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Comparison of the stability of glutathione and related synthetic tetrapeptides by HPLC and capillary electrophoresis‡

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Abstract: Glutathione and related peptides are interesting targets as protectors of biological systems against an oxidative injury. Two novel glutathione analogues, UPF1 and UPF15, have been designed and synthesised. As a result of different reactions taking place, the thiol-containing compounds oxidise to disulfides. In this study, the stability of UPF1, UPF15 and glutathione in various solutions was investigated by using HPLC and CE. The results showed that UPF1 and UPF15 are powerful hydroxyl radical scavengers and their dimerisation process velocity is higher than that of glutathione. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antioxidants; capillary electrophoresis; stability of peptides; hydroxyl radical scavenging capacity

INTRODUCTION

The thiol-containing compounds are central actors in many biochemical reactions taking place in the human organism. Reduced glutathione (GSH, L-γ-Glu-L-Cys-Gly) is the most abundant low molecular weight thiol that is present in most cells in millimolar amounts. In a reduced form, GSH is a major intracellular antioxidant. The molecules containing a cysteine residue (the sulphydryl group) easily participate in the thiol-disulfide exchange. In different conditions, GSH is oxidised to GSSG.

A considerable decrease in the level of GSH in aging organisms [1] and during the pathogenesis of different diseases has been observed. In recent years, the concentration of several thiols (glutathione, cysteine, etc.) in human plasma has determined that GSH is a biomarker of several diseases (Alzheimer's disease, diabetes, alcoholic liver disease, AIDS, etc.). Different researchers have shown that the low level of thiols in both fresh and preserved plasma is caused by their instability (i.e. after the addition of 300 μM GSH to human plasma, the level of GSH decreases to 50% in 15 min). The decrease in the level of GSH occurs because of the oxidising reactions taking place in human plasma [2].

Abbreviations: **GSH**, reduced glutathione, monomer; **GSSG**, oxidized glutathione, dimer; **UPF1**, 4-methoxy-L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine; **UPF15,** L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine; **THA,** terephthalic acid; **CE**, capillary electrophoresis.

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Carrying out the syntheses of various exogenic analogues of glutathione, whose stability and antioxidative capacity would be superior to the antioxidative capacity of GSH and which would be able to influence the level of GSH in cells, is highly justified. A major cellular non-enzymatic antioxidant, GSH eliminates the reactive species like a hydroxyl radical, peroxynitrite and peroxides, and plays a principal role in the cellular defence against high-grade oxidative and nitrosative stress. Owing to its special structural features, GSH is able to fulfil many different biofunctions. GSH is a cell redox regulator. It is used for the detoxification of several xenobiotics and is involved in the restoration of the thiol groups of proteins [3].

The most widely used methods for determining the concentration of GSH and GSSG are the fluorimetric assay [4] and high performance liquid chromatography [2,5,6].

Besides HPLC, CE has become a powerful tool for the analysis of polar peptides. It has been successfully applied to the separation of closely related peptides, such as the monomeric and dimeric forms of glutathione in biological systems [7–10], and to monitor the kinetics of their oxidation [11,12].

Compared to the other methods (HPLC, fluorimetric assay), the CE technique offers significant advantages, such as a high speed of separation and low sample and buffer requirement.

The aim of this study was to synthesise novel GSH analogues and investigate the stability of glutathione and related tetrapeptides in various media, such as water, a physiological solution, phosphate buffer, copper(II)sulphate solution and hydrogen peroxide solution, and investigate the scavenging ability of the hydroxyl radical.

MATERIALS AND METHODS

Synthesis

Glutathione is an essential antioxidant. At the same time, it is known that the methoxy group enhances its antioxidativity [13]. In the peptide UPF1, we added a non-coded amino acid residue, 4-methoxy-phenylalanine, to the *N*-terminus of the GSH trimer. UPF15 is a peptide with amino acid tyrosine in the *N*-terminus of glutathione.

The glutathione analogue UPF15 (MW 469.4) was synthesised with SPPS method as UPF1 (MW 483.5), as described in a previous study [14]. The crude peptides were purified by the reversed-phase HPLC (a 1100 Hewlett Packard column ZORBAX 300 SB-C18 9.4 mm × 25 cm) employing an acetonitrile–water mixture (containing 0.1% TFA) as an eluent at a flow rate of 4 ml/min and absorbance of 218 nm. The fractions were pooled together and lyophilised.

The molecular masses of peptides were determined by a MALDI-TOF mass spectrometer (Voyager DE-Pro, Applied Biosystem).

Stability Assay

Solutions of 1 mM GSH, UPF1 and UPF15 were prepared and incubated until the dimeric form appeared in water and in the physiological solution at room temperature. A sample (100 μl) of each solution was taken every day and analysed by HPLC. The samples were applied to a reverse-phase HPLC column (ZORBAX 300 SB-C18 4.6 mm × 15 cm) employing acetonitrile–water mixture (containing 0.1% TFA) as the eluent at a flow rate of 2 ml/min and absorbance of 218 nm. A linear gradient was carried out from 20 to 90% acetonitrile (v/v). The peaks were collected and the change in concentration of the components in the solution was observed.

The appearance of the oxidised forms of glutathione, UPF1 and UPF15 was established by a MALDI-TOF mass spectrometer.

Capillary electrophoretic analyses were performed using an in-house (laboratory-built) CE system equipped with a fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA), total length 55 cm, effective length 39 cm, 50 μm ID, high voltage power supply (Spellmann, Hauppauge, NY, USA), and a UV detector (Prince Technologies).

In the case of 15 mM phosphate buffer (pH 7.5), copper(II)sulphate (20 μM in 15 mM buffer) and hydrogen peroxide solutions (1 mM in 15 mM buffer), the oxidation process was monitored until the disappearance of the monomeric form of peptides was established by CE. The CE separation was performed with 25 mM phosphate buffer (pH 7.5) containing 50 mM SDS, using an applied voltage of 25 kV. The detection was performed at 200 nm. The peaks in the electropherogram were identified by a standard adding method.

Hydroxyl Radical Scavenging Assay

The antioxidativities of UPF1 and UPF15 were determined as described by Barreto *et al.* [15], using THA as a chemical dosimeter for the hydroxyl radicals. The THA dosimeter solution contained 10 mM THA in a 14.75 mM sodium phosphate buffer at pH 7.5. The buffer was used as a control. Hydroxyl radicals were generated via the Fenton reaction by adding $CuSO_4$ and H_2O_2 to the dosimeter solution to achieve the final concentration of 10 μM and 1 mM, respectively. Hydroxyl radical scavenging abilities of UPFs were measured by a Perkin–Elmer LS50B spectrofluorometer at the excitation of 312 nm and emission of 426 nm [16]. The EC_{50} values were determined by the sigmoid dose-response (a viable slope) analysis using the GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA, USA).

For monitoring the rate of the scavenging capacity of the hydroxyl radical by a CE assay, the peptides (250 μM) and a hydroxyl radical generating system consisting of $CuSO_4$ (10 μM) and H_2O_2 (1 mM) in 15 mM sodium phosphate buffer (pH 7.5) were used.

RESULTS AND DISCUSSION

The Stability of Analogues of Glutathione in Different Solutions and their Comparison

The stability of GSH and synthesised GSH analogues were examined in the following different solutions: water, a physiological solution, phosphate buffer, copper(II)sulphate solution and hydrogen peroxide solution. It is a well-known fact that glutathione and related peptides easily oxidise in the solution. The electronic structure of the sulfur atom underlies the high reactivity of the thiol group towards nucleophilic addition, redox reactions and metal chelation.

We observed that GSH is very stable in water and the physiological solution, unlike UPF1 and UPF15, at room temperature (Table 1). The oxidised form of GSH (GSSG) detected by MALDI-TOF appeared after 18 days of incubation. The appearance of disulfides of UPF1 and UPF15 was already observed after 2 days. In all cases, the oxidation in the phosphate buffer was considerably faster. The results showed that the addition of amino acid to the *N*-terminus of GSH increased the velocity of the dimerisation process of the analogues.

Using the reversed-phase HPLC, the separation of GSH and UPF15 monomers from the respective dimers failed, and we therefore studied the process using the CE assay. CE enabled the monitoring of the dimerisation process of GSH (Figure 1, peaks 1 and 2, respectively), UPF1 (Figure 1, peaks 3 and 5, respectively) and UPF15. HPLC and CE are orthogonal methods – in the case of reversed-phase HPLC, the separation was based on the difference

Table 1 Time of appearance of the dimeric forms of peptides

Peptide	Water[a] (days)	Physiological solution[a] (days)	Phosphate buffer[b] (15 mM, pH 7.5) (h)
GSH	18	18	16
UPF1	2	2	4
UPF15	2	2	4

[a] Measured by MALDI-TOF.
[b] Measured by CE.

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in the hydrophobicity of compounds, but in CE, the separation was based on the difference in the mass–charge ratio.

For all peptides, the rate of dimerisation in the solution of H_2O_2 was similar. Differences appeared in the $CuSO_4$ solution (Table 2) – the half-life of GSH was approximately 10 times and of UPF1 and UPF15 5 to 6 times longer in $CuSO_4$ than in H_2O_2. At the same time, formation of the dimers of UPFs took place about 2 times faster than GSH dimers. The oxidation reaction took place because of the reducing capacity of copper ions. It is based on the reduction of Cu(II) to Cu(I) by reductants (antioxidants). Under physiological conditions, the hazardous effect of H_2O_2 is realised in combination with Fe(II) or Cu(I) via the Haber–Weiss or Fenton reaction, producing a highly reactive hydroxyl radical. At low concentrations, H_2O_2 is rather inert, but in our experiments, the concentration was sufficient for oxidising the peptides.

We also investigated the behaviour of a mixture of GSH and UPF1. There was no difference in half-life between the GSH/UPF1 mixture and their pure components in hydrogen peroxide solution. As expected, the respective dimeric forms – GSSG and UPF1-UPF1 and the heterodimer GSH-UPF1 appeared–(Figure 1, peaks 2, 5 and 4, respectively).

The Study of Antioxidative Properties of Peptides via the Hydroxyl Radical Elimination

We tested the potential antioxidativity of UPF1 and UPF15 to verify the scavenging effects of the hydroxyl radical. We also compared the antioxidativity of UPF1 and UPF15 to GSH. For UPF1 and UPF15, changes in the velocity of elimination of the hydroxyl radical were observed in a concentration range of 5–100 μM. The solution with a concentration below 5 μM possessed no scavenging nature, and at concentrations above 100 μM there was minimal increase in the velocity of elimination of the hydroxyl radical. For GSH, the concentration range was wider, ranging from 10 μM to 10 mM.

UPF1 and UPF15 dissolved readily in water, and hence high concentrations of peptides can be used. In the present study, UPF1 and UPF15 showed a significantly higher antioxidativity than GSH, as measured by a THA dosimeter. We have previously shown that the increase in antioxidativity is caused by the methoxy group [14], but no difference in antioxidativity between the methoxy or

Table 2 The half-life times of peptides in various media

Peptide	Half-life, s		
	20 μM $CuSO_4$ in buffer	1 mM H_2O_2 in buffer	10 μM $CuSO_4$ and 1 mM H_2O_2 in buffer[a]
GSH	10 800 ± 520	810 ± 48	180.6 ± 8.3
UPF1	4 200 ± 180	738 ± 31	41.4 ± 4.1
UPF15	4 800 ± 210	768 ± 28	42.6 ± 4.8

[a] Peptide concentration 250 μM.

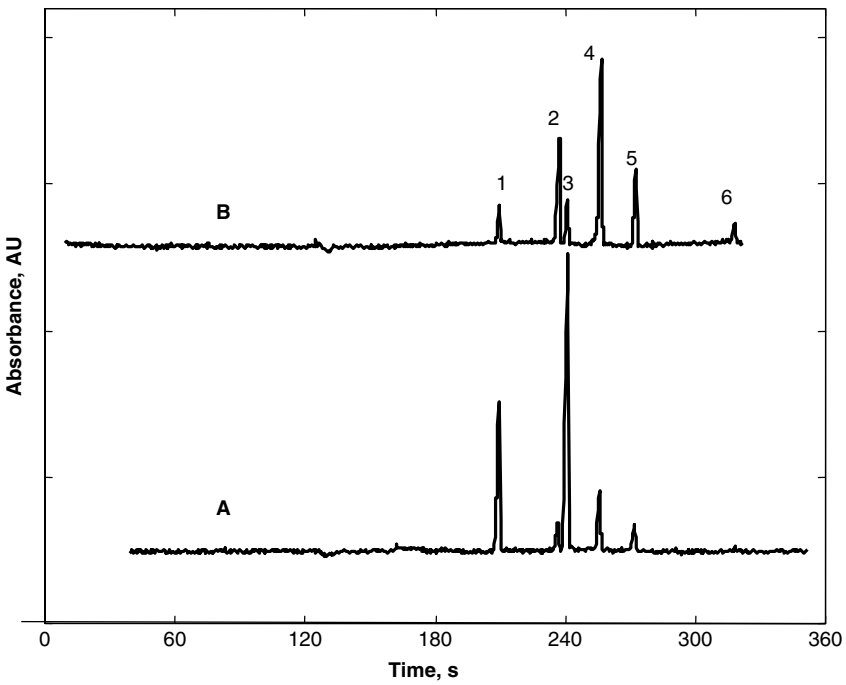

Figure 1 Monitoring the stability of the GSH/UPF1 mixture against hydrogen peroxide: 1, GSH; 3, UPF1; 2 and 5, their respective dimers; 4, heterodimer; 6, unknown. Reaction times: pherogram A, 4 min; pherogram B, 27 min. Separation buffer: 25 mM sodium phosphate, 50 mM SDS; pH 7.5. Applied voltage 25 kV, detection at 200 nm.

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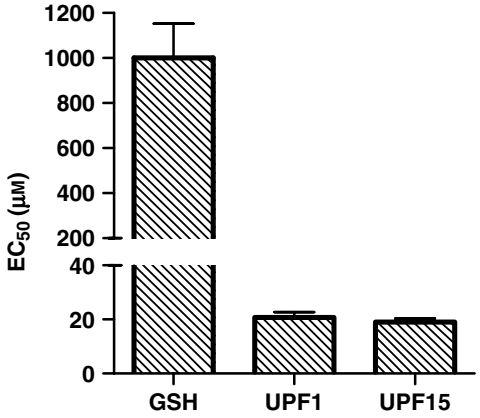

Figure 2 The comparison of the hydroxyl radical scavenging ability of GSH and synthetic peptides.

hydroxyl group carrying peptides was observed in this study.

As seen in Figure 2, the scavenging ability of UPF15 and UPF1 is very similar. From this we can conclude that the replacement of methoxytyrosine in UPF1 with tyrosine from UPF15 does not influence the antioxidative properties of their peptides.

Because the peptides investigated in the present study are the analogues of a natural antioxidant of GSH, the comparison of the scavenging capacity of the hydroxyl radicals was of interest. Our results showed that UPF1 and UPF15 possess a clear hydroxyl radical scavenging nature *in vitro*. The enhancement of the hydrophobic moiety to the *N*-terminus of GSH strengthened the antioxidative properties of the peptide. The EC_{50} of UPF1 (20.5 ± 2.3 μM) and UPF15 (19.0 ± 1.0 μM) was about 50 times lower than that of GSH (1000 ± 150 μM).

Investigation of the elimination velocity of the hydroxyl radical provides evidence of the difference in radical elimination ability between peptides. In CE experiments, the half-life of GSH in oxidising reactions was 180.6 ± 8.3 s, and that of UPF15 and UPF1 was 42.6 ± 4.8 and 41.4 ± 4.1 s, respectively.

The results obtained confirm that the hydroxyl radical elimination ability of synthetic peptides is higher than that of natural glutathione.

The exact mechanism of action of UPF1 is not known. UPF1 is an effective and potential agent that diminishes a neuronal injury in global cerebral ischemia [14]. It can act as a powerful free radical scavenger or a modulator of G proteins in frontocortical membrane preparations [17].

CONCLUSIONS

Glutathione analogues with high antioxidative capacity were designed and synthesised. Compared to glutathione, the stability of peptides was rather low, especially in $CuSO_4$ and H_2O_2 solutions. The scavenging capacity of the hydroxyl radical was 50 times higher than that of GSH.

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