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The use of 2D NMR to study β-cyclodextrin complexation and debittering of amino acids and peptides

Giani Andrea Linde [a,c,*], Antonio Laverde Junior [a], Eliete Vaz de Faria [b], Nelson Barros Colauto [a], Flávio Faria de Moraes [c], Gisella Maria Zanin [c]

[a] Laboratório de Biologia Molecular, Instituto de Ciências Exatas, Agrárias, Tecnológicas e Geociências, Universidade Paranaense, Umuarama, PR, Brazil
[b] Laboratório de Referência em Análises Físicas, Sensoriais e Estatística (LAFISE), Instituto de Tecnologia de Alimentos, Campinas, SP, Brazil
[c] Departamento de Engenharia Química, Universidade Estadual de Maringá, Maringá, PR, Brazil

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ABSTRACT

This work fully demonstrated the formation of amino acids complexes with β-cyclodextrin (β-CD) by the method of nuclear magnetic resonance with the rotating frame Overhauser effect spectroscopy (ROESY) and diffusion ordered spectroscopy (DOSY) techniques. The tested amino acids display the following decreasing order of affinity for β-CD: tryptophane > tyrosine > phenylalanine > proline > histidine > isoleucine. The influence of complexation on taste perception was determined with a trained panel to qualify taste alterations of single amino acids and quantify the debittering of soy protein hydrolysate by β-CD complexation. The results showed that β-CD complexation is effective for modifying single amino acids taste perception and debittering soy protein hydrolysate. Bitterness sensation of the latter is reduced by 90% when 5% β-CD was added, thus β-CD is recommended as a prospective additive for masking bitter taste of new functional food products.

1. Introduction

Unpleasant tastes have been a problem due to the call for healthier food. Consumption of active principles with health benefits used to be restricted to pharmaceutical products and now it is an important sector of the food industry. When there is reduction of sugar, fat and sodium, bitterness and astringency can also be intensified in healthy foods (Ley, 2008). Fortification of functional foods with polyphenols, phytosterols, vitamins minerals and soy products can cause serious taste deficiencies and reduce consumer demand for those products (Eckert & Riker, 2007).

A large and growing number of peptides have been used in therapy (Aachmann, Otzen, Larsen, & Wimmer, 2003; Wang & Mejia, 2005). Many of them are either naturally found in several foods or produced after food processing, as "food-derived bioactive peptides". In spite of the functional importance of these compounds, the hydrolysis of protein liberates peptides that are strongly bitter, limiting their use as food or therapeutic products. The degree of bitterness of a peptide is strongly correlated with the sequence, polarity and size of the amino acids that form the peptide (Saha & Hayashi, 2001).

A cyclodextrin (CD) is a cyclic oligosaccharide formed by glucopyranose units joined by α-(1, 4) bonds. It is conformed as a truncated cone, with a hydrophobic internal cavity and hydrophilic external circumference. The most interesting characteristic of the CDs are their capacity of forming inclusion complexes with a great number of organic or inorganic compounds (Szente & Szejtli, 2004). β-CD, which has seven glucopyranose units, is the CD most used in pharmaceutical applications and has inspired many detailed toxicological studies which show that it is safe for human consumption (Del Valle, 2004). It is not absorbed in the upper gastrointestinal tract but completely metabolized by the colon microbiota (Szente & Szejtli, 2004) acting as functional prebiotic fiber.

A number of studies have dealt with inclusion complex formation of amino acids and CDs, such as Miertus et al. (1999) and Yuexian, Yu, Shaomin, and Chuan (2005), but few of them have used conclusive techniques for the inclusion complex determination and quantified the effective alteration of taste caused by the complexation of amino acids with CD. Miertus et al. (1999) for instance, reported a study on the nature of the driving forces and mechanism leading to complex formation and the development of a computational model that allow estimating the affinity order of each of the 20 natural L-α-amino acids to form complexes with β-CD. Tamura et al. (1990) worked only with α-CD to reduce bitter taste in solutions of phenylalanine, isoleucine, arginine, valine, leucine and bitter peptides. They suggested the formation of an inclusion complex between α-CD and amino acids or peptides.

* Corresponding author. Address: Universidade Paranaense – UNIPAR, Laboratório de Biologia Molecular, CP 224, Umuarama, PR, Brazil. Tel.: +55 44 36212837; cel.: +55 44 88084006; fax: +55 44 36212830.
E-mail address: gianilinde@unipar.br (G.A. Linde).

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However, conformational alterations of the amino acids that modify taste might also have occurred by interaction of the amino acid aminic and carboxylic polar groups with the external surface of the CD. The natural sweet taste of CD might also have helped to mask the bitter taste of amino acids. According to Szejtli and Szente (2005) CDs cannot be considered a tasteless or only slightly sweet substance, because 0.5% of β-CD solution is as sweet as sucrose.

Nishijo and Tsuchitani (2001) have studied the formation of an inclusion complex between α-CD and L-tryptophane by nuclear magnetic resonance (NMR). However, L-tryptophane is just one of the many amino acids that are responsible for the bitter taste of peptides, as reported by Tamura et al. (1990), and other amino acids should be studied to determine which ones form inclusion complexes with CD and the effects on taste modification upon complexation. It is important as well to determine their inclusion constants and verify the real capacity of taste modification of protein hydrolysates for developing new food applications. Although some available papers are related to amino acid and peptide unpleasant taste reduction by addition of α-CD (Linde et al., 2009), there is little information about reduction of bitter taste by β-CD. Binello, Cravotto, Nano, and Spagliardi (2004) reported that β-CD at 0.4% is able to reduce about 90% of the bitterness of a 0.05% caffeine solution, while α- and γ-CD are less efficient. This indicates that β-CD has an important role on the development of functional foods with pleasant taste.

The conclusive interactions between amino acids and β-CD by NMR techniques are relevant for advancing the sensorial knowledge and the development of new technologies for masking the bitter taste of peptides in functional food products. There are few alternatives to NMR spectroscopy in the study of CD complexation. As with many carbohydrates it is often difficult, or too time consuming, to obtain single crystals of CD derivatives and then to analyze them by X-ray crystallography, and even more so by neutron diffraction. Other techniques such as fluorescence, UV/vis spectroscopy, calorimetry, among others, play a major role in measuring complexation energetics with CDs, but usually provide only very indirect and qualitative information about inclusion modes and geometries (Schneider, Hacket, Rudiger, & Ikeda, 1998).

In this study, it was firstly investigated the amino acid complexation with β-CD by different NMR experiments, such as diffusion ordered spectroscopy (DOSY) and rotating frame Overhauser effect spectroscopy (ROESY). DOSY experiments have been previously used for the analysis of a wide variety of complex processes, providing basic information on the diffusion characteristics of molecules (Johnson Jr., 1999). Self diffusion coefficients, D, of individual compounds from a mixture according to the differences in their effective sizes can be obtained with DOSY. In addition, ROESY technique can provide detailed information on the topology of the complexes, always keeping in mind the dynamic character of these systems. These studies give us information at a molecular level of the structure of the complexes and their association binding constants. Secondly it was investigated the effect of complex formation on the taste of amino acids and soluble protein hydrolysate was evaluated by sensorial analyses. A taste panel was used to qualify the overall taste perception of amino acids in the presence and absence of β-CD and quantify the bitterness reduction of hydrolyzed soy protein caused by β-CD complexation.

2. Materials and methods

2.1. Materials

The amino acids histidine (HIS), isoleucine (ILE), phenylalanine (PHE), proline (PRO), tryptophan (TRP) and tyrosine (TYR) were from Sigma Chemical Company (USA). β-CD was kindly donated by Cargill Food and Pharma Specialties North America. Amino acids and β-CD were dried for 24 h at 60 °C and 600 mm Hg of vacuum. Isolated soy protein was donated by the Solae Company (Esteio, Brazil) and alcalase® 2.4 L (EC 3.4.21.62) from Novozymes (Denmark). D_2O (99.8% purity) was from Cambridge Isotope Laboratories (USA).

2.2. NMR analysis

All proton NMR (^{1}H NMR) spectra were recorded at 500 MHz using a Varian Inova 500 spectrometer (11.75 T) from the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil, with a 5 mm z-gradient inverse probe at 25 °C. The resonance at 4.7 ppm was used as internal reference due to residual solvent water (H_2O and HDO). The complexation was investigated by ROESY method (Bax & Davis, 1985) using the *wg*-ROESY (*water-gate*-ROESY) pulse sequence. ROESY measurements were made with the experimental conditions as follows: 32 scans, acquisition time 0.150 s, pulse delay 2.3 s and 512 data points. The diffusion coefficients were investigated by DOSY experiment using the diffusion bipolar pulse pair stimulated echo (DBPPSTE) sequence (Wu, Chen, & Johnson Jr., 1995). Data was acquired using a 50 ms diffusion delay in all experiments, with bipolar gradient pulse duration of 2 ms and 5 ms eddy current delay. Thirty-two experiments (32 transients each) were recorded with gradient pulses amplitudes ranging from 0.000685 to 0.003427 T cm^{-1}, where an approximately 90–95% decrease in the resonance intensity was achieved. The complexed population (p_{compl}) and the association binding constant ($K_{ap'}$) of complexes were measured according to Laverde Jr., Conceição, Queiroz, Fujiwara, and Marsaioli (2002) and Linde et al. (2009).

2.3. Solution preparations

Equimolar mixtures of β-CD and each amino acid (15 mM) were made in D_2O and used for the NMR analyses. For the sensory analyses, a bitter peptide solution was made using hydrolyzed soy protein. Soy protein (20% w/w) with adjusted pH (5.9–6.0) was preincubated for 30 min at 37 °C, after that alcalase® was added in order to obtain an enzyme-substratum ratio of 1% (w/w); then the reaction proceeded until reaching the degree of hydrolysis of 15% which is the maximum bitter taste point according to Aubes-Dufau, Capdevielle, Seris, and Combes (1995). The degree of hydrolysis was determined in the supernatant liquid phase by the trinitrobenzenesulfonic acid method (Adler-Nissen, 1979). The mixture was heated (85 °C for 15 min) to denature the enzyme and then, the pH was adjusted to 4.5, to obtain maximum solubility of peptides, and centrifuged. The liquid phase was dried by circulation of air at 60 °C and used for the sensory analysis.

2.4. Sensory analysis

The sensory mouthfeel and taste characteristics were determined at the Physical, Sensory and Statistical Analysis Laboratory (LAFISE), Food Technology Institute (ITAL), Campinas, SP, Brazil by a panel of six tasters selected by their high sensory acuity, according to the ISO-8586-1 method (ISO-8586-1, 1993). Aqueous mixtures of amino acids (1 mM) were prepared with mineral water at room temperature and sensory evaluated in the absence and presence of β-CD (1 mM). The amino acids selected for sensory evaluation were those that formed inclusion complexes with β-CD, according to the NMR analyses. For the soy protein hydrolysate, the powder was suspended in mineral water at room temperature, obtaining a 5% (w/v) aqueous mixture, which was sensory evaluated in the absence and presence of β-CD (3% and 5%, w/v).

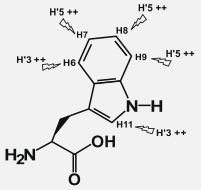
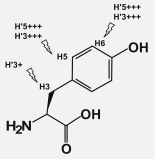
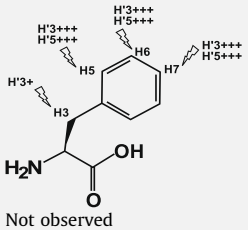
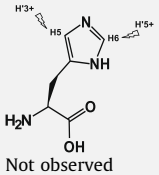
3. Results and discussion

The first step was to analyze the inclusion complex formation between amino acids and β-CD. Many techniques could have been used for inclusion complex characterization but only NMR provides conclusive data about the complexation at the molecular level (Schneider et al., 1998). In this work the advanced two-dimensional NMR (2D NMR) techniques (ROESY and DOSY) were used to provide unambiguous assignment of structure to the complex as well as the values of the parameters p_{compl} and $K_{ap'}$ (Johnson Jr., 1999; Laverde Jr. et al., 2002).

Intermolecular cross peak signals are obtained when the distance between the hydrogen nuclei from amino acid and β-CD were above 0.5 nm (Neuhaus & Williamson, 2000). Even when weak the signal presence of rotating frame Overhauser effect (ROE) evidences the formation of the inclusion complex. Fig. 1 presents the ROESY spectrum with cross peaks between the hydrogen protons of β-CD and PHE. Intermolecular correlations among internal H′3 and H′5 protons of β-CD and aromatic hydrogens (H3, H5, H6 and H7) of PHE were observed. These correlations prove that total complexation occurred between PHE and β-CD, as indicated by β-CD H′5 protons. Intermolecular cross peaks of internal H′3 and H′5 protons of β-CD with aromatic hydrogens H6, H7, H8, H9 and H11 of TRP, H3, H5, and H6 of TYR and H5 and H6 of HIS were observed in Table 1. Thus, in all cases that a complex between the amino acid and β-CD was clearly formed the topologies indicated that the non polar portion of the guest structure was encapsulated. In the ROESY spectra of β-CD with ILE or PRO intermolecular cross peaks were not observed, indicating that for these amino acids there was not complexation, or that a very weakly bound complex was formed.

The result for β-CD amino acid complexation in comparison with α-CD is more important in terms of industrial application in the food industry as well as in the pharmaceutical and cosmetics industries, because β-CD has a larger commercial distribution, as

Table 1
Hydrogen intermolecular rotating frame Overhauser effect (ROE) signals, values of the complexed population (p_{compl}) and constant of apparent association ($K_{ap'}$) of the complexes between β-cyclodextrin (β-CD) and some amino acids determined by nuclear magnetic resonance. The ROE's signals among amino acid hydrogens (H) and β-CD (H′) are indicated as: high +++, medium ++ and low +.

Complexes	Intermolecular ROE signal	p_{compl} (%)	$K_{ap'}$ (M^{-1})
β-CD-TRP		31.0	43.5
β-CD-TYR		30.0	40.7
β-CD-PHE		24.0	27.7
β-CD-PRO	Not observed	11.9	10.2
β-CD-HIS		8.8	7.0
β-CD-ILE	Not observed	5.5	4.1

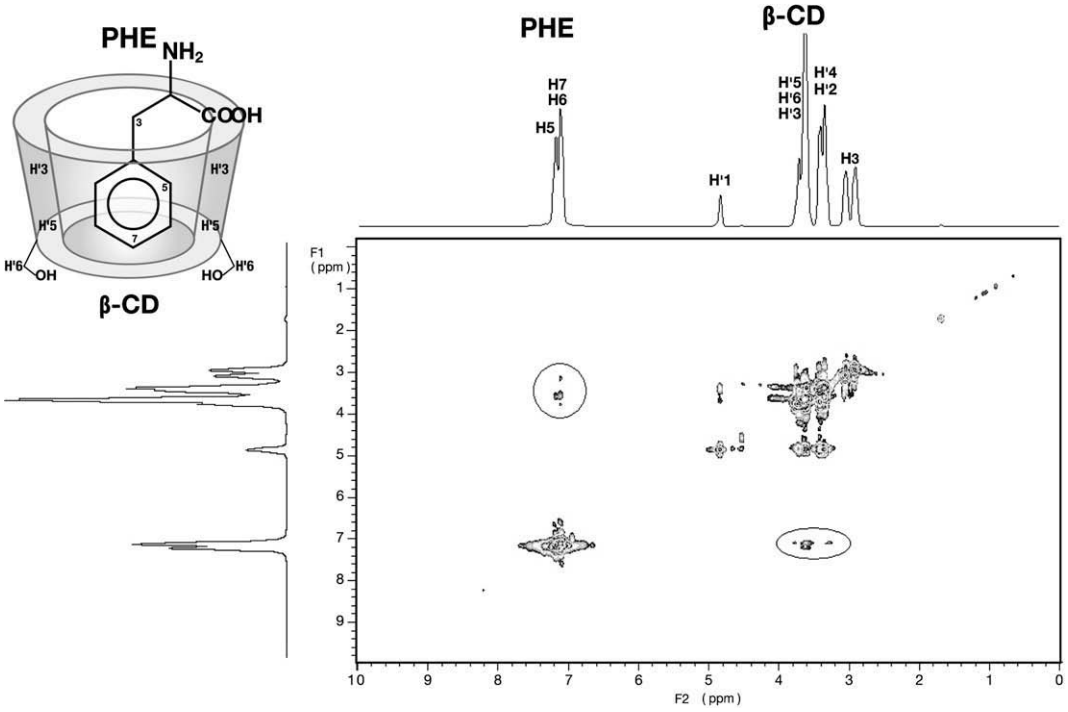


Fig. 1. Rotating frame Overhauser effect spectrum containing cross peaks between the β-cyclodextrin (β-CD) and phenylalanine (PHE) protons and the suggested topology of adducts.

it can be easily produced in comparison with the other CDs, and has a greater number of applications (Szente & Szejtli, 2004). These characteristics make β-CD ideal for industrial and commercial applications, many times becoming a decisive factor to make the CD application viable due to a profitable commercialization of the product.

In the case of the tested amino acids, favorable characteristics for industrial use of β-CD are compounded with technical evidence shown for the large number of amino acids that are able to accommodate, at different depths of burying, inside the β-CD cavity and hence form inclusion complex with it.

In this work the expected properties from the amino acid-β-CD complexes are that these derivatives pass by the tongue, without signaling their bitter taste. But it is very important that afterwards the amino acid can be easily liberated from the complex along the digestive system. According to Górnas, Neunert, Baczyński, and Polewski (2009) the complexation occurs through a non-covalent interaction between the molecule and the CD cavity. This is a dynamic process whereby the guest molecule continuously associates and dissociates from the host CD. The complexation dynamics process and the amino acid liberation along the digestive system can be inferred from the complexation constant obtained by the DOSY technique.

The p_{compl} and $K_{ap'}$ are estimated from the observed guest diffusion coefficient, D_{obs}, obtained by diffusion experiments. p_{compl} and $K_{ap'}$ values are important because they allow verifying the tendency for inclusion complex formation (Linde et al., 2009).

The results for p_{compl} and $K_{ap'}$ are presented in Table 1. TRP, TYR and PHE have shown p_{compl} values around 31–24% in presence of β-CD. These results indicate a low complexation affinity between the amino acids and β-CD, probably caused by the high solubility of the amino acids in aqueous solution. They suggest that the water polar environment is energetically more favorable to the whole molecule of the amino acids than the non polar ambiance of the CD cavity which captures only the non polar residues of the amino acids. Although the complexation was low, it could be sufficient to reduce the bitterness of low solubility peptides, which are the main responsible for the bitter taste of hydrolyzed proteins. This will be evaluated later on this work.

Table 1 shows the constants for the formation of the β-CD inclusion complexes in the following decreasing affinity order: TRP > TYR > PHE > PRO > HIS > ILE. These results are in partial agreement with Miertus et al. (1999) computational model to estimate the affinity order of each of the 20 amino acids with β-CD. When comparing our results for amino acids to their computational model, it was observed that only the position of PRO and HIS were inverted. Similar results were reported by Tang, Kong, Ou, Liu, and Zou (2006) that evaluated a cross-linked-β-CD polymer for adsorption of aromatic amino acids. The discrepancies between the experimental results and the previsions given by the computational model of Miertus et al. (1999) show that we still need to resort to accurate experimental methods, such as the ROESY and DOSY NMR techniques, to get completely reliable complexation results. The low affinity between the amino acids and β-CD found in our work demonstrate that the values obtained for the constants for the formation of the β-CD inclusion complexes, determined by NMR techniques, were adequate and similar to the literature.

The results for the p_{compl} (Table 1) for TRP, TYR, PHE and PRO, 31.0%, 30.0%, 24.0% and 11.9%, respectively, demonstrate that these amino acids are included in the β-CD cavity. They are the responsible for transmitting bitter taste to protein hydrolysates (Otagiri, Miyake, Ishibashi, Fukui, & Kaneshisa, 1983; Raksakulthai & Haard, 2003). Bitter molecules bind to a G protein-coupled receptor type T2R on the apical membrane of the taste receptor cells (TRC) located in the taste buds. In humans, roughly 25 different T2R are described. Additionally, several alleles are known and about 100 different bitter phenotypes exist in man. TRC are specialized to a certain taste quality (Ley, 2008).

The relative low percentage of the complexed population is important to keep the amino acid bioavailability, as the liberation of the included guest may become difficult during the digestive process due to the high values of $K_{ap'}$. However, molecules of many peptides and proteins are too hydrophilic and bulky to be included in the CD cavity; thus their interaction with CD could be local, and accessible only to hydrophobic side chains. Such interactions may alter the conformation of protein and peptides and thus alter their physicochemical parameters. In addition, complex formation may avoid detection of the amino acid taste by the tongue gustative buds, because the inclusion of the lateral chain in the CD cavity restricts amino acid access to the taste bud receptors.

Small structural variations can change the taste profile or strongly influence the bitter taste threshold. For example, the amino acid L-tryptophan is bitter but the D-enantiomer shows a distinct sweet taste (Belitz & Grosch, 1997). The conjunction of these effects – reduction of the bitter taste and a likely maintenance of the bioavailability of all the amino acids that form the original protein – may give rise to new opportunities for industrial applications of β-CD protein hydrolysate debittering.

Once the formation of the amino acid inclusion complex with β-CD was proven, then the effect of complex formation on the taste of amino acids was evaluated. For this sensory evaluation the amino acids chosen were PHE, PRO, TRP and TYR, because they formed inclusion complexes, as showed by the ROESY experiments. These amino acids are important for the formation of the bitter taste in peptides, mainly PRO that potentializes the bitterness, particularly when in pairs or localized at the center of a peptide. Another factor that enhances bitterness in peptides is the presence of two non polar or phenolic amino acids, such as PHE, PRO, TRP and TYR, at the C-terminal (Otagiri et al., 1983; Raksakulthai & Haard, 2003). In addition, aggravation of the bitter taste problem was found with protein hydrolysates made with endoproteases, because they have the tendency of hydrolyzing non polar amino acid residues, producing peptides with non polar C-terminals with high bitterness (Tamura et al., 1990). All the above mentioned factors strengthen the importance of choosing PHE, PRO, TRP and TYR for the sensory evaluation, seeking a debittering effect by complexation with β-CD.

Table 2 presents the description of the sensory characteristics concerning mouthfell and taste for solutions containing the chosen amino acids and β-CD in a molar ratio of 1:1. It can be observed that the presence of β-CD altered the mouthfeel sensation and reduced the astringency for PHE and TRP solutions. Regarding taste, the presence of β-CD changed the acid taste of PHE to a slightly sweet taste, with bitter aftertaste. This behavior may be associated

Table 2
Description of the mouthfeel characteristics and taste of amino acid (AA) aqueous solutions containing β-cyclodextrin (β-CD) at the 1:1 M ratio.

AA	β-CD	Mouthfeel characteristic	Taste
Tryptophane	Absence	Aqueous and slightly astringent	Slightly bitter
	Presence	Aqueous	Insipid
Tyrosine	Absence	Aqueous	Very slightly medicinal
	Presence	Aqueous	Insipid
Phenylalanine	Absence	Aqueous and slightly astringent	Slightly acid
	Presence	Aqueous	Slightly sweet with bitter after taste
Proline	Absence	Aqueous	Very slightly medicinal
	Presence	Aqueous	Insipid

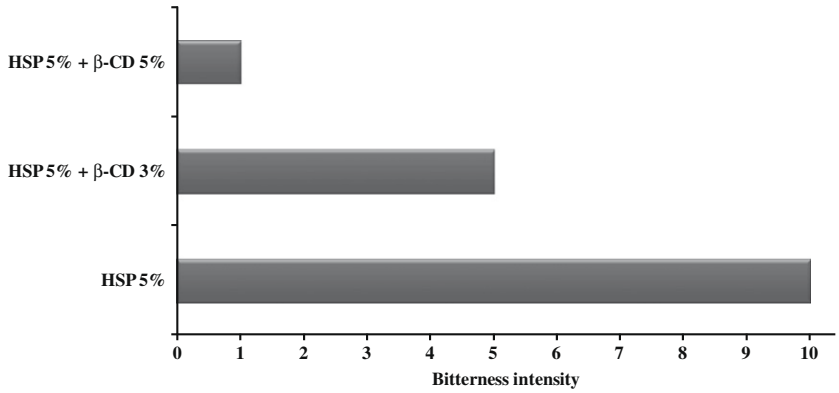


Fig. 2. Effect of the presence of β-cyclodextrin (β-CD) on the bitter taste of an aqueous solution of hydrolyzed soy protein (HSP).

with steric modifications of the amino acids as they are complexed with β-CD, exposing groups that show signaling of both sweet and bitter tastes (Chandrashekar, Hoon, Ryba, & Zuker, 2006).

For PRO and TYR solutions, the presence of β-CD altered the taste from very slightly medicinal to insipid (Table 2). The formation of the inclusion complexes of PRO and TYR with β-CD, showed interactions with the hydrogens H′5 and H′3 from the CD cavity and this may alter the conformation of the amino acids and expose amino acid groups that interact with different taste receptors, found at the plasmatic membrane (Chandrashekar et al., 2006). Consequently, it is possible that the presence of β-CD causes steric modifications in the amino acids, changing their sites of interaction with the taste receptors at the tongue.

This result obtained for the pure amino acids added further interest in testing the soy protein hydrolysate for evaluating the effect of β-CD on the perception of the bitter taste. The soy protein hydrolysate, after drying, had the aspect of an opaque solid with a light caramel color and a shining surface. When in water solution at 5% (w/v) it had a strong bitter taste that was classified as the maximum bitterness value in the sensory tests (10 points in a scale from 0 to 10 points, Fig. 2) and it had a residual umami taste.

The addition of 3% β-CD to the soy protein hydrolysate solution reduced both the bitter taste and the characteristic smell of soy. The addition of 5% β-CD to the hydrolysate solution reduced significantly its bitterness intensity by 90% (Fig. 2), and attenuated the characteristic soy odor.

Tamura et al. (1990) seems to be the first report on the use of α-CD for the alteration of the taste of amino acids and peptides, as previous reports refer to the use of CDs for the reduction of the bitter taste of juices. In Tamura's work it was necessary to add 150 g L^{-1} of α-CD to reduce adequately the bitter taste of the amino acids and peptides tested. Our work shows that for the alcalase soy protein hydrolysate, without removal of any peptides, the addition of only 5% β-CD is sufficient to reach a significant reduction of the bitter taste. Our work seems to be the first report on the addition of β-CD and reduction of the bitterness of soy protein hydrolysate, with various peptides with differentiated structures. In addition to the bitter taste reduction, the presence of β-CD may have functional effects on protein and peptide solutions as the increase of solubility (Irie & Uekama, 1999), stabilization of solution and emulsion (Hattori, Okada, & Takahashi, 2000), increase of the protein shelf life and decrease of denaturation (Branchu et al., 1999). Moreover, depending on the CD and the peptide, protection against enzymatic and chemical degradation has been reported (Koushik, Bandi, & Kompella, 2001) and it is related to specific interactions between CD and hydrophobic amino acids. Aachmann et al. (2003) studied the interactions between β-CD and non-carbohydrate-binding model proteins. Their results demonstrated that the interactions occurred with specific amino acids, and that TYR and PHE were the most important in all cases. They concluded that these interactions explains the wide range effects of CD on different proteins: aggregation (if residues responsible for aggregation are highly solvent accessible), protection against degradation (if the point of attack of a protease is sterically "masked" by CD), alteration of function (if residues evolved in function are "masked" by CD) and that the exact effect of a CD on a protein will always be given by the particular structure of this protein.

The main bitter peptides hold in their chains non polar amino acids situated at the middle or the extremity of the peptide chain (Raksakulthai & Haard, 2003; Saha & Hayashi, 2001). That structural characteristic is fundamental for the interaction of non polar amino acids with the taste sensitive cells at the tongue. Thus the higher proportion of β-CD (5%, Fig. 2), which have led to significant reduction in the bitterness of the soy hydrolysate, was sufficient to increase the amino acid and peptide complexed populations in the hydrolysate mixture up to the point of significantly lowering the solution bitterness intensity. In industrial applications it is hoped that the use of an acid pH, normally found in soft drinks and juices, in conjunction with a low temperature of refrigeration, would help the formation of inclusion complexes with β-CD. This should occur because the formation of inclusion complexes is increased by weakened interactions between water and peptides close to their isoelectric points and by reduced molecular vibration at lower temperatures, making β-CD a fundamental ingredient for the taste regulation of acid juices formulated with soy protein hydrolysates.

4. Conclusions

In the present study it was concluded that the amino acids form inclusion complexes with β-CD and the order of decreasing affinity for the β-CD cavity is TRP > TYR > PHE > PRO > HIS > ILE; NMR nuclear Overhauser effect (NOE) signals confirm inclusion complexes with β-CD and amino acids; β-CD alters the taste perception of the amino acids and reduces the bitter taste of hydrolyzed soy protein; β-CD is a potential additive for masking the bitter taste of acidic beverages made with hydrolyzed soy protein.

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