Bioorganic Chemistry 37 (2009) 126–132

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Mechanism and stoichiometry of 2,2-diphenyl-1-picrylhydrazyl radical scavenging by glutathione and its novel α-glutamyl derivative

Säde Viirlaid [b], Riina Mahlapuu [a], Kalle Kilk [a], Aleksei Kuznetsov [b], Ursel Soomets [a], Jaak Järv [b,*]

[a] Institute of Biochemistry, Medical Faculty, University of Tartu, The Centre of Excellence for Translational Medicine, 19 Ravila St, 50411 Tartu, Estonia
[b] Institute of Chemistry, University of Tartu, 2 Jakobi St, 51014 Tartu, Estonia

ARTICLE INFO

Article history:
Received 17 February 2009
Available online 12 May 2009

Keywords:
Antioxidant activity
Glutathione
GSH α-glutamyl analog
DPPH•
Kinetic mechanism
Scavenging stoichiometry

ABSTRACT

Kinetic mechanism and stoichiometry of scavenging the 2,2-diphenyl-1-picrylhydrazyl radical by glutathione and its novel analog, containing α-glutamyl residue in place of the γ-glutamyl moiety, were studied using different ratios of reagents. At low concentrations of the peptides, the process was described as a bimolecular reaction obeying the stoichiometric ratio 1:1. However, at excess of peptides the formation of a non-covalent complex between the reagents was discovered and characterized by dissociation constants $K = 0.61$ mM for glutathione and $K = 0.27$ mM for the glutathione α-glutamyl analog, respectively. The complex formation was followed by a reaction step that was characterized by the similar rate constant $k = 0.02\ s^{-1}$ for both peptides. Thus, the apparently different antioxidant activity of these two peptides, observed under common assay conditions, was determined by differences in the formation of this non-covalent complex.

1. Introduction

The thiol group of glutathione (GSH, Scheme 1) is the key intracellular scavenger of free radicals in biological systems, maintaining redox homeostasis for proper functioning of cellular processes [1]. This has generated a great interest in the design and pharmacological applications of GSH analogs as possible drugs and bioactive compounds [2]. In parallel, the same fact has also initiated investigations into the reaction mechanism of these compounds in non-biological model systems, often used for *in vitro* evaluation of their antioxidant properties. These data are clearly valuable for understanding the structure–activity relationship for antioxidants and elucidating the chemical mechanism behind their bioactivity. Moreover, linking the results with bioactivity data is generally believed to be a promising reference material for design studies since the *in vitro* assays are fast and less expensive if used for preliminary screening of drug candidates [3].

Previous studies have shown that alterations of the GSH structure at different positions of this mini-peptide may have a strong influence on the activity of these congeners, revealing also higher antioxidant and cell protecting effects [4]. Furthermore, a significant increase in hydroxyl radical scavenging activity has been demonstrated with GSH analogs belonging to a series of novel tetrapeptidic GSH analogs called UPF peptides [5]. In these compounds, the γ-glutamyl moiety of GSH has been replaced by the α-glutamyl group, corresponding to the "normal" peptide structure [5]. However, as several parts of the peptide structure were altered in UPF peptides, it was impossible to assess the specific effect of the γ to α replacement in the tripeptide structure. Therefore, the α-congener of GSH was subsequently synthesized (α-GSH, Scheme 1) and its reaction with the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•, Scheme 1) was investigated in the present paper.

The stable radical DPPH• was discovered by Goldschmidt and Renn in 1922 [6], and later it was introduced for the chemical assay of antioxidant properties by Blois [7]. Currently, this radical has become a popular tool in the antioxidant assays of synthetic and natural compounds and their mixtures [8,9]. DPPH• has a strong absorption maximum at $\lambda = 517$ nm, characterized with the molar extinction coefficient 9660 and this value decreases to 1640 if the odd electron of the stable radical becomes paired [8,10]. In summary, this process can be presented by the following equilibrium between DPPH• and the radical scavenging peptide (GSH or α-GSH):

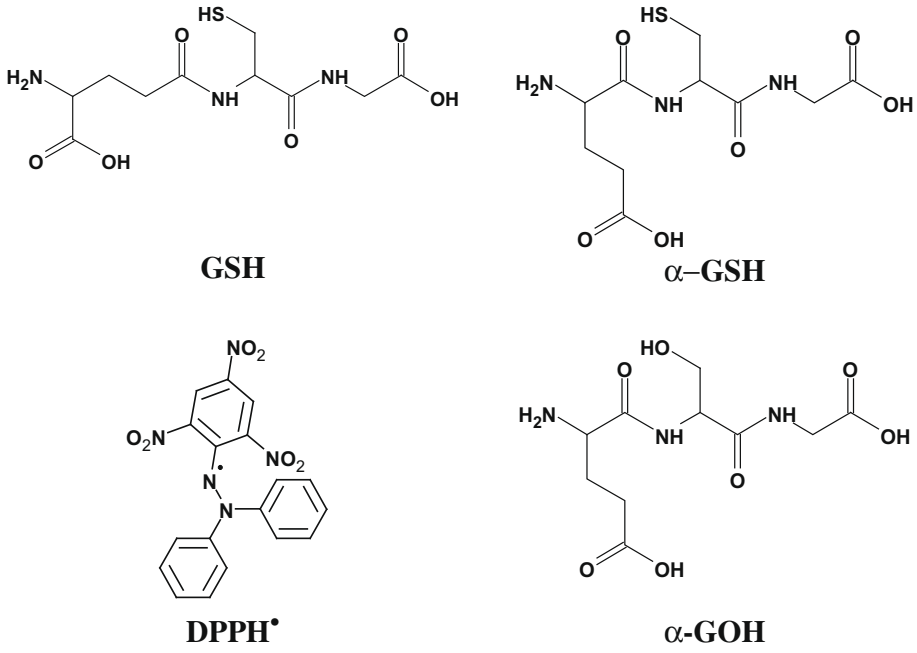
$$\text{DPPH}^{\bullet} + \text{Peptide-SH} \leftrightarrows \text{DPPH-H} + \text{Peptide-S}^{\bullet}. \quad (1)$$

In detail, the reaction (1) may proceed through different mechanisms. Firstly, it may involve the one-step abstraction of the hydrogen atom (H•) from the radical scavenging reagent and its

Abbreviations: DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; GSH, γ-L-glutamyl-L-cysteinylglycine; α-GSH, α-L-glutamyl-L-cysteinylglycine; α-GOH, α-L-glutamyl-L-serinylglycine; BDE, bond dissociation energy.

* Corresponding author. Fax: +372 7375247.
E-mail address: jaak.jarv@ut.ee (J. Järv).

0045-2068/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2009.05.001



Scheme 1.

direct transfer to DPPH•, named as the hydrogen atom transfer (HAT) mechanism [10,11]. Secondly, the concerted transfer of electron and proton may occur between the reagents [12–15]. The sequence of these events varies in different reaction mechanisms, depending on the reagent structure and the reaction medium, especially if protic and aprotic solvents are compared [12–15]. All these mechanisms, however, lead to the formation of the same product, the quenched radical DPPH-H as defined by Eq. (1).

Our preliminary experiments with the α-glutamyl analog of GSH (α-GSH) revealed that the DPPH• quenching rate for this derivative exceeded the rate at which GSH reacted with this radical under the same assay conditions. The different behavior of GSH and α-GSH in this assay was rather surprising, as the alteration of the position of the glutamyl residue in this peptide should not influence the reactivity of the thiol group of cysteine. For example, the energy of the homolytic dissociation of the S–H bond (BDE) was found to be close for the free cystein (373 kJ/mol, 298 K) and for the same amino acid in peptide structure (367 kJ/mol, the same temperature) [16]. These values also coincide with the theoretical and experimental BDE values for the S–H bond in various alkanethiols, ranging from 366 to 371 kJ/mol [17]. Based on the data, it has been recognized that the homolytic dissociation energy of a typical S–H bond attached to aliphatic structures does not vary much [17]. Therefore, it was intriguing to find the reasons why GSH and its α-glutamyl derivative α-GSH still react with DPPH• at different rates. To verify the role of the thiol group in these reactions, the serine analog of α-glutamyl GSH was synthesized (α-GOH, see Scheme 1) and its antioxidant activity was assayed under the same conditions.

The mechanism of DPPH• scavenging by GSH and α-GSH was studied kinetically and the experiments established that the reaction involved the formation of a reversible complex between the reactants, followed by a reaction step where the scavenging of DPPH• took place. Resolving of this reaction mechanism identified the different equilibria of the reversible complex formation step as the main reason of apparently different antioxidant properties of GSH and α-GSH in this assay system.

2. Materials and methods

2.1. Chemicals and peptide synthesis

Fmoc–Gly–Wang resin, Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Novabiochem, Switzerland; *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA) and acetonitrile from BDH Laboratory Supplies, England; trifluoroacetic acid (TFA) and triisopropylsilane (TIS) from Fluka, Switzerland; 1,2-ethanedithiol (EDT), glutathione and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) from Sigma-Aldrich, Germany.

The glutathione analogs were synthesized manually using Fmoc-chemistry starting from commercial Fmoc–Gly–Wang resin. Coupling of amino acid residues was carried out in a stepwise manner using the standard TBTU and HOBt activation protocol [18]. The peptides were removed from the resin and simultaneously deprotected with TFA in the presence of scavengers, 2% water (v/v), 2% EDT (v/v) and 2.5%TIS (v/v) for 90 min at room temperature. The purity of the synthetic peptides was >99% as demonstrated by HPLC on an analytical Nucleosil 120-3 C18 reversed-phase column (0.4 cm × 10 cm). The molecular masses of the peptides were determined by a MALDI–TOF mass spectrometry (Voyager DE Pro, Applied Biosystems) and the calculated values were obtained in each case.

2.2. DPPH• scavenging assay

Scavenging of DPPH• was monitored spectrophotometrically as described previously [5]. Briefly, 0.25 ml of GSH or α-GSH solution (within the concentration range of 5–2000 μM) in citric acid–sodium citrate buffer (20 mM, pH 3.9) was added to 0.25 ml of DPPH• solution in 95% ethanol. Freshly distilled solvents were used. Changes in the absorbance at 517 nm, occurring due to scavenging the free radical, were monitored in 1 cm thermostated quartz cells at 25 °C (UV–VIS spectrophotometer Unicam UV300, ThermoSpec-

tronic, USA). From these time courses, the kinetic curves of the scavenging process were obtained and used in kinetic analysis.

2.3. pK_a of thiol groups

The ratio of thiol and thiolate concentrations was measured spectrophotometrically at $\lambda = 240$ nm [19,20]. 1 ml of 50 μM peptide solution in phosphate buffered saline (PBS, Calbiochem, USA) was titrated with 5 μl volumes of 1 M NaOH and pH and absorbance changes were determined after each addition. The results were corrected to consider the dilution of the assay mixture. Absorbance was recorded on a PerkinElmer Lambda 25 spectrometer.

2.4. LC–MS analysis

GSH–DPPH$^{\bullet}$ complex formation was studied by liquid chromatography–mass spectrometry. 50 μl of the sample (DPPH$^{\bullet}$, GSH or their mixture) was injected automatically into a Shimadzu Prominence HPLC supplied with Phenomenex Luna $C_{18}(2)$ 3 μm column with 100 × 2 mm measurements. The gradient was built with solvents A: 99.9% H_2O + 0.1% HCOOH and B: 99.9% MeCN + 0.1% HCOOH. Gradient started at 100% A for 5 min, followed by a linear increase to 100% B in 20 min and finally 20 min wash at 100% B. The flow rate was 0.75 ml/min. The mass spectrometry detector was Q-Trap 3200 (Applied Biosystems, Inc., USA). Depending on the experiment, either Q1 scan from 50 to 1500 Da or MS2 scan on specific ions (306.0, 394.5 and 699.0) was used. The ionization mode was negative, at voltage −4500 V.

2.5. Structure modeling

Modeling studies were performed using the Spartan 5.0 software suite (Wavefunction, Inc., USA). Conformational searches for finding initial geometry were performed by using molecular mechanics with additional conditions for aqueous medium. The geometry of DPPH$^{\bullet}$ complexes with peptides were optimized by the PM3 semi-empirical method.

2.6. Data processing

Calculations and statistical analysis were made using the GraphPad Prism package version 4.0 (GraphPad Software Inc., USA) and the SigmaPlot software package (version 8.0, SPSS Inc., USA). The results reported are given with their standard errors.

3. Results

The reactivity of thiol groups in GSH and α-GSH was compared by determining their pK_a values. The analysis gave $pK_a = 9.0 \pm 0.2$ for GSH, in agreement with the pK_a values of 9.20 [19] and 8.75 [21] listed for the thiol group of GSH in other papers. The same titration method produced $pK_a = 9.1 \pm 0.1$ for α-GSH. The results show that the thiol groups have the similar acidity in GSH and α-GSH, and the mode of glutamyl residue coupling has no influence on chemical properties of the cystein residue. This conclusion was supported by the calculated GSH and α-GSH structures (Fig. 1), revealing that the thiol groups of these peptides were not involved in hydrogen bonding with other parts of these tripeptides. Moreover, such a conclusion has also been drawn by Rosei, proceeding from Raman spectroscopy data for GSH [22].

We tested the antioxidant properties of GSH, α-GSH and α-GOH by means of the spectrophotometric method [8], and the results were unambiguous for the Ser-containing peptide, indicating that no scavenging of the radical occurred in the presence of this compound. Conversely, in the presence of both thiol-containing ligands, there was a clear decrease in absorption of the reaction mixture and this change was monitored for kinetic analysis of the radical-scavenging reaction at different ratios of the reacting compounds.

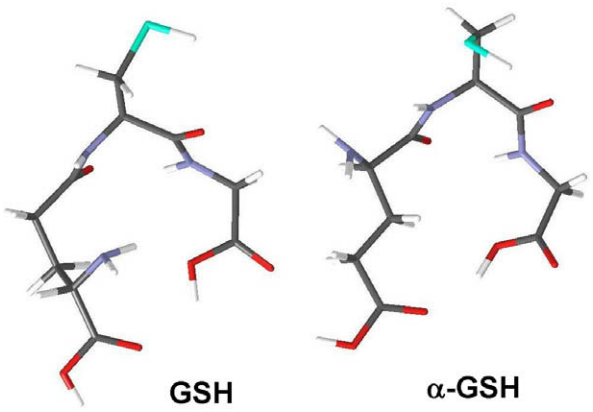


Fig. 1. Calculated structures for GSH (left) and α-GSH (right).

Firstly, we carried out experiments at excess of DPPH$^{\bullet}$ over the concentration of thiol-containing peptides, as commonly used for the assay of antioxidant properties of natural and synthetic compounds [9]. If 50 μM of DPPH$^{\bullet}$ reacted with 5 and 10 μM of peptides (Fig. 2), only part of the radical was consumed and the time course of this process followed the rate law for the first-order reaction:

$$[\mathrm{DPPH}^{\bullet}]_t = [\mathrm{DPPH}^{\bullet}]_{\mathrm{span}} e^{-k_{\mathrm{obs}} t} + [\mathrm{DPPH}^{\bullet}]_{\mathrm{plateau}}, \quad (2)$$

where $[\mathrm{DPPH}^{\bullet}]_{\mathrm{span}}$ is the maximal change of the radical concentration ("span" of the exponential function); $[\mathrm{DPPH}^{\bullet}]_{\mathrm{plateau}}$ indicates the concentration of the remaining reagent ("plateau"), $[\mathrm{DPPH}^{\bullet}]_t$ is the reagent concentration at time t and k_{obs} is the observed first-order rate constant. The k_{obs} values did not depend on the peptide concentration and were $0.0027 \pm 0.0005\ \mathrm{s}^{-1}$ and $0.0051 \pm 0.0006\ \mathrm{s}^{-1}$ for GSH and α-GSH, respectively. On the other hand, the $[\mathrm{DPPH}^{\bullet}]_{\mathrm{span}}$ values were equal to the peptide concentration in the reaction mixture. Consequently, these DPPH$^{\bullet}$ scavenging reactions had 1:1 stoichiometry. The conclusion was confirmed by other experiments performed at higher peptide concentrations (Fig. 3), where the "span" values were determined experimentally, measuring the concentration of DPPH$^{\bullet}$ at the end of the reaction. The concentration remained constant even if we incubated the reaction mixture during 45 min. The slope of the linear relationship was 0.98 ± 0.05 that was in good agreement with the 1:1 stoichiometry of these reactions. The result was in agreement with earlier data on the reaction of DPPH$^{\bullet}$ with butyl mercaptan and thiophenol in

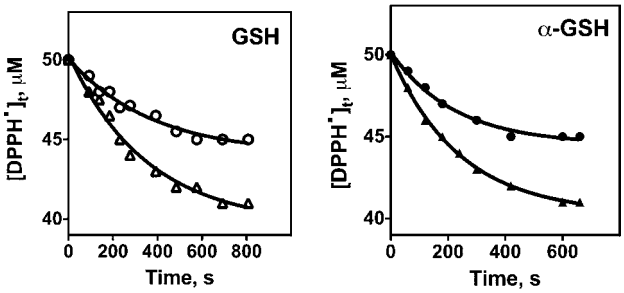


Fig. 2. Reaction of 50 μM DPPH with 5 and 10–15 μM scavenging peptides (GSH left panel and α-GSH right panel).

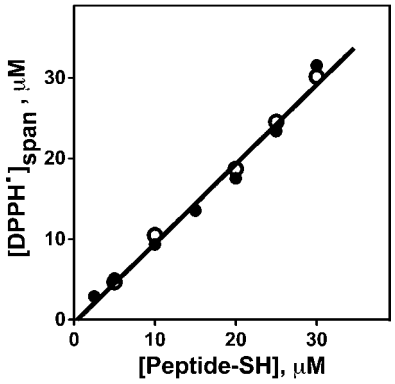


Fig. 3. Consumption of DPPH• in reaction with different amounts of GSH (open symbols) and α-GSH (filled symbols). Slope of this linear plot was 0.98 ± 0.05, confirming the 1:1 stoichiometry of the reaction.

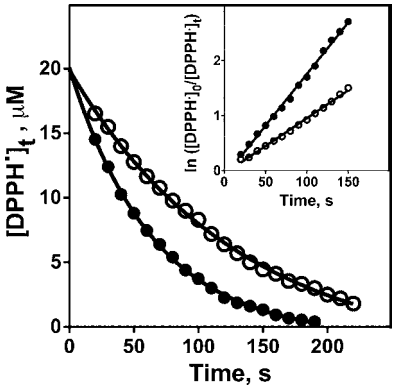


Fig. 5. Reaction of 20 μM DPPH with 500 μM GSH (open symbols) and α-GSH (filled symbols). Inserts present the linear transformations of the data following the rate equation for the first-order reaction.

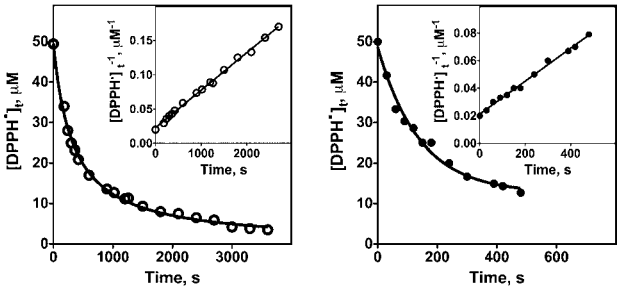


Fig. 4. Reaction of 50 μM DPPH with 50 μM GSH (left panel) and 50 μM α-GSH (right panel). Inserts present the linear transformations of the data following the rate equation for the second-order reaction.

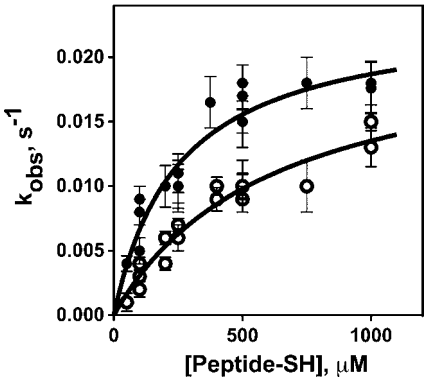


Fig. 6. Dependence of the observed rate constants k_{obs} for the DPPH scavenging reaction upon GSH (open symbols) and α-GSH (filled symbols) concentrations.

diluted solutions of these scavengers [23], and also with the 1:1 stoichiometry of the GSH reaction with this radical (10 mM citrate buffer, pH 3–4, 60% EtOH) reported by Takebayashi et al. [24].

Secondly, we measured kinetics of the radical scavenging at equal peptide and DPPH• concentrations (50 μM) (Fig. 4). Under these conditions, the process followed the rate law for a second-order reaction:

$$\frac{1}{[\mathrm{DPPH^{\bullet}}]_t} = \frac{1}{[\mathrm{DPPH^{\bullet}}]_{span}} + k_{II}t, \quad (3)$$

where $[DPPH^{\bullet}]_t$ and $[DPPH^{\bullet}]_{span}$ are as in Eq. (2) and k_{II} is the second-order rate constant. Here $k_{II} = 55 \pm 4\ M^{-1}\ s^{-1}$ and $k_{II} = 110 \pm 2\ M^{-1}\ s^{-1}$ were obtained for GSH and α-GSH, respectively. Consequently, the kinetic data gave evidence that under these conditions one peptide molecule scavenged one radical following the 1:1 stoichiometry of the process. The same results also indicated that the scavenging reaction was practically irreversible, and the equilibrium (1) should be significantly shifted to the right, either for thermodynamic reasons, or due to the fast removal of the oxidized product (Peptide-S•) from the reaction medium by some fast reactions with the secondary scavengers.

Thirdly, we made kinetic experiments at excess of peptide concentration ranging from 100 μM to 1000 μM over the DPPH• concentration at 20 μM. In these experiments DPPH• was completely consumed, as illustrated in Fig. 5 for the reaction of 500 μM GSH and α-GSH with the radical, and the observed rate constants k_{obs} were calculated by using the exponential rate Eq. (2). However, in this case the k_{obs} values had a different meaning if compared with the rate constants calculated at excess of DPPH•.

The observed rate constants k_{obs} of the DPPH• scavenging reaction were determined at peptide concentrations ranging from 100 μM to 1000 μM. It can be seen in Fig. 6 that the k_{obs} vs peptide concentration plots were not linear within the peptide concentration interval, as predicted for a simple bimolecular reaction, but revealed a clearly hyperbolic dependences for both scavengers. This means that at high concentrations of the scavengers the rate of the reaction became independent of the ligand concentration and approached some limiting value. Strickland et al. [25] have shown that such hyperbolic k_{obs} vs reagent concentration plots give evidence for a two-step reaction mechanism, which involves a fast reversible step of complex formation, followed by a slower conversion of this complex into the reaction products. Accordingly, the hyperbolic k_{obs} vs peptide concentration plots were analyzed by the following equation [25]:

$$k_{obs} = \frac{k[\text{Peptide-SH}]}{K + [\text{Peptide-SH}]}. \quad (4)$$

where K stands for the dissociation constant of the first equilibrium step and k is the rate constant of the following process. As there was no statistically relevant ordinate intercept in the k_{obs} vs peptide concentration plots in Fig. 6, the formation of the reaction product (DPPH-H) was described as an irreversible reaction [25]. Processing of the kinetic data shown in Fig. 6 yielded $k = 0.022 \pm 0.003\ s^{-1}$ and $K = 612 \pm 140$ μM for GSH, and $k = 0.024 \pm 0.003\ s^{-1}$ and $K = 272 \pm 61$ μM for α-GSH. Thus, the kinetic analysis revealed that GSH and α-GSH react with DPPH• through the same mechanism, characterized by the formation of a reversible complex between the reagents prior the radical scavenging takes place. The rate of this scavenging step was similar for both peptides, whereas the

effectiveness of the complex formation, as calculated from the kinetic data, was different.

One and perhaps the simplest kinetic mechanism, which explains the hyperbolic k_{obs} vs scavenger concentration plots, was presented by the following reaction scheme:

$$\mathrm{DPPH^{\bullet} + Peptide\text{-}SH} \overset{K}{\leftrightarrows} \{\mathrm{DPPH^{\bullet}}\cdots\mathrm{Peptide\text{-}SH}\} \overset{k}{\rightarrow} \mathrm{DPPH\text{-}H + Peptide\text{-}S^{\bullet}}, \quad (5)$$

where K stands for the dissociation constant of a reversible (non-covalent) complex of DPPH• with peptide, marked as {DPPH•···Peptide-SH}, and k is the rate constant of the DPPH• scavenging reaction taking place within this complex.

The formation of the reversible complex between DPPH• and GSH, predicted by the kinetic data, was verified by the LC–MS analysis. Samples of the reaction mixture, containing 50 μM of DPPH and 750 μM of the peptide, were taken from the reaction mixture at the beginning of the process and applied to a C_{18} column of a LC–MS equipment. If injected one by one, GSH eluted at >90% of water in the mobile phase, while DPPH• eluted at nearly 100% organic solvent in the mobile phase. However, in the mixture of GSH and DPPH• some of the GSH was eluted at high acetonitrile percentage, demonstrating that the retention time of this peptide was significantly affected in the presence of DPPH• (Fig. 7). This implies the formation of a reversible complex between GSH and DPPH•.

Besides the initial compounds, however, we also observed products of the secondary radical-scavenging reactions. As expected, we found GSH dimer formation (GS–SG) in these runs. However, when the excess of GSH over the radical concentration was used, we were able to discover the formation of an additional product, which had m/z = 699.0 (Fig. 7 insert). This product eluted following the last GSH peak, but before the DPPH• peak, implying its intermediate hydrophobicity. To further characterize the structure of the m/z = 699.0 Da peak, the fragmentation spectra of the novel m/z = 699 peak and the reagents were compared. At lower collision energies (>−40 V), only a few fragments were gained from the 699 Da compound, with masses 523.9, 466.1, 426.1, 379.3, 272.2, 225.8 and 196.1 Da. The two latter fragments were identical to the major peaks in the DPPH• mass spectra, whereas 272.2 was a fragment of GSH (see in [26]). Fig. 8 illustrates the low molecular mass fragments gained at a higher collision energy (−50 V). It was clear from this plot that the m/z = 699 fragment overlaps with DPPH• and GSH fragments. Thus, in parallel with the formation of the reversible complex between DPPH• and GSH, we observed the

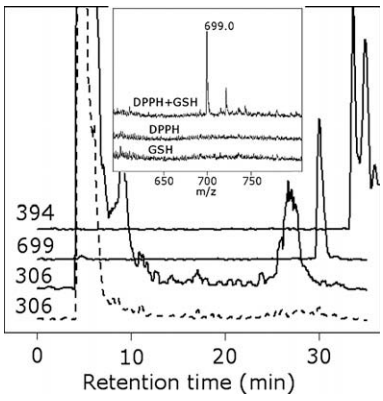


Fig. 7. Overlay of specific ion chromatograms extracted from DPPH and GSH mixture analysis (solid lines) and chromatogram of DPPH-free GSH solution (dashed line). The fragment with m/z = 699 corresponds to the putative DPPH-GSH covalent adduct, m/z = 394 corresponds to DPPH and 306 is the negative ion of GSH. Insert shows 600–800 Da mass range in mass spectra of GSH and DPPH solutions and their mixture.

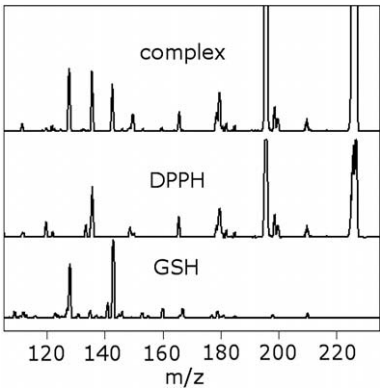


Fig. 8. Fragmentation spectra of GSH (bottom), DPPH (middle) and the proposed covalent DPPH-GSH adduct (upper). The peaks at m/z at 225.8 and 196.1 of the middle panel are capped at ca 30% of their height.

formation of a covalent adduct of the radical and GSH. Most probably, this is a product of recombination of DPPH• with GS•.

Further, the 306m/z peak at 9 min was generated by a dimer formed by GSH molecules, and we have observed this fragment while analyzing the GSH dimer before. It could be an isotopic form of a doubly-charged dimer, a product of partial fragmentation formed during dimer ionization, or both. At 394m/z we observed peaks probably due to the complex formation, and we considered the smallest one to be a noise caused split from the second major peak.

4. Discussion

The results of the present study confirmed that the thiol groups of GSH and α-GSH were responsible for the scavenging activity of these peptides in reaction with the DPPH• radical, and the kinetic mechanism of this process was similar for both antioxidants. At relatively low peptide concentration, one peptide molecule scavenged one radical and therefore the overall process could be described as an irreversible reaction. This, however, cannot be justified proceeding from the thermodynamic data, as the BDE value for the N–H bond of DPPH-H ranges from 333 to 334 kJ/mol [27,28] and these values are lower than the BDE value of 373 kJ/mol for the S–H bond in GSH [16]. Therefore, the apparent irreversibility of the process should be based on efficient secondary scavenging reactions, which quickly remove the Peptide-S• from the reaction medium and drive the overall reaction (1) to the right. There are multiple variants for these secondary reactions, including the recombination of the thiyl radicals, or their fast conjugation with oxygen [29]. However, as we measured the decrease in the DPPH• concentration and the formation of the quenched radical DPPH-H, we were not interested in the nature of these secondary scavenging reactions, especially until the 1:1 stoichiometry of the assay was not affected.

Under the excess of peptide over the DPPH• concentration, the stoichiometry of the scavenging reaction probably changed, as part of the radical combined with Peptide-S•, and we observed the formation of this product in the reaction mixture. Following the LC–MS analyses, the structure of this adduct was DPPH-SG. The formation of covalent adducts between DPPH• and thiyl radicals has been discussed before in the case of the reaction of this radical with thiophenol and butyl mercaptan [23] as well as with 2,4,6-tri-t-butylbenzenerhiol [30]. In all cases the possibility of formation the covalent adduct was connected with high thiol concentrations. Indeed, the same situation was also observed in this study, as we were able to detect the formation of the covalent adduct preferentially at high GSH concentrations. Under these conditions, a signif-

icant part of DPPH• was involved in the formation of the reversible complex with the peptide, as revealed by kinetic data, and confirmed by an HPLC analysis of the reaction mixture. Therefore, we suggested that the formation of the reversible complex might support the reaction of recombination of these two radicals, but was obviously not the necessary requirement of this process.

Kinetic evidence for the formation of the reversible complex between DPPH• and the peptides was obtained from the hyperbolic k_{obs} vs reagent concentration plots shown in Fig. 6 for both GSH and α-GSH. Following the results of the present kinetic analysis, we calculated kinetic parameters K and k for the studied peptides. Comparison of these parameters revealed that the ability of GSH and α-GSH to scavenge DPPH• was similar, as the reaction step of the scavenging process was characterized by the rate constants $k = 0.022 \pm 0.003\ s^{-1}$ and $k = 0.024 \pm 0.003\ s^{-1}$, respectively. In contrast, variation in positioning of the glutamyl residue in the peptide structure changed the stability of the non-covalent complexes, characterized by $K = 0.61$ mM and $K = 0.27$ mM for GSH and α-GSH, respectively. This indicated that the apparently different antioxidant activity of these peptides in model experiments with DPPH• is connected with the step of complex formation. Therefore, direct comparison of the reaction rate at low peptide concentrations did not characterize the genuine reactivity of the corresponding SH groups. It was obviously a somewhat surprising result that may have vast implication on the meaning of the results of this popular assay of antioxidant properties of various compounds.

We modeled non-covalent complexes between DPPH• and the scavenger peptides by docking calculations (Fig. 9). These calculations demonstrated, indeed, the possibility of a more intimate complex structure of α-GSH with DPPH• if compared with the GSH complex. This is in line with the results of the present kinetic study, where the complex of DPPH• with α-GSH had higher stability in comparison with GSH.

It is evident that under the conditions [Peptide-SH] < K the hyperbolic Eq. (4) transforms into a more simple relationship

$$k_{obs} = \frac{k}{K}[\text{Peptide-SH}], \tag{6}$$

where k/K has the meaning of the second-order rate constant. Consequently, at low concentration of ligands the process should behave as the second-order reaction between DPPH• and GSH or α-GSH. We observed this experimentally, when we studied the reaction at low peptide concentration interval. Therefore, the rate constants k/K in Eq. (6) and k_{II} in Eq. (3) should have the same physical meaning, and certainly the same value. On the other hand, however, this also means that we do not have a genuine bimolecular reaction and the scavenged radical DPPH-H should form "inside" the complex even at low concentration of reagents.

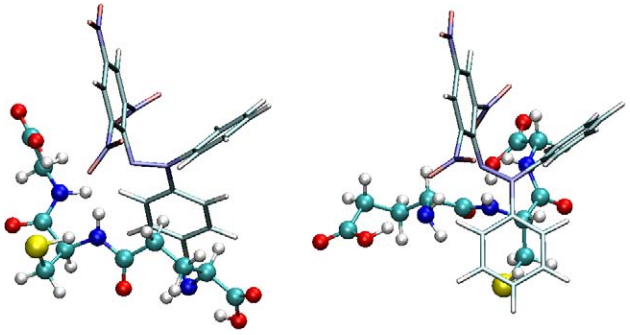


Fig. 9. Comparison of structures of non-covalent complexes between DPPH (rod model) and peptides (ball-stick models) for GSH (left) and for α-GSH (right). The picryl moiety of DPPH was fixed for clarity in the same position in both complexes, and different positioning of the two phenyl groups as well as peptides can be observed.

Sometimes this important aspect of the two-step reaction mechanism (5) is not thoroughly understood, and as proposed by our referee, is explained by two competing mechanisms: one involving the simple bimolecular reaction, and the other involving the preliminary formation of the reversible complex. Certainly, such a combination of different mechanisms is possible. However, under these conditions the reaction rate never levels off to a "plateau" at high reagent concentrations. This conclusion follows from general considerations of chemical kinetics, and has been thoroughly analyzed by Strickland et al. [25]. Although the distinction between the possibilities is not critical for the interpretation of kinetic data in this work, the formation of the reversible complex might affect the interpretation of the kinetic data used for analysis and modeling of the mechanisms of the hydrogen transfer reaction in general. Interestingly, Fori and Daquino have briefly discussed this situation in the case of the reaction of DPPH• with phenols [27].

The mechanism of the radical scavenging process described herein identified several limitations to the application of DPPH• in the assay.

Firstly, the time course of the process was dependent upon the ratio of the concentrations of the probe and the scavenger. If DPPH• is present in excess, the reaction follows the first-order kinetics and so the same assay time can be used for different scavenger concentrations. However, if the concentrations of the probe and the scavenger became close, the reaction followed the second-order rate law and the reaction time was increased. On the other hand, 1:1 stoichiometry of the reaction still simplifies the analysis, as one thiol group scavenges one radical, if the following secondary oxidation processes remove the peptide radical before it reacts with another DPPH• radical.

Secondly, this study has revealed that the formation of the non-covalent complexes between DPPH• and GSH or α-GSH molecules, characterized by the dissociation constants $K = 0.61$ mM and $K = 0.27$ mM, respectively, were responsible for the apparently different radical scavenging effectiveness of the two peptides, while the genuine reactivity of the thiol groups was practically the same. Therefore, based on our data, it would be impossible to draw significant conclusions about antioxidant properties of the peptides in biological systems, where their physiological activity should also be governed by different binding effectiveness with their target sites. However, the specificity pattern could be much more complex in the biological system.

Acknowledgments

The present investigation was supported by the Estonian Ministry of Education and Research (Grant SF0180064s08), the Estonian Science Foundation (Grants 6503, 7856 and 7494) and by the European Regional Development Fund.

References

[1] H. Sies, Naturwissenschaften 76 (1989) 57–64.
[2] M.S. Fernandez-Panchon, D. Villano, A.M. Troncoso, M.C. Garcia-Parrilla, Crit. Rev. Food Sci. Nutr. 48 (2008) 649–671.
[3] N. Masubuchi, C. Makino, N. Murayama, Chem. Res. Toxicol. 20 (2007) 455–464.
[4] M. Zilmer, U. Soomets, A. Rehema, Ü. Langel, Drug Des. Rev. – Online 2 (2005) 121–127.
[5] K. Ehrlich, S. Viirlaid, R. Mahlapuu, K. Saar, T. Kullisaar, M. Zilmer, Ü. Langel, Free Radical Res. 41 (2007) 779–787.
[6] S. Goldschmidt, K. Renn, Chem. Ber. 55 (1922) 628–643.
[7] M.S. Blois, Nature (London) 181 (1958) 1199–1200.
[8] L.M. Magalhães, M.A. Segundo, S. Reis, J.L. Lima, Anal. Chim. Acta 631 (2008) 1–19.
[9] D. Huang, B. Ou, R.L. Prior, J. Agri. Food Chem. 53 (2005) 1841–1856.
[10] K.E. Russell, J. Phys. Chem. 58 (1954) 437–439.
[11] M.I. de Heer, P. Mulder, H.-G. Korth, K.U. Ingold, J. Lusztyk, J. Am. Chem. Soc. 122 (2000) 2355–2360.
[12] J.M. Mayer, I.J. Rhile, Biochim. Biopys. Acta 1655 (2004) 51–58.

[13] G. Litwinenko, K.U. Ingold, J. Org. Chem. 70 (2005) 8982–8990.
[14] E. Baciocchi, A. Calcagni, O. Lanzalunga, J. Org. Chem. 73 (2008) 4110–4115.
[15] M.C. Foti, C. Daquino, I.D. Mackie, G.A. Dilabio, K.U. Ingold, J. Org. Chem. 73 (2008) 9270–9282.
[16] A. Rauk, D. Yu, D.A. Armstrong, J. Am. Chem. Soc. 120 (1998) 8848–8855.
[17] P.C. do Couto, B.J.C. Cabral, J.A.M. Simões, Chem. Phys. Lett. 421 (2006) 504–507.
[18] U. Soomets, M. Zilmer, Ü. Langel, Meth. Mol. Biol. 298 (2005) 241–257.
[19] R.E. Benesch, R. Benesh, J. Am. Chem. Soc. 77 (1955) 5877–5881.
[20] T. Kortemme, T.E. Creighton, J. Mol. Biol. 253 (1995) 799–812.
[21] J. Bjerrum, G. Schwarzenbach, L.G. Sillen, Stability Constants, Part I, Organic Ligands, The Chemical Society, London, 1957.
[22] M.A. Rosei, Cell Mol. Life Sci. 35 (1979) 1178–1179.
[23] A.G. Brook, R.J. Anderson, J.T. Van Patot, Can. J. Chem. 36 (1958) 159–166.
[24] J. Takebayashi, A. Tai, E. Gohda, I. Yamamoto, Biol. Pharm. Bull. 4 (2006) 766–771.
[25] S. Strickland, G. Palmer, V. Massey, J. Biol. Chem. 250 (1975) 4048–4052.
[26] C.M. Dieckhaus, C.L. Fernández-Metzler, R. King, P.H. Krolikowski, T.A. Baillie, Chem. Res. Toxicol. 4 (2005) 630–638.
[27] M.C. Foti, C. Daquino, Chem. Commun. (Camb) 39 (2006) 3252–3254.
[28] L.R. Mahoney, G.D. Mendenhall, K.U. Ingold, J. Am. Chem. Soc. 95 (1973) 8610–8614.
[29] P. Eyer, Environ. Health Perspect. 102 (S6) (1994) 123–132.
[30] J. Flood, K.E. Russell, Can. J. Chem. 53 (1975) 1123–1128.