Chelating properties of tripeptide-9 citrulline

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Propiedades de coordinación de Tripeptide-9-citrulline

Propietats de coordinació de Tripeptide-9-citrulline

Recibido: 4 de octubre de 2012; aceptado: 30 de octubre de 2012

RESUMEN

Tripeptide-9-citrulline (nombre INCI) es un péptido para el cuidado de la piel, usado en aplicaciones cosméticas. Para elucidar su mecanismo de acción en aquellas vías químicas que implican iones metálicos, se ha estudiado su capacidad para formar complejos metálicos. Dicho estudio se ha llevado a cabo utilizando técnicas espectrofotométricas, electroquímicas y electroforéticas. Los resultados obtenidos, usando Cu (II) como ión metálico, son consistentes con la formación de un complejo entre *Tripeptide-9-citrulline* y el ion metálico. La voltametría cíclica ha revelado un cambio significativo en los potenciales electroquímicos. Finalmente se ha demostrado mediante experimentos de electroforéticos que *Tripeptide-9-citrulline* podría proteger el ADN de la degradación inducida por los radicales producidos en la reacción de Fenton.

Palabras clave: Voltametría cíclica, DNA, Reacción de Fenton, Péptido

SUMMARY

Tripeptide-9 Citrulline (INCI name) is a peptide with skin care properties, used for cosmetic applications. In order to elucidate its mechanism of action in the chemical pathways that involve metal ions, its ability to complex such ions was investigated using spectrophotometrical, electrochemical and electrophoretical techniques. The obtained results using Cu(II) as metal ion were consistent with the formation of a complex between Tripeptide-9 Citrulline and Cu(II). Cyclic voltammetry revealed a significant change in the electrochemical potentials. In-vitro electrophoretic studies served as a proof of concept that Tripeptide-9 Citrulline may protect DNA from radical degradation induced by the Fenton reaction.

Keywords: Cyclic voltammetry, DNA, Fenton reaction, Peptide

RESUM

Tripeptide-9-citrulline (Nom INCI) és un pèptid usat en aplicacions cosmètiques que tenen cura de la pell. Per a elucidar el seu mecanisme d'acció en aquelles vies químiques que involucren ions metàl·lics, s'ha estudiat la seva capacitat per a format complexos metàl·lics. Aquest estudi s'ha dut a terme utilizant tècniques espectrofotomètriques, electroquímiques i electroforètiques. Els resultats obtinguts, emprant (II) com a ió metàl·lic, són consistents amb la formació d'un complex entre *Tripeptide-9-citrulline* i el ió metàl·lic. La voltametria cíclica revela un canvi significatiu entre els potencials electroquímics. Finalment, s'ha demostrat mitjançant experiments electroforètics que *Tripeptide-9-citrulline* podria protegir l'ADN de la degradació induïda pels radicals produïts en la reacció de Fenton.

Paraules clau: Voltametria cíclica, ADN, Reacció de Fenton, Pèptid

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INTRODUCTION

Skin is considered to be the largest organ in the human body weighing about 3 Kg spread over an area of roughly 1.5 m^2. Similar to other cells in the human body, skin cells breathe and consume energy and spontaneously generate reactive oxygen species (ROS)[1] which can be toxic and often cause cell damage due to various mechanisms mainly oxidation of membrane phospholipids, proteins, and nucleic acids.[2] Two groups of ROS species have been identified, radicals and non-radicals. The radicals group includes superoxides ($O_2^{\bullet\ominus}$), hydroxys ($HO^{\bullet}$), alkoxys ($RO^{\bullet}$), hydroperoxides ($HO_2^{\bullet}$) and peroxys ($RO_2^{\bullet}$),[3] whereas singlet oxygen ($^1O_2$), hydrogen peroxide H_2O_2, ozone (O_3), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{\ominus}$) represent the non-radicals. Among these species, H_2O_2 has special biological relevance due to its ability to interact with redox-active transition metals such as Cu(II), yielding other ROS products such as the hydroxy radical $HO^{\bullet}$,[4] commonly known as the most oxidative reactant as well as most damaging among all known ROS species,[5-6]. As depicted in Scheme I, H_2O_2 can react with transition metal ions to yield ROS via the Fenton reaction. in addition, H_2O_2 is freely diffusible into cells and can potentially reach the DNA in the nucleus.[7] DNA molecules, on the other hand, can also bind to metal cations at two binding sites: the anionic phosphate groups and the electron donor atoms on the heterocyclic bases[8] yielding structures such as those shown in Figure 1.

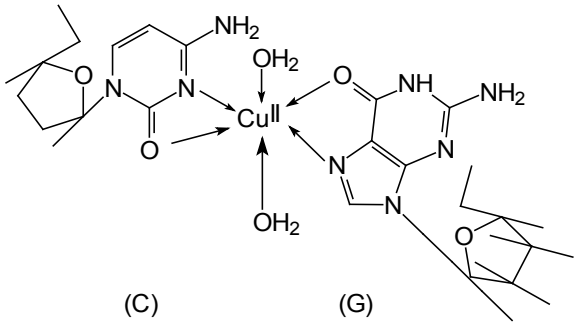


Figure 1. *Cu(II)-DNA coordination. Adapted from[8]*

To better appreciate the severity of this process, one must keep in mind the large amount of oxygen which enters the cells (10^{12} molecules /cell/day). Reactions of organic molecules (e.g. sugars) with molecular oxygen to generate reactive oxygen radicals do not occur because most organic molecules exist in the spin state whereas O_2 exists in the triplet spin state (reactions of a triplet with a singlet molecule are very unlikely as evidenced in their slow rate ($10^{-5}M^{-1}s^{-1}$)).[4] However, transition metals like iron or copper can bridge this kinetic restriction by reducing O_2 to form radical species that are capable of reacting with organic molecules.

With these considerations in mind, this investigation aims at studying the chelating properties of different compounds and their ability to reduce or eliminate interactions between ROS species, namely O_2 and H_2O_2 with metal ions to eliminate their oxidative stress and other harmful chemical reactions on biomolecules (such as DNA and proteins,. In this paper we report on the chelating ability of transition metals by Tripeptide-9 Citrulline using various techniques such as spectrophotometry, electrochemistry and electrophoresis. Cu(II) has been used routinely as a model cation due to its role as one of the most important transition metal ions in biological systems.[9]

MATERIALS AND METHODS

Materials

Tripeptide-9 Citrulline (CAS nr. 951775-32-9) was supplied by Lipotec S.A. Plasmid pBR322 as well as all other analytical grade chemicals were obtained commercially.

Methods

UV-VIS Spectrophotometry: Tripeptide-9 Citrulline and $CuSO_4$ solutions were mixed at different ratios and diluted to predetermined concentrations in a pH 5.5 acetate buffer with constant mechanical mixing. Absorbance spectra of peptide/Cu(II) mixtures were recorded with a UV-VIS Spectrophometer Shimadzu UV-1700 Series. The same acetate buffer was used for baseline readings.

Electrochemical studies: The electrochemical experiments were performed using a Metrohm 757 VA Computrace using a platinum wire as a working electrode, a platinum rod counter electrode and Ag/AgCl/KCl (3 M) reference electrode. All samples were placed in 10-ml conventional glass cells. Prior to electrolysis, the platinum working electrode was cleaned by heating the electrode using a Bunsen burner until incandescence. The experimental conditions of the cyclic voltammograms were: Mode DC, Equilibration time 5 s, Initial potential 1.25 V, Inversion potential -0.95V and sweep rate 100 mV/s. All cyclic voltammetry experiments were obtained beginning with a cathodic sweep. Electrolytes used in this experiment were aqueous solutions of 1 M Na_2SO_4 which have been degassed by N_2 bubbling for 5 min prior to the experiments.

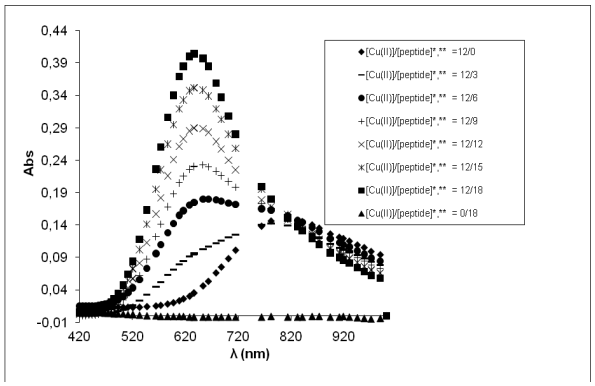
Electrophoresis: Aliquots of plasmid DNA pBR322 (0.70 mg/mL) were incubated in TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH = 7.5) at molar ratios r_i = 0.02 and 0.005 for electrophoresis experiments. Incubation of the samples was carried out at 37°C for 5 minutes in the absence of light. 1 **µL** marker was added to different aliquots (20 **µL**) of compound-DNA complex and free DNA was used as a control. Agarose gel electrophoresis (1% in TBE buffer, Tris-borate-EDTA) was run for 5 hours at 75 V/cm followed by staining in an ethidium bromide solution (0.75 mg / ml in TBE) for 6 hours. The experiment was carried out in a horizontal Ecogen tank connected to a Pharmacia GPS 200/400 with a variable power supply potential. The gel was photographed using a camera Image Master VDS (Pharmacia Biotech).

RESULTS AND DISCUSSION

Spectrophomometric studies of metal chelating properties of Tripeptide-9 Citrulline

In order to investigate the chelation of Cu(II) by Tripeptide-9 Citrulline, mixtures of these two components at different ratios were prepared with the Tripeptide-9 Citrulline used as a control. Mere visual inspection of the samples revealed that the intensity of the blue colour qualitatively increased with increased concentration of the Tripeptide-9 Citrulline (data not shown). UV-VIS absorbance scan spectra showed a maximum Cu(II) wavelength absorbance at 816 nm (Figure 2). Addition of the Tripeptide-9 Citrulline to

Cu(II) showed the emergence of a very intense new maximum peak at 635 nm, consistent with visual observations. Maximum absorbance peak shift for all concentrations of the Tripeptide-9 Citrulline used in this study are shown in Table 1.



**Peptide = Tripeptide-9 Citrulline; ** = Concentration is expressed in mM*

Figure 2. *UV-VIS Spectrophotometry spectra of mixtures of Cu (II) with Tripeptide-9 Citrulline at different ratios. For experimental conditions, refer to "Materials and Methods" section. Absorbance units are adimensional.*

Table 1. *Absorbance values of solutions containing Tripeptide-9 Citrulline and $CuSO_4$.*

[Tripeptide-9 Citrulline] (mM)	[$CuSO_4$] (mM)	Wavelenght of maximum absorbance (nm)	**Absorbance at 635 nm**
0	12	816	0.034
3	12	659	0.095
6	12	644	0.175
9	12	638	0.230
12	12	636	0.289
15	12	635	0.351
18	12	635	0.404
18	0	No absorption	No absorption

Taking into account that Tripeptide-9 Citrulline does not show any absorption in the UV-VIS range studied, and as the quantity of Cu(II) is constant in all the samples, in the absence of interaction between the two compounds the same UV-VIS spectrum corresponding to Cu(II) would be expected. On the contrary, the experimental behavior is consistent with the formation of a complex formed by Cu(II) and Tripeptide-9 Citrulline, and its intensity at different Cu(II) concentrations is reflected in the maximum peak absorbance at 635 nm (Table 1) as shown in Figure 3. A simple molecular modeling of the Tripeptide-9 Citrulline-metal complex (Figure 4) was calculated by Chem3D Ultra 10.0 with metal ion Cu(II) in solution. This model appears to indicate that interaction of the Trpeptide-9-Citrulline with the metal ion takes place at the amino group moieties. The structure shown in Figure 4 postulates a 1:1 molar ratio of Cu(II) and Tripeptide-9 Citrulline, however with this limited data, it is not possible to propose an accurate stoichiometry for the complex between the tripeptide and the copper ions.

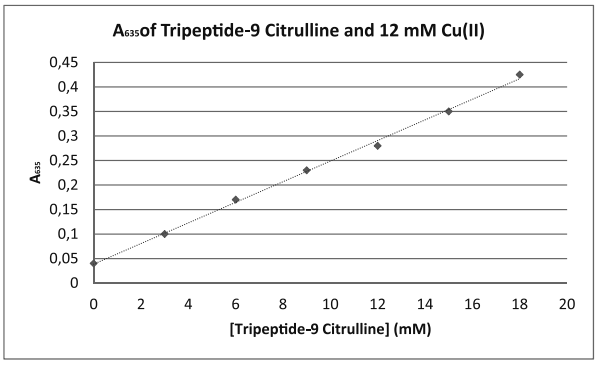


Figure 3. *A_{635} values corresponding to the spectra of Figure 2, listed in Table 1. Absorbance units are adimensional.*

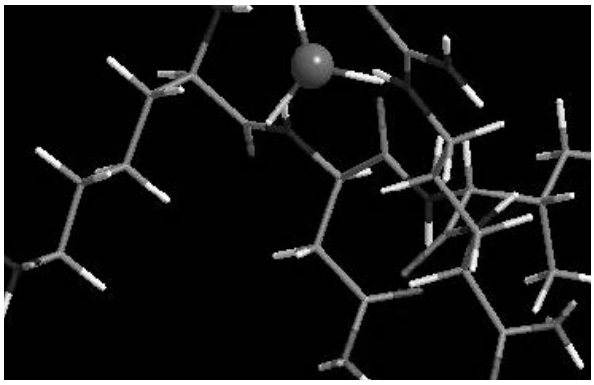


Figure 4. *3D structure of Tripeptide-9 Citrulline-Cu(II) 1:1 calculated by Chem3D Ultra 10.0*

As observed in Figure 3, the values of absorbance at 635 nm at increasing concentrations of Tripeptide-9 Citrulline show no saturation (at the observed range a linear relationship is obtained), even at 1:1 or higher molar ratios. This does not necessarily mean that the ratio in the complex is higher (e.g. 1 mol Cu(II): 2 mol Tripeptide-9 Citrulline), but it could easily be that the equilibrium constant is low. In such a case, saturation would never be achieved at the experimental values of the peptide /Cu(II) ratios. subsequently, despite the clear evidence of interaction, determination of the stoichiometry of the complex would require further experiments that we consider out of the scope of this work.
This equilibrium is also confirmed by the presence of an isosbestic point at *ca.* 820 nm. Isosbestic points[10] are commonly found when electronic spectra are recorded (a) for a solution in which a chemical reaction or interaction is in progress, or (b) for a solution in which the two absorbing components and their relative proportions are controlled by the concentration of some other component. In our experiments, the absorbance spectra of the mixtures of Tripeptide-9 Citrulline with Cu(II) have been carried out after sufficient interaction time in order to reach equilibrium, so we consider only possibility "(b)". According to that, an equilibrium between Cu(II), Tripeptide-9 Citrulline and the complex (or complexes) Cu(II) / Tripeptide-9 Citrulline is postulated, thus concluding that, whatever are the structure and molarity of the complex, Tripeptide-9 Citrulline is able to complex transition metal ions such as Cu(II).

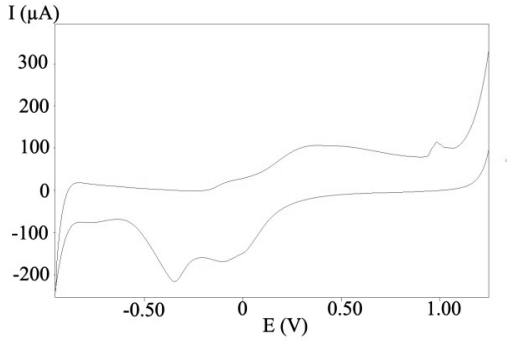
Electrochemical studies of metal chelating properties of Tripeptide-9 Citrulline

Cyclic voltammetry is the most versatile electroanalytical technique for studying electroactive species.[11] Therefore

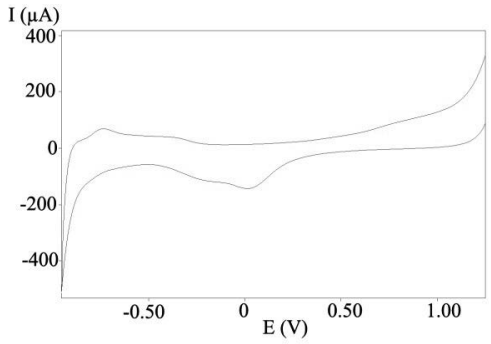
electrochemical measurements of the Tripeptide-9 Citrulline–Metal ion interactions is expected to complement other methods and to yield information on the mechanism of interaction as well as conformation of the adduct.[12]

Similar to earlier studies using cyclic voltammetry for investigating the ability of organic molecules to complex ions[13-16] we have performed electrochemical analysis of mixtures of Tripeptide-9 Citrulline and Cu(II). Figure 5A shows the cyclic voltammogram of 1 M Na_2SO_4 solution (blank). Two more peaks can be observed in the cathodic sweep, presumably due to the hydrogen adsorption on the platinum surface.[17] It is important to note that the potential window of the electrolyte used (1 M Na_2SO_4) is limited by oxygen evolution on the anodic region (E>1.2 V *vs.* Ag/AgCl/KCl (3 M)) and by hydrogen evolution on the cathodic region (E<-0.9 V vs. Ag/AgCl/KCl (3 M)). Figure 5B shows the cyclic voltammogram of 1 mM Tripeptide-9 Citrulline in 1 M Na_2SO_4 solution with two small peaks in the cathodic sweep due to the hydrogen adsorption on the platinum surface. In addition a small bump appears in the anodic sweep at -734 mV which could be due to oxidation of the Tripeptide-9 Citrulline. Figure 5C shows the cyclic voltammogram of 1 mM $CuSO_4$/1 M Na_2SO_4 solution (working solution) and corresponding peak potentials are listed in Table 2. In this same Figure, new peaks appear both in the cathodic and anodic sweep compared to the blank solution (see Figure 5A) with those of the cathodic sweep may be interpreted as due to reduction reactions of copper ions in the working electrode. In the anodic sweep, three peaks were observed (see Table 2) with two of these peaks likely corresponding to oxidation reactions complementary to the reduction processes mentioned above. The third peak may be attributed to an adsorption/desorption process of the copper ion at the electrode surface.

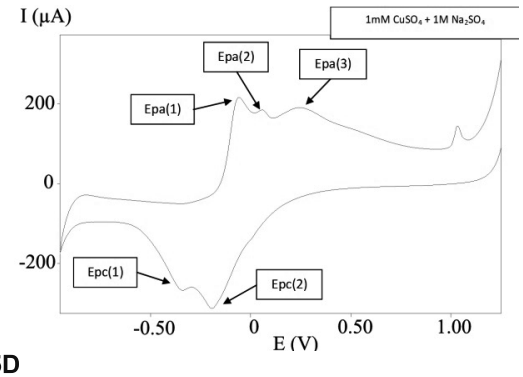
5A



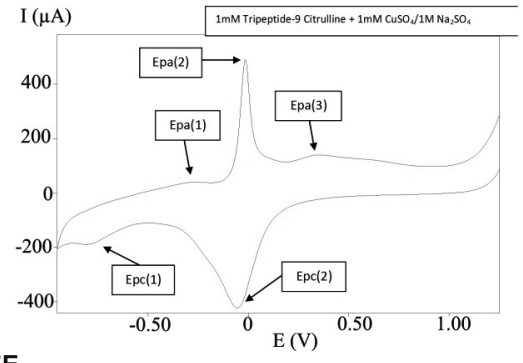
5B



5C



5D



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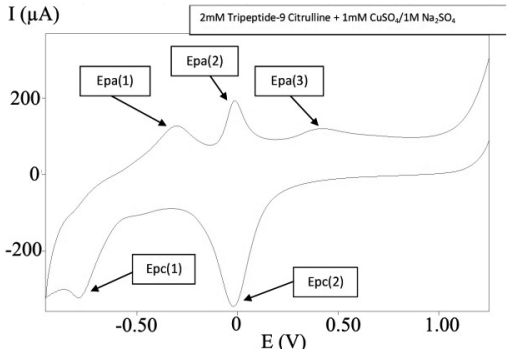

5F

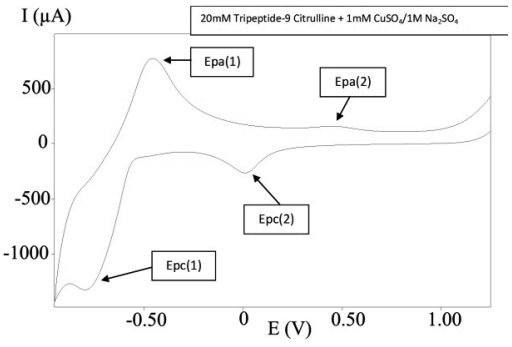

Figure 5. *Cyclic voltammograms of Tripeptide-9 Citrulline / $CuSO_4$ solutions at different concentrations in 1 M Na_2SO_4 (working solution).* **5A**, *Na_2SO_4 solution (blank);* **5B**, *1 mM Tripeptide-9 Citrulline;* **5C**, *1 mM $CuSO_4$;* **5D**, *1 mM Tripeptide-9 Citrulline + 1 mM $CuSO_4$;* **5E**, *2 mM Tripeptide-9 Citrulline + 1 mM $CuSO_4$;* **5F**, *20 mM Tripeptide-9 Citrulline + 20 mM $CuSO_4$. For experimental conditions, refer to "Materials and Methods" section.*

Table 2. *Peak potentials (Ep) of the cyclic voltammograms of Tripeptide-9 Citrulline at different concentrations in 1 mM $CuSO_4$ and 1 M Na_2SO_4 solution (working solution). "c" and "a" indicate cathodic and anodic sweep, respectively).*

[Tripeptide-9 Citrulline] (mM)	Epa (mV)	Epc (mV)	Voltammogram
0	Epa(1): -357 Epa (2): -186	Epc(1): -67 Epc(2): +60 Epc(3): +266	Figure 5C
1	Epa(1): -777 Epa(2): -55	Epc(1): -305 Epc(2): -15 Epc(3): +357	Figure 5D
2	Epa(1): -777 Epa(2): -15	Epc(1): -313 Epc(2): -12 Epc(3): +421	Figure 5E
20	Epa(1): -742 Epa(2): +12	Epc(1): -456 Epc(2): +464	Figure 5F

Figure 5D shows the cyclic voltammogram of 1 mM Tripeptide-9 Citrulline + 1 mM $CuSO_4$/1 M Na_2SO_4 solution with Table 2 listing the corresponding peak potentials. Two reduction peaks in the cathodic sweep and three oxidation peaks in the anodic sweep were observed. Compared to cyclcic voltammetry data of the 1 mM $CuSO_4$/1 M Na_2SO_4 solution (Figure 5C), a potential shift in peaks is evident in Figure 5.D. Epc(2) in 1 mM $CuSO_4$/1 M Na_2SO_4 was -186mV (Table 2) and when 1 mM Tripeptide-9 Citrulline was added to the working solution, the peak shifted to -55mV. In addition, the corresponding anodic peak shifted too: Epa(2) in 1 mM $CuSO_4$/1 M Na_2SO_4 was -67 mV and when 1mM Tripeptide-9 Citrulline was added the peak shifted to -15 mV.

The change in the peak potentials of the cyclic voltammograms when Tripeptide-9 Citrulline was added to the copper solution reveals that there is an interaction between the Tripeptide-9 Citrulline and the copper ions. It seems that when Tripeptide-9 Citrulline was added, the electrochemical processes of the copper reduction/oxidation became more reversible. Figure 5E shows the cyclic voltammogram of 2 mM Tripeptide-9 Citrulline + 1 mM $CuSO_4$/1 M Na_2SO_4 solution and the corresponding peak potentials are listed in Table 2.

It should be noted that when the concentration of the Tripeptide-9 Citrulline was increased to 2 mM the peak potentials changed again (Table 2). The main difference observed when the concentration of Tripeptide-9 Citrulline was increased was the change in Epc(2) and Epa(2). Note that both peak potentials, Epc(2) and Epa(2), evolved to more anodic potentials when the concentration of Tripeptide-9 Citrulline was increased. In addition, the gap between both potentials decreased as a function of the Tripeptide-9 Citrulline concentration. Again, it seems that the electrochemical reversibility of the copper reduction/oxidation processes increased as a function of the Tripeptide-9 Citrulline concentration. Figure 5F shows the cyclic voltammogram of 20 mM Tripeptide-9 Citrulline + 1 mM $CuSO_4$/1 M Na_2SO_4 solution and the corresponding peak potentials are listed in Table 2.

DNA protection by metal chelating ability of Tripeptide-9 Citrulline

The damage to DNA produced by Fe(II)-mediated Fenton reactions, with sequence-specific cleavage, has been reported.[18] Moreover, exposure to Fenton reagents leads to the formation of in-strand cross-link lesions where the neighboring nucleobases in the same DNA strand are covalently bonded.[19]

The spectrophotometric and electrochemical investigations described above give information about the complexation of metal transition ions by Tripeptide-9 Citrulline; in order to study the protective effect of this chelating ability of DNA against stress conditions of the Fenton reaction (Scheme 1), a proof of the concept of tertiary-structured DNA protection was designed. This proof was provided by electrophoresis experiments using commercially available plasmid pBR322. The electrophoretic pattern of pBR322 (Figure 6, Lane 3) shows two bands with different mobilities corresponding to covalently closed (CCC) and open form (OC). pBR322 was incubated in the Fenton reaction conditions with Cu(II) and H_2O_2 (see Materials and Methods for details) in the absence (Figure 6, Lane 1) or the presence (Figure 6, Lane 2) of the Tripeptide-9 Citrulline. In the absence of the Tripeptide-9 Citrulline H_2O_2 (Figure 6, Lane 1), the bands CC and OC bands are no longer observed, and a single diffuse band was observed instead. This is attributed to the Fenton reaction that has taken place, resulting in the emergence of various free radicals such as hydroxyl radicals, which ultimately have degraded the plasmid. However, results of the experiment performed in the presence of Tripeptide-9 Citrulline are completely different: when Tripeptide-9 Citrulline is present at the beginning of the incubation with the Fenton reagents, the electrophoretic profile (Figure 6, Lane 2) is similar to the one of untreated plasmid (figure 6, Lane 3), indicating that DNA remains virtually unchanged.

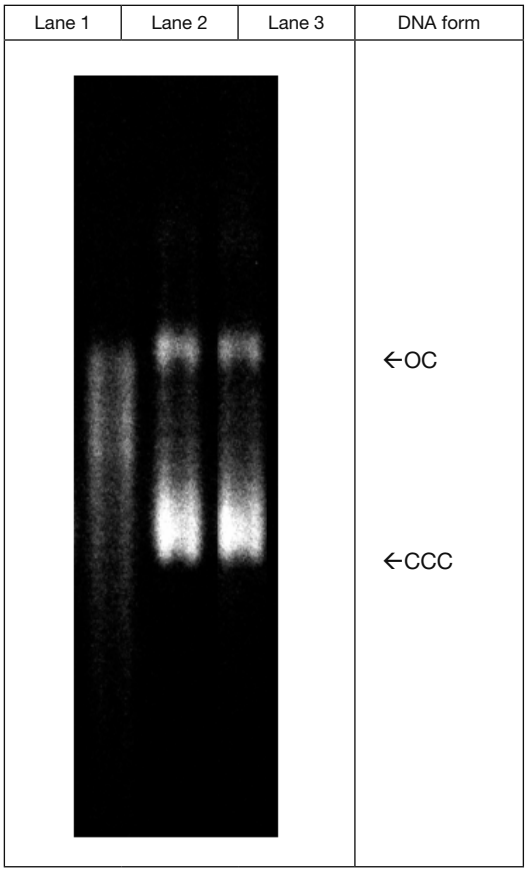

Figure 6. *Effect of the Fenton reaction in the electrophoretic mobility of DNA pBR322 plasmid. For experimental details, refer to "Materials and Methods" section.*

This constitutes a proof of the ability of Tripeptide-9 Citrulline to complex metal ions present in solution, preventing them from reacting with the hydrogen peroxide present in the medium and the subsequent inhibition of free radicals formation, thus offering an effective protection of DNA against Fenton degradation reaction.

Scheme 1. *Reaction of metal species with H_2O_2 generating ROS, adapted from literature [8,9].*

$Me^{n\oplus} + H_2O_2 \rightarrow Me^{(n-1)\oplus} + O_2^{\bullet\ominus} + 2H^{\oplus}$ adapted from[8]

$Me^{(n-1)\oplus} + H_2O_2 \rightarrow MeOOH^{\oplus} + H^{\oplus}$ adapted from[8]

$Me^{(n-1)\oplus} + H_2O_2 \rightarrow Me^{n\oplus} + HO^{\bullet} + OH^{\ominus}$ adapted from[9]

CONCLUSIONS

UV-VIS Spectrophotometry and cyclic voltammetry have shown to be valuable tools for demonstrating the ability of a peptide to chelate transition metal ions. The presence of isosbestic point in UV-VIS spectra at different ratios of peptide/metal and the change in the peak potentials of the cyclic voltammograms when Tripeptide-9 Citrulline was added to the copper solution are two independent confirmations of the presence of interaction between the Tripeptide-9 Citrulline and Cu(II) ions in aqueous solution. Interactions of Tripeptide-9 Citrulline with other transition metals of similar electronic configuration such as Fe(II) are expected. Furthermore, the ability of Tripeptide-9 Citrulline to complex metal transition ions provides protection of biomolecules from Fenton degradation reaction involving the formation of several ROS. Electrochemical reversibility of the copper ions reduction/oxidation processes increased with the Tripeptide-9 Citrulline concentration. According to this interaction, the introduction of this peptide in cosmetic preparations can provide protection against cellular damage by different reactive species formed between metal ions and biological molecules.

ACKNOWLEDGEMENTS

The authors thank Dr. Virtudes Moreno (Universitat de Barcelona, Departament de Química Inorgànica) for helpful discussions.

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