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Products of Cu(II)-catalyzed oxidation in the presence of hydrogen peroxide of the 1–10, 1–16 fragments of human and mouse β-amyloid peptide

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Abstract

The interactions of proteins with reactive oxygen species (ROS) may result in covalent modifications of amino acid residues in proteins, formation of protein–protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation. In an attempt to elucidate the products of the metal-catalyzed oxidation of the human (**H**) and mouse (**M**) (1–10**H**), (1–10**M**), (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide, the high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) methods and Cu(II)/H_2O_2 as a model oxidizing system were employed. Peptide solution (0.50 mM) was incubated at 37 °C for 24 h with metal:peptide:H_2O_2 molar ratio 1:1:1 for the (1–16**H**), (1–16**M**) fragments, and 1:1:2 for the (1–10**H**), (1–10**M**) peptides in phosphate buffer, pH 7.4. Oxidation targets for all peptide studied are the histidine residues coordinated to the metal ions. For the (1–16**H**) peptide are likely His^{13} and/or His^{14}, and for the (1–16**M**) fragment His^6 and/or His^{14}, which are converted to 2-oxo-His. Metal-binding residue, the aspartic acid (D^1) undergoes the oxidative decarboxylation and deamination to pyruvate. The cleavages of the peptide bonds by either the diamide or α-amidation pathways were also observed.
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Keywords: β-Amyloid peptide; Metal-catalyzed oxidation; Copper(II) complexes; Products of oxidation; MALDI-TOF MS

1. Introduction

The oxidative modification of proteins by reactive oxygen species (ROS) is implicated in the etiology or progression of a number physiological disorders and diseases. The ROS may be formed by any one of a large number of physiological and nonphysiological processes. Among others these include the formation of hydrogen peroxide by endogenous oxidases and the conversion of H_2O_2 to hydroxyl radical (OH·) by metal-catalyzed oxidation (MCO) systems [1]. The principle source of OH· under normal physiological conditions is via the metal-catalyzed cleavage of H_2O_2. In the presence of the low concentrations of the red-ox active transition metals (Fe^{2+}, Cu^{2+}) and H_2O_2, as might occur under physiological conditions, the modification of proteins is restricted to amino acids residues at metal binding sites on the protein [2–4]. A role of protein oxidation in the etiology and/or progression of the disease is indicated by the fact that the level of oxidized protein in tissues of subjects having the disease is higher than that of normal subjects. The elevated levels of oxidized protein can be measured by the carbonyl content [5].

Alzheimer's disease (AD) is a common neurodegenerative disorder that is accompanied by loss of memory, speech, cognitive function, and behavioral activity. Age-dependent progression of the disease is accompanied by

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a loss of synapses and neurons, overproduction of the amyloid precursor protein (APP), increase in the intracellular Ca^{2+}, altered phosphorylation of brain enzymes, loss or abnormal distribution of several enzymes, and also development of neuritic plaques, consisting of aggregated β-amyloid protein fragments and various proteins, and of neurofibrillary tangles, consisting of the tau protein and other cytoskeletal proteins [6,7]. The involvement of ROS in AD is indicated by the observation that the levels of nitrotyrosine [8], protein carbonyl [5,9,10], and malondialdehyde [11] are elevated in patients with AD. Abnormal processing of APP may be involved in the development of neuritic plaques since the β-amyloid peptide (Aβ), which is a 40–43 amino acid fragment of APP, is present in neuritic plaques in an aggregated form and has been shown to be cytotoxic to cultured neurons [12]. A role of free radicals in AD pathology is supported also by results of in vitro studies with a synthetic 40-amino acid peptide (Aβ1–40) possessing the same amino acid sequence as endogeneous Aβ. When added to aqueous solutions, this peptide undergoes slow fragmentation and generation of peptide radicals [13]. A synthetic peptide Aβ25–35, corresponding in sequence with the C-terminal portion of Aβ1–40, promotes production of free radicals and ROS, initiates synaptosomal lipoprotein oxidation, and causes inactivation of glutamine synthetase and creatine kinase [13–15]. The hypothesis that AD is a disease associated with oxidative stress [16–18] is consistent with the studies that demonstrate that a free radical scavengers like vitamin E [19] or melatonin [20] could mitigate the damage free radical-mediated to a considerable extent. A recent report demonstrated beneficial effects in AD patients of a high-dose vitamin E treatment [21].

The large body of evidence indicates that the homeostases of zinc, copper and iron, and their respective binding proteins, are significantly altered in the AD brain and that these metals are present in the neuropathology [22]. It was recently reported that Zn/Cu-selective chelators markedly enhance the resolubilization of Aβ deposits from post-mortem AD brain samples, supporting the possibility that Cu and Zn ions play a significant role in assembling these deposits [23]. The metals may play more of a role than assembling Aβ alone. It was reported that Aβ is redox active, and reduces Cu^{2+} or Fe^{3+} and then produces hydrogen peroxide by electron transfer to O_2 [24], leading to the hypothesis that Aβ–Cu^{2+} interaction is a source for oxidative stress. Although Cu^{2+} is essential for life and the function of numerous enzymes of interest to neurobiology, such as tyrosinase, ceruloplasmin, cytochrome *c* oxidase, and dopamine hydroxylase, free or incorrectly bound Cu^{2+} can also catalyze the generation of the most damaging radicals, such as the hydroxyl radical ($OH^{\cdot}$) [22]. It is suggested that the copper(II) ions may play the important role in AD and other neurodegenerative diseases [25,26]. In vitro experiments have confirmed that Aβ is indeed sensitive to metal catalyzed oxidation [27]. Aβ peptide contains side chains of amino acid residues (Tyr^{10}, Met^{35}, His^{6}, His^{13} and His^{14}) that are susceptible to metal-catalyzed oxidation. The solid-state NMR experiments showed that copper(II) ions are reduced to copper(I) and this reduction is coupled with the oxidation of Met^{35} of Aβ1–39 to give the sulfoxide [28]. It was recently reported that for the systems Cu(II)/Aβ1–28, Aβ1–40, Aβ1–42 in the presence of H_2O_2 the formation of dityrosine-cross-linked was observed [29]. It was also demonstrated that the ascorbate-dependent copper(II)-catalyzed oxidation of Aβ1–28 and Aβ1–40 primarily targets the residues His^{13} and His^{14} in a site specific process, but that the metal-catalyzed oxidation of Tyr^{10} and (for Aβ1–40) Met^{35} is negligible [30].

The studies of the copper(II)-catalyzed oxidation process of the (1–10) and (1–16) fragments of human and mouse β-amyloid peptide in the presence of hydrogen peroxide at pH 7.4 is described in this paper. The oxidation products of these peptides have been identified and characterized by reversed-phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) methods. The stability constants and the metal-binding sites for the complexes formed in a function of pH of the (1–10**H**) DAEF*R*HDSG*Y*-NH_2, (1–10**M**) DAEF*G*HDSG*F*-NH_2, (1–16**H**) DAEF*R*-HDSG*Y*EV*H*HQK-NH_2, (1–16**M**) DAEF*G*HDSG*F*-EV*R*HQK-NH_2 fragments of human (**H**) and mouse (**M**) β-amyloid peptide were determined [31,32]. β-Amyloid deposition does not occur in rat and mouse cerebral tissue [33]. The rat amyloid sequence is similar to human, but contains three amino acid substitutions (Arg → Gly, Tyr → Phe, His → Arg at positions 5, 10 and 13, respectively), which influence its coordination properties [31,32]. Because the mouse and human fragments of β-amyloid peptide form the complexes with different binding sites of copper(II) ions at physiological pH, and the metal-catalyzed oxidation is viewed as a caged process [1,34] in which the active oxygen species preferentially reacts with functional groups at the metal binding site, it may expect the different products of metal-catalyzed oxidation. This study was performed in order to examine the difference of the products of copper(II)-catalyzed oxidation of human and mouse fragments at physiological (7.4) pH in the presence of the hydrogen peroxide.

2. Experimental

2.1. Materials

All measurements in 0.1 M phosphate buffer (pH 7.4) were performed. Deionized and triply distilled water was

used, and phosphate buffer was treated with Chelex 100 resin (sodium form, Sigma-Aldrich) to remove trace metals. Hydrogen peroxide was obtained from Fluka (Perhydrol, 30%) and 0.10 M stock solution in phosphate buffer (pH 7.4) was prepared. $Cu(NO_3)_2 \cdot 3H_2O$ (Merck) was used as the source of the metal ions. The methods of synthesis and purification of the (1–10**H**), (1–10**M**), (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide were described in previous reports [31,35].

2.2. Spectroscopic measurements

EPR spectra were recorded on a Brucker ESP 300E spectrometer at X-band frequency (9.3 GHz) at 120 K. Absorption spectra were recorded on a Beckman DU 650 spectrophotometer. Circular dichroism (CD) spectra were obtained on a JASCO J-715 spectropolarimeter in 750–250 nm range. The metal concentration in all spectroscopic measurements was adjusted to 1×10^{-3} M and the metal-to-ligand molar ratio was 1:1.

2.3. Oxidation of the (1–10*H*), (1–10*M*), (1–16*H*) and (1–16*M*) fragments of β-amyloid peptide

Copper(II)-catalyzed oxidation of peptides in the presence of hydrogen peroxide was monitored by analytical RP-HPLC on Waters 600 apparatus with Waters 996 PDA Detector using C_8 Kromasil column (4.6 mm × 250 mm, 5 μm) by using a linear gradient of 0–80% acetonitryle in 0.1% aqueous TFA as a mobile phase. For reaction mixtures containing 5×10^{-4} M of complex (1:1 metal to ligand molar ratio) and 1:10 complex to H_2O_2 molar ratio, the peptides were completely fragmented after 3 h of incubation of samples at 37 °C (excess of hydrogen peroxide). Therefore, lower complex-H_2O_2 molar ratio was employed. A reaction mixture (5 cm^3) containing 5×10^{-4} M of peptide and metal-to-ligand molar ratio 1:1 in 0.1 M phosphate buffer (pH 7.4) was incubated at 37 °C for 24 h in the presence of hydrogen peroxide at metal-to-H_2O_2 molar ratio 1:2 for (1–10**H**), (1–10**M**) and 1:1 for (1–16**H**), (1–16**M**). The reaction was started by the addition of hydrogen peroxide, which was freshly prepared. For these reaction mixtures after 6, 10 and 24 h of incubation at 37 °C the chromatograms indicated no significant difference (the shape, intensity of peaks and retention times) suggesting the end of reaction after 6 h. After incubation, the reaction was stopped by addition of nitric acid to a final pH 2–3. For desalted of the reaction mixture a Sep-Pak C-18 cartridge (solid phase extraction, Baker) was used. The column first was equilibrated with 0.1% TFA (trifluoroacetic acid) in water and then the reaction mixture was applied. The inorganic salts were removed by this solution. The peptides were recovered from the cartridge by washing with 80% ACN (acetonitrile) in 0.1% aqueous TFA. After desalted of reaction mixtures the samples were analyzed by RP-HPLC. There was no significant difference between chromatograms (shape, intensity of peaks and retention times) before and after removal of the salts by gel filtration. The eluent containing the products of oxidation was evaporated in vacuo and lyophilizated.

2.4. Isolation of oxidation products

The oxidized mixture was dissolved in 0.1% TFA water solution and separated by RP-HPLC semipreparative C_8 Kromasil column (20 mm × 250 mm, 5 μm). The separation of mixture was performed on Knauer HPLC Instrument (K-1001 2-pomp system) equipped with multivariable K-2500 UV-detector. HPLC was performed with the buffer system 0.1% TFA in water (buffer A) and 0.1% TFA in 80% acetonitrile (buffer B) using linear gradient of buffer B. The flow rate of 10 cm^3/min was maintained. The elution of peptides was monitored by UV absorbance 223 nm.

The major oxidized products were collected and analyzed by analytical RP-HPLC on Waters 600 apparatus with Waters 996 PDA Detector using C_8 Kromasil column (4.6 mm × 250 mm, 5 μm) by using a linear gradient of 0–80% acetonitryle in 0.1% aqueous TFA as a mobile phase during 60 min at a flow rate of 1 cm^3/min. Collected fractions at the same retention time were drier by Speed-Vac centifugal evaporator and analysed by MALDI-TOF MS spectrometer.

2.5. Mass spectrometry

The composition of products of peptide oxidation separated by HPLC method was established by means of MALDI-TOF MS. The analysis was performed on a Bruker Biflex III MALDI-TOF mass spectrometer with a nitrogen laser with 337 nm wavelength and 3 ns pulse. In a typical experiment, peptide sample was mixed with of matrix solution consisting of α-cyjano-4-hydroxycinnamic acid (50% ACN, 49% H_2O, 1% TFA), applied into ionization chamber.

3. Results and discussion

The metal ion-catalyzed oxidation (MCO) of biomolecules (lipids, nucleic acids, proteins) is thought to involve the formation of OH· by means of the ''Fenton reaction'' when iron is the metal ion. The reaction involves reduction of Fe^{3+} or Cu^{2+} by a suitable electron donor such as NADH, NADPH, ascorbate, or mercaptane. Fe^{2+} and Cu^{+} ions bound to specific metal-binding sites on proteins react with H_2O_2 to generate OH· [36,37], which immediately oxidizes neighboring amino acid residues. Above mentioned free radical generating systems can be replaced by metal(Cu^{2+})/

H_2O_2 system [38–42]. For the Cu^{2+} complexes with peptides the generation of hydroxyl radicals was observed [41], and the following reactions have been proposed for this process [39,41,43].

$$\text{peptide-}Cu^{2+} + H_2O_2 \rightarrow \text{peptide-}Cu^{+} + O_2^{-\cdot} + 2H^{+}$$

$$\text{peptide-}Cu^{2+} + O_2^{-\cdot} \rightarrow \text{peptide-}Cu^{+} + O_2$$

$$\text{peptide-}Cu^{+} + H_2O_2 \rightarrow \text{peptide-}Cu^{2+} + OH^{\cdot} + OH^{-}$$

It is assumed that H_2O_2 reduces peptide-Cu^{2+} to peptide-Cu^{+}, this is followed by the reaction of Cu^{+} with H_2O_2 to give $OH^{\cdot}$ [44,45].

MCO of proteins is mainly a site-specific process in which only one or a few amino acids at metal-binding sites on the protein are preferentially oxidized [1,40,42,46,47]. The oxidation of proteins by MCO can lead to oxidation of amino acid residue side chains, cleavage of peptide bonds and formation of covalent protein–protein cross-linked derivatives. Results of the studies indicate that the most common pathway for the oxidation of simple aliphatic amino acids involves the hydroxyl radical-mediated abstraction of hydrogen atom to form a carbon-centered radical at the α-position of the amino acid or amino acid residue in polypeptide chain [1,48]. Cleavage of the peptide bond (fragmentation of the peptide) may undergo by either the diamide or α-amidation pathways (Fig. 1) [37]. Upon cleavage by the diamide pathway, the peptide fragment derived from the N-terminal portion of the protein possesses a diamide structure at the C-terminal end, whereas the peptide derived from the C-terminal portion of the protein possesses an isocyanate structure at the N-terminal end. The hydrolysis reaction of isocyanate follows essentially instantaneously (Fig. 1) [49]. In contrast, upon cleavage by the α-amidation pathway, the peptide fragment obtained from the N-terminal portion of the protein possesses an amide group at the C-terminal end, whereas the C-terminal portion of the protein exists as an N-α-ketoacyl derivative.

The spectroscopic data for the copper(II) complexes of the (1–10**H**), (1–10**M**), (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide in 0.1 M phosphate buffer at pH 7.4 are summarized in Table 1. These results suggest that the coordination mode of these fragments to copper(II) ions is similar to those obtained in water solution at pH 7.4 [31,32] except the (1–16**M**) fragment. The parameters of CD spectra for the (1–16**M**) peptide are comparable to that of the (1–10**M**) fragment suggesting rather the $\{NH_2, COO^{-}, N^{-}, N^{6}_{Im}\}$ than $\{NH_2, COO^{-}, N^{-}, N^{14}_{Im}\}$ binding mode to the metal ion. The stability constants for both complexes are comparable to each other [31,32] and the equilibrium of these complexes in solution may be proposed.

Fig. 2 presents the chromatograms of the (1–16**H**) peptide incubated with copper(II) alone, H_2O_2 only and with Cu(II)/H_2O_2 at 37 °C. For all peptides studied after 24 h incubation when Cu(II) ions plus H_2O_2 were used then the lower molecular fragments of the peptides were observed. Copper(II) complexes and H_2O_2 alone are not able to damage peptides and it is clear that both the Cu(II) complexes and H_2O_2 are necessary for the induction of peptides damage. Copper ion can catalyze a Fenton-like reaction to produce $OH^{\cdot}$ radical from H_2O_2 [34]. Although, the identification of these radicals was not the subject of this work, it may be supposed that the oxidation and fragmentation of peptides induced by H_2O_2 is due to hydroxyl radicals formed by a copper-dependent Fenton-like reaction [41].

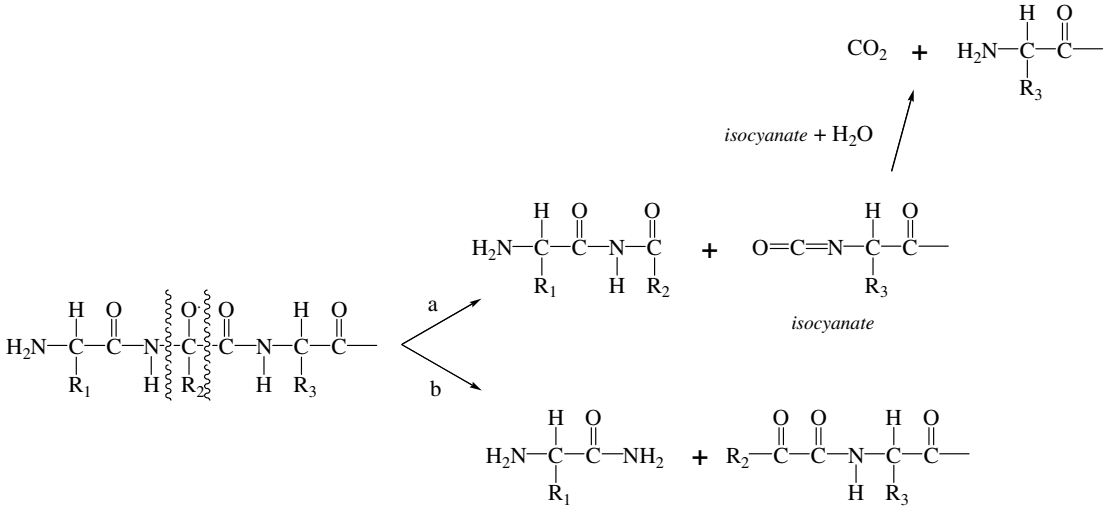

Fig. 1. Peptide bond cleavage by the diamide (a) and α-amidation (b) pathways.

Table 1
Spectroscopic data for copper(II) complexes of (1–10**H**), (1–10**M**), (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide in 0.1 M phosphate buffer (pH 7.4)

Ligand/species	UV–Vis		CD		EPR	
	λ (nm)	ε ($M^{-1}\,cm^{-1}$)	λ (nm)	$\Delta\varepsilon$ ($M^{-1}\,cm^{-1}$)	A_{II} (G)	g_{II}
1–10**H**	619[a]	139	592[a]	−0.290	150	2.227
			317[b]	+0.360		
1–10**H**[e] {NH_2, N^-, CO, N_{Im}}	615[a]	120	590[a]	−0.494	154	2.229
			316[b]	+0.630		
1–10**M**	613[a]	129	672[a]	−0.480	150	2.229
			528[a]	−0.052		
			309[b]	+0.397		
1–10**M**[e] {NH_2, COO^-, N^-, N_{Im}}	614[a]	100	669[a]	−0.793	156	2.228
			532[a]	−0.090		
			310[b]	+0.532		
1–16**H**	615[a]	128			171	2.267
1–16**H**[f] {NH_2, COO^-, $2N_{Im}$}	615[a]	120			175	2.262
1–16**M**	627[a]	132	673[a]	−0.343	150	2.226
			535[a]	−0.056		
			311[b]	+0.264		
1–16**M**[f] {NH_2, COO^-, N^-, N_{Im}}	613[a]	113	677[a]	−0.331	157	2.229
			542[a]	+0.263		
			350[c]	−0.384		
			307sh[b]	+0.485		
			276[d]	+1.775		

The copper(II) complexes formed at pH 7.4 in aqueous solution for these fragments for comparison are also given.
[a] d–d transition.
[b] N^-(amide) → Cu(II) charge transfer transition.
[c] N_{Im} → Cu(II) charge transfer transition.
[d] $N_{Im}\pi_2$ → Cu(II) and NH_2 → Cu(II) charge transfer transitions.
[e] Ref. [29].
[f] Ref. [30].

*3.1. Identification of the copper(II)-catalyzed oxidation products of the (1–10**H**) and (1–10**M**) fragments of β-amyloid peptide*

The spectroscopic data for the Cu(II)–(1–10**H**) system in phosphate buffer at pH 7.4 clearly indicate the formation of the 3N complex with the {NH_2, N^-, CO, N_{Im}} coordination mode (Table 1). According to the proposition that the metal ion catalyzed reactions are "caged" processes in which most of the oxygen radicals produced are not released in solution but react directly with amino acid residues in the complex [1,34,48], the modifications of the His and N-terminal aspartic acid residues are expected. An ion at m/z 1211.8 Da appeared in the HPLC fraction eluted at retention time 19.65–19.88 min (Table 2, Fig. 3) is 16 Da heavier than that of nonoxidized peptide (1195.8 Da). It may correspond to the peptide containing the 2-oxohistidine in the sixth position of amino acid sequence. 2-oxohistidine [1,40, 50,51], aspartic acid and asparagine [1,52,53] as oxidation products of histidine were detected. Little is known about the oxidation products of glutamic and aspartic acids, though it is likely that alcohols and carbonyl-containing materials are formed [49]. The oxalic acid and pyruvate adducts as the oxidation products of glutamic acid were observed [49], while the oxidative decarboxylation and deamination of aspartic acid to pyruvate was detected [54]. A peak occurring at m/z 1150.6 Da in the HPLC fractions eluting at 22.62–23.25 min (Table 2) may correspond to the peptide containing pyruvate at N-terminal part of the peptide after oxidative decarboxylation and deamination of the aspartic acid (D^1) of the (1–10**H**) fragment. The A^2–Y^{10} and E^3–Y^{10} fragments formed by cleavage of the D^1–A^2 and A^2–E^3 peptide bonds by diamide pathway were observed (Table 2). MALDI-TOF mass spectrometry of the HPLC fraction eluted at 19.65–19.88 min yielded masses of 1080.7 and 1007.6 Da supporting the formation of the A^2–Y^{10} and E^3–Y^{10} fragments, respectively (Table 2, Fig. 3). The others degradation products were also observed, and the modifications of the (1–10**H**) peptide in copper(II)-catalyzed oxidation in the presence of hydrogen peroxide in Table 2 are proposed.

In phosphate buffer (pH 7.4) the (1–10**M**) fragment of β-amyloid peptide forms the 3N complex with the coordination of the metal ions by the amine nitrogen and carboxylate oxygen atoms of the aspartic acid residue (D^1), and amide and imidazole nitrogen atoms of the histidine residue (H^6, Table 1) [31]. MALDI-TOF mass spectrometry of the HPLC fraction eluting at 9.84–10.73 min (Table 3, Fig. 4) revealed a peak representing the mass of oxidized peptide containing 2-oxohistidine

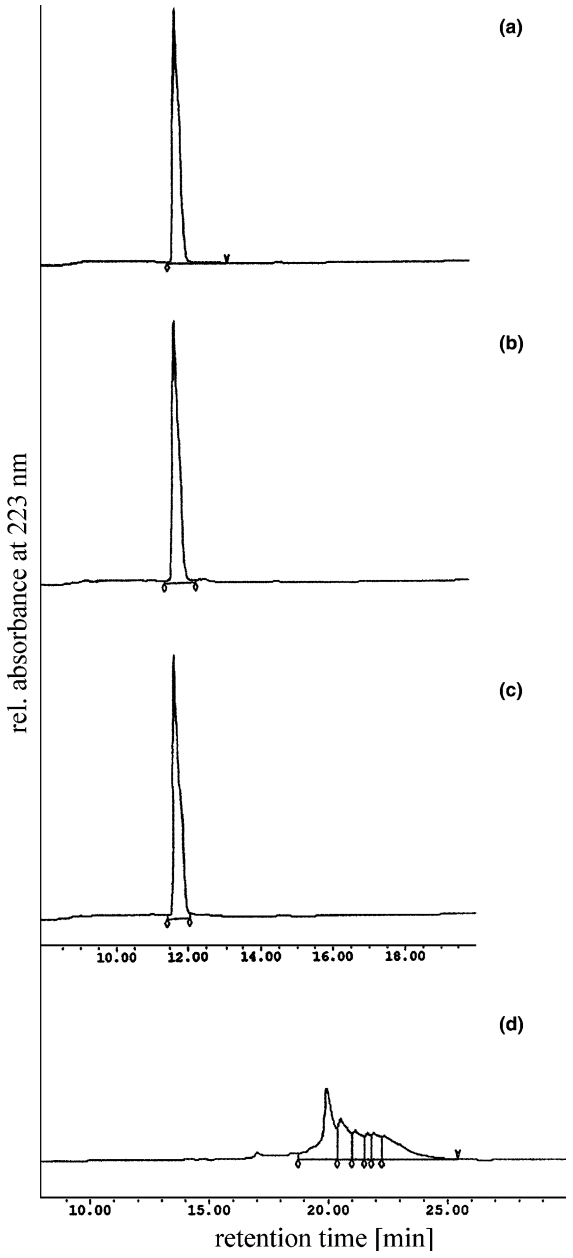

Fig. 2. Chromatograms of the (1–16**H**) peptide after 24 h incubation at 37 °C for the peptide alone (a), peptide with copper(II) (1:1 molar ratio) (b), peptide with H_2O_2 (1:1 molar ratio) (c) and peptide with copper(II) and H_2O_2 (1:1:1 molar ratio) (d). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–16**H**) peptide, Cu(II) and H_2O_2, 0.50 mM. Incubation at 37 °C.

(1096.4 Da). The oxidative decarboxylation and deamination of the aspartic acid residue (D^1) coordinated to copper(II) ions were also observed. The mass of 1035.4 Da observed for HPLC fraction eluting between 15.50 and 15.70 min may be assigned to the peptide with modification of the D^1 residue to pyruvate (Table 3). Moreover, the fragment of the (1–10**M**) peptide formed by cleavage of the D^1–A^2 peptide bond by α-amidation pathway with oxidized H^6 residue was detected. A peak at m/z 979.4 Da in the HPLC fraction at 14.57 min is observed (Table 3) and this mass may correspond to that of the A^2–F^{10} fragment. Although, the coordination of the amide nitrogen atom of the histidine residue (H^6) was proposed [31], under our experimental conditions the fragmentations by cleavage of peptide bonds the G^5–H^6 by α-amidation and/or H^6–D^7 by diamide pathways were not detected. The others metal-catalyzed oxidation products in the presence of hydrogen peroxide were also formed, and proposed modifications of the (1–10**M**) peptide in Table 3 are presented.

*3.2. Identification of the copper(II)-catalyzed oxidation products of the (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide*

According to the spectroscopic data (Table 1) the (1–16**H**) fragment of β-amyloid peptide forms with copper(II) ions, in phosphate buffer at pH 7.4, the 3N $\{NH_2, COO^-, 2N_{Im}\}$ complex [32]. The metal ion is coordinated by the nitrogen atom of amine group and oxygen atom of carboxylate group of aspartic acid residue (D^1) and imidazole nitrogen of two histidine residues (likely H^{13} and H^{14}). The reaction of H_2O_2 with Cu(II)–(1–16**H**) complex may involve site-specific modifications of peptide dictated by reaction at the active copper-binding site. MALDI-TOF MS revealed that HPLC fractions eluting between 10.56 and 12.38 min (Table 4) contained the peptide with one and two 2-oxohistidine residues corresponding to a mass of 1969.3–1970.7 and 1985.2–1986.7 Da compared to the nonoxidized peptide with a mass of 1953.4–1954.9 Da (Fig. 5). The range of the observed mass for the same molecular ion may result from mass accuracy of the instrument used. The peptide containing three 2-oxohistidine residues was not detected supporting the involvement only two imidazole nitrogens, likely of His^{13} and His^{14} residues, in coordination of the metal ion. Moreover, the fragmentations of the peptide by the cleavage of the V^{12}–H^{13} and H^{13}–H^{14} peptide bonds by α-amidation pathway were observed. MALDI-TOF MS showed that the corresponding HPLC fractions eluting between 10.86 and 11.94 min contained ions the molecular masses of 1424.3–1424.6 and 1562.3–1562.4 Da (Table 4) which could be assigned to D^1–V^{12} and D^1–H^{13} fragments, respectively. Ascorbate/Cu(II)-induced metal-catalyzed oxidation of the (1–16**H**), (1–28**H**) and (1–40**H**) revealed that initial oxidation targets are His^{13} and His^{14}, which are converted to 2-oxo-His [30]. The aspartic acid (D^1) residue coordinated to copper(II) ions may undergo the oxidative decarboxylation and deamination to pyruvate [48,53]. The MALDI-TOF MS spectra for the HPLC fraction eluting at 12.38 min (Table 4, Fig. 5) showed the existence of species of molecular mass 1911.1 Da which can correspond to the A^2–K^{16} peptide containing the N-terminal pyruvate. In MS spectra the fragments with molecular masses of the

Table 2
Products of oxidized (1–10**H**) fragment (DAEF*R*HDSG*Y*-NH_2) analyzed by HPLC and theirs observed molecular weights in MALDI-TOF MS spectra

Determination of peptide modification (peptide fragment)	MW_{calcd}	t_R (min), MW_{obsd}			
		19.65–19.88	20.73–20.96	22.03–22.50	22.62–23.25
M(1–10**H**)	1195.2	1195.8			
Oxidation of $H^6 \rightarrow$ 2-oxo H^6	1211.2	1211.8			
Decarboxylation and deamination of D^1 to pyruvate	1150.2				1150.6
Cleavage of D^1–A^2 by α-amidation pathway, (A^2–Y^{10})	1079.1			1079.6	
Cleavage of A^2–E^3 by diamide pathway, (E^3–Y^{10})	1009.0	1007.6			
Cleavage of D^1–A^2 by α-amidation pathway, oxidation of $H^6 \rightarrow$ Asp, (A^2–Y^{10})	1057.0				1057.5
Cleavage of D^1–A^2 by diamide pathway, (A^2–Y^{10})	1080.1	1080.7			
Deamination of NH_2–D^1	1194.2		1193.7		
Deamination of NH_2–D^1, oxidation of $H^6 \rightarrow$ 2-oxo H^6	1210.2		1209.7		

t_R – retention time, MW_{calcd} – calculated molecular weight.

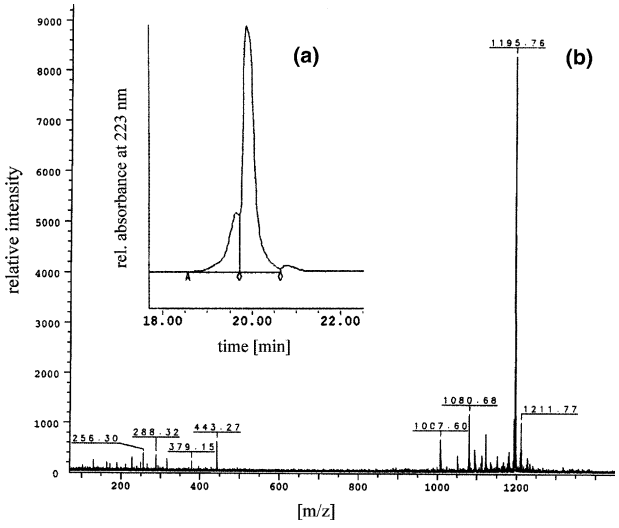

Fig. 3. Chromatogram of the (1–10**H**) oxidized peptide eluted at 19.65–19.88 min (a) and mass spectrum of this HPLC fraction (b). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–10**H**) peptide and Cu(II) 0.50 mM; H_2O_2 1 mM. Incubation at 37 °C.

A^2–V^{12} and A^2–K^{16} formed by the cleavage of the D^1–A^2 peptide bond by α-amidation pathway and modified at the histidine residues were also observed (Table 4). It should be mentioned that in amyloid deposits many N-terminal residues are usually missing [55], with the major component (64% total) lacking D^1–A^2–E^3 [56].

The spectroscopic data in phosphate buffer at pH 7.4 for the Cu(II)–(1–16**M**) system (Table 1) indicate the formation of the 3N complex with {NH_2, COO^-, N^-, N_{Im}} binding mode [32]. Although the CD spectrum of the Cu(II)–(1–16**M**) complex in phosphate buffer is similar to the Cu(II)–(1–10**M**) species suggesting the coordination of His^6, it may be also suggested that in solution there is the equilibrium of the complexes with coordinated His^6 or His^{14} residues. In MALDI-TOF MS spectra for the HPLC fractions eluting between 11.24 and 13.25 min (Table 5) the molecular ions with 1873.1–1875.5 and 1888.9–1891.6 Da masses were detected (Fig. 6). The masses of these ions are 16 and 32 units higher than that of the nonoxidized (1–16**M**) peptide (1857.3–1860.0 Da), corresponding to the addition of one and two oxygen atoms to the (1–16**M**) fragment. The involvement of the histidines in binding of copper(II) ions may suggest that the His^6 and/or His^{14} residues could be converted to 2-oxo-histidines. The fragmentation of the peptide by cleavage of the peptide bonds was also observed. MALDI-TOF mass

Table 3
Products of oxidized (1–10**M**) fragment (DAEF*G*HDSG*F*-NH_2) analyzed by HPLC and theirs observed molecular weights in MALDI-TOF MS spectra

Determination of peptide modification (peptide fragment)	MW_{calcd}	t_R (min), MW_{obsd}				
		9.84–10.73	11.41–13.91	13.58–14.35	14.57	15.50–15.70
M(1–10**M**)	1080.1		1080.4			
Oxidation of $H^6 \rightarrow$ 2-oxo H^6	1096.1	1096.4				
Decarboxylation and deamination of D^1 to pyruvate	1035.0					1035.4
Cleavage of A^2–E^3 by α-amidation pathway, (E^3–F^{10})	892.9			892.4	892.4	
Deamination of NH_2–D^1	1079.0			1078.5		
Cleavage of A^2–E^3 by diamide pathway, oxidation of $H^6 \rightarrow$ 2-oxo H^6 (E^3–F^{10})	909.9				908.4	
Cleavage of D^1–A^2 by α-amidation pathway, oxidation of $H^6 \rightarrow$ 2-oxo H^6 (A^2–F^{10})	980.0				979.4	

t_R – retention time, MW_{calcd} – calculated molecular weight.

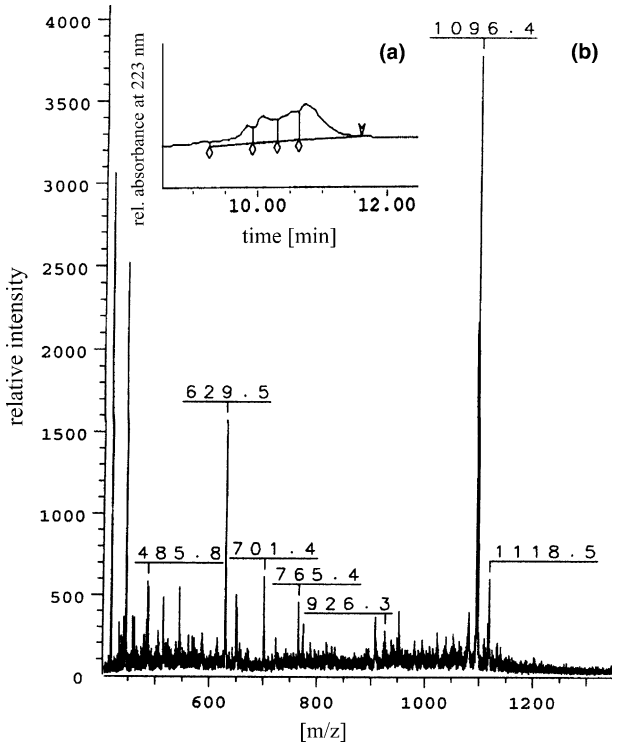

Fig. 4. Chromatogram of the (1–10**M**) oxidized peptide eluted at 9.84–10.73 min (a) and mass spectrum of this HPLC fraction (b). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–10**M**) peptide and Cu(II) 0.50 mM; H_2O_2 1 mM. Incubation at 37 °C.

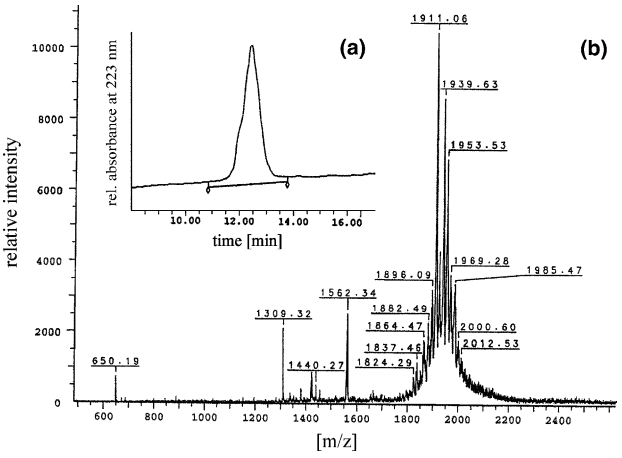

Fig. 5. Chromatogram of the (1–16**H**) oxidized peptide eluted at 12.38 min (a) and mass spectrum for this HPLC fraction (b). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–16**H**) peptide, Cu(II) and H_2O_2, 0.50 mM. Incubation at 37 °C.

spectrometry for the HPLC fractions eluted at 9.37–9.90 min and 13.91–14.44 min yielded masses of 1338.4 and 1465.7–1466.0 Da, respectively (Table 5). These masses may be assigned to the H^6–K^{16} and D^1–R^{13} fragments of the (1–16**M**) peptide obtained by α-amidation process of the G^5–H^6 and R^{13}–H^{14} peptide bonds, respectively. The molecular ions with lower molecular masses in HPLC fraction with 9.37–9.90 min were also detected

Table 4
Products of oxidized (1–16**H**) fragment (DAEF*R*HDSG*Y*EV*H*HQK-NH_2) analyzed by HPLC and theirs observed molecular weights in MALDI-TOF MS spectra

Determination of peptide modification (peptide fragment)	MW_{calcd}	t_R (min), MW_{obsd}					
		8.51	10.56	10.86	11.94	12.38	12.80–13.23
M(1–16**H**)	1954.0	1954.2	1954.3	1953.4	1954.9	1953.5	1955.3
Oxidation one of H → 2-oxo H	1967.0		1970.7	1970.0	1969.5	1969.3	
Oxidation of H^{13}, H^{14} → 2-oxo H^{13}, H^{14}	1986.0		1986.7	1986.1	1985.2	1985.5	
Cleavage of V^{12}–H^{13} by α-amidation pathway, (D^1–V^{12})	1423.5			1424.6	1424.3		
Cleavage of H^{13}–H^{14} by α-amidation pathway, (D^1–H^{13})	1560.6			1562.4	1562.4	1562.3	
Decarboxylation and deamination of D^1 to pyruvate	1909.0					1911.1	
Cleavage of V^{12}–H^{13} and D^1–A^2 by α-amidation pathway, (A^2–V^{12})	1307.4					1309.3	
Cleavage of D^1–A^2 by α-amidation pathway, oxidation of H^{13}, H^{14} → 2-oxo H^{13}, H^{14}, (A^2–K^{16})	1870.0				1869.7		
Cleavage of H^{13}–H^{14} by diamide pathway, α-amidation of A^2–E^3, oxidation of H^{13} → 2-oxo H^{13}, (E^3–H^{13})	1360.4	1362.6					
Cleavage of H^{13}–H^{14} by α-amidation pathway, decarboxylation and deamination of D^1 to pyruvate, (D^1–H^{13})	1515.6						1517.0
Oxidation one of H → Asp	1932.0		1931.6				
Decarboxylation and deamination of D^1 to pyruvate, oxidation of H^{13}, H^{14} → 2-oxo H^{13}, H^{14}	1941.0			1939.2	1939.5	1939.6	

t_R – retention time, MW_{calcd} – calculated molecular weight.

Table 5
Products of oxidized (1–16**M**) fragment (DAEF*G*HDSG*F*EV*R*HQK-NH_2) analyzed by HPLC and theirs observed molecular weights in MALDI-TOF MS spectra

Determination of peptide modification (peptide fragment)	MW_{calcd}	t_R (min), MW_{obsd}					
		9.37–9.90	11.24	12.60–12.77	13.25	13.91	14.44
M(1–16**M**)	1858.0			1860.0	1857.3		
Oxidation one of H → 2-oxo-H	1874.0		1875.5	1875.5	1873.1		
Oxidation of H^6, H^{14} → 2-oxo H^6, H^{14}	1890.0		1890.4	1891.6	1888.9		
Cleavage of G^5–H^6 by α-amidation pathway, (H^6–K^{16})	1337.4	1338.4					
Cleavage of R^{13}–H^{14} by α-amidation, (D^1–R^{13})	1464.5					1465.7	1466.0
Cleavage of H^{14}–Q^{15} by diamide pathway, α-amidation of G^5–H^6, oxidation one of H → Asp, (H^6–H^{14})	1030.0	1028.2					
Cleavage of G^5–H^6 by α-amidation pathway, oxidation of H^6, H^{14} 2-oxo H^6, H^{14}, (H^6–K^{16})	1369.4		1370.3				
Cleavage of H^{14}–Q^{15} by diamide pathway, α-amidation of A^2–E^3, (E^3–H^{14})	1385.4		1385.4				
Cleavage of H^{14}–Q^{15} and A^2–E^3 by α-amidation pathway, oxidation one of H → Asp, (E^3–H^{14})	1392.4		1393.4				
Cleavage of H^{14}–Q^{15} by diamide pathway, deamination of NH_2–D^1, (D^1–H^{14})	1571.6						1569.0
Cleavage of A^2–E^3 by α-amidation, (E^3–K^{16})	1670.8					1670.6	
Cleavage of A^2–E^3 by diamide pathway, oxidation one of H → 2-oxo H, (E^3–K^{16})	1687.8		1689.1				
Cleavage of D^1–A^2 by α-amidation pathway, oxidation of H^6, H^{14} → 2-oxo H^6, H^{14}, (A^2–K^{16})	1773.9				1771.5		
Decarboxylation and deamination of D^1 to pyruvate	1813.0					1811.7	1811.0
Decarboxylation and deamination of D^1 to pyruvate, oxidation one of H → 2-oxo H	1828.9					1828.2	
Decarboxylation and deamination of D^1 to pyruvate, oxidation of H^6, H^{14} → 2-oxo H^6, H^{14}	1844.9					1842.8	1842.6

t_R – retention time, MW_{calcd} – calculated molecular weight.

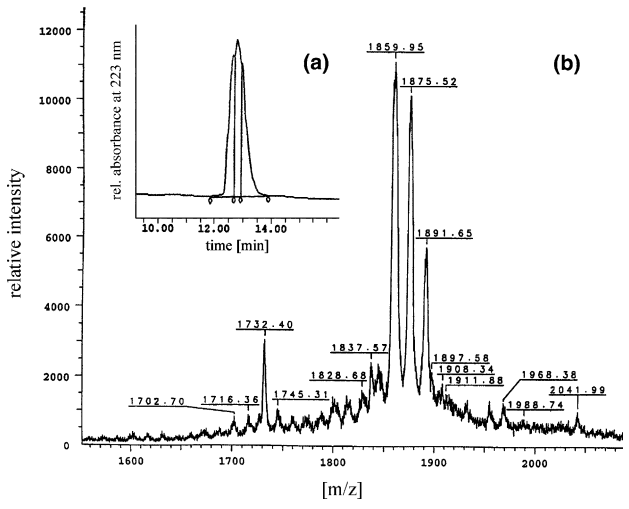

Fig. 6. Chromatogram of the (1–16**M**) oxidized peptide eluted at 12.60–12.77 min (a) and mass spectrum of this HPLC fraction (b). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–16**M**) peptide, Cu(II) and H_2O_2, 0.50 mM. Incubation at 37 °C.

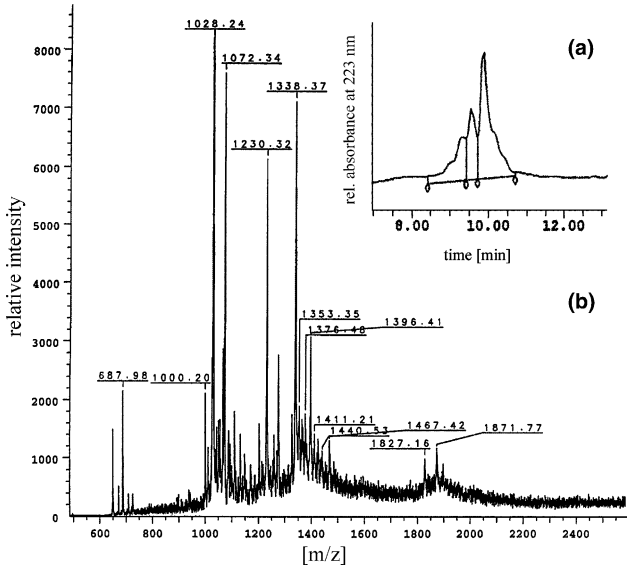

Fig. 7. Chromatogram of the (1–16**M**) oxidized peptide eluted at 9.37–9.90 min (a) and mass spectrum of this HPLC fraction (b). Solution contained 0.1 M phosphate buffer (pH 7.4); concentrations of (1–16**M**) peptide, Cu(II) and H_2O_2, 0.50 mM. Incubation at 37 °C.

(Fig. 7, Table 5). A peptide fragment at m/z 1028.2 Da may correspond to the H^6–H^{14} fragment with oxidized one histidine residue to aspartic acid (Table 5). The fragmentations of this peptide by cleavage of the G^5–H^6, R^{13}–H^{14} and H^{14}–Q^{15} peptide bonds may support the coordination mode of copper(II) ions to the (1–16**M**) fragment. Moreover, the N-terminal aspartic acid residue of the peptide undergoes the modifications in copper(II)-catalyzed oxidation in presence of H_2O_2. An ion at m/z 1811.0–1811.7 Da appeared in the HPLC fractions at 13.91–14.44 min (Table 5) may correspond to the A^2–K^{16} peptide containing pyruvate at N-terminal of peptide after oxidative decarboxylation and deamination of D^1 [48,54]. The A^2–K^{16} fragment formed by cleavage of the D^1–A^2 peptide bond by α-amidation fragmentation mechanism with modified histidine residues was also observed (Table 5).

The others molecular ions of copper(II) catalyzed oxidation products for the (1–16**H**) and (1–16**M**) fragments of β-amyloid peptide were also detected and the modifications of these peptides are proposed in Tables 4 and 5, respectively.

4. Conclusions

The results suggest that the oxidation of Cu(II)-β-amyloid fragments complexes by H_2O_2 catalyzed by bound metal ions occurs at the copper-binding site of peptides. It is in agreement with suggested mechanisms for oxygen radical-mediated cleavage reactions of polypeptide chains and a site-specific generation of OH$^{\bullet}$, which is likely the most important mechanism of protein damage [3,57]. These studies indicate that histidine coordinated to copper(II) ions is the residue in β-amyloid fragments most susceptible to metal-catalyzed oxidation. This observation is in agreement with previous reports, indicating that His is one of the preferred targets in proteins under conditions of site-specific metal-catalyzed oxidation [40,58]. For the (1–16**H**) peptide, likely His^{13} and/or His^{14}, while for the (1–16**M**) fragment of β-amyloid peptide His^6 and/or His^{14} are converted to 2-oxo-His. The (1–16**H**) and (1–16**M**) peptides are coordinated to copper(II) ions by the amine nitrogen and carboxylate oxygen atoms of aspartic acid (D^1) and this residue in metal-catalyzed oxidation by the oxidative decarboxylation and deamination is converted to pyruvate. The fragments A^2–V^{12} and A^2–H^{13} of the (1–16**H**) peptide were also observed supporting the coordination of His^{13}, His^{14} and D^1 residues to the copper(II) ions. Under our experimental conditions the (1–16**M**) fragment of β-amyloid peptide also undergoes the fragmentations by cleavage of the G^5–H^6 and R^{13}–H^{14} peptide bonds, and the H^6–K^{16}, D^1–R^{13} fragments in MALDI-TOF MS spectra were detected. These studies clearly indicate that the radicals formed in peptide/copper(II)/H_2O_2 system attack the specific site in protein molecule near the place of complex formation. In our experimental conditions (copper(II)-fragment of β-amyloid peptide-H_2O_2, 1:1:1 or 1:1:2 molar ratio) the copper-catalyzed oxidation of Tyr^{10} and the formation of dityrosine cross-linked were not observed. The formation of dityrosine cross-linked was detected [29] in different experimental conditions (copper(II)–Aβ–H_2O_2, 5:1:50). Hydrogen peroxide is produced in vivo [59], either accidentally from electron transport chains, or through specific pathways [60]. If both H_2O_2 and copper(II) ions are available in vivo, then radicals will form. Metal catalyzed oxidation of specific amino acid residues has been proposed to be an important physiological mechanism of marking proteins for proteolytic degradation [61].

Acknowledgements

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