chromatographic peak 2. The fragmented ions $b_7(O)$ and b_6 with m/z of 893.45 and 746.44 respectively clearly indicated that the oxidation reaction took place on the Met residue of EL2 peptide. And the appearance of MS signals attributing to $b_8(O)$, $b_9(O)$ and $a_9(O)$ fragments also evidenced the oxidation of the Met residue in EL2 peptide. In tandem mass peptide sequencing, it is often difficult to differentiate between methionine sulfoxide and phenylalanine, because both residues have the same nominal mass of 147 Da. In this study, a neutral loss of 64 Da from the molecular ion was detected in the fragmentation pathway of collision induced dissociation, which is characteristic of the decomposition of methanesulfenic acid (CH_3SOH) from the side chain of methionine sulfoxide residue [40]. The mechanism of the fragmentation pathway was elucidated in Fig. 2b. With the fact that no phenylalanine was included in the native sequence of EL2 peptide, the existence of methionine sulfoxide residue can be clearly identified. The inset of the Fig. 2a shows an isotopic pattern of divalent ions ($[M+O+2H]^{2+}$).

The species in the chromatographic peak 3 was also characterized by ESI-MS/MS (Fig. 3). The results showed that this product had the same mass spectra and fragmentation pathway as peak 2. Hence, the peak 3 fraction was modified at the same site with peak 2 giving methionine sulfoxide residue after oxidation. Regarding these identical products showing different chromatographic property, this phenomenon most probably attributed to the formation of the sulfoxide diastereoisomers. The oxidation of the sulfur atom in the Met residue often results in the formation of isomers, which was also observed in the reported work [41]. The mechanism of the formation of the sulfoxide diastereoisomers is illustrated in Fig. 4.

3.4. Mild and efficient reduction of the oxidized EL2

For methionine sulfoxide reduction, various protocols have been previously reported. However, many of them are time-consuming and limited by the incompatibility with cysteine and aromatic amino acids [20]. Moreover, irritating organic reagents with poor biocompatibility such as 1,2-ethanedithiol, *N*-methylmercaptoacetamide and dimethyl sulfide were usually employed by these methods. The addition of NH_4I into the cleavage cocktail as the protecting reagent has been utilized to prevent the Met residue from oxidization during the solid phase peptide synthesis. Herein, to reduce the oxidized EL2, KI was used as the reductant. A brown precipitate of iodine was observed immediately after the addition of KI powder, representing the effectiveness of redox reaction. And ascorbic acid was subsequently used to dissolve the precipitate. The consumption of the generated iodine further drove the reduction reaction forward. According to some reported data [42], a proposed mechanism of reduction reaction is demonstrated in Scheme 2, which is based on a nucleophilic iodide attack on the protonated sulfoxide leading to the reduced methionine sulfide.

The reaction process was monitored by RP-HPLC analysis. From the chromatogram (Fig. 5a), only one main peak was detected which had identical retention time with the original synthesized peptide EL2 (peak 1). This main peak was collected and further subjected to MALDI-TOF MS and ESI-MS/MS characterization. As shown in Fig. 5b, peak 1 separated from the reduction mixture gave fragmentation ions of m/z 877.58 (b_7) and 746.51 (b_6), which clearly confirmed the existence of Met residue (residue mass: 131 Da). Isotopic pattern of divalent ions ($[M+2H]^{2+}$) was also illustrated in the inset of Fig. 5b. These data were consistent with that from the original peptide EL2. Although sulfoxide diastereoisomers were detected during the oxidation of the Met residue, EL2 peptide regained its original structure after reduction. The disappearance of peaks 2 and 3 representing two oxidized forms of EL2 indicated our method could completely reduce the methionine sulfoxide residue back to methionine form. The usage of inorganic reagents KI for the reduction of EL2 peptide is not only rapid, but also environmental friendly and compatible with other amino acids, showing distinct advantage for practical application.

4. Conclusions

In this study, cyclic oxidation and reduction of a methionine containing peptide EL2 was realized and analyzed. RP-HPLC and MS were employed to monitor and characterize the redox reactions. Even with a moderate H_2O_2 oxidation condition, the high tendency of EL2 peptide to oxidation was still observed, implying the readily modification of this peptide to *in vivo* oxidative stress. By using an inorganic reductant KI, the oxidized sulfoxide diastereoisomers can be conversed back to EL2 completely and rapidly. The

interconversion between the oxidized and reduced forms of EL2 may play an important role during the regulation of physiologic processes. This investigation into the interconversion of EL2 allows further study of its function and mechanism during cancer cell signaling processes. Our method also provides a new perspective to study biochemically active Met containing peptides and proteins in various biological systems.

Acknowledgements

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