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2-Oxo-histidine as a novel biological marker for oxidatively modified proteins

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We report a promising finding that oxidative modification of proteins by free radicals could be monitored by the formation of oxidized histidine that is detectable by reverse-phase HPLC with electrochemical detection (HPLC-ECD). When the *N*-protected histidine derivative (*N*-benzoyl-histidine) was exposed to a free radical-generating system (copper/ascorbate), a number of products were detected by HPLC-ECD and the main product among them was found to be identical to *N*-benzoyl-2-oxo-histidine. The acid hydrolysis of *N*-benzoyl-2-oxo-histidine provided a single product (2-oxo-histidine) that was detected sensitively by HPLC-ECD. Thus 2-oxo-histidine was indeed generated as the main product in the oxidatively modified proteins by free radicals. Taken together, 2-oxo-histidine may be a useful biological marker for assessing protein modifications under oxidative stress.

2-Oxo-histidine; HPLC-ECD; Oxidative modification of protein; Biological marker

1. INTRODUCTION

A number of systems that generate oxygen free radicals catalyze the oxidative modification of proteins [1–3]. Protein oxidation by free radicals contributes to the pool of damaged proteins, which increases in size during aging and in various pathological states [4]. It has been established that oxidative modification of proteins involves the conversion of amino acids to their oxidized forms [2]. Histidine, in general, is one of the amino acids most vulnerable to metal-catalyzed free radical reactions. The conversion of histidine to asparagine has been established [2]; however, asparagine may not be an appropriate marker for monitoring oxidatively modified proteins because it is detected as aspartate in the amino acid analysis following acid hydrolysis of oxidized proteins. These situations prompted us to search for a novel biological marker to monitor oxidized histidine in proteins.

In this communication, we report a promising procedure to monitor the oxidized histidine using HPLC with electrochemical detection (HPLC-ECD) that permitted the detection of the oxidized product with the highest specificity.

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Abbreviations: HPLC-ECD, high performance liquid chromatography with electrochemical detection.

2. MATERIALS AND METHODS

2.1. *Materials*

N-α-Benzoyl-L-histidine and bovine serum albumin were obtained from Sigma. L-Ascorbate, trifluoroacetic acid, and tetrafluorobutyric acid were obtained from Wako Pure Chemical Industries Ltd. All other reagents were of the highest grade commercially available.

2.2. *Preparation of authentic* N-*benzoyl-2-oxo-histidine*

The reaction mixture (300 ml) containing 50 mM *N*-benzoylhistidine, 50 mM ascorbate, and 0.5 mM $CuSO_4$ in 50 mM sodium phosphate buffer (pH 7.2) was incubated at room temperature. Oxygen gas was bubbled into the mixture for 48 h. Isolation of *N*-benzoyl-2-oxo-histidine was performed with reverse-phase HPLC on a Lop ODS-5 column (2 × 25 cm). The reaction mixture was concentrated and applied to the column equilibrated in a solution of 25% methanol in 0.1% trifluoroacetic acid. Products were eluted at a flow rate of 6 ml/min, the elution being monitored by absorbance at 230 nm. The main product was isolated (yield, 35.7 mg) and its chemical structure was characterized by fast atom-bombardment mass spectrometry and ^1H- and ^{13}C nuclear magnetic resonance spectrometry [5,6].

2.3. *Preparation of authentic 2-oxo-histidine*

Purified *N*-benzoyl-2-oxo-histidine was hydrolyzed with 6 N HCl in vacuo at 105°C for 24 h. The hydrolysate was concentrated and dissolved with 50 mM sodium phosphate buffer (pH 7.2). The product was purified with reverse-phase HPLC on a TSK-GEL ODS-80TM column (0.46 × 25 cm) (TOSOH). The sample was applied to a column equilibrated in a solution of 5% methanol in 0.1% heptafluorobutyric acid and eluted at a flow rate of 0.8 ml/min, the elution being monitored by absorbance at 210 nm. HPLC analysis of the hydrolyzed sample revealed the formation of a single product and its chemical structure was characterized by fast atom-bombardment mass spectrometry and ^1H- and ^{13}C nuclear magnetic resonance spectrometry [7].

2.4. *Oxidation of* N-*benzoylhistidine by copper/ascorbate*

N-Benzoylhistidine (1 mM) was incubated with 50 μM $CuSO_4$ and 5 mM ascorbate in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) at 37°C. An aliquot of the reaction mixture was taken and analyzed

by HPLC. The column (TSK-GEL ODS-80TM) was equilibrated and the products were eluted with 20% methanol in 50 mM NaCl solution containing 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The elution was monitored at 0.85 V of applied oxidation potential on the electrochemical detector.

2.5. *Detection of 2-oxo-histidine in bovine serum albumin treated with copper/ascorbate*

Bovine serum albumin (1 mg/ml) in 50 mM sodium phosphate buffer (pH 7.2) was treated with 50 μM $CuSO_4$ and 5 mM ascorbate at 37°C. The reactions were stopped by the addition of 0.1 mM EDTA. The reaction mixtures were then treated with 10% trichloroacetic acid. After centrifugation at 10,000 × *g* for 3 min, the proteins were hydrolyzed with 6 N HCl for 24 h at 105°C. The hydrolysates were concentrated and dissolved with 50 mM sodium phosphate buffer (pH 7.2).

The amount of 2-oxo-histidine present in the hydrolysates of the protein samples was measured by HPLC-ECD. The column (TSK-GEL ODS-80TM) was equilibrated and the products were eluted with 50 mM NaCl solution containing 0.1% heptafluorobutyric acid at a flow rate of 0.5 ml/min. 2-Oxo-histidine was detected at 0.85 V of applied oxidation potential on the electrochemical detector.

2.6. *Amino acid composition*

The amino acid analysis was performed with a JEOL JLC-300 amino acid analyzer equipped with a JEOL LC30-DK20 data analyzing system.

3. RESULTS AND DISCUSSION

As a preliminary experiment, the histidine derivative (*N*-benzoyl-histidine) oxidatively modified by a free radical-generating system (copper/ascorbate) was analyzed by HPLC-ECD in order to determine whether or not any oxidized products could be detected electrochemically. As shown in Fig. 1A, a number of products were found to have significantly electrochemical activity. Although most of the products have not been identified chemically, the main product eluted at 18.2 min on the HPLC chromatogram was found to be identical to *N*-benzoyl-2-oxo-histidine that had been discovered previously amongst the oxidation products of the histidine derivative and the histidine-containing peptides exposed to the copper/ascorbate-free radical-generating system [5–7]. The fact [7] that the 2-oxo-histidyl moiety is resistant to the acid hydrolysis conditions of proteins led to the assumption that 2-oxo-histidine, if it is generated in proteins, can be monitored by HPLC-ECD. In fact, the acid-hydrolysis of *N*-benzoyl-2-oxo-histidine generated a single product (Fig. 1B) that was identical to 2-oxo-histidine, and the electrochemical response of the hydrolyzed samples increased in a concentration-dependent manner for *N*-benzoyl-2-oxo-histidine (data not shown).

We then attempted to quantify the 2-oxo-histidine generated in proteins that had been treated with metal-catalyzed free radical-generating systems. As shown in Fig. 2, the reaction of copper/ascorbate toward bovine serum albumin was selective for histidine residues. We found that, concurrent with the loss of histidine, approximately 25% of histidine residues was converted to

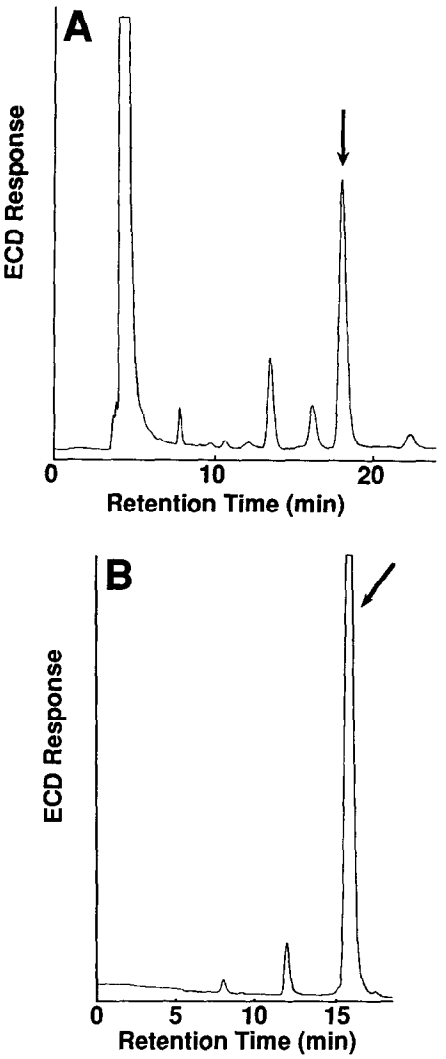

Fig. 1. Detection of *N*-benzoyl-2-oxo-histidine (A) and its hydrolyzed product 2-oxo-histidine (B) by HPLC-ECD. (A) *N*-benzoylhistidine (1 mM) was incubated with 50 μM $CuSO_4$ and 5 mM ascorbate in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) for 1 h at 37°C. The main peak indicated by an arrow at 18.2 min represents *N*-benzoyl-2-oxo-histidine. The starting material (*N*-benzoylhistidine) was eluted at 13 min while it did not show ECD response. (B) Authentic *N*-benzoyl-2-oxo-histidine (0.1 mg) was hydrolyzed with 6 N HCl for 24 h at 105°C. The hydrolysate was concentrated and dissolved with 0.5 ml of 50 mM sodium phosphate buffer (pH 7.2). An aliquot (10 μl) was taken and subjected to the HPLC-ECD analysis. The main peak indicated by arrow at 15.8 min represents 2-oxo-histidine.

2-oxo-histidine after 30 min of incubation. The yield of 2-oxo-histidine in this reaction reached a maximum (3.78 mol/mol protein) after 1 h of incubation and decreased gradually thereafter, suggesting that 2-oxo-histidine underwent secondary free radical reactions.

The 2-oxo-histidine is possibly a ubiquitous oxidized material since copper/ascorbate could be replaced by Fenton's reagents (metal/hydrogen peroxide) and Udenfriend's reagents (iron/ascorbate) which have been implicated as potent free radical-generating systems in biological systems [8,9], although the maximum yield of

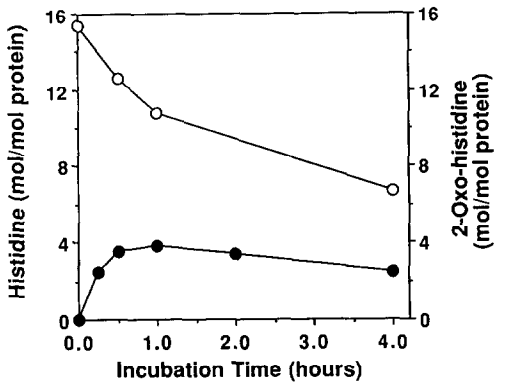

Fig. 2. Loss of histidine and concomitant formation of 2-oxo-histidine in bovine serum albumin treated with copper/ascorbate. The amount of histidine (○) and 2-oxo-histidine (●) was quantitated by amino acid analysis and HPLC-ECD, respectively.

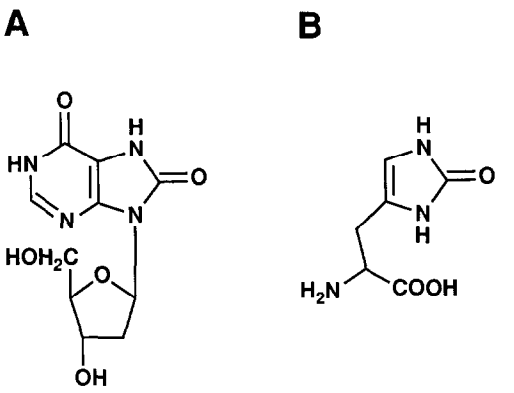

Fig. 3. Chemical structure of 8-oxodeoxyguanosine (A) and 2-oxo-histidine (B).

2-oxo-histidine in those systems were lower than that in copper/ascorbate: the reaction of bovine serum albumin with Fenton's reagents (0.1 mM Cu^{2+} and 5 mM H_2O_2) yielded 0.53 mol of 2-oxo-histidine per mol of protein after 30 min of incubation, whereas 0.51 mol of 2-oxo-histidine per mol of protein was formed when the protein was incubated with Udenfriend's reagents (0.1 mM Fe^{2+} and 5 mM ascorbate) for 1 h. Furthermore, 2-oxo-histidine has so far been detected in a number of proteins exposed to oxidative stress in vitro: for example, metal-catalyzed oxidative modification of low-density lipoprotein (LDL) resulted in the generation of 2-oxo-histidine (0.30 nmol/mg LDL) when LDL was incubated with 10 μM Cu^{2+} for 24 h, suggesting relevance to atherosclerosis.

Taken together, we have developed a useful probe (2-oxo-histidine) that might be applicable to monitoring oxidized proteins generated during oxidative stress. Several lines of evidence indicate that protein oxidation by free radicals and the following accumulation of oxidatively modified proteins, possibly an early indication of oxygen radical-mediated tissue damage, have been implicated in cells during aging, oxidative stress, and in various pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis. In this regard the procedure using HPLC-ECD is crucial since ECD in combination with HPLC can raise the specificity in detecting the oxidized product. This HPLC-ECD technique has been commonly applied to monitoring 8-oxo-deoxyguanosine, which is an important biological marker for oxidatively damaged DNA under oxidative stress [10–14]. It is of interest to note that 8-oxo-deoxyguanosine has a similar 2-oxo-imidazole moiety in its molecule (Fig. 3).

In conclusion, it is not unlikely that, during aging and oxidative stress, the formation of 2-oxo-histidine may be involved in the accumulation of modified proteins in mammalian cells. However, an assessment of the true physiological significance of the conversion of histidine to 2-oxo-histidine awaits extension of the study to mammalian sources known to accumulate modified forms of proteins during aging and in various pathophysiological states.

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