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Gamma Ray Irradiation of the Vasoactive Peptide Bradykinin Reveals a Residue- and Position-Dependent Structural Modification

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Abstract In this work the effect of radical species generated by gamma ray irradiation of aqueous solution upon structure of vasoactive peptide bradykinin (BK, RPPGFSPFR) was investigated. Increasing doses of 1–15 kGy Co^{60} gamma radiation were applied to BK solutions and a progressive degradation of its structure in a non-linear mode was observed. Two main peptide derivatives generated by these treatments were isolated and characterized through a combined amino acid analysis and daughter ion scanning mass spectrometry approach. Notably, it was observed that only the Phe residue located at position 8 and not 5 of BK was oxidized by reactive hydroxyl radical species given rise to Tyr^8-BK and *m*-Tyr^8-BK analogues. Comparative circular dichroism (CD) experiments of these peptides revealed that BK presents greater conformational similarity to Tyr^8-BK than to *m*-Tyr^8-BK. These results are in agreement with the biological potencies of these compounds measured in rat uterus and guinea pig ileum muscle contractile experiments. In summary, gamma irradiation of BK solutions revealed a residue- and surprisingly, position-structural modification effect of reactive radicals even in small peptides. Also of value for peptide chemistry field, the approach of applying controlled strong electromagnetic radiation in solution seems to be an alternative and unique strategy for generating, in some cases, peptides derivatives with uncommon structures and valuable for their further therapeutic potential evaluations.

Keywords Peptide · Bradykinin · Gamma radiation · Biological activity

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Introduction

A large amount of works reporting the effect of radiolysis on macromolecules has been described to date. Most of them have focused mainly on the role played by the reactive oxygen species (ROS) generated by strong electromagnetic upon protein structures in solution (Butler et al. 1984; Stadtman and Levine 2003; Nascimento et al. 1966; Hawkins and Davies 2001; Garrison 1987). However, many aspects regarding the mechanism of this radiolytic effect are still unclear. This fact seems to be due to a complexity of factors that come into play during the interaction between reactive species and the target macromolecules. Such factors may include not only the type and intensity of radical species but also the structure and conformation of the compound, the solvent system and its accessibility, etc. Needless to emphasize the relevance of this type of investigation as many types of physiological disorders are related to the action of these reactive compounds (Simic 1994; Selkoe 1994; Romero and Reckelhoff 1999; Opara 2004).

Recently we reported (Nardi et al. 2008) an evaluation of the effect of controlled gamma radiation on the structure

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of small peptides in solution, starting with the potent vasoconstrictor peptide angiotensin II (DRVYIHPF, AngII) (Catt et al. 1993; Oliveira et al. 2007). In order to gain further insight to this line of research, we now decided to study the effect of this strong electromagnetic radiation on bradykinin (RPPGFSPFR, or BK) structure. This relevant vasoactive peptide is known to be involved in various physiological processes including hypotension, inflammation, vasodilatation and pain (Bhoola et al. 1992; Regoli and Barabe 1980). For years, attention has given by our group to examine the structure–function relationship of this nonapeptide but applying the electron paramagnetic resonance spectroscopy of paramagnetically labeled BK analogues (Nakaie et al. 2002; Schreier et al. 2004; Vieira et al. 2009). The labeling probe used for these experiments was the stable amino acid-type radical 2,2,6,6-tetramethyl-1-oxyl-4-amino-4-carboxylic acid (TOAC), previously incepted in the peptide chemistry field (Nakaie et al. 1981; Marchetto et al. 1993; Toniolo et al. 1998).

Thus, the present work follows with BK studies but now focusing on the possible occurrence of its structure modification induced by controlled gamma irradiation experiments. After this step, two main detected peptide derivatives were purified and further characterized in order to initiate the classical structure-function relationship study.

Materials and Methods

Materials

The *tert*-butyloxycarbonyl (Boc) amino acids were purchased from Bachem (Torrance, CA, USA). Solvents and reagents were from Aldrich or Sigma (St. Louis, MO, USA). Prior to their use, N,N′-dimethylformamide (DMF) was distilled over P_2O_5 under reduced pressure. All solvents and chemicals met the standards established by the American Chemical Society.

Methods

Peptide Synthesis and Purification

BK was synthesized manually according to the standard Boc-protocol (Barany and Merrifield 1980; Kates and Albericio 2000) and analogues produced by gamma irradiation experiments were purified through HPLC on a semi-preparative C_{18} column (Vydac, Hesperia, CA, USA). In this step, solvents A and B were aqueous 0.02 M ammonium acetate (pH 5) and 60% acetonitrile in solvent A, respectively (linear gradient of 30–70% B for 2 h, flow rate of 10 ml/min at 220 nm). In the other hand, the analytical HPLC analyses of peptides were performed in a system consisting of two model 515 HPLC Waters pumps, a Waters 717 auto sampler plus and a Waters 2487 dual λ absorbance detector (Waters, Milford, USA) and UV detection at 220 nm. The samples were analyzed on an analytical C_{18} column (Waters, Wilford, USA), using the following solvent systems: solvent A (H_2O containing 0.1% TFA); and solvent B (60% acetonitrile in solvent A). A linear gradient of 5–95% B in 30 min was employed at a flow rate of 1.5 ml/min and detection at 220 nm. HPLC injections were evaluated in duplicate for each sample with similar results.

Peptide Irradiation

Purified BK solutions were prepared in Eppendorf tubes with Milli-Q water at neutral pH to a final concentration of 1 mg/ml and irradiated in the presence of oxygen, at room temperature. Doses of 1–15 kGy Co^{60} gamma radiations were emitted by a Gammacell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) at a fixed rate of 3.32 kGy/h at the Institute for Energy Research and Nuclear Science in São Paulo University, Brazil. The electromagnetic radiation experiments with BK solutions were carried out in triplicate with similar results.

Amino Acid Analysis

The amino acid analyses of peptide samples were performed in duplicate after standard acid hydrolysis of each sample. A Biochrom 20 Plus Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK) was employed.

Mass Spectrometry

The LC/ESI-MS experiments were performed on a system consisting of a separation module Waters Alliance model 2690 and a photodiode array detector model 996, both from Waters (Milford, USA). These equipments were coupled to a mass detector model ZMD from Micromass (Altrincham, Cheshire, UK) and were controlled by a Compaq AP200 workstation. The samples were automatically injected onto a Waters narrow bore Nova-Pak column C_{18} (2.1 × 150 mm, 60 Å pore size, 3.5 µm particle size). The elution was carried out with solvents A (0.1% TFA/H_2O) and B (60% acetonitrile/0.1% TFA/H_2O) at a flow rate of 0.4 ml/min using a linear gradient of 5–95% B in 30 min and UV detection at 220 nm. The condition used for mass spectrometry measurements was a positive ESI.

Peptide sequencing determinations were carried out in a electrospray triple-quadrupole Quattro II equipment (Micromass, Manchester, UK) by applying the daughter ion scanning by collision induced dissociation method (Johnstone and Rose 1996). Each sample was analyzed in duplicate, with similar results.

CD Experiments

CD measurements were performed on a Jasco J-810 spectropolarimeter at room temperature and continually flushed with ultra-pure nitrogen. CD spectra were recorded using a 1 mm path length rectangular quartz cell, with four accumulations at 50 nm/min scan speed, 8 s response time, 0.5 nm spectral bandwidth and wavelength range of 260–190 nm. The spectra were obtained in 0.1 mM phosphate buffer (pH 7) and with 0.1 mM of the sample. The results are expressed in terms of molar ellipticity (θ) in units of degree cm^2 $dmol^{-1}$.

Bioassays

The biological experiments followed the previously reported method (Nakaie et al. 2002; Nardi et al. 2008). Muscle contractile responses were measured in rat uterus and guinea pig ileum and recorded by means of force-displacement transducers (model FTA-10; Hewlett-Packard, Andover, MA, USA) through an amplifier (model E805: Hewlett-Packard) and a potentiometric recorder (ECB model RB102). All pharmacological experiments followed current guidelines for the care and use of laboratory animals, as well as ethical guidelines for investigations and were pre-approved by the Animal Care Committee of the Federal University of São Paulo. All bioassay experiments were carried out in duplicate with equivalent results. The concentration-response curves were obtained by administration of increasing concentrations of the sample (90-s treatments), at 5-min intervals. The biological potencies were calculated in relation to the non-irradiated BK sample, taken as 100%.

Results and Discussion

Aqueous BK solutions (1 mg/ml) were exposed to controlled doses of 1, 2, 4, 6, 8, 10 and 15 kGy gamma radiation in the presence of oxygen, at room temperature and at a 3.32 kGy/h constant dose rate. The HPLC profiles of non-irradiated and irradiated BK solutions are displayed in Fig. 1.

By comparing these HPLC chromatograms and taking the peak area of non-irradiated BK sample (Fig. 1, panel A)—retention time (RT) of 13.6 min—as a control, it was possible to observe significant degradation of the peptide as the radiation doses increased. Possibly, the gamma ray irradiation induces the generation of a large amount of unknown aggregated materials arising mainly from radical-radical termination (Garrison 1987; Hawkins and Davies 2001). This hypothesis is supported by the progressive appearance of a broad peak centered at near 17 min in the HPLC chromatogram (mainly above 4 kGy) in parallel with the decrease in the height of BK peak. This peptide degradation process seems to initiate early, at approximately 1 kGy (Fig. 1b) and it was possible to observe the presence of some few isolated side products detected as small peaks having RT values of about 12–13 min (Figs. 1a–c).

Quantitatively, a typical non-linear decreasing curve was observed for this BK degradation process as a function of the increase in the dose of strong electromagnetic radiation (Fig. 2). Notably, these degradation products were no longer observed at 6–8 kGy, when the amount of remaining BK dropped to a minimum and reaching the 50% degradation yield after about 2 kGy irradiation dose. Of note, this finding indicated that the BK structure is less stable towards gamma radiation than that of AngII. In fact, for the latter compound, the degradation level reached value of about 50% only when a dose of approximately 4 kGy was applied (Nardi et al. 2008).

Structural Determination of Analogues I and II

In order to conduct an in-depth analysis of structural modifications that might be occurring in the BK molecule under gamma irradiation, we decided to isolate the two main side products observable mainly when 1 or 2 kGy radiation doses were applied (Fig. 1, panel 1B and 1C; peaks with RTs near 12–13 min). For this experiment, 50 mg of previously purified BK were thus submitted to 2 kGy radiation dose and the components of the treated solution were fractionated by semi-preparative HPLC as detailed in Methods. The two mentioned side products were successfully isolated and presented RT of about 12 and 13 min, respectively, in comparison with 14 min of BK. Amounts of 20.8 mg of BK and 2.3 mg and 1.9 mg of henceforth denoted analog I and analog II, respectively, were obtained.

To characterize these purified BK analogues, they were initially examined by their amino acid composition. The Table 1 displays the amino acid proportions found for the three peptide samples. It is noteworthy that for both analogues, only one Phe amino acid was detected concomitantly to the appearance of a Tyr residue in the case of analogue I. For the analogue II, an unidentified peak was detected in the amino acid analysis chromatogram with elution at 28.1 min against 29.3 min of Tyr, under the same chromatographic amino acid analysis conditions (data not shown).

To elucidate the exact structures of both BK analogues, we first decided to examine both isolated derivatives using an electrospray triple-quadrupole mass spectrometer through direct infusion of the peptide solution (at flow rate of 300 µl/min). Since the mass spectra profiles of the both BK analogues were identical, the analogue I data was taken

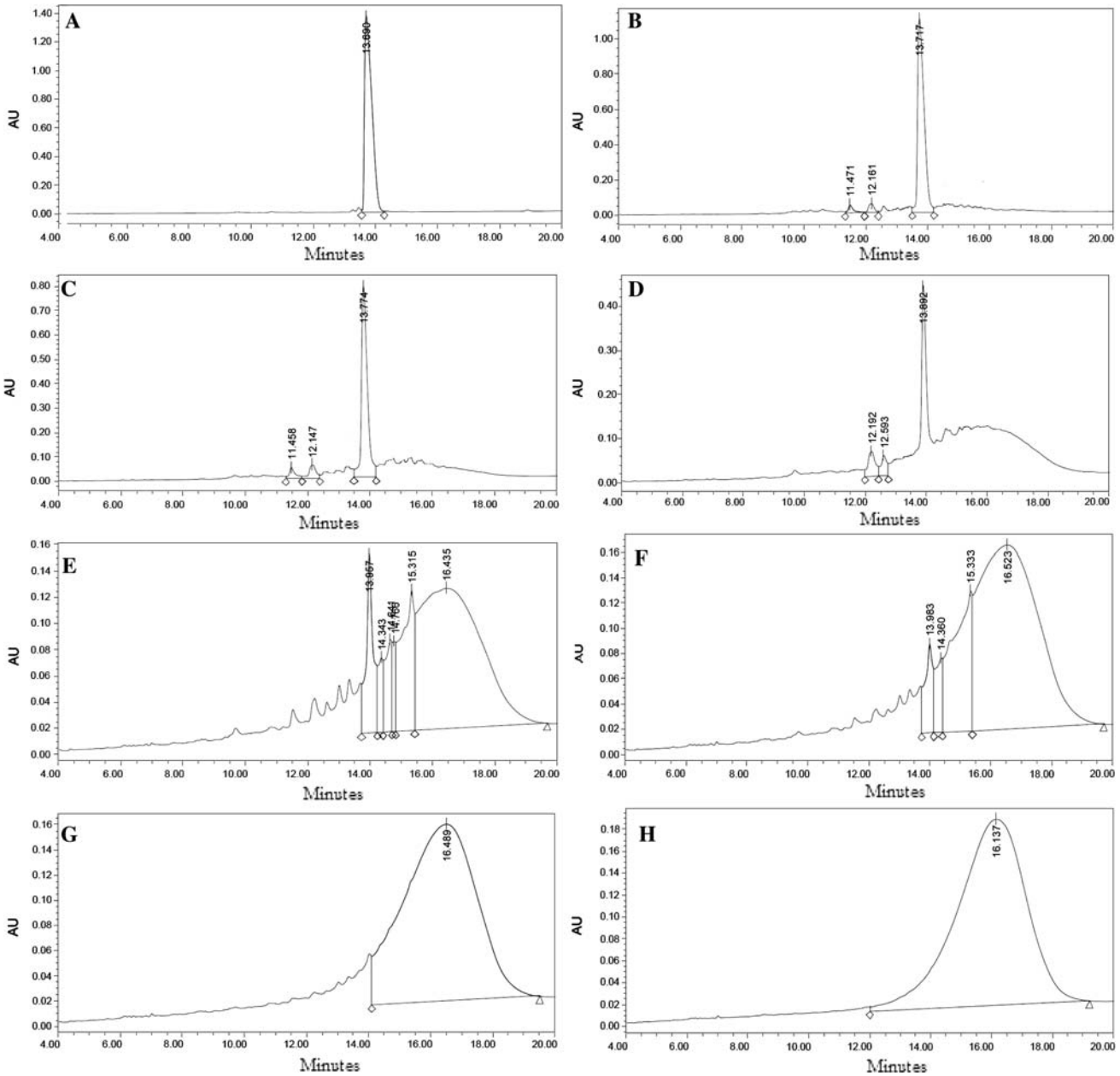


Fig. 1 HPLC profiles of solutions of non-irradiated BK (**a**) and irradiated with 1 (**b**), 2 (**c**), 4 (**d**), 6 (**e**), 8 (**f**), 10 (**g**) and 15 (**h**) kGy of gamma radiation doses

as representative of both altered peptides and was displayed in Fig. 3. As can be seen from this figure, there was a peak with $m/z = 539.0$ [M + $2H^+$], which corresponds to a molecular weight of 1076 Da, (Fig. 3a) i.e., 16 Da greater than that of BK (1060 Da). Taking into account the amino acid analysis results previously detailed (Table 1), one can suppose that this 16-Da increase in mass seems to be due to an oxygen atom addition at a single Phe residue yielding Tyr (analogue I) or an other compound (X) but with the same molecular weight of Tyr and present in the structure of analogue II.

At this point, the following item to be addressed argues whether it was the Phe residue at position 5 or 8 that was oxidized to Tyr or X in the analogues, since only one Phe residue was modified after the radiation experiment. To gain insight into this issue, we next analyzed the peptides through collision induced dissociation mass spectrometry (CID-MS/MS)(Johnstone and Rose 1996). Using this technique, it was possible to monitor sequentially the entire fragmentation pattern of the peptide, thus indicating which Phe residue was modified in the BK sequence. In this context, the CID-MS/MS spectrum of analogue I (similar

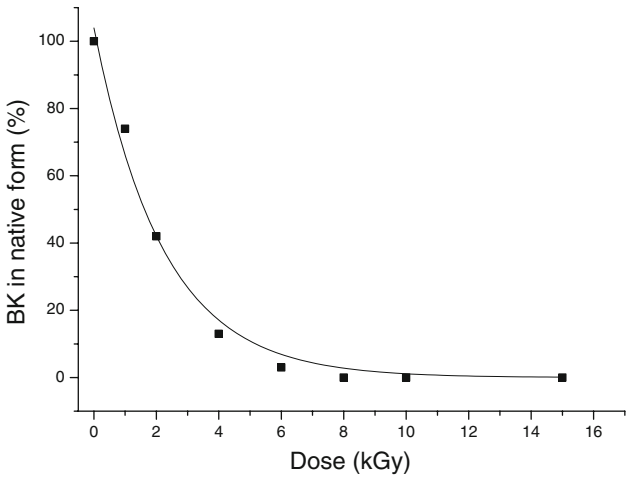


Fig. 2 Quantity of remaining (BK) (%) as a function of the gamma radiation dose

Table 1 Calculated amino acid proportion of BK and analogues I and II

Amino acid	BK	Analogue I	Analogue II
Ser	1.02	0.98	1.01
Gly	1.01	1.02	0.97
Tyr	0.0	0.98	0.0
Phe	1.97	1.03	1.02
Arg	2.02	1.97	2.01
Pro	2.96	3.00	2.97

to analogue II), with this respective prediction of ion fragmentations, is shown in Fig. 3b.

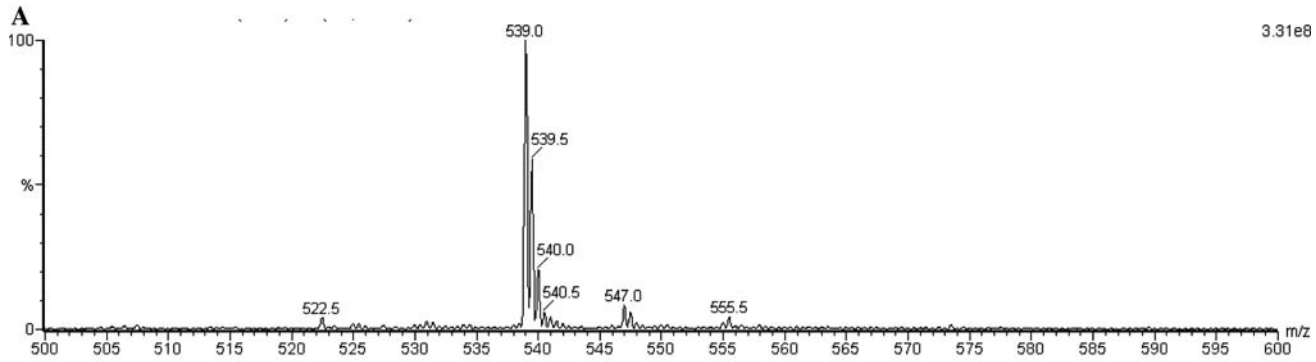
The $m/z = 539$ precursor ion was selected to be the most abundant during the fragmentation and a series of type *b* and *y* ions (Roespstorff and Fohlman 1984) were used for amino acid sequence deduction of each sample (see Fig. 3b and its corresponding table of CID-MS/MS data). The presence of fragment ion b6 ($m/z = 642.6$, corresponding to the RPPGFS sequence) demonstrated that the Phe^5 residue was present in both BK analogues. Otherwise, the presence of fragment ion y8 ($m/z = 920.7$, corresponding to the PPGFSPYR sequence), as well as that of ion b8 ($m/z = 903$, corresponding to the RPPGFSPY sequence), indicated that the Phe^8 residue was oxidized to Tyr (analogue I) or X (analogue II). These ions can be compared directly to predicted peaks of the BK sequence, in which y8 and b8 should be m/z 904.5 and $m/z = 886.5$, respectively (data not shown). Based on these findings, it was possible to conclude that analogues I and II have the same molecular weight (1076 Da) and with modification only at the Phe^8 residue, yielding RPPGFSPYR (analogue I) and RPPGFSPXR (analogue II).

Thus, the last issue to be solved was the structural identity of the X residue. In the previously discussed amino acid analysis results, we had confirmed that there was one less Phe residue in both peptides, as well as that the natural Tyr residue was only presented in analogue I. To clarify this point, we compared *p*-Tyr with its *o*-Tyr and *m*-Tyr isomers in terms of elution time in the amino acid chromatogram. The RT of 28.1, 29.3 and 30.2 min were obtained for *m*-Tyr, *p*-Tyr and *o*-Tyr amino acids, respectively. This finding demonstrates that the mentioned unknown peak appearing only in analogue II does in fact correspond to the *m*-Tyr derivative, and that its sequence is therefore *m*-Tyr^8-BK against Tyr^8-BK for analogue I.

The conversion of Phe to Tyr residue but yielding also the latter *meta* and *ortho* isomers has been already described in the literature when the Phe residue, either free in solution or inserted into a protein sequence is submitted to gamma irradiation (Biondi et al. 2006; Miyahara et al. 2000; Stadtman and Levine 2003). However, the amount of Phe to Tyr conversion and also the two Tyr isomers produced seem to be clearly dependent on experimental details regarding the radiolytic protocol applied. Different factors come into play to explain these results such as whether the Phe residue is free in solution or inserted in a macromolecular backbone, the type of solvent system including its pH and salt composition, the radiation intensity, etc. Moreover, depending upon the radiation strategy, some other amino acid residues can be modified through different mechanism as already reported (Xu et al. 2003; Xu and Chance 2004; Garrison 1987).

Nevertheless, the fact that gamma radiation affected only the Phe^8 and not the Phe^5 residue in the case of the BK structure, points to a surprising position-dependent effect for this amino acid. Interestingly, the Phe residue was also sensitive to gamma radiation in the AngII sequence, also giving rise in this preliminarily report (Nardi et al. 2008) to its substitution by Tyr. By comparing the sequences of these peptides (RPPGFSPFR for BK and DRVYIHPF for AngII), one can hypothesize that the Phe to Tyr transformation is more facilitated when containing the Pro imino acid coupled to its amine function. The steric constraints that the Pro residue in either *trans* or *cis* conformation might impose on its immediate neighbors has been already reported (Pullmann and Pullmann (1974). This factor would explain an apparent difference in the reactivity of Phe residue when located at position 5 or 8 in the BK structure. In close accordance with these findings, previous conformational work with this peptide (Lintner et al. 1977) has demonstrated that its Phe^8 residue seems to be more rigidly coupled to the peptide backbone than its Phe^5 partner thus facilitating the hydroxyl radical attack in the aromatic moiety of the former compound.

To reinforce this observed Phe residue position-dependence towards hydroxyls radicals attack, careful electrospray ionization mass scanning of each irradiated BK



a	129.1	226.2	323.2	380.2	527.3	614.3	711.4	874.5	-
b	157.1	254.2	351.2	408.2	555.3	642.3	739.4	902.5	-
c"	174.1	271.2	368.2	425.3	572.3	659.4	756.4	919.5	-
i	129.1	70.1	70.1	30.0	120.1	60.0	70.1	136.1	129.1
	1	2	3	4	5	6	7	8	9
	Arg	**Pro**	**Pro**	**Gly**	**Phe**	**Ser**	**Pro**	**Tyr**	**Arg**
	9	8	7	6	5	4	3	2	1
x	-	946.4	849.4	752.3	695.3	548.2	461.2	364.2	201.1
y"	-	920.5	823.4	726.4	669.3	522.3	435.2	338.2	175.1
z	-	903.4	806.4	709.3	652.3	505.2	418.2	321.2	158.1

Fig. 3 Mass spectra of analogue I (identical to analogue II) pointing out the $m/z = 539$ [M + $2H^+$] (**a**); CID-MS/MS ion fragmentation data (**b**) and table containing the expected a and y ion fragment series (**c**)

solutions did not detect peptides corresponding to Tyr^5-BK or even Tyr^5-Tyr^8-BK analogues (data not shown). Only two mass peaks i.e., 1060 and 1076 Da which correspond to BK (RT = 14 min) and its two oxidized analogues (RTs = 12 and 13 min) were detected in our experimental conditions (data not shown). The fact that already published results indicated similar reactivity for two adjacent Phe residues in the Glu-fibrinopeptide (Maleknia et al. 1999) or more recently, a clear radical attack dependence to the presence of neighboring negative groups (Sharp and Tomer 2006) only come into play for arguing in favor of the complexity of factors that might govern the radical reactivity outstanding issue.

Analogues I and II: Structure–Functional Relationship

To gain insight to structure-function relationships of both identified BK analogues, CD experiments were carried out in order to acquire structural data for further correlation with their corresponding biological potencies. Figure 4 displays comparatively the CD curves obtained for the samples and as previously reported (Denys et al. 1982; Lintner et al. 1977), a population of extended and flexible conformations was observed for BK. Comparatively, greater similarity in the shape of the spectra was observed between this peptide and that of analogue I (Fig. 4a and b, respectively) rather than that of analogue II (Fig. 4c), thus

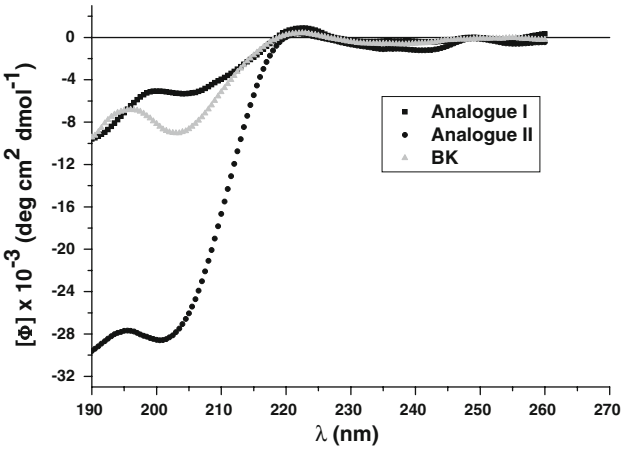


Fig. 4 Circular dichroism spectra of BK and isolated analogues I and II in PBS solution, pH 7.0

suggesting different conformations for the BK derivative However, one must to be aware that besides the conformational factor, these CD spectral features could also be indeed, induced by different electronic transitions properties of the *para*- and *meta*-phenol chromophores in the amide region and present in the structures of the analogues I and II, respectively.

In agreement with these peptide structural CD data, the results of the biological experiments showed that analogue I presented 50% (in rat uterus) and 10% (in guinea-pig ileum) of the activity observed for BK, respectively. These values are greater than those measured for analog II (8% in both muscle preparations). These results are in accordance with earlier reports which described the importance of the Phe^8 residue for the maintenance of BK biological potency (Bhoola et al. 1992, Regoli and Barabe 1980). In addition, these initial results also strongly suggest the existence of a direct relationship between the structure and biological properties of these two BK analogues.

Concluding Remarks

The vasoactive peptide bradykinin (RPPGFSPFR, or BK) was submitted to gamma radiation doses of 1–15 kGy and a non-linear process of its degradation occurs. Surprisingly, although inserted internally at the BK structure, only the Phe residue at position 8 (and not that at position 5) was modified, generating Tyr^8-BK and *m*-Tyr^8-BK derivatives. This indicates that, even in small peptides, this radiation has a residue- and more importantly, sequence-dependent effect. The presence of a Pro residue neighboring the N-terminal extremity of Phe residue seems to facilitate this aromatic residue oxidation by reactive hydroxyl radicals. In complement, circular dichroism revealed that BK presents greater structural similarity to Tyr^8-BK than to *m*-Tyr^8-BK, which is in agreement with their biological potencies. The present strategy of applying controlled gamma irradiation to a peptide structure might be therefore relevant as a different experimental strategy to deliberately produce new and, in some cases, uncommon peptide derivatives not so easily obtained through standard peptide synthesis methodology.

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