

Structural and physical–chemical evaluation of Bradykinin Potentiating Peptide and its high soluble supramolecular complex

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Abstract The supramolecular interactions between a Bradykinin Potentiating Peptide (BPP10c) and β -cyclodextrin (β CD) have been investigated by using several techniques. These new properties acquired by the inclusion phenomena are important in developing a strategy for pharmaceutical formulation. The BPP10c structural elucidation and its inclusion complex formed have been investigated using Nuclear Magnetic Resonance techniques. The peptide secondary structure was investigated using infrared spectroscopy in solution, Circular Dichroism and NMR. In addition, the thermodynamic parameters of the inclusion process were also evaluated using Isothermal Titration

Calorimetry. The results obtained by these physical–chemical techniques suggested a 1:1 complex formed by interaction between the Tryptophan amino acid residue and the β CD cavity. The peptide secondary structure was not substantially modified for the inclusion process. In addition, the inclusion process proved to be spontaneous ($\Delta G^\circ = -2.53 \text{ kcal mol}^{-1}$), with an enthalpy reduction ($\Delta H^\circ = -3.72 \text{ kcal mol}^{-1}$) and a favored entropic variation ($T\Delta S^\circ = -1.19 \text{ kcal mol}^{-1}$).

Keywords Peptides · β -cyclodextrin · Bradykinin Potentiating Peptides · Supramolecular complexes · Intermolecular interactions · Secondary structure

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Introduction

In 1965 Sergio Ferreira et al. demonstrated that the venom of Brazilian snake *Bothrops jararaca* contained small toxins, called Bradykinin Potentiating Peptides (BPPs), which greatly enhance the smooth-muscle contraction, induced by Bradykinin [1]. Later, other experiments developed by the same research group demonstrated the activity of these peptides on the Angiotensin-I Converting Enzyme (ACE) inhibition [2]. ACE acts by generating angiotensin II and degrading Bradykinin, playing a key role in regulating the humoral blood pressure [3, 4].

The BPPs are peptides of 5–14 amino acids residues with Pyroglutamyl and Proline residues at the N-terminal and C-terminal, respectively, and contain several internal Proline residues [5]. Recently, it has been demonstrated that the BPPs, including BPP10c, when produced at low doses create potent and sustained anti-hypertensive effects in spontaneously hypertensive rats [6].

The shorter BPPs are particularly more susceptible to enzymatic hydrolysis, rendering them impracticable for oral routes [7]. Clinical studies verified that the therapeutic use of the BPPs were limited by lack activity after oral route, in animal models and human subjects [8–10]. From one viewpoint, the peptides and proteins in pharmaceutical formulations and their use through oral route can be a challenge, once these molecules become both enzymatic and chemical instable. These compounds present poor absorption through biological membrane, peculiar dose–response curves, and rapid plasma clearance among others, mainly because the great number of peptides and proteins are preferentially absorbed by the colon [11–15].

In this sense, cyclodextrins (CDs) could be a strategic alternative as peptide and protein carrier systems once the cyclodextrin–drug conjugate is able to survive passage through stomach and small intestine, and release the drug at the colon where it is not only the preferential site of peptides or proteins adsorption, but also the CDs enzymatic degradation [13]. Recently, it was demonstrated that association between a Bradykinin Potentiating Peptide (BPP5a) and β CD was able to reduce the blood pressure in spontaneously hypertensive rats. This observation was attributed to the increase of the BPP5a biodisponibility caused by its inclusion in CD cavity [16].

The CDs have a toroidal shape with a hydrophobic cavity, in which can be inserted a variety of organic and inorganic guest molecules, with formation of inclusion complexes (ICs) [17–20]. The hydrophilic nature of the outer surface of the cyclic structure makes the CDs somewhat water-soluble [21]. The widest natural CDs molecules investigated capable of forming ICs are α -, β - and γ -cyclodextrins with six, seven and eight glucopyranose units, respectively.

Supramolecular matrixes using CDs have attracted extensive attention because of their potential in several areas of science and technology [18, 22]. CDs, in particular, have pharmaceutical applications as high-performance biomaterials in drug delivery systems, due to their multifunctional characteristics and bioadaptability [23–25]. The study of drug complexation with CDs is one of the most extensively researched fields. CDs offer several advantages in drug delivery including improved drug solubilization and protection against physical–chemical and enzymatic degradation, as well as their potential for enhanced absorption [26]. In addition, investigation of complexes in solution at different molar ratios with high soluble drugs has been also reported in the literature. These assemblies in solution could prove to be a promising drug controlled release system [27, 28].

Another relevant characteristic of the CDs is its ability to possibly recognize not only the size and the shape, but also the chirality of amino acids [12, 29, 30]. However,

structures of many peptides and proteins are too hydrophilic and bulky to be wholly included in the CD cavity, and the topological constraints of peptide backbone might reduce the formation of inclusion complexes. Thus the host–guest interactions could be local with hydrophobic amino acid residue, forming the supramolecular system [12].

The non-covalent interactions established between the CDs with peptides and proteins could be responsible for affecting the three-dimensional structure of the guest molecules [31] or inhibiting their intermolecular association, modifying the secondary structure of the peptide, and thus changing their chemical and biological properties [12, 13]. For this reason, understanding the nature of interactions in host–guest systems is fundamental to the design and control of supramolecular systems.

Thus, in this article, we report the decapeptide (Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro-OH, BPP10c) structural and physical–chemical characterization (Fig. 1). In addition, the interaction of this peptide with β -cyclodextrin (β CD) was also evaluated to propose the stoichiometry and topological structure of the supramolecular complex formed in solution.

Firstly, the pure BPP10c was characterized by 1D (^1H and ^{13}C) and 2D (COSY $^1\text{H}/^1\text{H}$, TOCSY $^1\text{H}/^1\text{H}$ and HSQC $^1\text{H}/^{13}\text{C}$) Nuclear Magnetic Resonance (NMR) techniques. The supramolecular complex β CD/BPP10c was investigated using 1D and 2D NMR experiments. In this context, the NOe effects are one of the most important in providing deeper insights regarding the topology of the supramolecular complex [20, 27, 32–34].

The Horizontal Attenuated Total Reflectance (FTIR-HATR), in solution, was also used to evaluate the BPP10c secondary structure in presence and absence of β CD. This spectroscopic approach has been used to characterize peptides and proteins structurally, by evaluation of the amide I band, and their results are commonly compared with those obtained by Circular Dichroism spectroscopy [35–38]. For this reason, Circular Dichroism was carried out in order to evaluate the peptide and its IC secondary structure. Isothermal Titration Calorimetry (ITC) experiments were also performed to obtain the thermodynamic parameters for the BPP10c complexation process with β CD [27, 39].

Results

Nuclear Magnetic Resonance

^1H NMR spectrum of BPP10c in D_2O and $\text{D}_2\text{O}/\text{H}_2\text{O}$ solution are depicted in Fig. 2a and b, respectively, and hydrogen peptides chemical shift is presented in Table 1.

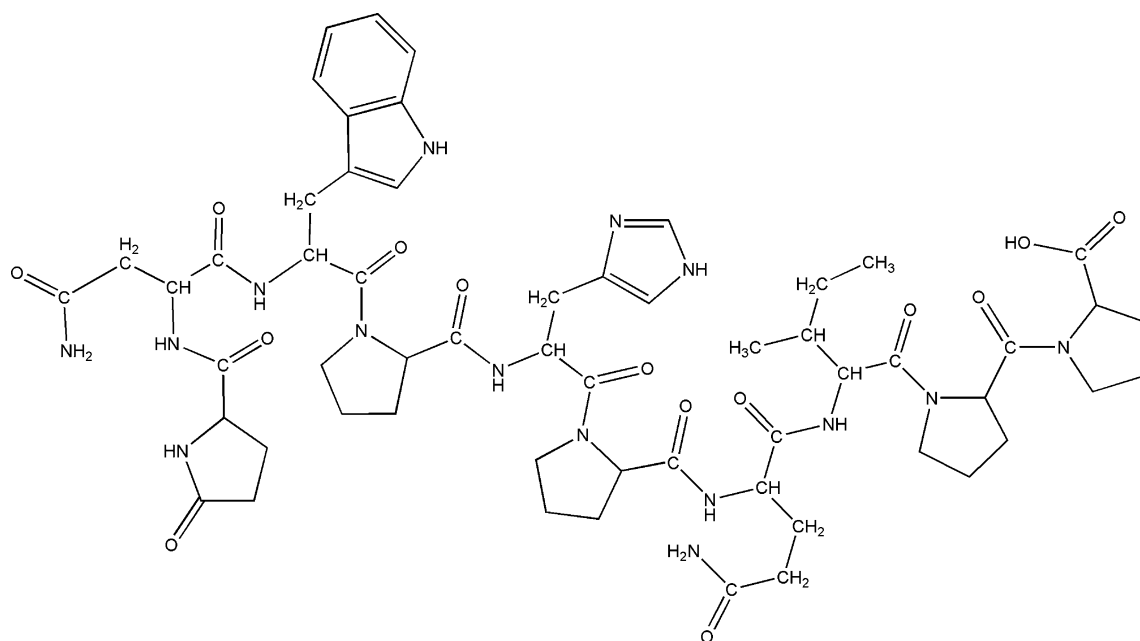


Fig. 1 Chemical primary structure of BPP10c, (Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro-OH)

Fig. 2 ^1H NMR spectrum for BPP10c in (a) 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ (500 MHz) and (b) pure D_2O (400 MHz)

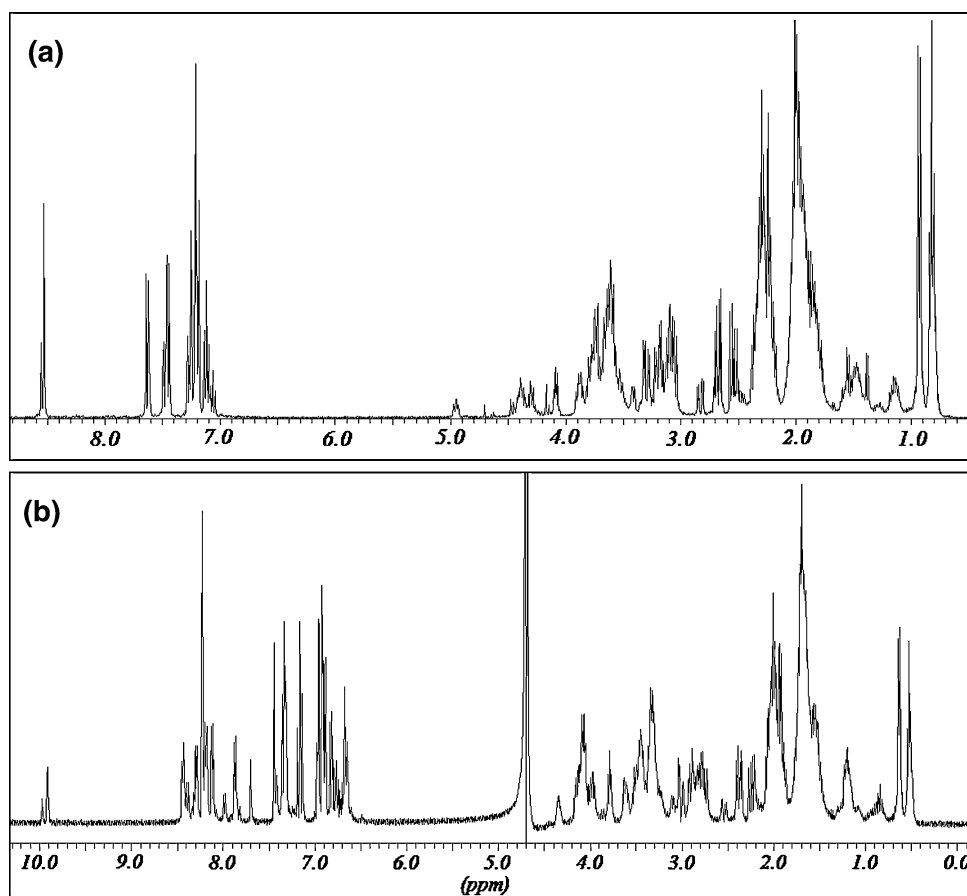


Figure 3 presents the TOCSY $^1\text{H}/^1\text{H}$ contour map, which was used to identify the chemical shifts of each amino acid residue. The presence of more than one set of chemical

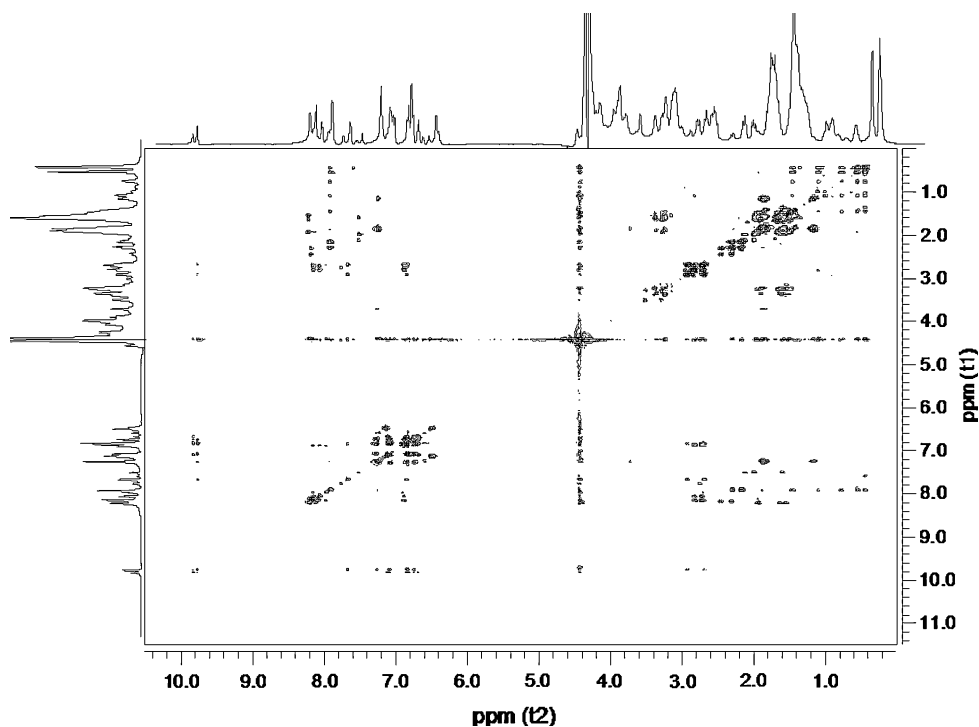
shifts for each amino acid residue was the main difficulty in assigning all hydrogen peptides, mainly for the Proline (Pro) residues. These different structures can be better verified by

Table 1 NMR ^1H chemical shifts of BPP10c (400 MHz at 5 °C) in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$

Residue	δ_{NH} ($J_{\text{NH-H}\alpha}$)	$\delta_{\alpha\text{-H}}$	$\delta_{\beta\text{-H}}$	δ others
Pyr	8.43	3.98	1.62 (1.77)	γ (CH_2) 2.03
Asn	8.13	4.36	2.35 (2.24)	
Trp	7.98	4.11	2.84 (2.90)	1(NH) 9.91 2(CH) 6.92 4(CH) 7.16 5(CH) 6.75 6(CH) 6.83 7(CH) 7.16
Pro ^a	8.42	4.04	1.66	γ (CH_2) 2.07 δ (CH_2) 3.34
His	7.87	4.68	3.03 (2.79)	2(CH) 8.24 4(CH) 6.97
Pro ^b				
Gln	8.45	3.98	1.63 (1.20)	γ (CH_2) 2.01
Ile	8.19	4.15	1.54	γ (CH_3) 0.62 γ (CH_2) 1.18 (0.85) δ (CH_3) 0.52
Pro ^b				
Pro ^b				

^a Hydrogen chemical shifts assigned using 1D projections obtained by the TOCSY contour map

^b Proline residues could not be distinguished one from each other

Fig. 3 TOCSY $^1\text{H}/^1\text{H}$ contour map for the BPP10c (500 MHz) using 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ 

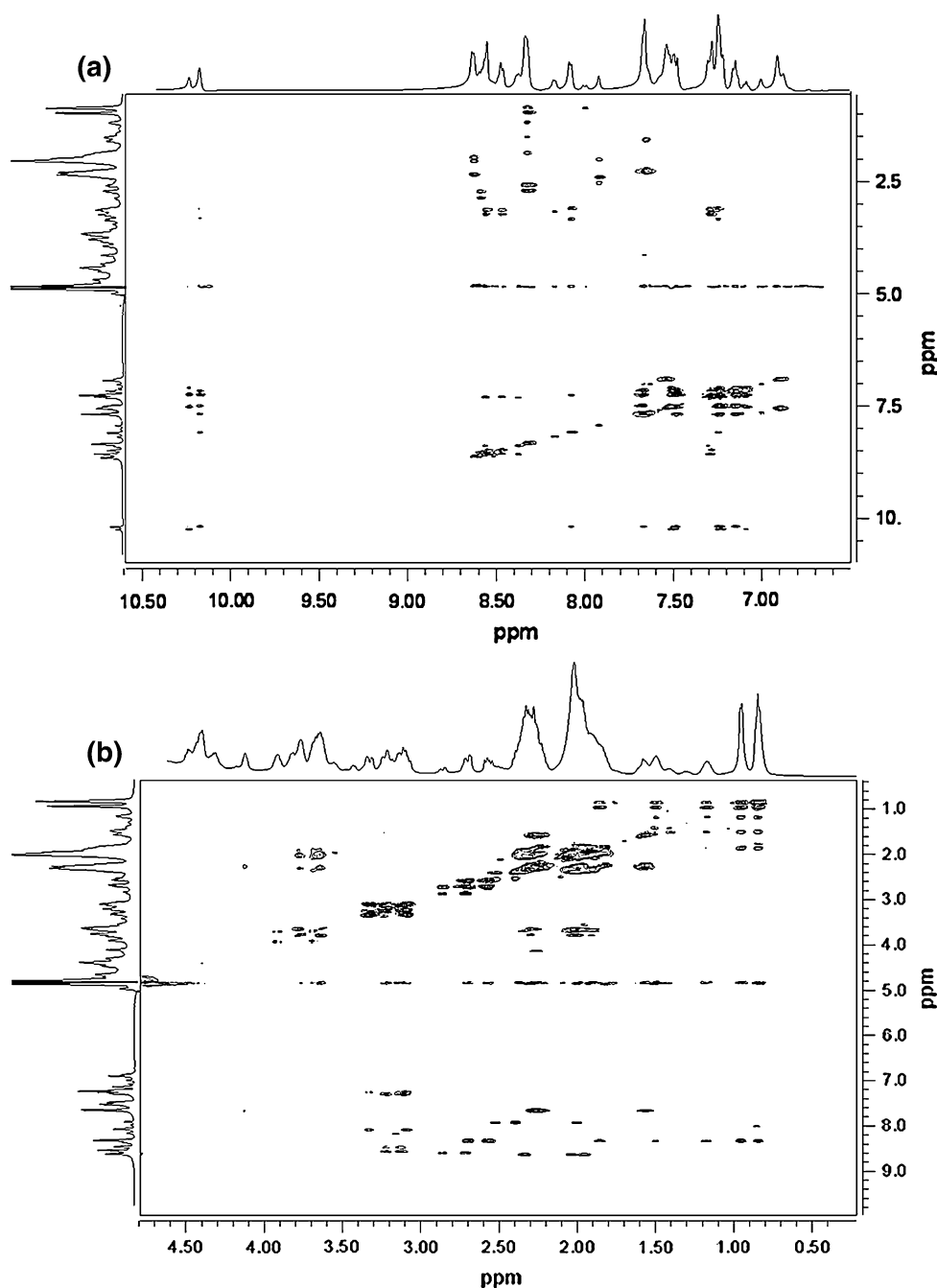
the existence of two different signals for the Tryptophan (Trp) N–H at $\delta \approx 9.91$ and three different β_{H} signals for the Asparagine (Asn) residue at: 2.24, 2.37 and 2.54, Fig. 4a and b. Thus, Pro hydrogens of each residue could not be assigned separately, once an overlap of these hydrogens was observed. However, these hydrogens were identified for their regions: β_{H} (δ 1.60–1.80), γ_{H} (δ 1.83–2.14) and δ_{H} (δ 3.32–3.52). The existence of more than one peptide secondary structure in solution could also be verified in HSQC $^1\text{H}/^{13}\text{C}$, in which more than an expected number of α_{H} was verified, Fig. 5a. HSQC $^1\text{H}/^{13}\text{C}$ was also extremely useful in assigning the hydrogens of the aromatic region, Fig. 5b.

Figure 6 presents the ^1H NMR spectrum for the $\beta\text{CD}/\text{BBP10c}$ complex. The presence of two different peptide secondary structures could also be confirmed by the presence of two distinguishable hydrogen signals for the Trp N–H at $\delta = 9.82$, as it was observed for the pure BPP10c.

Moreover, a slight chemical shift variation was verified for the majority of the hydrogens signals. This change in the hydrogen chemical shift was mainly observed for the aromatic residues Trp and Histidine (His), and also for the N–H ($J_{\text{NH-H}\alpha}$), Fig. 6.

In order to confirm the interaction in this supramolecular system, 2D NOESY experiments were carried out. Figure 7 presents the 2D NOESY contour map for this system, in which one can verify dipolar correlations between the aromatic hydrogens of the Trp residue and the internal hydrogens of the βCD .

Fig. 4 TOCSY $^1\text{H}/^1\text{H}$ contour map for the BPP10c (500 MHz) using 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ (a) aromatic region and (b) aliphatic region



DOSY experiment, Fig. 8, was carried out in order to investigate the interaction among the species in solution and in the DOSY contour map. Only one diffusion coefficient time was identified, $\log D \approx -9.48 \text{ cm}^2 \text{ s}^{-1}$.

Isothermal Titration Calorimetry

ITC was used to evaluate the thermodynamic parameters of the supramolecular system formation. ITC is a powerful technique used to study the host guest non-covalent

interactions [40]. In addition, information about the peptide aggregation process can also be obtained using ITC curves [41]. However, ITC results must be interpreted carefully, as changes in enthalpy are likely to arise from several phenomena, namely: binding of CD to peptide, aggregation of the complexes, and also conformation changes of the peptide structure, and peptide self-association/dissociation [42].

Figure 9 shows the BPP10c dilution curve in water and peptide titration in βCD aqueous solution. The BPP10c dilution process in water exhibits an exothermic profile,

Fig. 5 HSQC $^1\text{H}/^{13}\text{C}$ contour map (400 MHz) at 5 °C in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ (a) for the aliphatic region including α -H and (b) for the aromatic region

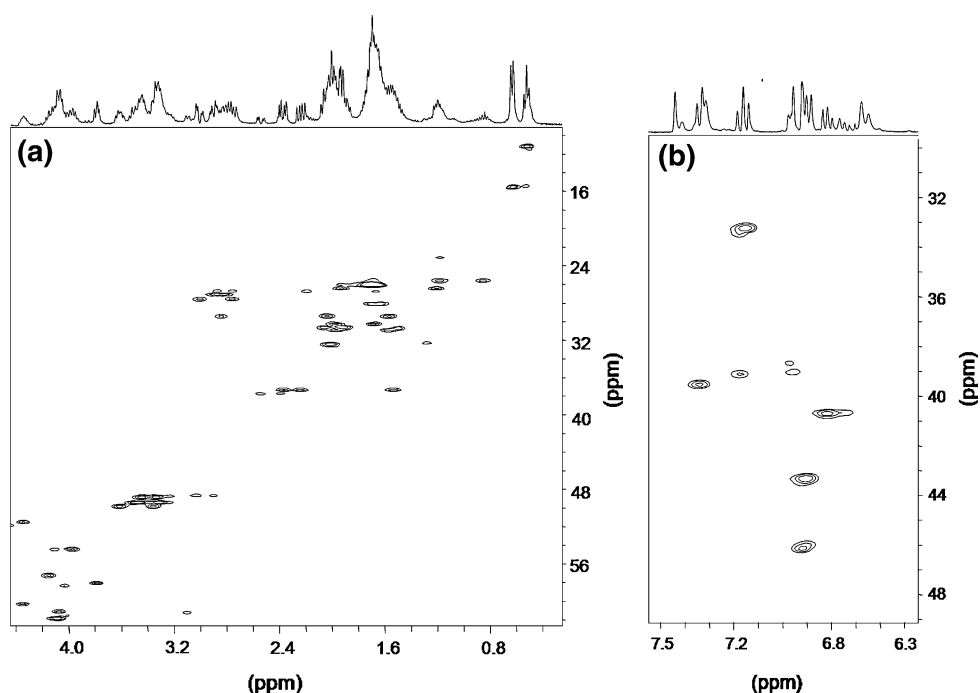
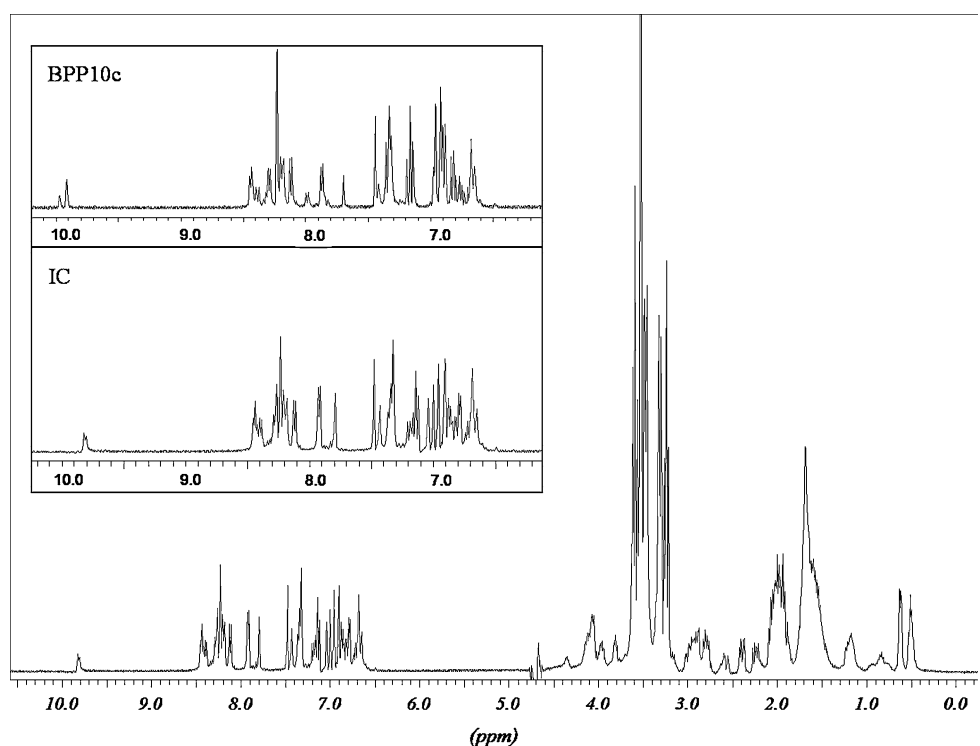


Fig. 6 ^1H NMR spectrum for IC at 1:1 M ratio (500 MHz) in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ and the expansion for the aromatic region, comparing pure peptide and its IC



and this result could be associated with the peptide self-aggregation. The BPP10c titration in βCD solution also assumes exothermic values.

The titration of βCD with BPP10c does not present an inflection for the equivalence titration point. Thus, the thermodynamic parameters were calculated through the

non-linear fit assuming 1:1 stoichiometry, as previously suggested by NMR results, in one set sites model present in Microcal Origin for ITC [43]. Using this approach the enthalpy change observed was ($\Delta H^\circ = -3.72 \pm 0.02 \text{ kcal mol}^{-1}$), the entropic contribution was ($T\Delta S^\circ = -1.19 \text{ kcal mol}^{-1}$) with a $K = 70 \pm 22$.

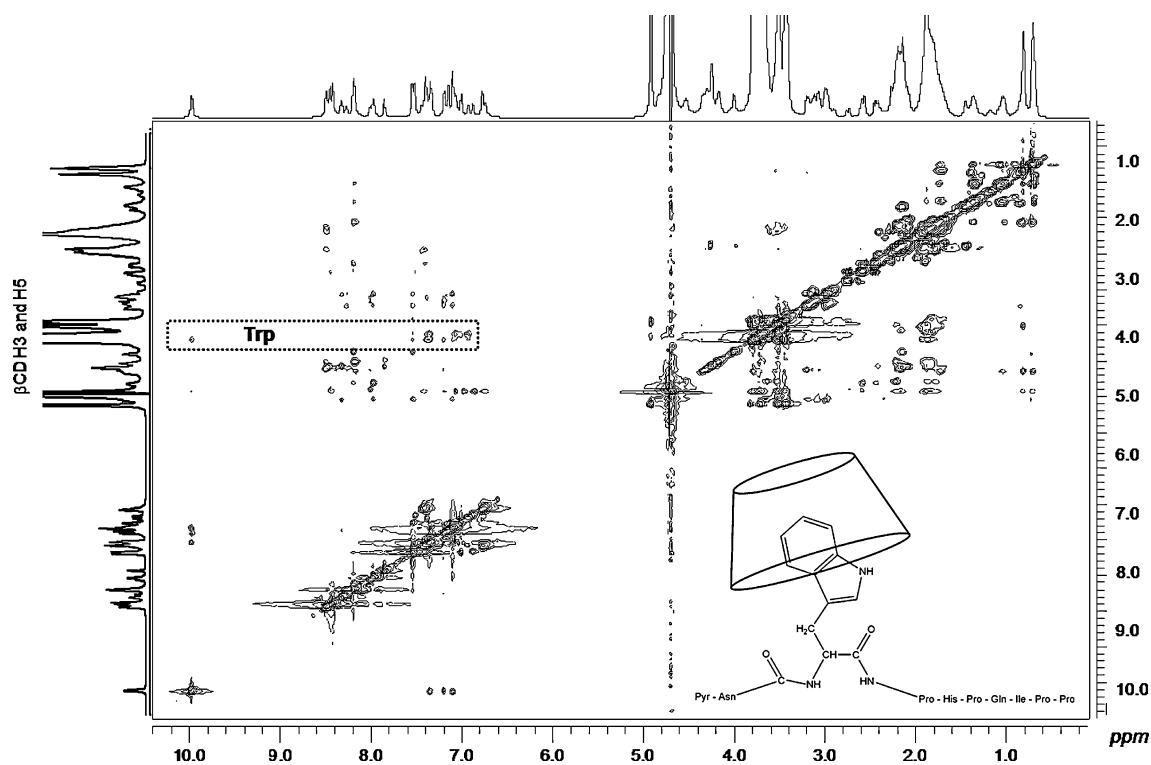
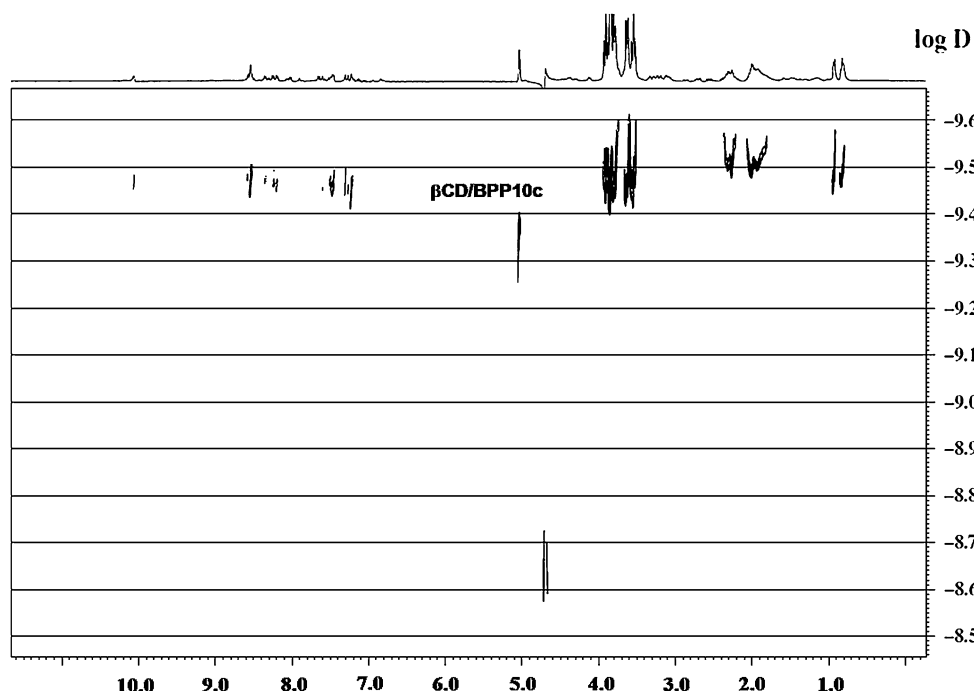


Fig. 7 2D NOESY contour map (500 MHz, mixing time = 200 ms) at 20 °C in 10% D₂O/H₂O for the β CD/BPP10c complex

Fig. 8 DOSY spectrum at 400 MHz in D₂O/H₂O for the supramolecular system β CD/BPP10c at 1:1 M ratio



Horizontal Attenuated Total Reflectance

In order to investigate changes in the peptide secondary structure in presence and absence of β CD, FTIR-HATR in

solution was used. FTIR-HATR of the amide I band for BPP10c in DMSO is displayed in Fig. 10a. This spectrum presents a characteristic carbonyl single band around 1663 cm^{-1} .

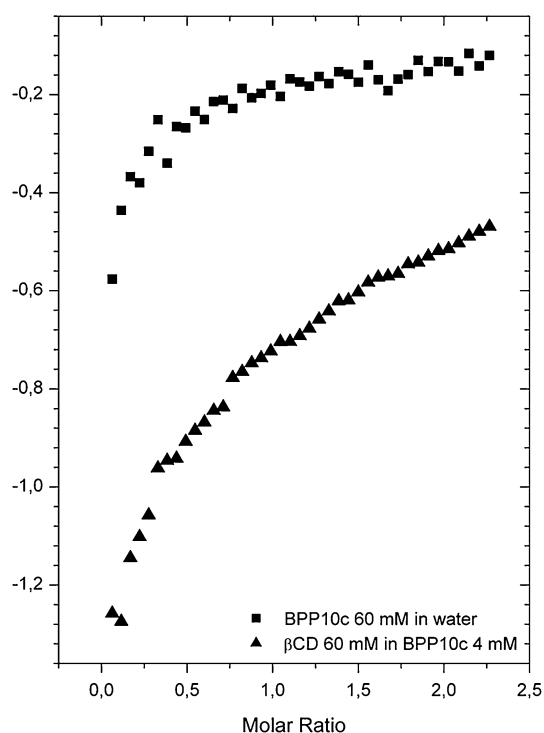


Fig. 9 ITC experiments for BPP10c, 60.0 mM (filled triangle) in water and (filled square) in β CD 4.0 mM

However, in the FTIR-HATR spectrum for the BPP10c in DMSO and in presence of the β CD (1:1 IC), different carbonyl bands were observed using the second derivate resolution enhancement technique, Fig. 10b. Table 2 presents the assignments for the peptide conformation in presence of β CD and their respective contribution of each structure.

The infrared bands for the IC at 1624, 1675 and 1688 cm^{-1} in DMSO solution results from the carbonyl groups involved in the hydrogen-bonding in β -sheet structures [44]. The presence of turns structures is confirmed by the infrared absorption bands at 1641 and 1661 cm^{-1} [38]. In addition, the absorption band observed at 1607 cm^{-1} corresponds to the $(\text{NH}_2)_\delta$ of the Asn amino acid side chain [45]. The spectrum of pure β CD, in DMSO solution, does not present a significant band in the region between: 1750 and 1550 cm^{-1} , used to investigate the secondary structure of the BPP10c, Fig. 10c.

The FTIR-HATR spectrum of the BPP10c in water is depicted in the Fig. 11a. The absorption bands observed in this spectrum at 1673 and 1631 cm^{-1} referred to the β -sheet and 1661 and 1645 cm^{-1} corresponding to the turns structures. The band at 1611 cm^{-1} corresponding to the β -sheet conformation structure also contributes to peptide self-aggregation, as previously reported [38, 46]. Although the band position of the Gaussian fit curve for the BPP10c and β CD/BPP10c spectra are quite similar, a small

difference among the profile of each contribution: β -sheet (80.89–62.80%), unordered (7.70–17.05%) and turns (11.41–20.15%) for the secondary structures were observed, resulting in a variation of each structure percentage, Table 2.

Figure 12a and b present the FTIR-HATR spectra for the BPP10c and the 1:1 β CD/BPP10c IC in D_2O . Both spectra were acquired after 24 h to guarantee the complete H–D exchange. It has been reported in the literature a shift at the absorption bands frequencies when D_2O is used as a solvent [38, 47]. Table 2 presents the assignments and the percentage of each secondary contribution for the peptide and IC in D_2O . The band at 1654 and 1656 cm^{-1} in the BPP10c and β CD/BPP10c spectra, respectively, in D_2O demonstrate the existence of the α -helix conformation. Thus, using D_2O solutions might make observing α -helix contributions easier [38].

The β -sheet contributions in the BPP10c and in its IC are associated with the bands at 1622/1623, 1626/1625 and 1639 cm^{-1} , respectively. The turns conformation could be attributed to the bands at 1671 and 1673 cm^{-1} for the pure peptide and its respective complex with β CD. The contribution of each structure varied from: β -sheet (52.18–57.85%), α -helix (17.07–22.57%) and turns (30.75–19.58%) as observed for the spectra obtained using water solution.

Circular Dichroism

The BPP10c and β CD/BPP10c 1:1 IC Circular Dichroism spectra are shown in Fig. 13. The spectrum of pure BPP10c presents a characteristic standard of unordered secondary structure for shorter peptides. However, according to the peptide deconvolution spectrum, carried out using PEPFIT software [48], a more accurate secondary structure could be obtained.

According to the BPP10c deconvolution spectrum, it was possible to observe 50% for the *gfas*, and 25% for the β -turns and *gbt* structures. The spectrum of the IC at 1:1 M ratio showed a more similar profile than the pure peptide ratio, however, an unordered structure (7%) was also observed. Thus, a small change in the percentage of *gfas* (52%), *gbt* (21%) and β -turns (20%) secondary structure were verified for the supramolecular complex using the spectrum deconvolution analysis.

Discussion

The peptide NMR spectrum carried out in D_2O solution was crucial in identifying the aromatic hydrogens, considering the peptide obtained in $\text{D}_2\text{O}/\text{H}_2\text{O}$. The 1D and 2D NMR experiments were useful in assigning the hydrogen

Fig. 10 FTIR-HATR spectra of BPP10c in DMSO (—) and the non linear fitting, Gaussian fitting (---) **a**) in absence of β CD, **b**) in presence of β CD and **c**) comparison between BPP10c in presence and absence of β CD and pure β CD

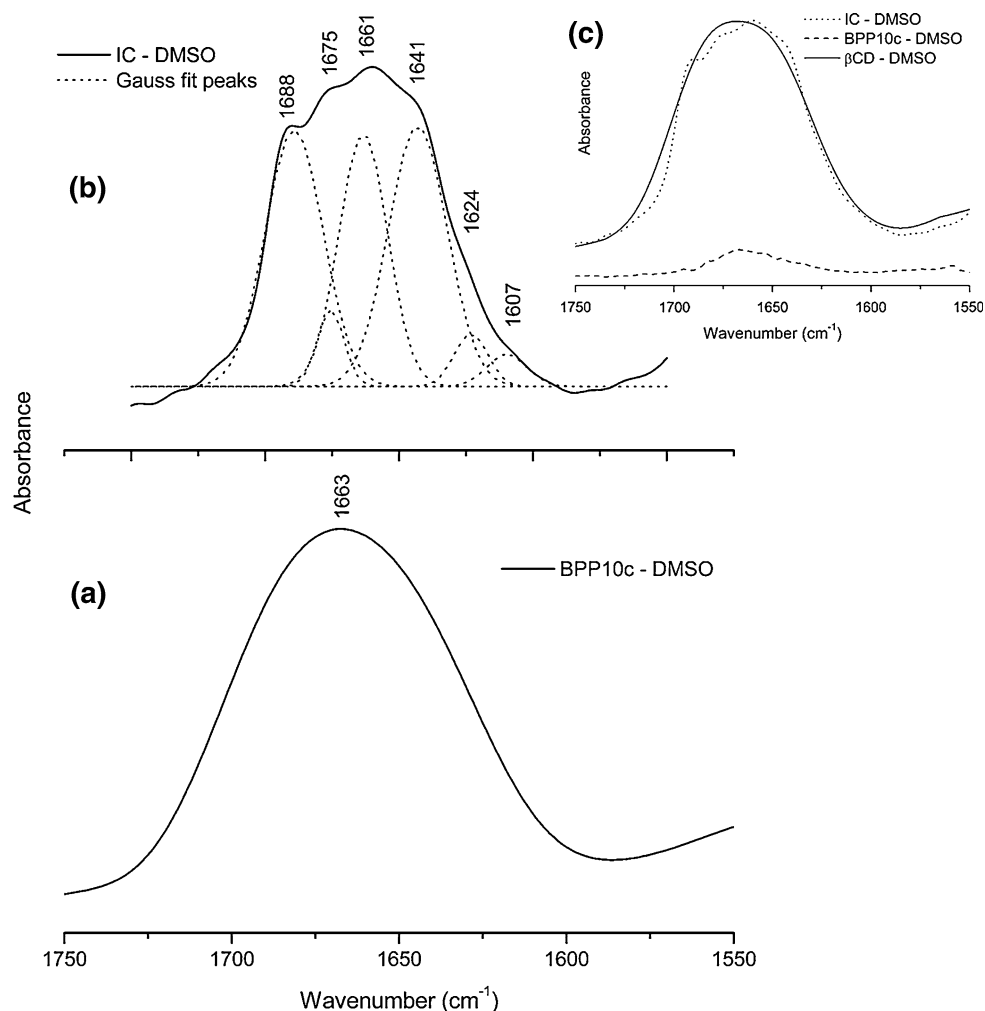
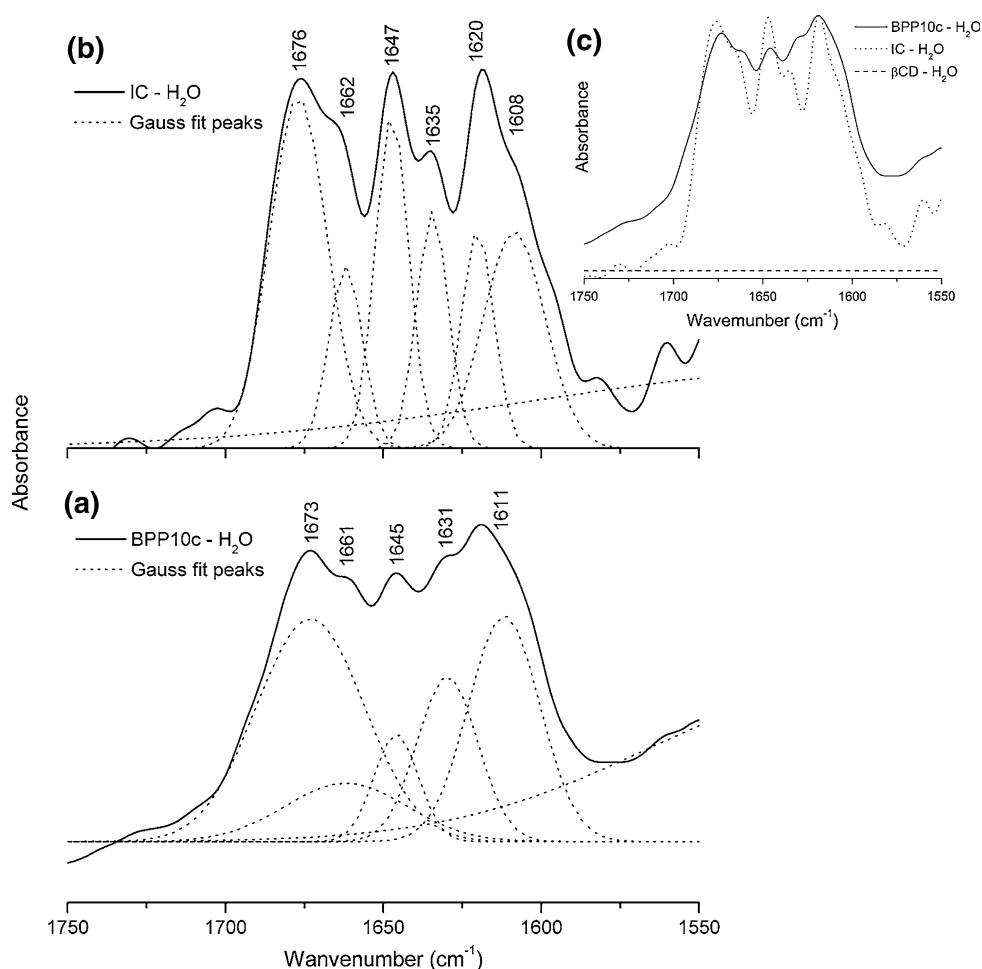


Table 2 FTIR-HATR band positions (in wavenumbers, cm^{-1}) determined with the aid of second derivative technique for amide I and side chain spectral regions for BPP10c and inclusion compound in DMSO, H_2O and D_2O

Sample	DMSO		H_2O		D_2O	
	ν (cm^{-1})	Assignment	ν (cm^{-1})	Assignment	ν (cm^{-1})	Assignment
BPP10c	1663 (100)	—	1611 (25.99)	β -sheet Aggregation	1622 (17.20)	β -sheet
			1631 (16.05)	β -sheet	1625 (18.55)	β -sheet
			1645 (7.70)	Unordered	1639 (16.43)	β -sheet
			1661 (11.41)	Turns	1654 (17.07)	α -helix
			1673 (38.85)	β -sheet	1671 (30.75)	Turns
			1688 (31.22)	β -sheet		
IC	1607 (2.67)	Side chain	1608 (20.34)	β -sheet Aggregation	1622 (10.23)	β -sheet
	1624 (3.72)	β -sheet	1620 (10.83)	Turns	1625 (36.20)	β -sheet
	1641 (32.13)	Turns	1635 (11.47)	β -sheet	1639 (11.42)	β -sheet
	1661 (25.92)	Turns	1647 (17.05)	Unordered	1656 (22.57)	α -helix
	1675 (4.34)	β -sheet	1662 (9.32)	Turns	1673 (19.58)	Turns
			1676 (30.99)	β -sheet		

Italic numbers in brackets show tentative percentage of each structure obtained from curve fitting. — All hydrogen C=O–H–N bond in the peptide structure were disrupted

Fig. 11 FTIR-HATR spectra of BPP10c in H₂O (—) and the non linear fitting, Gaussian fitting (---) **a**) in absence of β CD, **b**) in presence of β CD and **c**) comparison between BPP10c in presence and absence of β CD and pure β CD



chemical shifts of the amino acids residues. However, the existence of more than one possible peptide secondary structure made it impossible to identify each hydrogen of the Proline aminoacids residues.

The existence of these different peptide structures in solution was confirmed by the complementary information acquired from the NMR experiments, such as the existence of a greater number of α H hydrogens and a duplet of the N–H of Trp at 9.91, suggesting that at least two different secondary structures in solution could be observed.

The interaction between β CD and the BPP10c was confirmed based on the NMR experiments, in which a change in all peptide chemical shifts was observed, which might indicate the interaction between host and guest molecules in solution. This phenomenon was already reported in the literature for other different systems [27, 28, 31, 49]. This chemical shift variation was more evident in the aromatic region of the β CD/BPP10c spectrum, indicating that the interaction among the species could occur specifically by the aromatic portion of the peptide. Moreover, the presence of two distinguished signals for the Trp N–H at $\delta = 9.82$ suggests that the secondary peptide

structure was not so affected by the presence of β CD molecule. In addition, an overlap of the β CD internal hydrogens was observed indicating an inclusion of a peptide portion in the β CD cavity, as reported for different guest molecules [50].

The correlation cross peak verified in the 2D NOESY between the Trp amino acid residue and the hydrophobic cavity of the β CD confirms the interaction of these molecules and also explains the greater variation in the hydrogen chemical shift for this spectrum region, observed in the ¹H NMR spectrum. In addition, the DOSY result also indicates that an interaction between host and guest molecules occurs in solution, once both molecules present a diffusion coefficient time.

The ITC curve for the β CD/BPP10c titration process exhibits a typical profile of weak host:guest interactions with $K = 70 \pm 22$ when compared to other systems reported in the literature ($K \approx 7,000$) [27, 28] or enzyme-substrate interactions ($K > 20,000$) [51, 52]. This equilibrium constant suggests that a small fraction of the peptide will be included into the β CD cavity. However, this K is equivalent to other ones already related for β CD/peptide

Fig. 12 FTIR-HATR spectra of BPP10c in D₂O (—) and the non linear fitting, Gaussian fitting (---) **a**) in absence of βCD, **b**) in presence of βCD and **c**) comparison between BPP10c in presence and absence of βCD and pure βCD

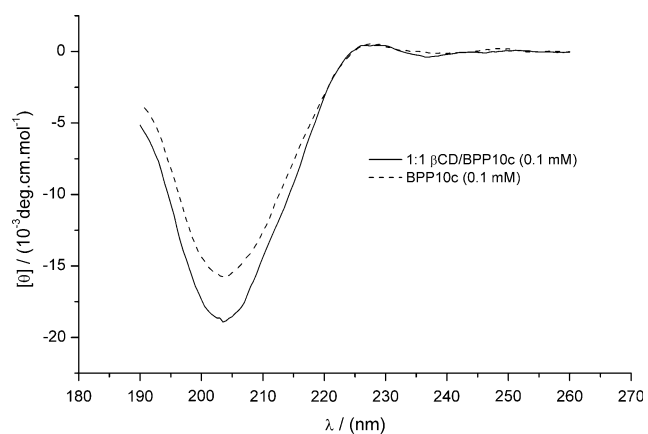
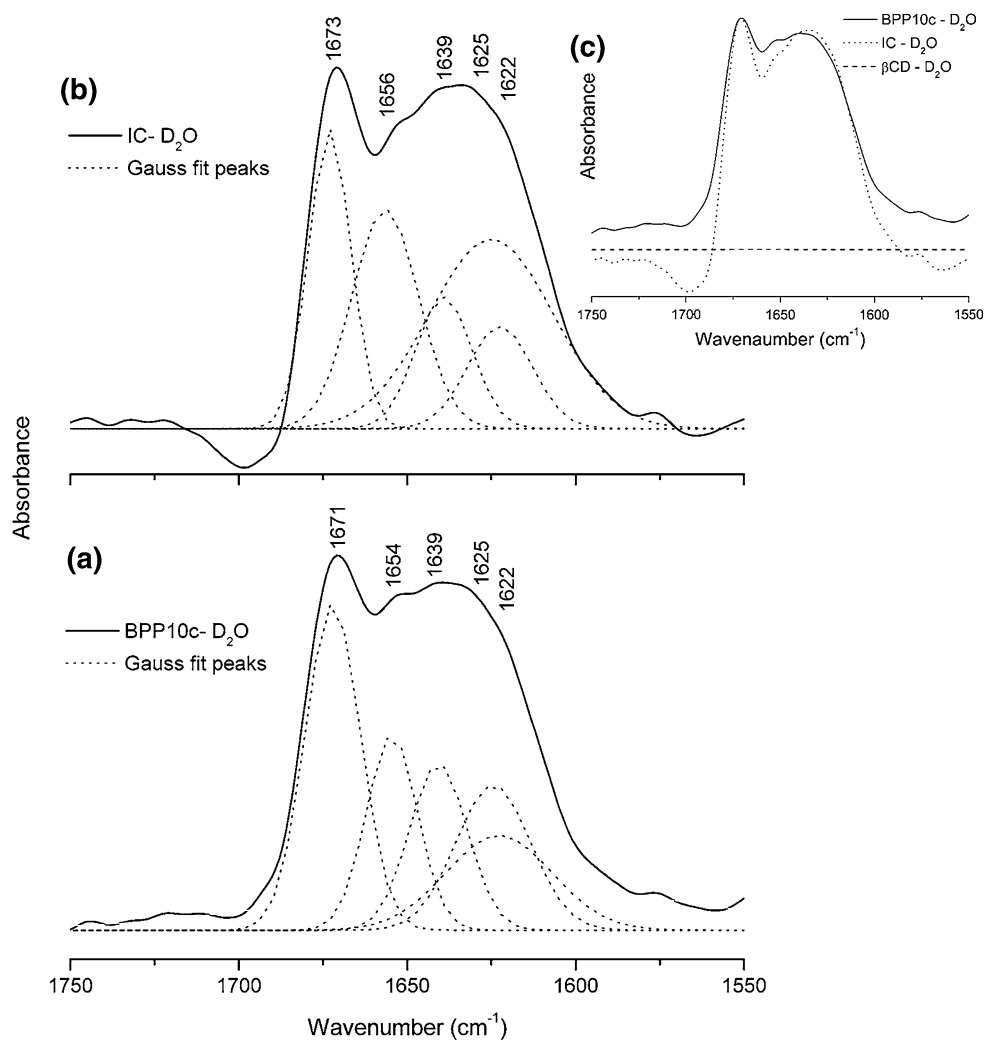
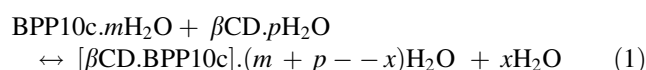


Fig. 13 Circular Dichroism spectra of BPP10c (0.1 mM) and βCD/BPP10c complex (0.1 mM) in buffer K₂HPO₄/H₃PO₄ (pH 4.0)

system. [16, 31] According to the thermodynamics parameters obtained by the titration curve, the process is enthalpic driven and entropically unfavorable.

The complexation process may be described in a simplified mode from Eq. 1:



According to Eq. 1, the thermodynamic of complexation depends on two simultaneous processes featuring non-covalent bond formation and solvent reorganization. From the enthalpic viewpoint, the formation of non-covalent interactions is exothermic ($\Delta H_{\text{intrinsic}} < 0$), while the disruption of well-defined solvent shells is endothermic ($\Delta H_{\text{desolvation}} > 0$). In this sense, the βCD/peptide interactions play a predominant role in the supramolecular complex formation, according to the observed negative enthalpy changes. It has been proposed that during the formation of supramolecular interactions, solvent reorganization accounts for great contributions to enthalpy variation [53]. Nevertheless, the complex formation generally reduces the solvent-accessible surface area, resulting in the release of highly ordered solvent molecules in bulk

solution. Consequently, the observed enthalpy variation is the compensatory outcome of desolvation enthalpy and intrinsic enthalpy, as already described in the literature [53].

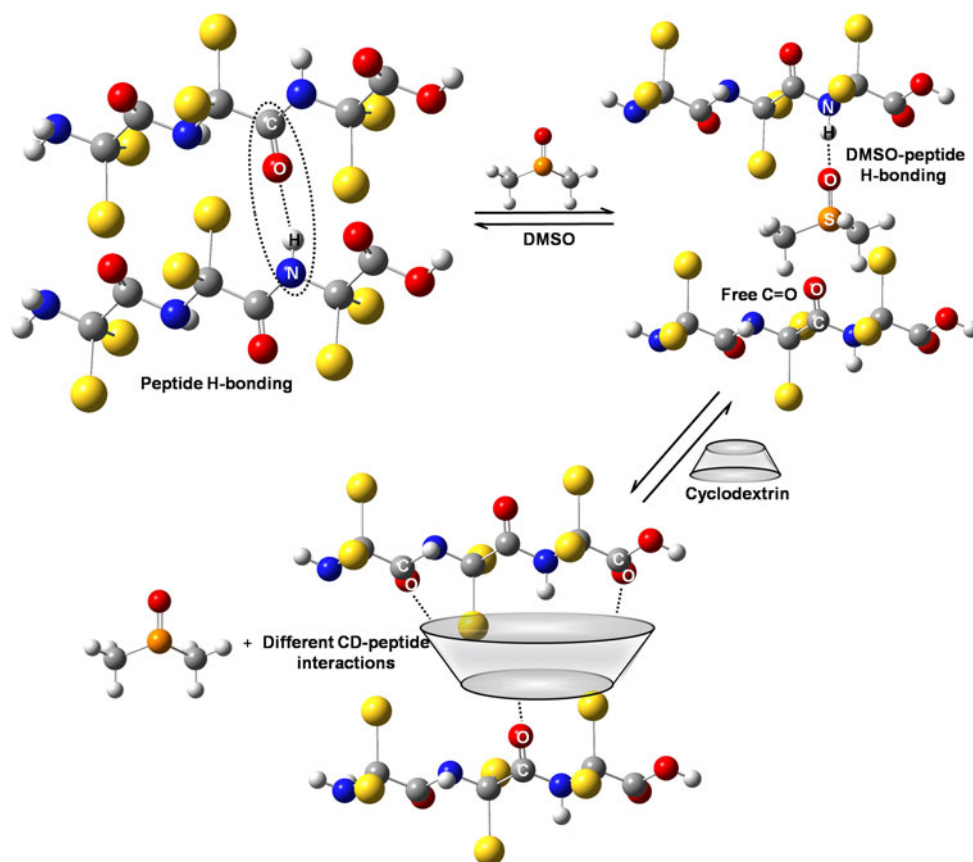
The negative entropy changes do not necessarily indicate that the hydration of the supramolecular complex interface remains unchanged or increases in comparison with the free peptide and CDs molecules. Another important unfavorable contribution to the entropy change may arise from the conformational restriction of the amino acid residues upon the complexation.

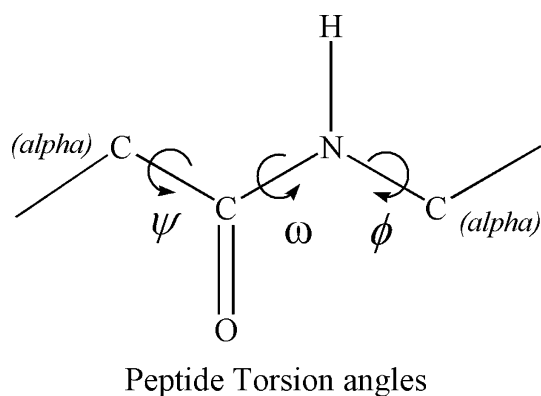
The presence of only one carbonyl band in the peptide-DMSO FTIR-HATR spectrum can be explained by the competition to form hydrogen bonding with N–H of the peptide chain by the S=O group in DMSO solvent, a strong hydrogen bonding acceptor, and the C=O groups of their own peptide [38, 44]. In DMSO solution, sufficient C=O–H–N peptides intramolecular bonds are broken due to competition of S=O to form S=O···H–N, Scheme 1. This phenomenon makes the C=O groups “free”, favoring this unique carbonyl band in the FTIR-HATR. This disruption in the hydrogen bonding of BPP10c also indicates the solvent accessibility to the peptide structure, forming an extended structure as previously reported in the literature for different peptides [54, 55].

However, in presence of β CD, six new bands are observed in the peptide-DMSO solution, indicating that the β CD is able to compete with the DMSO solvent molecule in order to interact with the peptide with non-covalent forces. Moreover, the interactions between BPP10c with the host molecule may favor different neighborhoods for the carbonyl group. This allows the one single band (at 1663 cm^{-1} , Fig. 10a) to develop in six distinguished bands (Fig. 10b), as represented in the Scheme 1. In addition, these new six bands indicate that the intermolecular interactions between β CD and BPP10c were able to modify the peptide secondary structure in DMSO solution.

A small change in the BPP10c vibrational modes were observed for the peptide in presence of the β CD, Fig. 11b, when these bands are compared to that verified for pure peptide using water as solvent (Fig. 11a). These small variations in the BPP10c bands frequencies in presence of β CD suggest an interaction between the species what could be responsible to cause a change in the peptide secondary structure. A similar behavior for the peptide and its IC were observed using D_2O as solvent, Fig. 12a and b. In this case, α -helix structure could be verified in both, presence and absence of β CD. The spectrum of pure β CD in water (Fig. 11c) and using D_2O (Fig. 12c) did not present a

Scheme 1 Representation of the non-covalent interactions for the equilibrium between peptide-peptide, DMSO-peptide and cyclodextrin-peptide





Scheme 2 Representation of the peptide torsion angles ψ , ϕ and ω

significant band at the spectrum region used to characterize the peptide secondary structure.

The BPP10c and β CD/BPP10c presented a characteristic unordered secondary structure, according to the circular dichroism spectra. However, using the spectra deconvolution process, it was possible to identify different structures in each case as well as their contribution.

The *gfas* is a structure observed in presence and absence of β CD in a typical unordered conformation, in which a red-shift of about 10 nm is verified. This phenomenon might be explained by the presence of the Pro residues, which can prevent the α -helix conformation, and due to the small size of the peptide. The *gbt* structure could be included as a potential representative of cases in which turns structures is present, but possibly in rapid equilibrium between ϕ and ψ torsion angles, Scheme 2 [48].

The circular dichroism spectra are in accordance with the results observed by the FTIR-HATR results, verifying that there is little difference among deconvolution spectra, resulting in small difference for the secondary structure in presence and absence of β CD. In addition, the dichroism deconvolution proposes a secondary structure combination also verified using FTIR-HATR methodology, suggesting an agreement among these spectroscopic techniques.

Experimental section

Reagents and inclusion compounds preparation

BPP10c (Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro-OH) and β CD were purchased from Bachem Bioscience Inc., Pennsylvania (USA) and from Xiamen Mchem, Xiamen (China), respectively. Milli-Q[®] water was used to prepare all solutions in the experiments. IC at 1:1 M ratio was prepared by the freeze-drying method, in which the aqueous solutions of the materials (β CD and BPP10c) were stirred for 4 h. After, this mixed solution was frozen in

liquid nitrogen and lyophilized (Savant ModulyoD—Freeze Dryer, Thermo Electron Corp., Waltham, MA, USA) for 48 h [27, 32].

Nuclear Magnetic Resonance

NMR spectra were recorded at 5 °C on a Bruker DRX 400—AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 400 MHz, and also at 20 °C on a Varian INOVA—500 AS spectrometer operating at 500 MHz, equipped with a 5 mm inverse probe with z-gradient coil. The NMR BPP10c samples were prepared in D₂O (Cambridge Isotope Laboratories, Inc.—99.9% of isotope purity) and 10% D₂O/H₂O to obtain the NH NMR spectra. ¹H NMR spectra were achieved using the WATERGATE technique for the suppression the residual water signal [56, 57]. The Nuclear Overhauser Enhancement Spectroscopy (2D NOESY) experiments, mixing time 300 ms on Bruker DRX 400—AVANCE spectrometer and 200 ms on Varian INOVA—500 AS spectrometer, were acquired using standard experiments from the spectrometers library. TOCSY contour maps (spin lock time of 80, 120 and 220 ms) were acquired using MLEV-17 pulse sequence.

The interaction between BPP10c and β CD was monitored by ¹H NMR spectra and 2D NOESY experiments. In addition, a Diffusion-ordered spectroscopy (DOSY) experiment was executed to investigate the interaction among molecules in solution by the diffusion coefficient times. DOSY experiments were performed using the simulated echo with a bipolar gradient pulse pair sequence, modified with an introduction of a presaturation pulse for solvent signal suppression (STEBPGP1S) [28, 58]. Data were processed using 1D Win NMR 5.1 and 2D Win NMR 5.1 (Bruker spectra) and MestReC 4.9.9.6 (Varian spectra).

Isothermal Titration Calorimetry

ITC were performed in duplicate with a VP-ITC Microcalorimeter (Microcal Company, Northampton, MA, USA) at 25 °C. Each titration experiment consisted of 41 successive injections of an aqueous solution of BPP10c (60.0 mM) into the reaction cell charged with 1.5 mL of β CD aqueous solution (4.0 mM) and with time intervals of 300 s. These intervals were sufficiently long enough for the signal to return to the baseline, in order to ensure equilibrium for the system. The first injection of 1 μ L was discarded to eliminate diffusion effects of the material from syringe to calorimetric cell. Afterwards, the subsequent additions were injected at constant volume of 5 μ L of BPP10c and the time of injection was 2 s.

The β CD concentration in the calorimeter cell varied from 4.0 to 3.5 mM and the concentration of the BPP10c

from 0.0 to 7.1 mM. The ITC data were analyzed by the software supplied with the calorimeter (Microcal Origin 5.0 for ITC).

The peaks produced during titration were converted to heat output per injection by integration and correction in order to determine the cell volume and sample concentration. The dilution process of BPP10c was carried out in water to evaluate the heat of dilution of the molecule. In addition, the heat dilution curve was subtracted from the titration curve (BPP10c in β CD) to eliminate interactions between BPP10c and water. Binding enthalpies, stoichiometry and binding constant were obtained by nonlinear fitting (Wiseman isotherm) [52], incorporated in the software, which assumes a single set of identical binding sites.

Horizontal Attenuated Total Reflectance

Peptide samples were dissolved in DMSO, H₂O or D₂O, at a concentration of 10.0 mg mL⁻¹. Solutions of the IC, at 1:1 M ratio, were prepared using the same solvents in which the BPP10c concentration was kept at 10.0 mg mL⁻¹.

The peptide and IC samples were deposited at FTIR-HATR crystal, ZnSe, for measurements. All experiments were carried out in a Perkin Elmer spectrophotometer (Boston, MA, USA) model, Spectrum GX, with a KBr beam splitter, equipped with a HATR accessory. All spectra were recorded within the range of 4000–590 cm⁻¹, with a 4 cm⁻¹ resolution and co-addition of 128 scans. To reduce the atmosphere effects, the sample detector was purged with N₂ before the analysis. In the presence of β CD, the solvent spectra were obtained under the same conditions and were subtracted from sample spectra.

The amide I region (1700–1600 cm⁻¹) was used for peptide structure determination. The second derivate resolution enhancement technique (Spectrum v5.0.1, Perkin Elmer Instruments LLC) was applied to determine the number and positions of the overlapped bands. The presence of certain peptide secondary structure elements were determined based on the positions of the component bands according to the literature [38, 44, 47, 59–61]. These bands were used as input parameters for the curve Gaussian fitting and the all spectra were also normalized (Origin[®] 7.0, OriginLab Corporation, Northampton, MA, USA) to make the direct comparison among them possible. The area of all the component bands assigned to a certain conformation were integrated using program AutoCAD 2007 (Autodesk) and their percentage in the total area of all bands were considered to reflect the approximate amount of each structure, as previously proposed in the literature [38].

Circular Dichroism

Circular Dichroism spectra of BPP10c (0.1 mM) and β CD/BPP10c complex (0.1 mM) solutions were recorded in duplicate with a JASCO spectrophotometer Model J-720 at 25 °C, in buffer K₂HPO₄/H₃PO₄ (pH 4.0), below its isoelectric point. The BPP10c isoelectric point was identified using the “compute pI/M_w” described at (http://www.us.expasy.org/tools/pi_tool.html), which is a tool that allows the computation of the theoretical pI (5.25).

The wavelength was scanned from 190 to 320 nm at a run time of 100 nm min⁻¹ with a resolution of 0.5 nm and a bandwidth of 1 nm. The spectra were the average of four scans. It used a quartz cell with 0.1 cm of optical length. The Circular Dichroism spectra were previously processed with JASCO software, with each spectrum subtracted from the blank. The final figures were edited with Microcal Origin 7.0.

Conclusion

In this work the Bradykinin Potentiating Peptide BPP10c was structurally elucidated using a combination of 1D and 2D NMR experiments. In addition, NMR techniques were able to identify at least two different secondary structures for this peptide in solution. The interaction of the BPP10c with β CD was also confirmed by NMR techniques, mainly by 2D NOESY and DOSY. These experiments indicate a dipolar correlation between Trp residue and the internal hydrogens of β CD and only one diffusion coefficient time for both molecules (β CD and BPP10c), suggesting that a 1:1 stoichiometry is formed. Although this interaction occurs in solution, the peptide secondary structure was not substantially affected in the β CD presence.

This 1:1 β CD/BPP10c stoichiometry was confirmed by ITC curves. The inclusion process demonstrated to be spontaneous ($\Delta G^\circ = -2.53$ kcal mol⁻¹) with an enthalpy variation of $\Delta H^\circ = -3.72 \pm 0.02$ kcal mol⁻¹, entropic contribution of $T\Delta S^\circ = -1.19$ kcal mol⁻¹ and equilibrium constant ($K \approx 70 \pm 20$) were also obtained.

The peptide secondary structures were also observed by FTIR-HATR in solution by the second derivate resolution enhancement technique. In DMSO solution, an extended structure for the peptide might be proposed, however in presence of β CD a combination of structure was observed, indicating the supramolecular interaction and the competition between β CD and DMSO to interact with BPP10c. In addition, a combination of at least three different BPP10c secondary structures was also verified in presence of β CD, both in DMSO, D₂O and H₂O. The Circular Dichroism was essential to confirm these secondary structures and the

results obtained are in accordance with that verified by FTIR-HATR spectroscopy.

Further *in vivo* studies with the complex β CD/BPP10c will be necessary to verify anti-hypertensive efficacy after oral administration and to confirm the absorption of the peptide in the colon.

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