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Pro-oxidant activity of histatin 5 related Cu(II)-model peptide probed by mass spectrometry

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Abstract

Histatin 5 is a cationic salivary peptide with strong candidacidal and bactericidal activity at physiological concentration. In this paper we demonstrate by optical spectroscopy and ESI-IT-MS experiments that a synthetic peptide related to the N-terminus of histatin 5 specifically binds copper ions *in vitro* and that the complex metal–peptide generates reactive oxygen species at physiological concentration of ascorbate, leading to significant auto-oxidation of the peptide within short reaction time. The oxidative activity of this peptide is associated to the presence of a specific metal binding site present at its N-terminus. The motif is constituted by the amino acid sequence NH_2-Asp-Ser-His, representing a copper and nickel amino terminal binding site, known as "ATCUN motif". The results of the study suggest that the production of reactive oxygen species can be an intrinsic property of histatin 5 connected to its ability to bind metals.

Keywords: Histatin 5; Reactive oxygen species (ROS); Metals; "ATCUN" motif; ESI-IT-MS spectrometry

Histatins are a family of histidine-rich salivary peptides probably present only in higher primates [1]. Until now, only two human genes HTN1 (HISI) and HTN2 (HIS2) localized on chromosome 4q13, have been recognized as responsible for their synthesis [1,2]. The products of the two genes are histatin 1 and histatin 3, respectively. The former is a peptide of 38 amino acids, phosphorylated at Ser-2, whereas the latter, 32 amino acids long with sequence very similar to histatin 1, is not phosphorylated. Many other peptides of this family have been identified in human saliva, all sharing a sequence common to the two parent peptides. Recently, it has been suggested that histatin related-peptides rather than by a random process are generated by a sequential fragmentation pathway, which is under the principal action of a furin-like pro-protein convertase of the kexin-subtilisin family [3].

One of the most interesting histatin 3 fragment is represented by histatin 5 (Hst 5), which corresponds to the first 24 amino acids of the parent peptide. Hst 5 is usually present in human saliva at a concentration higher than that of the other fragments and it was established to be the most efficient salivary antimicrobial peptide in killing *Candida albicans* at physiological concentration (15–30 μM) [4].

Studies performed to elucidate the molecular events underlying Hst 5 antifungal activity evidenced that they are significantly different from that proposed for azole-based drugs, which exert their activity by inhibiting biosynthesis of ergosterol, the main sterol of fungi membranes [5]. Two different mechanisms have been proposed to rationalise the antifungal activity of Hst 5. The first hypothesizes that the peptide, after crossing the plasma membrane,

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reaches the mitochondrion by an unknown mechanism [6] and interacts with the respiratory chain. Probably impairing the electron transfer, by interacting with coenzyme Q, it induces the generation of reactive oxygen species (ROS) with the concomitant non-lytic release of ATP [7].

In the second proposed mechanism, the first molecular event responsible for the candidacidal activity of Hst 5 is its binding at the level of the cell wall with the heat shock proteins Ssa1/2, which drive the internalization [8]. This event should be followed by the interaction of Hst 5 with the potassium transporter TRK1, accompanied by loss of cell volume, loss of cytoplasmic small molecules and ions, including ATP and K^+, leading to the cell cycle block [9].

It has been previously demonstrated that Hst 5 is a metal binding peptide due to the presence of specific motifs for the binding of zinc, copper and nickel [10–12]. Considering that complexes between NH_2-X-X-His peptides and Cu(II) ions can generate reactive oxygen species [13], we decided to study by mass spectrometry the ability of a peptide analogous to the N-terminal sequence of Hst 5 to produce ROS in the presence of copper and ascorbate *in vitro*.

The results obtained evidence the role of the "ATCUN" motif [14] in the pro-oxidant activity of the Hst 5 related peptide, suggesting that this property can be linked to increased ROS production following histatin 5 cellular internalization.

Material and methods

Reactants. All common reagents were of analytical grade.

Synthetic P1 (DSHAKRAHGY) and P2 (DSAAKRAHGY) peptides were purchased from Peptide Specialty Laboratories GmBH (Germany). Purity was >98%.

ESI-IT-MS and MS/MS experiments. The mass spectrometer was an LCQ Deca-XP Plus (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ion source (ESI) and an ion-trap analyser.

Electronebulization was performed at a flow rate of 10 μl/min with a sheath gas flow rate of 34.3 arbitrary units. The electrospray capillary temperature was 280 °C, the source voltage was 5 kV and the capillary voltage 30 V. The ion-trap analyzer operated in positive mode in the 300–2000 m/z range and spectra were acquired every 3 ms. MS–MS experiments were performed by isolating the ions at 2–4 m/z width and using 40% of the maximum activation amplitude.

Deconvolution of average ESI mass spectra was automatically performed either by the software provided with the Deca-XP instrument (Bioworks Browser) or by MagTran 1.0 [15]. Experimental mass values were compared with the expected average and monoisotopic values calculated using the Peptide Mass program. Theoretical MS–MS spectra were generated utilizing the MS-Product program. Both programs are available at the Protein Prospector site (http://prospector.ucsf.edu/).

Determination of molecular mass and sequence of P1 and P2 peptides. Peptides were dissolved in 10 mM phosphate buffer, pH 7.4, to 0.175 mM final concentration. 200 μl of the solution of the peptide were mixed with 800 μl of 0.2% acetic acid in water/methanol 50/50 (v/v) to perform ESI-MS and MS–MS analysis.

Spectrophotometric metal binding characterization. P1 and P2 peptides were dissolved in H_2O to a concentration of 0.5 mM, $CuCl_2$ or $NiCl_2$ were added at 1:1 molar ratio with respect to the peptides. The pH of the solution was raised up by adding 0.2 M NaOH. Solutions were equilibrated for 30 min at each pH before spectrophotometric measurements. The UV-vis spectra of the metal–peptide complexes were registered on a Perkin-Elmer spectrophotometer Lambda-Bio.

Oxidation reaction. All reactions were performed in 1 ml teflon vials, which allows for sufficient oxygen in headspace. Peptide P1 (100 μM in 20 mM phosphate buffer, pH 7.4) was incubated in the presence of 100 μM $CuCl_2$ for 5 min. Metal-catalyzed oxidation was initiated by exposure of P1–Cu(II) complex to 70 μM ascorbate at 25 °C. Control experiments were performed in the absence of ascorbate. The reactions were stopped by the addition of concentrated acetic acid at a final concentration of 1% (v/v). Reaction products were investigated by ESI-IT-MS and MS–MS experiments.

Results

The synthetic peptides utilized in this study were: P1—DSHAKRAHGY and P2—DSAAKRAHGY.

They correspond to the sequence of the first ten N-terminal amino acid residues of Hst 5 with the exception of a substitution His/Ala at the residue 7 (P1) and His/Ala at the residue 3 and 7 (P2), respectively. Experimental mass values of P1 and P2 peptides ($[M + H]^{1+}$; monoisotopic) determined by ESI-IT-MS corresponded to 1141.6 ± 0.2 Da and 1075.5 ± 0.2 Da, respectively. Sequences of the two peptides were confirmed by MS–MS experiments. Fig. 1 shows the fragmentation pattern of P1 peptide obtained by SIM (Selected Ion Monitoring)-MS–MS experiments performed by selecting the bi-charged ion at 571.3 m/z.

The interaction between peptide P1 and P2 and divalent cations, such as Cu(II) and Ni(II) has been studied by following the absorption in the visible region of the spectrum between 300 and 800 nm. Fig. 2A shows that the complex P1–Cu(II) reaches an absorption maximum at 525 nm when the pH is raised from 3.0 to 11.0. It is relevant to note that the major increase in the absorbance is evident in the pH range 3.3–5.4. In Cu(II)–P1 binding experiments, the absorption maximum at 525 nm reached at low pH values and maintained through a wide range of pH, indicates a specific amino terminal copper binding site. Fig. 2B shows the spectra of Ni(II) binding to P1 peptide. In the spectra an absorbance increase at 420 nm is observed when the pH is raised from 6.0 to 11.0, characteristic of a planar co-ordination [16]. Conversely, non-specific Ni(II)-binding is usually octahedral [17]. The pH of binding is indicative of a high affinity metal–peptide P1 complex, whom specific spectral properties evidence a co-ordination of the metals with the ATCUN motif constituted by the N-terminal sequence NH_2-Asp-Ser-His (Fig. 3).

The visible spectrum of P2 peptide, in the presence of Cu(II) shows a maximum at 600 nm, that shifts at 525 nm only at pH higher than 10 (Fig. 2C). Similarly the binding of Ni(II) to the same peptide shows a maximum shifted at 420 nm only at pH values higher than 9 (Fig. 2D). The spectra of P2 peptide are indicative of an aspecific binding between the metal ions and the negative form of the peptide prevalent at high pH.

The different spectroscopic behaviour of P1 and P2 peptides in the pH-dependent binding experiments with metal

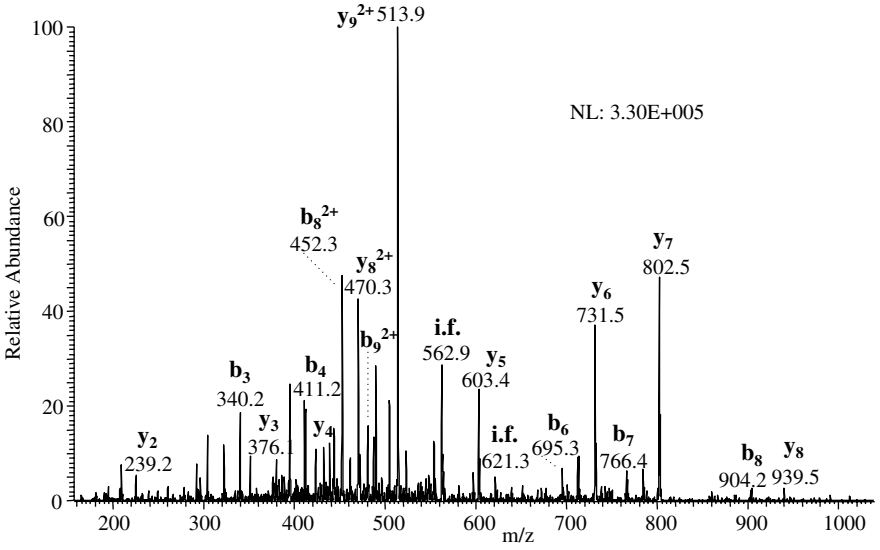

Fig. 1. ESI-MS–MS spectrum of P1 peptide. The spectrum was obtained by direct injection as described in Materials and methods, following a SIM-MS–MS experiment selecting the bi-charged ion of P1 at 571.5 *m/z*. Fragments of *b* and *y* series are in agreement with P1 sequence (i.f.: internal fragment).

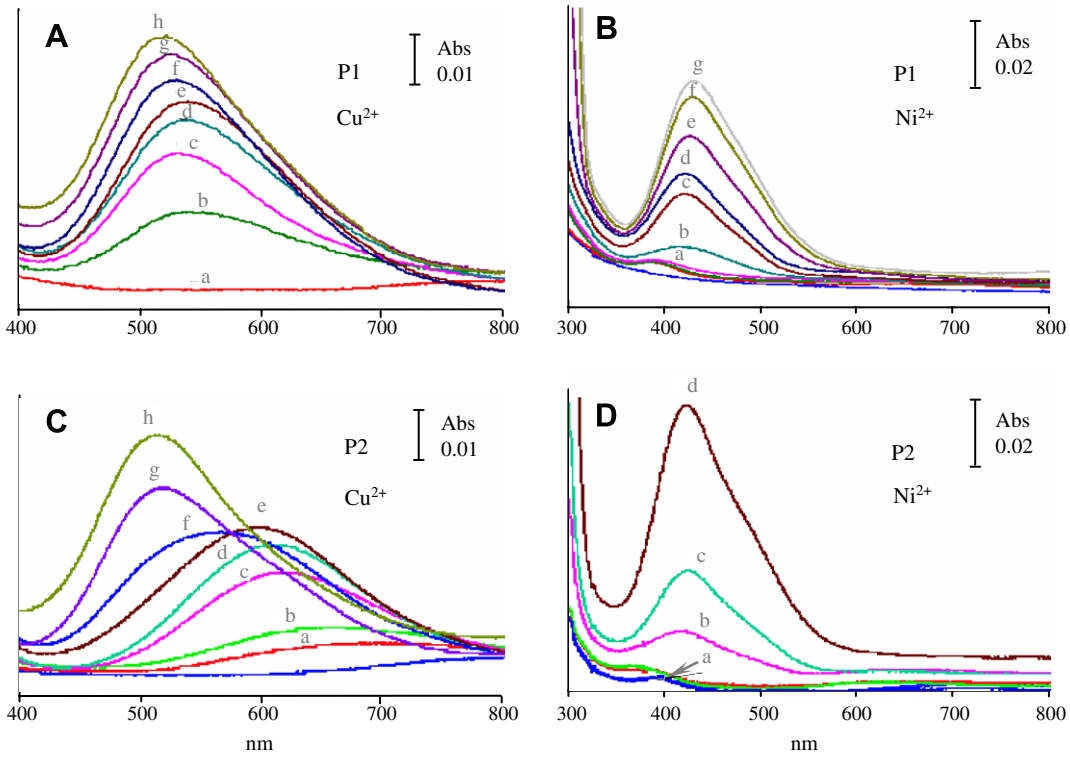

Fig. 2. Optical spectra of Cu(II) and Ni(II) binding to P1 and P2 peptides. (A) Optical spectra of 0.5 mM P1 peptide in the presence of an equivalent of Cu(II) at different pHs. The spectra indicated were measured at pH 3.3 (a), pH 4.3 (b), pH 5.4 (c), pH 6.4 (d), pH 7.8 (e), pH 9.1 (f), pH 10.0 (g), and pH 11.0 (h). (B) Optical spectra of 0.5 mM P1 peptide in the presence of an equivalent of Ni(II) at different pHs. The spectra indicated were measured at pH 5.2 (a), pH 6.0 (b), pH 7.4 (c), pH 8.1 (d) pH 9.2 (e), pH 10.0 (f), and pH 11.0 (g). (C) Optical spectra of 0.5 mM P2 peptide in the presence of an equivalent of Cu(II) at different pHs. The spectra indicated were measured at pH 4.8 (a), pH 5.1 (b), pH 6.3 (c), pH 7.2 (d), pH 8.1 (e), pH 9.0 (f), pH 10.0 (g), and pH 11.0 (h). (D) Optical spectra of 0.5 mM P2 peptide in the presence of an equivalent of Ni(II) at different pHs. The spectra indicated were measured at pH 3.6 (a), pH 9.0 (b), pH 9.3 (c), and pH 10.5 (d).

ions demonstrates that a histidine in third position is essential for the correct topology of the ATCUN motif in the formation of a complex with high-stability.

Electrospray ionization mass spectrometry (ESI-MS) experiments were carried out to further investigate the binding of Cu(II). Fig. 4 shows the ESI mass spectrum

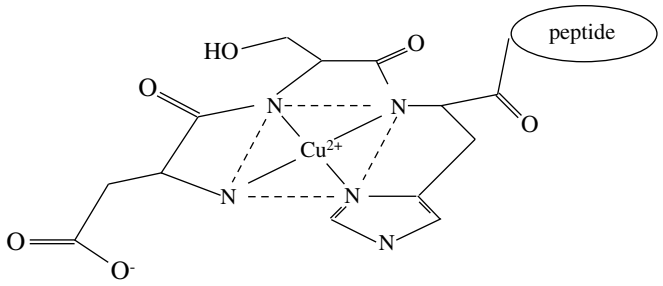

Fig. 3. Stereo-model of the ACTUN-Cu^{2+} complex.

obtained by direct infusion of peptide P1 (100 μM in 20 mM phosphate buffer, pH 7.4) incubated in the presence of 100 μM Cu(II) for 5 min. By deconvolution of the ESI spectrum two masses were obtained: the value of 1140.3 ± 0.2 Da was attributed to the free peptide, the value of 1202.3 ± 0.2 Da, increased by 62.0 Da, was in agreement with the formation of a complex with Cu (II) showing 1:1 stoichiometry. Remarkably, no masses attributable to oxidation products of peptide P1 were detected.

The pro-oxidant properties of the peptide–Cu(II) complex were investigated by adding 70 μM ascorbate to the mixture and analysing aliquots of the solution by mass spectrometry. Fig. 5 shows the results obtained after 5 min incubation at 25 °C. Several masses attributable to oxidised derivatives were detected together with those of the free peptide (1140.5 Da) and the complex Cu(II)–peptide (1202.5 Da). The masses were 1156.6 Da, showing an increment of +16 Da with respect to the mass of the free peptide, and 1218.4 Da, consistent with the mass of the mono-oxidized form of the complex peptide–Cu(II) (1202.5 + 16 Da). Thus, both derivatives corresponded to mono-oxidized P1 peptide products. Negligible quantity of bi-oxidized P1 peptide with a mass value of 1172.4 Da was also observed.

To identify the amino acid target of the oxidation we submitted the mono-oxidized peptide P1 to Tandem-MS experiments. The bi-oxidized form of the peptide could not be investigated due to the low intensity of its ions. SIM MS–MS experiments were performed by selecting the bi-charged ion at 579.5 *m/z* and obtained results are shown in Fig. 6. The experimental fragmentation pattern was compared with the theoretical ones simulated considering all the tyrosines and histidines present. The solely simulation which provided results comparable with the experimental data located the oxidation at His 8 (Figs. 6 and 7). In fact, the fragments ^{1}DSHAKRAHG^{9} and

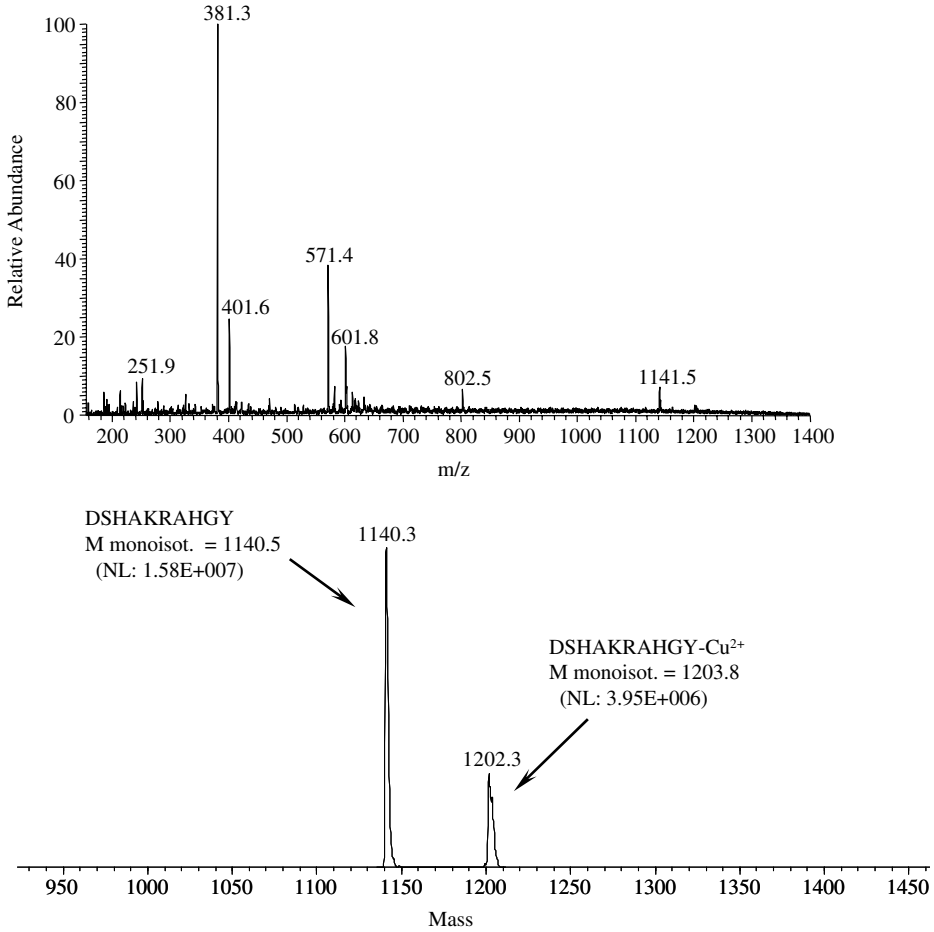

Fig. 4. ESI-MS spectrum of the P1 peptide in the presence of Cu(II). Upper diagram: ESI spectrum of the incubation mixture (100 μM Peptide P1 in 20 mM phosphate buffer, pH 7.4 incubated with 100 μM $CuCl_2$). Bottom diagram: deconvolution of the ESI-spectrum.

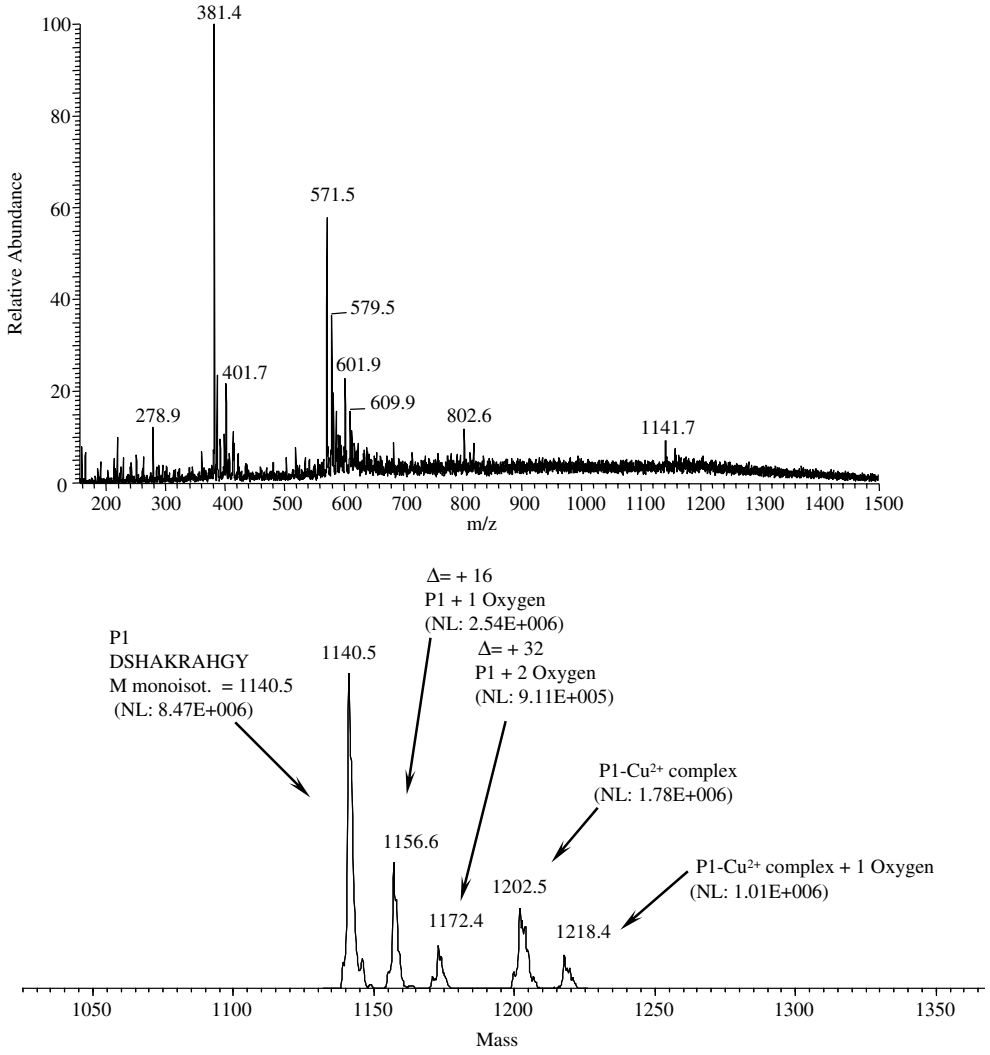

Fig. 5. ESI-MS spectrum of the P1 peptide in the presence of Cu(II) and ascorbate. Upper diagram: ESI spectrum of the incubation mixture (100 μM peptide P1 in 20 mM phosphate buffer, pH 7.4 incubated with 100 μM $CuCl_2$ and 70 μM of ascorbate). Bottom diagram: deconvolution of the ESI-spectrum.

^{1}DSHAKRAH^{8} were detected at 488.9 *m/z* (b_9^{2+}) and 460.3 (b_8^{2+}). They differ by 8 *m/z* from the fragments of the non oxidized peptide (b_9^{2+} *m/z* 481.0 and b_8^{2+} *m/z* 452.3, respectively), not detected at all in the MS–MS fragmentation pattern of the oxidized peptide. These results indicated the presence of an additional oxygen atom in the first 9 residues excluding the oxidation on Tyr-10. The ions of the *b* series from b_7 to b_3, corresponding to fragments of P1 peptide up to Ala-7 residue, did not show any mass/charge increase with respect to the non oxidized P1 peptide and thus excluded His 3 as possible site of oxidation. The mass/charge increase of 16 *m/z* of the ions from y_8 to y_3 (*y* series) confirmed that His 3 was not involved in the oxidation (Figs. 6 and 7).

Experiments carried out on P2 peptide did show neither any evidence of Cu(II) binding, under the experimental conditions used to perform the ESI-MS analysis, nor peptide auto-oxidation by ascorbate (data not reported), even after 24 h of incubation. Thus, it can be concluded that the formation of a metal–peptide complex is the necessary prerequisite for the oxidative activity of P1 peptide.

Discussion

Histatins are small histidine-rich salivary peptides which exhibit activity against *C. albicans*. This activity has been partly attributed to the high content of basic amino acids. Differently from most other antimicrobial proteins, histatins are known to participate in metal ions co-ordination.

In a previous paper [11], some of us found for the first time that Hst 5, a major salivary component of the family, is able to bind zinc ions to a specific "HEXXH zinc binding motif" present in the C-terminal region of the peptide and that the metal binding induced the aggregation and fusion of negatively charged vesicles.

Later, the metallopeptide nature of the histatins has been confirmed by studies performed by electrospray ionization mass spectrometry, which demonstrated the

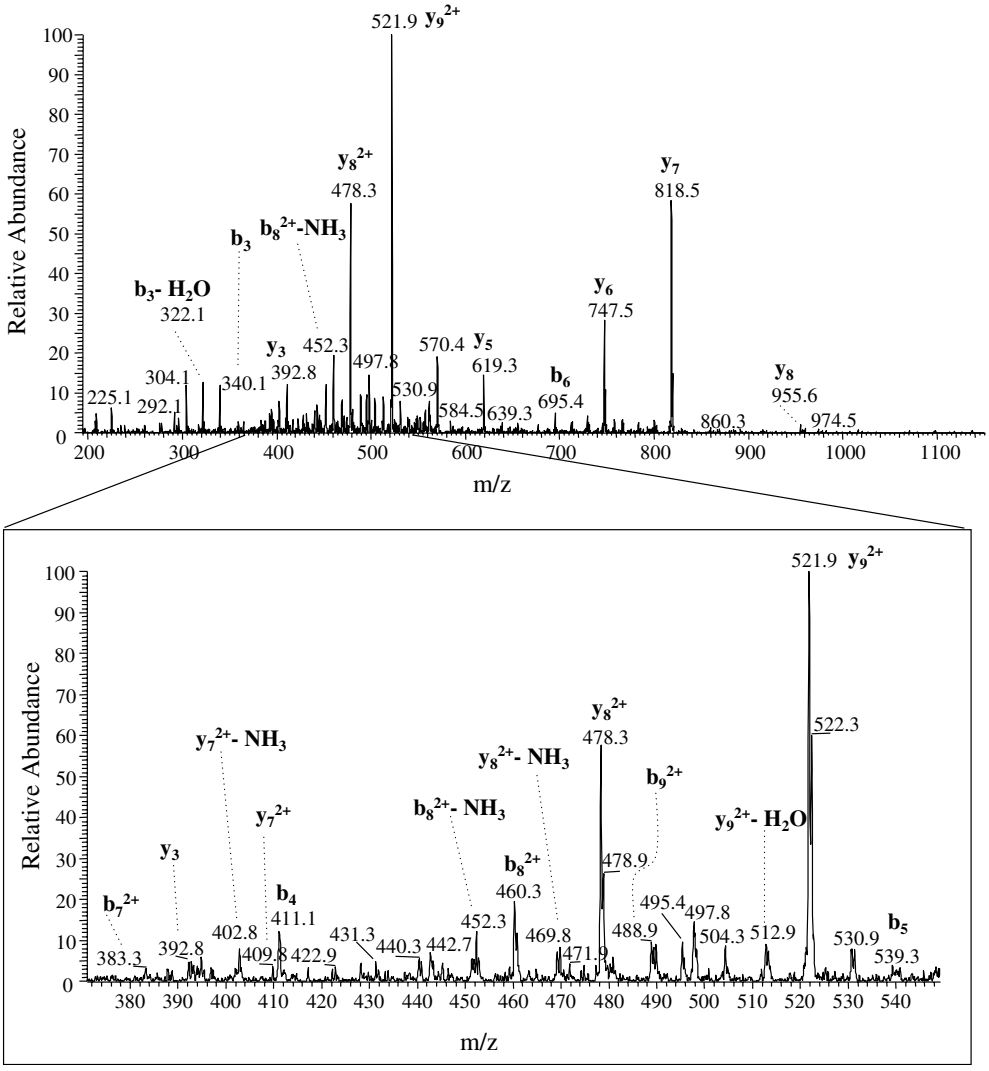

Fig. 6. ESI-MS–MS spectrum of mono-oxidized P1 peptide. SIM-MS–MS experiment performed selecting the bi-charged ion of mono-oxidated P1 peptide at 579.5 *m*/*z*.

b^{2+}- NH_3				261.7	339.9	375.4	452.0	480.5	
b - NH_3				522.5	678.7	749.8	902.9	960.0	
b^{2+}- H_2O		161.7	197.2	261.3	339.4	374.9	451.5	480.0	
b - H_2O	185.2	322.3	393.4	521.6	677.7	748.8	902.0	959.0	
b^{2+}		170.7	206.2	270.3	348.4	383.9	460.5	489.0	
b	203.2	340.3	411.4	539.6	695.7	766.8	920.0	977.0	

1	2	3	4	5	6	7	8	9	
D	S	H	A	K	R	A	H(ox)	G	Y
9	8	7	6	5	4	3	2	1	

1043.1	956.1	818.9	747.8	619.7	463.5	392.4	239.2	182.2	y
522.1	478.5	409.9	374.4	310.3					y^{2+}
1026.1	939.0	801.9	730.8	602.6					y - NH_3
513.6	470.0	401.5	365.9	301.8					y^{2+}- NH_3
1025.1									y - H_2O
513.1									y^{2+}- H_2O

Fig. 7. Theoretical fragmentation of mono-oxidized P1 peptide. Mono- and bi-charged theoretical *b* and *y* series (also after H_2O and NH_3 neutral losses) of P1 peptide mono-oxidized on histidin 8 residue are shown. The grey boxes underline the fragments detected in the ESI-MS–MS spectrum of Fig. 6.

formation of complexes with copper and nickel [18]. Although, the co-ordination of these metals is particularly interesting, especially for copper and zinc, which are metals present in salivary secretions and whole saliva, the precise biological significance of this binding has not yet been clearly established.

Recently, the exogenous addition of histatin 5 to *C. albicans* isolates was found to specifically triple the total intracellular levels of ROS and a similar increase in total cellular ROS was found in DB9 cells, after induction of the expression of the Hst 5 [19], in agreement with the results of a previous study [7]. No significant increase in ROS levels was detected following the induction of nontoxic protein in DB10 cells, suggesting that elevated ROS levels are specific to the intracellular expression of Hst 5. To explain the cellular and mitochondria increase of ROS following Hst 5 addition, it has been postulated that the peptide inhibits the respiratory chain, possibly at the co-enzyme Q level, thus inducing ROS formation [7].

Recently, it has been demonstrated that the yeast mitochondrion contains a pool of copper distinct from that associated with the cuproproteins. This copper is localized within the matrix as a soluble, low molecular weight complex and is accessible to the cuproproteins [20]. It should be outlined, that the copper binding to Hst 5 occurs to a single high affinity site ($K = 2.6 \times 10^{-7}$ M) corresponding to an ATCUN motif. Thus, it cannot be excluded the formation of Cu(II)–peptide complex inside the mitochondrial matrix.

The link between the copper–peptide complex formation and the ROS generation is demonstrated by the oxidation observed in P1 peptide, but not in P2 peptide, missing the pivotal histidine 3 residue. The net addition of one oxygen could be consistent with the hydroxylation of histidine 8 to 2-oxo-histidine by hydroxyl radicals as previously reported for the Cu(II)-catalyzed oxidation of some histidines present in β-amyloid peptide [21]. Metal-catalyzed oxidation has been recently utilized to identify the amino acids participating to the metal binding sites in copper metalloproteins [22,23].

The ability of the Cu(II)/Ni(II)–peptide complex to produce oxidative damage has been also recently utilised to cleave nucleic acids in presence of co-reactants [24–26] or in presence of O_2 and by light activation in the case of peptide–Co^{2+} complexes [27], thus demonstrating that these molecules can be used as artificial nuclease.

Moreover, the potential of Cu(II) complex with N-terminal ATCUN motif of human protamine HP2, to mediate oxidative DNA damage by oxygen activation has been also demonstrated [28].

The metals binding co-ordination by these peptides is particularly interesting, because Hst 5 and its fragments have been found in whole saliva [3], where probably they exert a protective action against oral increase of metal concentration. In fact, it has been reported that copper at concentrations ranging from 0.1 to 50 μM is implicated in the pathogenesis of several fibrotic disorders and *in vitro*, at a concentration of 50 μM it upregulates the collagen production in oral fibroblasts [29].

Acknowledgments

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