MARA ROSE DE OLIVEIRA

CONTRIBUIÇÃO AO ESTUDO DA SEPARAÇÃO E DA IDENTIFICAÇÃO DE PEPTÍDEOS COM POTENCIAL ANTI-HIPERTENSIVO OBTIDOS DA TRIPSINÓLISE DE CASEÍNAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Magister Scientiae*.

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APROVADA: 21 de fevereiro de 2017.

Maria Cristina Baracat Pereira
(Coorientadora)

Jane Sélia dos Reis Coimbra
(Coorientadora)

Edvaldo Barros

Eduardo Basílio de Oliveira
(Orientador)

Dedico este trabalho à minha família,

por todo amor e confiança

em mim depositados.

"É melhor tentar e falhar, que preocupar-se e ver a vida passar;
é melhor tentar, ainda que em vão, que sentar-se fazendo nada até o final.
Eu prefiro na chuva caminhar, que em dias tristes em casa me esconder.
Prefiro ser feliz, embora louco, que em conformidade viver ..."

Martin Luther King

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BIOGRAFIA

MARA ROSE DE OLIVEIRA, filha de Fernando de Oliveira e Maria Trindade de Oliveira, nasceu no dia 21 de outubro de 1988, em Viçosa, MG.

Em março de 2008, ingressou no curso de Ciência e Tecnologia de Laticínios da Universidade Federal de Viçosa, MG, graduando-se como Bacharela em Ciência e Tecnologia de Laticínios em maio de 2013.

Ao final da graduação, julho de 2012, desempenhou atividades na área de assuntos regulatórios no Laticínios Tirolez Ltda, unidade de Monte Aprazível, SP, sendo essa experiência profissional finalizada em junho de 2013. Em julho de 2013, trabalhou na empresa Microvet – Microbiologia Veterinária Especial Ltda, localizada em Viçosa, MG, onde permaneceu até agosto de 2014. Em setembro de 2014, foi bolsista de apoio técnico a pesquisa, pelo CNPq, no laboratório de Leite e Derivados (Inovaleite) do Departamento de Tecnologia de Alimentos da Universidade Federal de Viçosa, MG, sendo essas atividades concluídas em fevereiro de 2015.

Em março de 2015, ingressou no Programa de Pós-Graduação *Stricto Sensu* em Ciência e Tecnologia de Alimentos, em nível de mestrado, na Universidade Federal de Viçosa, MG, submetendo-se à defesa da dissertação em fevereiro de 2017.

SUMÁRIO

LISTA DE FIGURAS

Estudo Bibliográfico

Estudo Experimental

LISTA DE TABELAS

Estudo Bibliográfico

Estudo Experimental

RESUMO

OLIVEIRA, Mara Rose, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Contribuição ao estudo da separação e da identificação de peptídeos com potencial anti-hipertensivo obtidos da tripsinólise de caseínas.** Orientador: Eduardo Basílio de Oliveira. Coorientadoras: Jane Sélia dos Reis Coimbra, Maria Cristina Baracat Pereira e Monique Renon Eller.

Determinados peptídeos, após serem liberados da estrutura proteica, podem exercer funções fisiológicas benéficas no corpo humano. A literatura tem evidenciado a caseína como substrato para obtenção de peptídeos com diversas atividades biológicas. Dentre essas, a atividade anti-hipertensiva é uma das bioatividades mais investigadas. A hipertensão está relacionada a uma das principais causas de mortes por doenças cardiovasculares no mundo. O principal tratamento dessa patologia consiste na administração de fármacos. No entanto, efeitos colaterais decorrentes de longos períodos de tratamento indicam a necessidade de pesquisas para o emprego de compostos naturais capazes de auxiliar no controle da hipertensão com pouco ou nenhum efeito colateral. Diante do exposto, foi realizado um estudo bibliográfico com análise crítica das informações apresentadas na literatura, objetivando evidenciar aspectos relacionados à obtenção de peptídeos derivados de caseína com potencial anti-hipertensivo. Neste trabalho, foi observado que pesquisas sobre peptídeos bioativos representam uma área altamente promissora e o uso de peptídeos anti-hipertensivos na saúde humana é caracterizado por oportunidades e desafios. A obtenção e purificação industrial de compostos bioativos (rendimento), bem como a atuação dos mesmos nos tecidos-alvo (dosagem e viabilidade biológica), são assuntos que ainda necessitam ser mais explorados. No estudo experimental, foi proposto um protocolo diferenciado de separação do hidrolisado de caseína bovina sob condições otimizadas e identificou-se os peptídeos contidos nas frações cromatográficas obtidas. Os resultados confirmaram algumas características apresentadas na literatura em relação à alta atividade anti-hipertensiva de peptídeos e a estrutura dos mesmos. Além disso, foi possível evidenciar uma expressiva correlação entre a sequência tripeptídica da região C-terminal com a alta atividade biológica avaliada.

ABSTRACT

OLIVEIRA, Mara Rose, M.Sc., Universidade Federal de Viçosa, February, 2017. **Contribution to the study of the separation and identification of peptides with antihypertensive potential obtained from casein trypsinolysis.** Advisor: Eduardo Basílio de Oliveira. Co-advisors: Jane Sélia dos Reis Coimbra, Maria Cristina Baracat Pereira and Monique Renon Eller.

Certain peptides, after released from protein structure, can exert beneficial physiological functions in human body. Literature has been evidencing the casein as a substrate for obtaining peptides with several biological activities. Among these, antihypertensive activity is one of the most investigated bioactivities. Hypertension is related to one of the leading causes of cardiovascular disease deaths worldwide. The main treatment of this pathology is the administration of drugs. However, side effects resulting from long periods of treatment indicate the need for research for employment of natural compounds capable of assisting in the control of hypertension with little or no side effect. Based on the above exposed, it was carried out a bibliographic study with a critical analysis of information showed in the literature, aiming to evidence aspects related to the obtaining of peptides derived from casein with antihypertensive potential. In this study, it was observed that research on bioactive peptides represents a highly promising area and the use of antihypertensive peptides in human health is characterized by opportunities and challenges. The obtaining and industrial purification of bioactive compounds (yield), as well as their performance in the target tissues (dosage and bioavailability) are issues that still need to be further explored. In the experimental study, it was proposed a unusual protocol for the separation of bovine casein hydrolysate under optimized conditions and identified the peptides contained in the chromatographic fractions obtained. The results confirmed some characteristics presented in the literature regarding the high antihypertensive activity of peptides and their structure. In addition, it was possible to evidence an expressive correlation between the C-terminal tripeptide sequence and the high biological activity evaluated.

CAPÍTULO 1

Introdução Geral

Proteínas alimentares de origem animal (carne, peixes, ovos e leite) e vegetal (amendoim, soja e glúten) podem originar, após digestão (*in vivo)* e/ou hidrólise enzimática (*in vitro),* determinados fragmentos que, para além do seu valor nutricional, são capazes de exercer atividades biológicas diversas no corpo humano. Tal capacidade se expressa por meio da regulação de processos fisiológicos. Sendo assim, os referidos fragmentos são comumente qualificados como "peptídeos bioativos". Estes compostos são geralmente constituídos por 2 a 20 resíduos de aminoácidos e apresentam massa molecular $\leq$ 6 kDa.

Proteínas do leite bovino são reconhecidas como excelentes fontes de peptídeos bioativos. Compreendidas em dois principais grupos, as proteínas do leite são classificadas em caseínas e proteínas do soro. Compostas essencialmente de quatro frações (α_{S1}, α_{S2}, β e k-caseína), as caseínas correspondem a 80% do conteúdo proteico total no leite bovino (Saito, 2008[1]). Essa proteína é reconhecidamente um substrato para a formação de peptídeos com diferentes bioatividades. Dentre estes, se destacam aqueles com potencial anti-hipertensivo, pois inibem a enzima que, no organismo, catalisam reações fisiológicas que desencadeiam a elevação da pressão arterial (Korhonen & Pihlanto, 2006[2]; Saito, 2008[1]).

A hipertensão é uma doença crônica definida por valores de pressão arterial sistólica ≥140 mmHg e/ou e diastólica ≥90 mmHg. Cerca de 55% das mortes ocasionadas por doenças cardiovasculares no mundo têm sido atribuídas a essa patologia como causa primária (WHO, 2013[3]). Seu principal tratamento consiste na administração de fármacos, sendo o captopril e o enalapril dois exemplos muito conhecidos. Em função de efeitos colaterais frequentemente associados a esse tipo de medicação, compostos nutracêuticos, como peptídeos bioativos são pesquisados como possíveis coadjuvantes de drogas sintéticas. Com o propósito de contribuir cientificamente para o avanço do conhecimento na área de peptídeos derivados de caseína com potencial anti-hipertensivo, um projeto de pesquisa nesta área vem sendo desenvolvido por nossa equipe nos últimos 5 anos. Inicialmente foi identificada

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[1] Saito, T. (2008). Antihypertensive peptides derived from bovine casein and whey proteins. Advances in Experimental Medicine and Biology, 606, 295–317.

[2] Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. International Dairy Journal, 16(9), 945–960.

[3] WHO - World Health Organization, (2013). A global brief on hypertension: Silent killer, global public health crisis. Available at: http://www.who.int/campaigns/world-health-day/2013/en/ (Acessed January 10, 2017).

a atividade inibitória da enzima conversora de angiotensina (ECA) *in vitro* em hidrolisados de caseína bovina, obtidos utilizando-se como como biocatalisador a tripsina de pâncreas bovino (Souza et al., 2014[4]). Em seguida, esse mesmo processo de hidrólise foi reproduzido e otimizado, sendo identificado o tempo de reação ótimo (120 minutos) para obtenção do hidrolisado contendo peptídeos com elevada atividade inibitória de ECA (≈ 80%) (Silva, 2016[5]).

Constituindo uma etapa adicional desse projeto, o presente estudo visou contribuir com a proposta de um protocolo diferenciado de separação de peptídeos anti-hipertensivos, em relação àqueles apresentados na literatura, sendo assim adotados métodos cromatográficos com emprego de uma coluna para cromatografia em fase líquida (C4). Além disso, análises estruturais por espectrometria de massas dos peptídeos contidos nas frações cromatográficas de maior potencial anti-hipertensivo (inferido a partir da atividade inibitória *in vitro* de ECA) foram empreendidas, a fim de identificar a composição e a sequência aminoacídica dos peptídeos ali contidos. Os resultados do trabalho desenvolvido estão apresentados nesta dissertação, que está assim estruturada:

1) Primeiramente, um estudo bibliográfico, sob a forma de um manuscrito de artigo de revisão de literatura, construído a partir de dados compilados por Silva (2016), e que foi extensivamente revisado e atualizado. O tema central desse manuscrito é “peptídeos inibidores de ECA, derivados de caseína”, detalhando estudos *in vitro*, *in vivo* e patentes relacionadas ao tema.
2) Em seguida, também estruturado sob a forma de um manuscrito de artigo, é apresentada a parte experimental do trabalho. Foi proposto um protocolo cromatográfico de separação de peptídeos com potencial anti-hipertensivo, usando uma coluna C4, cuja constituição se diferencia da tradicional C18 empregada na maioria dos estudos encontrados em que se utilizou coluna de fase reversa. Adicionalmente a esse aspecto diferenciado, são apresentados os resultados de análises de espectrometria de massas, com a identificação estrutural dos peptídeos que compunham todas as frações cromatográficas.

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[4] Souza, E. C., Coimbra, J. S. D. R., de Oliveira, E. B., & Bonomo, R. C. F. (2014). Recovery of casein-derived peptides with in vitro inhibitory activity of angiotensin converting enzyme (ACE) using aqueous two-phase systems. Journal of Chromatography B, 973, 84–88.

[5] Silva, T. J., (2016). Obtenção e identificação de peptídeos derivados da tripsinólise de caseínas com atividade inibitória de enzima conversora de angiotensina I (ECA). Dissertação de Mestrado. Universidade Federal de Viçosa – Programa de Pós-Graduação Stricto Sensu em Ciência e Tecnologia de Alimentos. Viçosa, Minas Gerias. Brasil.

CAPÍTULO 2

Estudo Bibliográfico

Antihypertensive peptides from caseins: production, mechanisms of physiological actions and challenges for food applications

Mara Rose de Oliveira[a], Thaís Jordânia da Silva[a], ...

[a] *Departamento de Tecnologia de Alimentos (DTA), Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

ABSTRACT

This review is focused on the state-of-art of peptides with angiotensin I -converting enzyme (ACE) inhibitory activity, thus with antihypertensive potential derived from casein enzymatic hydrolysis. Firstly, molecular characteristics of caseins relevant to a better understanding of this subject were concisely commented and, then casein-derived antihypertensive peptides were addressed. Next, a brief presentation of the pathophysiology of hypertension regarding the ACE activity on two main systems that regulate blood pressure in the human body are explored. Parameters of enzymatic hydrolysis processes, such as casein substrates, enzymes, pH, temperature and times of reactions of hydrolysis were compiled. The main *in vitro* and *in vivo* bioassays for antihypertensive peptides have been presented and discussed, including the advantages and limitations of such approaches. Characteristics related to the amino acid composition of peptides with high ACE-inhibitory potential were also referenced in this work. Several recent studies and patents dealing with casein-derived antihypertensive peptides derived from caseins were examined in terms not only of hydrolysis process parameters, but also reconsidering amino acid sequences and taking into account effectiveness of peptides, compared to classical antihypertensive drugs. Finally, some trends, challenges and opportunities were presented in the field of casein-derived antihypertensive peptides, inferred from the literature analysis.

KEYWORDS*:*

Bioprocesses
Milk proteins
Proteolysis
Angiotensin-Converting Enzyme (ACE)
Hypertension;
Nutraceutical

41 **SUMMARY**

42

54

ABBREVIATIONS

AC: Affinity Chromatography
ACE: Angiotensin Converting Enzyme
AHTPDB: Database of Antihypertensive Peptides
Ang I: Angiotensin I
Ang II: Angiotensin II
ESI: Electrospray Ionization
E:S: Enzyme-to-Substrate Ratio
GMP: Glycomacropeptide
GRAS: Generally Recognized as Safe
HHL: Hippuryl-L-Histidyl-L-Leucine
HILIC: Hydrophilic Interaction Liquid Chromatography
HPLC: High Performance Liquid Chromatography
HT: Hypertension
IEC: Ion-Exchange Chromatography
IEF: Isoelectric Focusing
KKS: Kallikrein-Kinin System
LC: Liquid Chromatography
MALDI: Matrix-Assisted Laser Desorption/Ionization
MRM: Multiple Reaction Monitoring
MS: Mass Spectrometry
MS/MS: Tandem Mass Spectrometry
NO: Nitric Oxide
PGI_2: Prostaglandins 2
QIT: Quadrupole Ion Trap
RAS: Renin-Angiotensin System
RP-HPLC: Reversed Phase-HPLC
SEC: Size-Exclusion Chromatography
SHR: Spontaneously Hypertensive Rat
TOF: Time-of-Flight
UF: Ultrafiltration
UHPLC: Ultra High Pressure Liquid Chromatography
α_{s1}-CN: alpha$_{S1}$-casein
α_{s2}-CN: alpha$_{S2}$-casein
β-CN: beta-casein
κ-CN: kappa-casein

Symbols
λ: Wavelength (nm)

1. Introduction

Dietary proteins are very diverse in terms of both amino acid composition and three-dimensional structures. Many of them, beyond their well-known nutritional role (to provide essential amino acids to the metabolism), can exert benefic health effects on human body, due to the physiological action of peptides released during their digestion [1]. Food proteins, as those found in eggs [2, 3], soybean [4, 5] and milk [6, 7], to cite

only some of most emblematic examples, can originate one or more of these bioactive peptides. This expression, “bioactive peptides”, is often used to refer to biologically active molecules originated from protein hydrolysis, usually composed by 2-20 amino acid residues and presenting molecular mass ≤ 6 kDa [8]. The first report of a bioactive peptide derived from a food was introduced by Mellander, who demonstrated an increase in bone calcification in stunted newborns after ingestion of phosphopeptides from casein [9]. Since then, several studies about these peptides have been developed and numerous bioativities demonstrated for the major systems of the human body. These peptides, to effectively present benefits to the body, must withstand the whole digestion process, be absorbed by gastrointestinal tract, and achieve their action site, also referred as “biological target” [10].

Bioactive peptides derived from food proteins have been the focus of intensive research in last years, as recently reviewed by Castro & Sato [8]. Milk is considered a good source of protein and essential amino acids [11]. After the cleavage, the milk proteins can provide small peptide fragments with different physiological activities, being that used as a strategic material for research involving bioactive peptides. In fact, a great variety of peptides derived from milk proteins has been obtained and their numerous bioactivities have been demonstrated [12]. Also, studies demonstrating the antioxidant [13], immunomodulatory [14], cytomodulatory [15], anti-inflammatory [16] and ACE-inhibitory peptides [17] derived from hydrolysis of milk caseins are available. More particularly, the inhibitory activity of casein-derived peptides towards angiotensin converting enzyme (ACE) is nowadays well-recognized and intensively investigated. ACE is widespread in human body and is involved in a cascade of metabolic reactions that trigger increased blood pressure. For this reason, inhibitors of this enzyme are putative antihypertensive and, thus, they have great practical relevance.

Hypertension (HT) is a chronic medical condition in which blood vessels are persistently submitted to greater pressures as the blood flows within them. Formally, it is characterized by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg at rest [18]. It is estimated that more than 20% of adults worldwide have HT [19, 20]. Often termed as “silent killer” because it can be asymptomatic, this medical condition has been pointed out as the cause of at least 45% of deaths from cardiovascular diseases and 51% from stroke, being responsible for about 9.4 million deaths due to cardiovascular disease worldwide each year [19, 21]. A healthy lifestyle – reduced dietary sodium intake, control of body weight, regular aerobic physical activities, limited alcohol consumption and diets rich in fruit and vegetables – strongly helps preventing HT. However, once a HT diagnostic is established, lifestyle changes are not sufficient to control it, and the use of

antihypertensive drugs becomes necessary [22]. Various side effects have been associated with the use of typical antihypertensive drugs, such as increased potassium levels, reduced renal function, cough, angioedema, skin rashes, and even fetal abnormalities [23]. That is the reason why the search for natural compounds, such as peptides, able to aid controlling HT with less or none undesirable secondary effects has become an increasingly relevant applied research field.

The recently created online database AHTPDB (http://crdd.osdd.net/raghava/ahtpdb) contains information about all peptides already identified and validated as antihypertensive in literature [24]. In February 2017, this database has been contained around 6000 entries, constituting a very valuable online tool to easily follow the discovery of new peptides or sources. Nevertheless, to the best of our knowledge, a recent review paper analyzing the research results for antihypertensive peptides derived from caseins is not available. In addition, a critical analysis of patents about productions of this bioactive peptides composed the present work. Hence, this review intended to provide a state-of-art of research on casein-derived antihypertensive peptides during the last years. A summary explanation of relevant aspects on hydrolysis of caseins, molecular composition, structures, and *in vitro* and *in vivo* evaluations was firstly presented. Next, patents dealing with production of such peptides by protease-catalyzed hydrolysis, as well as their amino acid sequence determination and their bioactivities assessment were tabulated, described and commented. Finally, based on these literature data, some opportunities and challenges in this field were discussed.

2. Caseins

Cow milk contains about 32 $g \cdot L^{-1}$ of protein, with variation according to factors such as species, breed, feed, season, diseases and lactation stage [25]. Milk proteins are usually grouped into two major classes: “whey proteins” and “caseins”. The whey proteins (which include mainly β-lactoglobulin, α-lactalbumin, serum albumin and lactoferrin) account for 20% of total milk proteins. The majority of them are globular proteins soluble in water over a wide pH range, formed by compactly coiled peptide chains [26, 27]. The caseins have their average isoelectric point around pH 4.6, and their molecules do not present well-defined tridimensional structures. Instead, they are found organized into micelles which remain in suspension in milk [28, 29]. A common aspect of all caseins is the fact that these proteins are mostly linked to phosphate groups (esterified to side chains of serine residues). In addition, their high content of proline residues leads to a particular bending of protein chains and prevents the formation of close-packed, ordered secondary and tertiary structures [30]. Then, there

is considerable exposure of hydrophobic residues, favoring protein-protein interactions over protein-water interactions and rendering the proteins practically insoluble in water, forming micelles.

Casein micelles aggregate calcium phosphate (more than 90% of the Ca^{2+} in skim milk is associated with casein micelles) and thousands of molecules of different casein of which approximately 80% of the total protein content in bovine milk are α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN fractions in the approximate ratio of 4:1:3.5:1.5 (by weight) [32]. The main molecular characteristics of these four casein fractions are summarily described in Table 1 and a schematic representation of the casein micelle is shown in Figure 1.

Table 1 - Composition and mains characteristics of casein fractions.

Fraction (%)	Molecular mass	Residues of amino acid (nº)	Residues of proline (nº and %)	Sites of action for trypsin (nº)	Main physicochemical characteristics
α_{s1}-CN (38.5%)	≈ 23 kDa	199	17 (8.54%)	21 (15 K + 6 R)	• Presents two hydrophobic regions separated by a more hydrophilic region containing seven of eight phosphate groups • It has elevated net negative charge and low hydrophobicity
α_{s2}-CN (10.1%)	≈ 25 kDa	207	10 (4.83%)	31 (25 K + 6 R)	• Presents several positive charges near C-terminus and negative charges near N-terminus regions • It easily aggregates in the presence of Ca^{2+} ion
β-CN (38.7%)	≈ 24 kDa	209	35 (16.75%)	16 (11 K + 5 R)	• Presents a charged N-terminal region and a hydrophobic C-terminal region • This molecule is amphiphilic
κ-CN (12.6%)	≈ 19 kDa	169	20 (11.83%)	15 (10 K + 5 R)	• The main fraction responsible for interactions and structural stability of casein micelles • Rennet hydrolysis of the peptide bond Phe105-Met106 releases a hydrophobic portion (para- κ-CN) and a hydrophilic portion (GMP)

Legend: Lysine “K” and Arginine “R”.

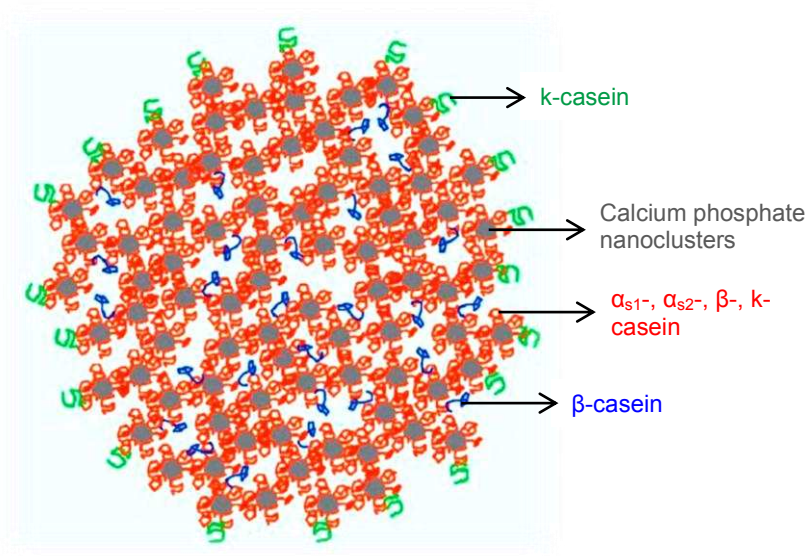


Figure 1 – Structural representation of the model casein micelle. Calcium phosphate (grey) with their attached caseins (red) and the k-casein (green) located on the surface. The mobile β-casein (blue) is shown within the water channels inside the micelle [30].

Although there are some models proposed for casein micelle, its structure is still under investigation. However, there is a consensus that this protein in micelle form is stabilized by hydrophobic and electrostatic interactions, whereas calcium phosphate clusters (contained in αs-CN and β-CN) bind to the phosphoseryl residues. The κ-CN on the surface of the casein micelle (negatively charged) provide the stabilization of adjacent casein micelles against aggregation [31, 32].

Organization of caseins in milk micelles has been correlated to different biological functions, such as the transport of high concentrations of Ca^{2+} and phosphates within mammary ducts (avoiding the calcification of mammary glands), and to the retention of proteins in the stomach of the newborn long enough to ensure a complete digestion (improving amino acid bioavailability). In addition to the high biological and nutritional value of caseins, the hydrolysis of these proteins can originate various bioactive peptides with biological roles, exerting a protective action on human health [27, 33, 34].

As caseins naturally form micellar structures, the susceptibility of these proteins to hydrolysis is dependent on their aggregation states [35, 36]. For several proteins, aggregation states are affected by physical-chemical properties of the medium (pH, ionic strength, solvent polarity etc.), exposure to heat or to heat-cold cycles, and, eventually, adsorption of proteins at interfaces [33, 37, 38]. The relative resistance of casein to hydrolysis can be attributed to their ability to form micelles and aggregates. Peptide bonds hidden within these supramolecular structures remain inaccessible to hydrolytic enzymes [36]. Hence, before being used as substrates for hydrolysis, caseins should be adequately solubilized by specific processes of separation and purification.

Different approaches can be used for separate caseins from milk, basing on differences: of density, such as ultracentrifugation [39]; of molecular size, such as centrifugation combined with filtration techniques (ultrafiltration or microfiltration) [40]; and of balance between repulsive/attractive intermolecular interactions, as in isoelectric precipitation [41]. Preliminaries techniques of separation can provide a great cost-benefit ratio on preparation of the bioactive peptides before the purification step. A strategy commonly employed in these processes is based on membrane pressure technique, such as ultrafiltration and nanofiltration, which are employed for enrichment and fractionation of bioactive peptides from protein hydrolysates under lower cust when compared with chromatographic techniques [42, 43].

Roach & Harte reported the separation and isolation of micellar casein from raw skimmed milk by ultrafiltration, combining a regenerated cellulose membrane (nominal cut off of 30 kDa) with a hollow fiber microfiltration module (0.2 μM cut off polyethersulfone membrane) [40]. Unlike this example, in the majority of analyzed studies dealing with bioactive peptides production, pre-purified casein fractions (β, κ, α_{s1} and/or α_{s2}-CN) are usually obtained from specialized suppliers, being adequately solubilized prior to hydrolysis. In Figure 2, a general depiction of controlled biochemical processes to produce peptides with putative bioactivities is shown.

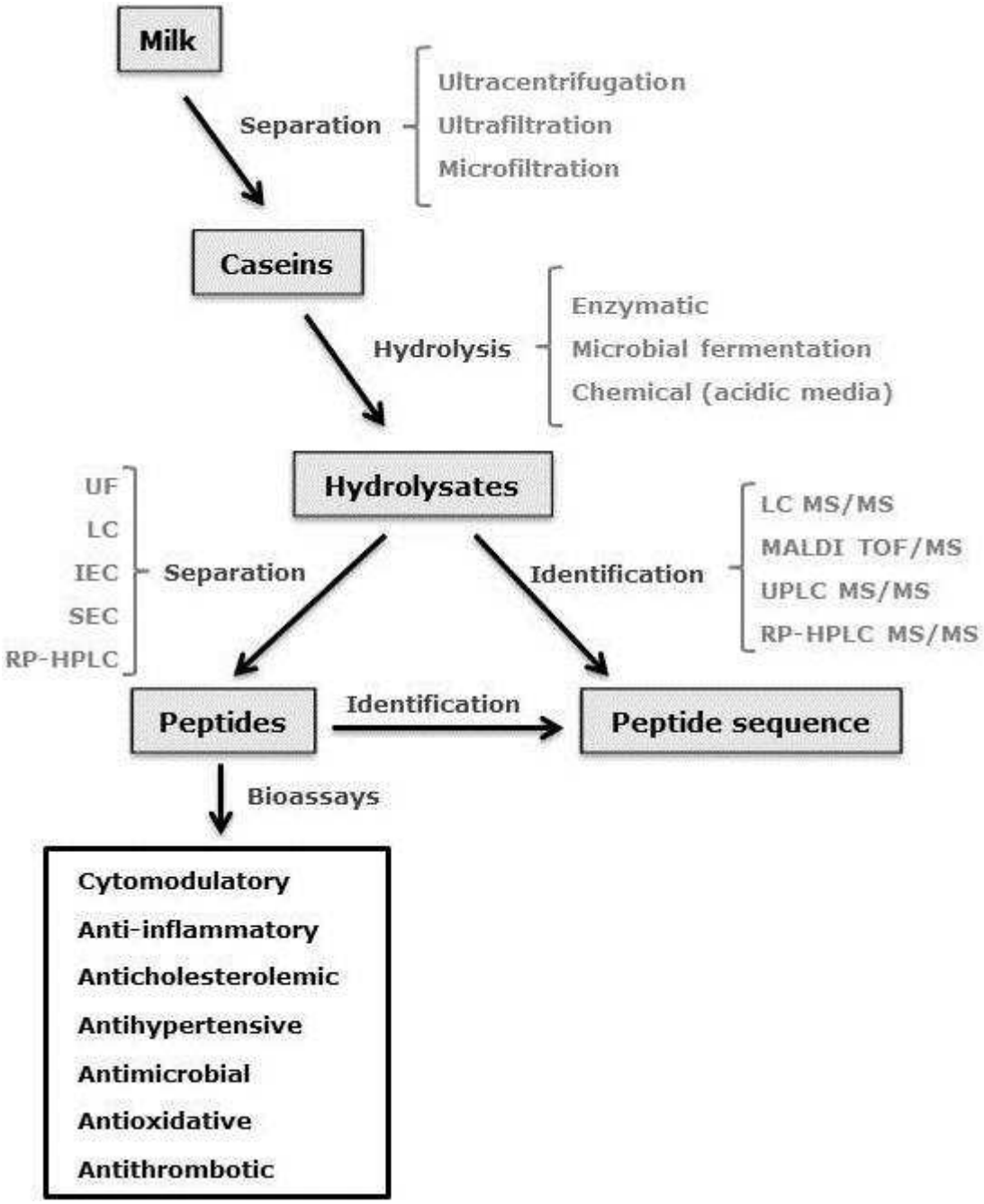


Figure 2 – A schematic representation of the processes sequence for production, separation, identification and bioactivity assessment of peptides derived from bovine casein hydrolysis.

Casein hydrolysis is usually performed by microbial fermentation [15, 44, 45] or, more frequently, enzyme-catalyzed processes, using proteases obtained from the gastrointestinal tract of humans and other mammalians (e.g. pepsin, trypsin, chymotrypsin) [17, 46], plants (e.g. *Ficus carica* L. latex proteinase) [47], and microorganisms (e.g. *Bifidobacterium longum*) [48].

The hydrolysates contain a large quantity and diversity of peptides, but only few of them are expected to present bioactivity whatever the protein sources or hydrolysis parameters. Preliminary bioactivity assays can be carried out directly on hydrolysates; however, in subsequent steps peptides must be recovered, isolated and separately

tested, to identify those which have biological activity. In a recent literature review, Castro & Sato summarized the main characteristics of different analytical methods frequently used for purification and identification of bioactive peptides derived from food proteins, emphasizing their mechanisms, advantages and limitations [8]. These methods include electrophoretic (IEF), chromatographic (HILIC, UHPLC, SEC, IEC, AC, RP-HPLC) and spectrometric (ESI/MS, MALDI-TOF/MS) approaches.

3. Casein-derived peptides with ACE-inhibitory activity

In the present topic, a more specific state-of-art analysis is focused on casein-derived peptides inhibitors of the ACE, thus acting as potential antihypertensive agents. Firstly, some physiological aspects of HT are summarily described, aiming at providing to readers a view of the main mechanisms underlying the action of antihypertensive peptides in human body. Next, approaches reported in literature to evaluate ACE-inhibitory activity of peptides are described and commented. Finally, in the last two sections, scientific studies (Tables 2 and 3) and patents (Table 4) dealing with the production, characterization and identification of casein-derived peptides with antihypertensive potential were presented and discussed.

3.1. ACE involvement in hypertension: a brief recapitulative

HT is a chronic medical condition in which the blood pressure in blood vessels is elevated, so that they are persistently submitted to a greater mechanical stress for pumping the blood. It appears as a consequence of a disruption in the balance of Na^+/K^+, leading to an increased fluid volume within blood vessels [18]. This physiological dysfunction is one of the consequences of consumption of food containing too much salt and fat, in a diet lacking fruit and vegetables, harmful levels of alcohol consumption, physical inactivity and poor stress management. Furthermore, these causes are intensified by smoking, obesity, high blood cholesterol levels and diabetes mellitus. HT substantially increases the probability of heart attacks, stroke and kidney failure, as it progressively weakens the resistance of blood vessels walls [19].

Physiologically, regulation of arterial blood pressure is mainly performed by metabolic, hormonal and nervous systems, in particular by the action of vasopressor compounds. Among these systems, the Renin-Angiotensin System (RAS) is known to play an essential role in blood pressure control. HT is strongly related to an increased activity of ACE and/or to the deactivation of the Kallikrein-Kinin System (KKS) [21, 49, 50]. ACE is a multifunctional zinc peptidase, whose activity is dependent on chloride ions, found in different tissues of the human body (heart, kidney, brain, reproductive organs and notably in endothelial cells of pulmonary capillaries) [51, 52]. This enzyme

was identified and isolated for the first time from horse plasma by Skeggs, Ph, Marsh, Kahn, & Shumway and its inhibition by casein-derived hydrolysates was firstly demonstrated by Yamamoto, Akino, & Takano [53, 54]. It plays key roles in both KKS and RAS (Figure 3).

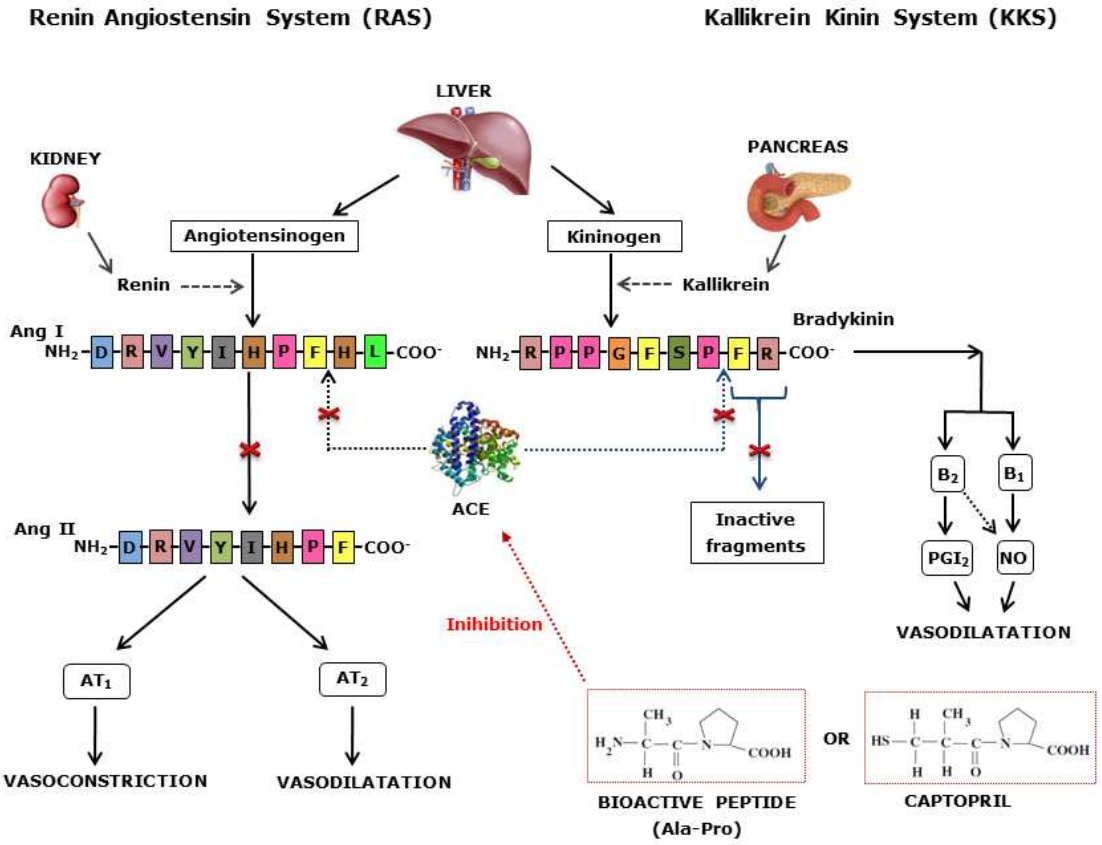


Figure 3 – Renin-angiotensin system (RAS) and kallikrein kinin system (KKS) to regulate of blood pressure. Ang I: Angiotensin I, Ang II: Angiotensin II, ACE: Angiotensin converting enzyme, AT_1: Angiotensin receptor 1, AT_2: Angiotensin receptor 2, B_1: Bradykinin receptor 1, B_2: Bradykinin receptor 2, NO: Nitric oxide, PgI_2: Prostaglandins 2.

In KKS, the kininogens (proteins produced in the liver and found in bloodstream) undergoes the hydrolytic action of kallikreins (enzymes originated mainly in the pancreas), forming bradykinin, which is a peptide composed of nine amino acid residues (RPPGFSPFR). This peptide presents vasodilating activity through its binding to two different receptors, type 1 (B_1) and type 2 (B_2) [21]. Both B_1 and B_2 induce the production of vasodilator nitric oxide (NO) in endoepithelial cells. In addition, B_2 receptor activates phospholipase A_2 that releases arachidonic acid, leading to the formation of other vasodilators, among which prostaglandins 2 (PGI_2) [21]. As ACE has high affinity for bradykinin, conditions that favor their interaction promotes the cleavage of bradykinin into two inactive molecules: the heptapeptide RPPGFSP and the dipeptide FR [23, 55].

In RAS, angiotensinogen (a glycoprotein originated in the liver and found in bloodstream) is hydrolyzed, releasing the decapeptide NRVYIHPFHL, called angiotensin I (Ang I), in a reaction catalyzed by renin (enzyme synthesized by kidneys). Ang I is subjected to ACE activity, generating the dipeptide HL and the octapeptide DRVYIHPF, also known as angiotensin II (Ang II) [21, 56, 57]. Ang II is able to bind two receptors, type 1 (AT_1) and type 2 (AT_2), both present in the endothelium of lung vessels. The biding of Ang II and AT_1 causes vasoconstriction in vascular smooth muscle cells, leading to an increment on the extracellular fluid volume through the stimulation of adrenal aldosterone released by sympathetic system, due to increased sodium concentration in blood vessels. On the other hand, interactions of Ang II with AT_2 in endothelial and vascular tissues and smooth muscle cells promote vasodilatation [21, 51, 52].

From these descriptions, it can be noted that ACE activity may lead to increased blood pressure through mechanisms involving both KKS and RAS: in KKS, increase of blood pressure results from the degradation of a compound which triggers vasodilation (bradikynin), whilst in RAS increased blood pressure comes from production of a compound (Ang II) able of rether inhibiting vasodilation or promoting vasoconstriction. Thus, ACE inhibitors not only block the generation of Ang II, but also prevent the degradation of bradykinin, exerting an antihypertensive effect [55]. It is important to keep in mind, however, that ACE inhibition is not the only way to control HT. Antihypertensive effects may be achieved by using drugs with other pharmacodynamics, alone or in combinations, such as diuretics, angiotensin receptor blockers, aldosterone receptor antagonists, renin inhibitors and calcium channel blockers acting on endothelial muscle cells [57, 58].

There is a notable structural similarity between molecules of captopril and the Ala-Pro peptide (Figure 3), a well-known ACE inhibitor [52, 56, 59]. In fact, because of the structural similarity between these two compounds, the antihypertensive activity of Ala-Pro is supposed to be due to a similar binding within the catalytic site of ACE. Other peptides have been proven *in vivo* as effective antihypertensive compounds, as the tripeptides FAP, VPP and IPP. When *in vitro* analyses unexpectedly indicated low ACE-inhibitory activity of these tripeptides, they were supposed to act as "pro-drugs", i.e., in a physiological environment, they are likely to undergo further cleavage, releasing dipeptides effectively able to inhibit ACE [21, 60].

The first mention in literature to bioactive peptides derived from caseins was done by Mellander, who reported a positive effect of peptides from phosphorylated casein on bone calcification, independent of vitamin D, in rickety children [9]. The antihypertensive potential of casein-derived peptides was reported by Yamamoto,

Akino and Takano who used a purified proteinase from *Lactobacillus helveticus* CP790 to hydrolyze milk previously fermented by the same bacteria, and demonstrated the antihypertensive activity of these hydrolysates in hypertensive rats [54].

From then on, the use of microorganisms was successfully applied to produce antihypertensive peptides by the action of their proteases. In fact, some foodstuffs formulations (yogurts, milk beverages etc.) contain microorganisms capable of hydrolyze caseins and form antihypertensive peptides, such as *Lactobacillus casei* c*asei*, *Lactobacillus casei Shirota* and *Streptococcus thermophillus* [45, 61]. Also, fungi species have been identified as able to promote the hydrolysis of caseins generating antihypertensive peptides, such as *Aspergillus niger prolyl* (T = 55 °C, pH = 6.0) [62, 63] and *Debaryomyces hansenii* (T = 28 °C, pH = 5.5) [64]. Although such fermentation processes to obtain antihypertensive peptide are undeniably important, enzymatic processes reports are more abundant in literature and remain the focus of the present review.

ACE-inhibitory peptides can be obtained from all the four casein fractions; however most of those reported in recent literature were obtained from β-CN or α_{s1}-CN. The identified peptides are formed by 3-19 amino acid residues. One of main advantages of using casein individual fractions during hydrolysis is the predictability of which peptides should be expected from a given enzyme with known specificity. For example, pepsins catalyzes the cleavage of peptide bonds between Phe and Leu residues [65]. Thus, according to the known amino acid sequence of α_{s1}-CN and β-CN, the peptides LHLPLP, RYLGY, AYFYPEL and YQKFPQY can be in fact expected to be formed from these substrates hydrolyzed with pepsin, as reported.

Srinivas & Prakash compared enzymes from various sources – a bacterial (*Streptomyces griseus*) protease, a fungal protease (*Aspergillus oryzae*) endoproteinases (aminopeptidase, carboxypeptidase and chymotrypsin) – to hydrolyze α-CNs [17]. These authors reported chymotrypsin as more efficient than the others to produce peptides presenting antihypertensive activity. Some antihypertensive peptides have been obtained by hydrolysis using other microbial proteases: ITP and WIQP [66]; LHLPLP and HLPLP [64]; IQP and VEP [62]; YLGYLEQLLR, HIQKEDVPSER and EPMIGVNQELAYFYPELFR [61]; MKP [67]; YQEPVLGPVRGPFPIIV [45]. In these cases, authors emphasized the lack of specificity of these microbial proteases during the hydrolysis as a non-negligible disadvantage to process control, as it enables the concomitant formation of several other irrelevant peptides, thus requiring additional separation steps.

3.2. Mechanisms of physiological action

Studies related to molecular recognition between bioactive peptides and ACE corroborate that hypotensive peptides usually have hydrophobic amino acids, such as proline, near the C-terminal region [68]. This characteristic gives them an expressive potential for ACE-inhibition, mainly defined by the tripeptide composition at the C-terminal position, as well as resistance to digestive enzyme degradation [42]. These evidences justify the significant numbers of studies on caseins as a substrate for the generation of peptides with ACE-inhibitory activity, since this protein contains in its composition many hydrophobic amino acids.

Some studies use molecular dynamics simulation to evaluate the level of interaction of possible hypotensive peptides with ACE [69]. This molecular docking simulation allows the quantification of the degree of interaction of ACE-peptide being that hydrogen bonds, electrostatic bonds and Pi bonds are usually related to the structural stability of interaction of ACE-peptide. There is also evidence that metal carboxylic bond formation between the ACE zinc atom and the hypotensive compound (peptides or drugs anthypertensives) causes the observed behavior of inhibition of ACE [70]. This interaction with ACE may become stronger with the increase in the number of hydrogen bonds [69].

3.3. In vitro studies

Peptides can have their antihypertensive potential evaluated through rapid and relatively low cost *in vitro* assays. It is important to keep in mind that these compounds will not have necessarily the same effect *in vivo* [71], because of the huge complexity of interactions between them and thousands of molecules in real biological systems. Despite of this, *in vitro* tests have been very frequently used to assess the antihypertensive potential of casein-derived peptides. Indeed, such assays have proven quite accurate, without the above mentioned difficulties regarding to expensiveness and time for execution of *in vivo* studies. Table 2 presents some data compiled from the literature concerning studies of antihypertensive peptides *in vitro.*

405 **Table 2** – *In vitro* studies dealing with antihypertensive peptides obtained from enzymatic hydrolysis of caseins.

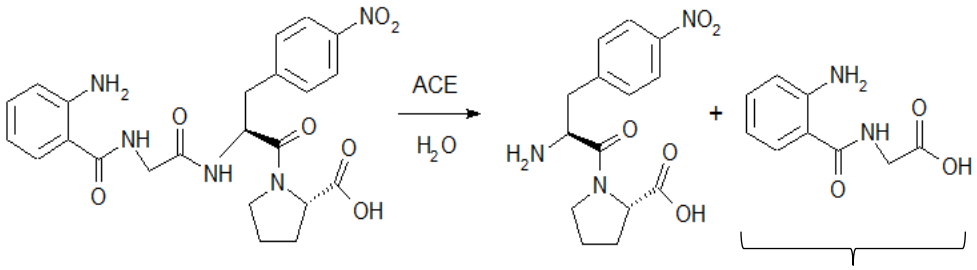
Casein (supplier)	Reaction conditions	Bioactivity assay	Identification technique	Peptide sequence♦	Reference
β-casein (Extracted by authors)	*Aspergillus niger* prolyl endoproteinase 55 °C / 0, 4 and 24* h pH = 6.0 E:S = 2.5% (w/w)	Fluorometric microtitre assay	UHPLC-ESI-TOF MS/MS	IQA VEP	[62]
Bovine casein (Sigma Aldrich)	Trypsin 37 °C / 1, 2*, 5, 8 and 24 h pH = 8.0 E:S = 1:150 (w/w)	Spectrophotometric assay (Quantification of hippuric acid at 228 nm).	-	-	[72]
Micellar casein and β-casein* (Extracted by authors)	Trypsin 37 ± 0.5 °C pH = 8 ± 0.05	Spectrophotometric assay (Quantification of hippuric acid at 228 nm).	UHPLC-ESI-MS/MS	44 peptides (18 from Micellar casein and 16 from β-casein)	[73]
Bovine casein (Extracted by authors)	Neutral protease (AS1.398) 45 °C / 1, 3, 6, 12* and 24 h pH = 7.0 E:S = 5% (w/w)	Spectrophotometric assay (Quantification of hippuric acid at 228 nm).	MALDI-TOF/TOF	RYPSYG DERF	[71]

Casein (supplier)	Reaction conditions	Bioactivity assay	Identification technique	Peptide sequence♦	Reference
α-casein from bovine milk (Sigma Aldrich)	Aminopeptidase, carboxypeptidase, bacterial protease, fungal protease and chymotrypsin* 37 °C / 120 min pH = 7.0 E:S = 1:150 (w/w)	Spectrophotometric assay (Quantification of hippuric acid at 228 nm).	RP-HPLC MALDI-TOF MS/MS	QKALNEINQF TKKTKLTEEEKNRL	[17]
Bovine casein (Extracted by authors)	Porcine pepsin A 37 °C / 3 h pH = 3.0 E:S = 3.7% (w/w)	Spectrophotometric assay (Quantification of hippuric acid at 228 nm).	-	-	[74]
Bovine casein (Extracted by authors)	Porcine pepsin A 37 °C / 1, 2, 3*, 4, 5, 6, 7 and 24 h pH = 2.0 - 2.5 E:S = 3.7 % (w/w)	Spectrophotometric assay (Quantification of bicinchoninic acid by HPLC).	RP-HPLC-ESI-QIT-MS	RYLGY AYFYPEL YQKFPQY	[75]

406 * Experimental conditions providing peptides with better antihypertensive potential.

407 ♦ Peptides that displayed ACE-inhibitory activity

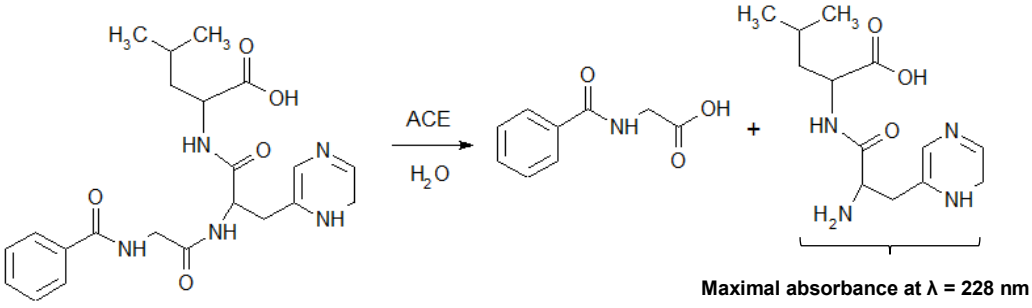
The two *in vitro* methodologies most commonly used to evaluate the antihypertensive potential of peptides are based on the inhibitory activity of such peptides towards ACE [76, 77]. The most recent of them is a fluorescence spectroscopy approach which uses the substrate aminobenzoylglycyl-p-nitrophenylalanylproline (Abz-Gly-Phe(NO_2)-Pro). ACE hydrolyzes this substrate forming a fluorescent product, aminobenzoylglycine (Abz-Gly) (Equation 1), with excitation and emission at respectively 355 and 405 nm. The inhibitory activity of the peptides is then quantified based on the decrease of the fluorescence intensity [77–80].



(Equation 1)

Fluorescent (excitation: λ = 355 nm; emission: λ = 405 nm)

Despite the quickness and sensitivity of the fluorescence spectroscopy approach, the UV-vis method proposed more than four decades ago by Cushman & Cheung is still widely used to evaluate *in vitro* inhibition of ACE activity by casein-derived peptides [76]. Cushman & Cheung defined one unit of catalysis as the amount of enzyme that catalyzes the formation of 1 μM hippuric acid from HHL in 1 min at 37 °C [76]. As previously presented, ACE acts in body on Ang I, to converting it into Ang II and simultaneously releasing the dipeptide HL. This *in vitro* test is grounded on a similar reaction, in which the ACE acts on the hippuryl-L-histidyl-L-leucine (HHL) substrate, releasing hippuric acid and the dipeptide HL (Equation 2). The released hippuric acid is then extracted and spectrophotometrically quantified by measuring the intensity of its absorbance at 228 nm [2, 45, 61, 72, 81, 82].



(Equation 2)

Maximal absorbance at λ = 228 nm

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Adaptations and/or minor modifications in this experimental methodology have given rise to different variants of the original protocol, although the conceptual basis remains the same. The adaptations observed in this protocol mainly regarded the

composition of the buffers used, temperature/time binomial, ACE:HHL ratio, and the adoption, or not, of a pre-incubation step before adding ACE to start the reaction.

3.4. In vivo studies

In vivo studies directly involving animals or humans to evaluate the bioactivity of a given compound certainly provide more realistic results. Nonetheless, studies addressing the antihypertensive potential of casein-derived peptides have been less frequently found in literature reports, which may be attributed mainly to their expensiveness, bioethics issues, and the delays sometimes very long needed to obtain statistically reliable results [83]. Table 3 shows some *in vivo* studies of peptides from casein with antihypertensive activity.

442 **Table 3** – *In vivo* studies dealing with antihypertensive peptides obtained from enzymatic hydrolysis of caseins.

Casein (supplier)	Reaction conditions	Bioactivity assay	Identification technique	Peptide sequence♦	Reference
Camel* and bovine casein (Extracted by authors)	Trypsin*, α-chymotrypsin and pepsin 37 °C / Overnight pH = 7.8 (trypsin and α-chymotrypsin) and 2.0 (pepsin) E:S = 2 %	Evaluation of the vasorelaxant effect of hydrolysates in Wistar Kyoto rats.	-	-	[84]
α_{S1}-casein and α_{S2}-casein (Ingredia)	Trypsin 37 °C / 24 h pH = 8.1	Evaluation of the hydrolysates aortic vasoconstriction and aortic tissues *ex vivo* on Wistar rats.	RP-HPLC-ESI-MS/MS	TTMPLW; FFVAPFPEVFGK; TVY; YL; ALNEINQFYQK; NMAINPSK; FPQYLQY; FALPQYLK; LYQGPIVLNPWDQVK	[85]
Bovine casein (Extracted by authors)	Neutral protease (AS1.398) 45 °C / 1, 3, 6, 12* and 24 h pH = 7.0 E:S = 5% (w/w)	Oral administration of the hydrolysates in SHR and measurement of their systolic blood pressure	MALDI-TOF/TOF	RYPSYG DERF	[71]
Bovine casein (Extracted by authors)	Porcine pepsin A 37 °C / 3 h pH = 3.0 E:S = 3.7% (w/w)	Oral administration of the hydrolysates by gastric intubation in SHR and measurement of their systolic and diastolic blood pressure	-	-	[74]

Casein (supplier)	Reaction conditions	Bioactivity assay	Identification technique	Peptide sequence♦	Reference
Bovine casein (Extracted by authors)	Porcine pepsin A 37 °C / 1, 2, 3*, 4, 5, 6, 7 and 24 h pH = 2.0 - 2.5 E:S = 3.7 % (w/w)	Oral administration of the peptides in SHR and measurement of their systolic blood pressure	RP-HPLC-ESI-QIT-MS	RYLGY AYFYPEL YQKFPQY	[75]

443 * Experimental conditions providing peptides with better antihypertensive potential.

444 ♦ Peptides that displayed antihypertensive activity

Most of *in vivo* studies found aiming to evaluate the antihypertensive potential of a peptide were carried out on SHRs, to which one or more doses of the compound (normally dissolved in water) were given, by oral administration, under adequately controlled conditions. As usual in this kind of study, the animals were clustered in a "control group", which received a traditional antihypertensive medicine (positive control) and a "test group", which received the peptides to be evaluated. The blood pressures of animals in both groups were measured before the oral administration of peptides or drugs, and compared to those measured periodically after this. For instance, the antihypertensive effect of casein hydrolysates (α_{s1}-CN or α_{s2}-CN; doses ranged from 10 to 1,000 mg·L^{-1}) in Wistar rats was compared to that of the antihypertensive medicine perindopril (doses ranged from 1 nM to 0.1 mM) [85]. The authors reported maximal decreasing of the systolic blood pressures of 11 mmHg (group treated with 1,000 mg·L^{-1} of α_{s1}-CN hydrolysate), 6 mmHg (group treated with 1,000 mg·L^{-1} of α_{s2}-CN hydrolysate) and 63 mmHg (group treated with perindopril). Miguel et al. compared the antihypertensive effects of a casein hydrolysate (400 mg·kg^{-1}) and captopril (50 mg·kg^{-1}), orally administrated to SHR [74]. The animals treated with hydrolysates and captopril showed a reduction of systolic blood pressure of, respectively, 28 mmHg (after 2 h) and 55 mmHg (after 6 h), while their diastolic blood pressure were, respectively, 40 mmHg (after 2 h) and 41 mmHg (after 4 h). Contreras et al. treated SHR (initial systolic blood pressure = 178 mmHg) with a single dose of 5 mg·kg^{-1} of the peptides RYLGY, AYFYPEL, YQKFPQY, VPP, RYLG, RY, YLGY, or zofenopril [75, 86]. The maximal blood pressure reductions observed with each compound were, respectively, 23 mmHg (after 8 h), 21 mmHg (after 4 h), 16 mmHg (after 2 h), 20 mmHg (after 4 h), 18 mmHg (after 6 h), 19 mmHg (after 8 h), 13 mmHg (after 4 h) and 31 mmHg (after 4 h). All these cases illustrate that the effectiveness of casein peptides in reducing SHR blood pressure can be sometimes comparable to classical ACE inhibitors drugs (zofenopril, perindopril or captopril). Nevertheless, in several cases, results suggested that peptides have particularities in their pharmacokinetics, as the effective dose and the time to reach maximal antihypertensive effects were different from classical drugs and variable among them.

Studies describing *in vivo* tests of antihypertensive peptides in humans are scarce; only some a few reports were found. For instance, Jauhiainen et al. administrated milk fermented with *Lactobacillus helveticus* containing peptide IPP (7.5 mg.100 mL^{-1}) and VPP (10 mg.100 mL^{-1}) to 94 hypertensive subjects (systolic blood pressure ranged 140 from 180 mmHg; diastolic blood pressure ranged from 90 to 110 mmHg) [87]. After 10 weeks, their average systolic and diastolic blood pressures were 132.6 mmHg and 83.0 mmHg, respectively. In another study, Mizuno et al. evaluated the effects in human directly intake of 1 to 8 mg of VPP and IPP, obtained from

hydrolysis of casein with *Aspergillus oryzae*. These authors worked with 136 healthy volunteers aged between 30 and 57 years, unmedicated, and presenting systolic blood pressure from 140 to 159 mmHg [88]. Volunteers received daily doses of 1 to 8 mg of a VPP+IPP mixture, during 6 weeks. After this time, their final systolic blood pressure was ranged from 133.2-134.8 mmHg.

4. Patents and products

There are nowadays a lot of scientific and technological information contained in patents, distributed on standardized schemes to facilitate the identification and examination of the inventions [89]. So, an insightful state-of-art analysis must consider patent databases, in addition to scientific periodicals. The current number of patents related to bioactive peptides is growing and standing out in the international patent database (http://www.freepatentsonline.com). In December 2015, an advanced search on this patent database using the keywords combination “peptides antihypertensive casein” returned 2,185 entries related to this subject. Some of them were selected and described in Table 4, which includes with the patent claim, hydrolysis conditions, identification techniques used, and the peptides identified.

500 **Table 4** – Some patents of antihypertensive peptides obtained from casein hydrolysis and products thereof.

Casein Source	Claim	Reaction Conditions	Identification technique	Peptides	Reference
Casein	Use of novel strains of *Lactobacillus helveticus* to produce high amounts of hypotensive peptides (IPP, VPP and LPP).	Enzyme: strain of *Lactobacillus helveticus* T: 38-42 °C / 15-50 h E:S: 10^7-10^9 cells/mL	Quantification of the VPP and IPP by LC-MS	Fermented milk product comprising a lactic acid bacteria strain of *Lactobacillus helvelicus*	[90]
α_{s2}-casein	Bioactive peptide isolated with antimicrobial, antihypertensive and/or antioxidant activity *in vitro* and/or *in vivo*	Enzyme = pepsin T = 37 °C / 3 h pH = 3.0 E:S = 3.7/100 (p/p)	RP-HPLC-MS / MS	LKKISQ, PYVRYL, HLPLPLL	[91]
Caseinate	High yield generation of IPP and VPP peptides by a single enzymatic step	Enzyme: Proline specific endoprotease from *Aspergillus niger* T: 50 °C / 4 h	LC - MS / MS	VPP, IPP, LPP	[92]
Casein	To provide a food product having a high content of tripéptidos	Enzyme: Proteases from *Aspergillus oryzae* T: 50 °C / 6 h pH: 7.0 E:S: 2-10 wt. % based on casein	HPLC-MRM-MS	VPP, IPP, LPP	[93]
Bovine casein	Use of a casein-derived peptide with antioxidant and ACE-inhibitory activity *in vitro* and *in* mammals, and compositions thereof.	Enzyme = pepsina porcina A T = 37 °C / 1, 2, 3*, 4, 5, 6, 7 e 24 h pH: 2.0-2.5 E:S = 3.7% (w/w)	RP-HPLC-MS / MS	RYLGY, AYFYPEL, YQKFPQY, HLPLPLL, VAPFPEVF, FVAPFPEV	[94]
Casein	Hydrolysis of casein and chemical synthesis of a peptide with high potential antihypertensive and its application in hypotensive drugs	Enzyme: Biopuraze sp-20 + Protease N + PTN6.0S T: 45-55 °C pH: 9.5 E:S: 1:1.200 + 1:2000 + 1:7.000	RP-HPLC-MS / MS	MKP	[95]

Casein Source	Claim	Reaction Conditions	Identification technique	Peptides	Reference
Casein	Develop an industrially usefull method for production of IPP and VPP tripeptides	Enzyme: Proteases from *Aspergillus oryzae, Aspergillus melleus, Lactobacillus helveticus*, papain T: 37 °C / 3 h	HPLC	VPP, IPP	[96]
β-casein from goat	Getting a new antihypertensive peptide as active ingredients in pharmaceutical preparations, dietary supplements, or as food ingredients	Enzyme: subtilisin enzyme from *Bacillus amyloliquefaciens* pH : 3.0 T: 55 °C / 3 h	RP-HPLC-MS	YGPIPN, SERK, SLPE	[97]
Casein	Invention of a process for preparing a product containing antihypertensive peptides by fermenting molecular casein containing lactic acid bacteria.	Enzyme = several strains *by Lactobacillus helveticus* T = 37 °C / 24 h pH = 6.7	-	VPP, IPP	[98]

The casein-derived peptides LKKISQSEQ, PYVRYL, HLPLPLL RYLGY, AYFYPEL, YQKFPQY, HLPLPLL, VAPFPEVF, FVAPFPEV were proposed as ingredients for pharmaceuticals, cosmetics, dermatologic preparations, dietary supplements or food formulations [91, 94]. It was claimed that these peptides enhance the natural defenses of the body, facilitating the control of blood pressure and/or bacterial infections.

Patents addressing the tripeptide MKP have also been found due to its antihypertensive potential *in vitro* and *in vivo* (rats and human) [95]. In SHR, the animals receiving 0.05 mg/kg of MKP showed a reduction in systolic blood pressure, after 2 hours of administration, of 42 mmHg, while the control group showed a reduction of only 7 mmHg. In humans (male volunteers at the age of 30-58, who suffered from mild HT, systolic blood pressure of 140-165 mmHg), the daily oral intake of MKP during 3 weeks led to a decrease of systolic blood pressure by 18 mmHg. Accordingly, this peptide is an excellent alternative as a new natural antihypertensive agent, low toxicity and high safety for the population.

As previously mentioned, IPP, VPP and LPP were the first casein-derived peptides proven as antihypertensive agents. The IPP peptide may be obtained from β-CN (fragment 74-76) or κ-CN (fragment 108-110), while VPP and LPP can be obtained only from β-CN (VPP = fragment 84-86; LPP = fragments 135-137 and 151-153) [99]. These peptides VPP and IPP have been object of several patents. The novelty claimed by the authors in such cases is not the peptides themselves, but the specificities and advantages of the alternative enzymes and bioprocesses conditions developed to obtain them, as for example, using proteases from *Lactobacillus helveticus* (40 °C / 24 h) [90]; *Lactobacillus helveticus* LBK-16 H (37 °C / 24 h) [98]; *Aspergillus niger* (55 °C / 24 h) [92]; *Aspergillus oryzae* (50 °C / 6 h) (Burg-Koorevaar et al., 2010); *Aspergillus* sp. + *Lactobacillus helveticus* (37 °C / 12 h) [96].

VPP, IPP and LPP were identified in fermented milk product obtained using *Lactobacillus helveticus* [90]. The patented production process requires milk with solid content comprised between 5 and 20 (w/w%). The fermentation may be carried out at 38-42 °C (temperature range within which the highest amount of tripeptides VPP, IPP and LPP is formed), for 15-50 hours. The concentrations of VPP, IPP and LPP after storage at 8 °C for one month were only slightly lower than those measured at the end of fermentation, demonstrating the stability of these compounds within the formulation. Similarly, Edens et al. developed a process using an endoprotease from *Aspergillus niger* to produce a beverage containing high concentrations of VPP and IPP, as this proteolytic enzyme was shown to catalyze specifically the cleavage of C-terminally proline [92]. This invention also relates the use of these peptide compositions for the

manufacture of a nutraceutical formulation to be used as a dietary supplement, with health claims such as prevention and/or treatment of HT, cardiac insufficiency, diabetes, obesity or stress. These products must contain a dose of 0.005-70 g per day of IPP and VPP (each), for a 70 kg person. Also, Yamamoto et al. developed a method for high-performance, large-scale production of tripeptides IPP and VPP. Peptidase derived from *Lactobacillus helveticus* was used, alone and combined with other fungal aminopeptidases or carboxypeptidases [96]. Casein hydrolysis was performed using 150 mg of casein and 0.2 mg of proteases from *Aspergillus oryzae* and *Aspergillus melleusum*. The mixture was allowed to react at 37 °C for 12 hours. After finishing the reaction, 1 mL of the purified peptidase extracted from *Lactobacillus helveticus* was added and the system was again left for 12 hours at 37 °C. According to the document, this process yielded 18.5% (w/w) of VPP and 25% (w/w) of IPP.

Geerlings, Hidalgo, Boza, & Jimenez described the use of a protease from *Bacillus amyloliquefaciens* to proceed to the hydrolysis of goat milk casein pre-concentrated by evaporation [100]. Hydrolysis was carried out at pH 3.0 and 55 °C, for 3 h, generating a hydrolysate containing the antihypertensive peptides YGPIPN (β-CN; f78-83), SERK (β-CN; f84-87) and SLPE (β-CN; f181-184). A fruit juice added of this hydrolysate (20 $g \cdot kg^{-1}$) was prepared and its processing (pasteurization, homogenization and packaging) was also reported in the patent. This case exemplifies a non-dairy food product enriched with antihypertensive peptides derived from casein.

In general, there is a consensus between researchers about antihypertensive peptides constitution. This agreement points that most of casein-derived peptides as good ACE inhibitors presented hydrophobic or basic amino acid residues at the C-terminal position. Indeed, different authors had already stated that peptides possessing at their C-terminal position a proline residue, or an aromatic residue (tryptophan, tyrosine and phenylalanine), or a positively charged residue (glutamine and lysine) bind more favorably within the active site of ACE [101–104]. In contrast, a negatively charged amino acid residue (aspartate and glutamate) at the C-terminal region of peptides have been correlated to poorer inhibitory activity of ACE [105].

5. Trends and concluding remarks

This literature analysis confirms bioactive peptides as a promising research area. The direct use of antihypertensive peptides for improving human health represents, however, a broad set of both an opportunity and a challenge. Indeed, it is reasonable to expect that a regular intake of such compounds may be a strategic way to prevent (or at least to reduce the risk) of HT. However, this expectation may be considered with caution, due to the lack of more exhaustive clinical studies, proving the

efficacy and long term safety of these peptides, characterizing their pharmacodynamics and pharmacokinetics, establishing their optimal doses, and dressing a precise panel of their possible secondary effects. The legal status for commercialization of these compounds (nutraceuticals, supplements, functional ingredients, or others) should also be established based on data from such clinical studies, and in agreement with the legal exigencies of each country. For people who were already diagnosed with HT, a strict dietary control, lifestyle changes and synthetic drugs remain the best combination, to date, to circumvent the deleterious effects of this medical condition.

Most of analyzed patents dealing with antihypertensive peptides derived from caseins claim novelty in industrial or semi-industrial processing, and not in peptides themselves. In fact, there are a limited number of peptides that can be obtained from a same protein. Thus, patented production processes aimed to obtain antihypertensive peptides with improved reaction yields generally catalyzed by enzymes with different regioselectivities, or to reduce costs/time by adjusting operational conditions of some processing step rather than the discovery of new molecules.

One possible solution for future industrial application that has been explored for antihypertensive peptides production is the use of membrane bioreactors [106]. In this process the enzyme membrane reactor system offers control of the reaction with, minimized substrate, catalyst losses, faster reactions, higher yields, and lower operating costs [107]. Another approach is the recombinant DNA technologies, using generally recognized as safe (GRAS) microorganisms as expression vectors, are nowadays industrial tools largely applied to produce techno-economically relevant molecules, as for example insulin and penicillin. There are evidences that these technologies are applicable to the production of peptides with high yields [108]. Nevertheless, contrarily to what was observed for antihypertensive peptides production by enzymatic hydrolysis of caseins, few papers and patents addressing the use of recombinant DNA tools with this aim were found within the databases examined.

Despite of all these advances, the industrial obtaining and purification of these peptides still represent major challenges, because of limited reaction yields and complexity of unit operations and apparatus to separate and isolate them. Hence, the techno-economic viability of such processes is another aspect to be continuously and carefully studied. In the chapter 3 of this dissertation we search among other objectives to contribute to the development of information about purification of antihypertensive peptides in order to enable the use of C4 column in this procedure.

An alternative explored in some patents seems promising and deserves more attention by food product developers: the improvement of processes to produce

simultaneously certain bioactive peptides directly within food formulations, instead to produce and subsequently add them into such formulations. This appeared specially promising concerning fermented milks and products derived thereof.

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7. References

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CAPÍTULO 3

Estudo Experimental

Combined RP-HPLC and MALDI-TOF/TOF analysis for separating and identifying casein-derived peptides with antihypertensive potential

Mara Rose de Oliveira[a], Thaís Jordânia da Silva[a],

Edvaldo Barros[b], Maria Cristina Baracat Pereira[c], Valéria Monteze Guimarães[c],

Monique Renon Eller[a], Jane Sélia dos Reis Coimbra[a], Eduardo Basílio de Oliveira[a] ✉

[a] *Departamento de Tecnologia de Alimentos (DTA), Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

[b] Núcleo de Análise de Biomoléculas (NuBioMol)*, Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

[c] Departamento de Bioquímica e Biologia Molecular (DBB)*, Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

✉ Corresponding author: eduardo.basilio@ufv.br

ABSTRACT

Bovine casein was hydrolyzed through an enzymatic bioprocess, using pancreatic trypsin as biocatalyst in aqueous medium, at 37 ºC, during 120 minutes. The peptides in this hydrolysate were separated by ultrafiltration, and those smaller than 5 kDa were subsequently purified into six fractions through reversed-phase high-performance liquid chromatography, using a C4 column, differently of similar literature reports found, which use other columns or even other chromatographic approaches. All of these fractions were individually evaluated in terms of their *in vitro* inhibitory activity towards the angiotensin I-converting enzyme (ACE). Three fractions displayed higher ACE-inhibition (80.68% in Fraction 1, 79.00% in Fraction 2 and 62.44% in Fraction 4). The amino acid sequence of the peptides contained in all of six fractions fraction was assessed by matrix assisted laser desorption/ionization time-of-flight/time-of-flight in tandem mass spectrometer. The amino acid composition of peptides contained in three of these fractions (NAVPITPTLNR and ALNEINQFYQK in Fraction 1, FALPQYLK in Fraction 2 and FALPQYLK and HQGLPQEVLNENLLR in Fraction 4) suggested that the C-terminal tripeptide sequence have a more significant influence on ACE-inhibition effect than the overall hydrophobicity of the peptide itself. The fraction F3 (HQGLPQEVLNENLLR and, HPIKHQGLPQEVLNENLLR) showed greater relative ACE-inhibition.

KEYWORDS

Bioprocesses

Trypsin

Angiotensin I-converting enzyme

Reverse phase chromatography

Mass spectrometry

ABBREVIATIONS AND SYMBOLS

ACE	Angiotensin I-Converting Enzyme
ACN	Acetonitrile
AH	Antihypertensive
BCHs	Bovine Casein Hydrolysates
ESI-QTOF	Electrospray Ionization and Quadrupole Time-of-Flight Mass Spectrometry
E:S	Enzyme:Substrate ratio
GRAVY	Grand Average of Hydropathy
HCl	Hydrochloric Acid
HHL	N-hippuryl-His-Leu hydrate
HPLC	High-Performance Liquid Chromatography
I_{ACE}:A	Percentage of ACE-inhibition:percentage of peak area ratio
IEC	Ion-Exchange Chromatography
KKS	Kallikrein-Kinin System
MALDI	Matrix Assisted Laser Desorption/Ionization
MALDI-TOF/TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight
MS	Mass Spectrometry
NMWL	Nominal Molecular Weight Limit
PVDF	Polyvinylidene fluoride
RAS	Renin-Angiotensin System
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
SEC	Size Exclusion Chromatography
TFA	Trifluoroacetic Acid
UF	Ultrafiltration
UV	Ultraviolet
λ	Wavelength (nm)

1. INTRODUCTION

Lifestyle changes due to an expressive economic development in several countries have aroused great concern regarding hypertension. In 2000, 972 million adults worldwide were affected by hypertension, and this amount is predicted to reach 1.56 billion by 2025 [1]. Responsible for about 55% of deaths caused by cardiovascular diseases in the world [2], this pathology has been treated mainly by means of the administration of drugs, such as captopril and enalapril. Due to undesirable side effects caused by the chronic use of these drugs (e. g., cough, taste disturbances and skin rashes) [3], bioactive compounds of natural origin have been widely researched as possible coadjuvants and/or substitutes for such synthetic drugs [4–6].

Technically, the hypertension is defined by values of systolic and diastolic blood pressure equal to or higher than 140 mmHg and/or 90 mmHg, respectively [2]. In biochemical terms, blood pressure regulation, mainly comprised by the Renin-Angiotensin System (RAS) and the Kallikrein-Kinin System (KKS), is associated with the control of a zinc metallopeptidases known as angiotensin I-converting enzyme (ACE; 3.4.15.1). On these systems, ACE plays a crucial role in increasing blood pressure, promoting vasoconstriction (conversion of the decapeptide angiotensin I – DRVYIHPFHL – to the potent vasoconstrictor angiotensin II – DRVYHIPF – by cleavage of a dipeptide at the C-terminal site) and hindering vasodilation (degradation of vasodilator bradykinin, into inactive peptide) [7–9].

Many ACE-inhibitory peptides have been obtained from the enzymatic hydrolysis of food proteins [4–6]. At present, milk proteins have been recognized as excellent sources of peptides with different bioactivities including antihypertensive (AH) activity [10]. In particular, caseins are strategic sources of AH peptides, which seems to be related to their amino acid residues composition, comprising a hydrophobic region whose molecular mass is relatively low (<10 kDa) [11]. The AH activity of peptides is often associated with their amino acid composition and sequence to which usually they are featured with a high concentration of proline residue preceded by hydrophobic residues. Besides that, these active sequences varies in size (usually, from 2 to 20 amino acid residues; molecular mass ≤6 kDa) [12,13]. It is noteworthy that other biological activities, such as antioxidant [14], antimicrobial [15], cytomodulatory [16],

antithrombotic [17], anti-inflammatory [18], hypocholesterolemic [19] and AH [17] have been attributed to casein-derived peptides.

Identifying peptides with AH potential within a hydrolysate is an essential step for producing such compounds, along with the steps of separation and purification. However, the identification is not a trivial procedure, since these bioactive compounds are often present in low concentrations in the hydrolysates [20]. For this reason, classical separation methods, such as ultrafiltration and nanofiltration, are often employed as a strategy to promote the previous enrichment and fractionation of peptides in the hydrolysate medium [10,20–22]. The filtration through membrane technique despite comprising a relatively simple mechanism can become complex depending on the material of interest that will be filtered. For bioactive peptides, the transmission through membrane should be carefully evaluated because it involves not only exclusion by size, but also electrostatic repulsion and ionic and hydrophobic interactions between peptides and membrane [21,22]. In a more robust approach, chromatographic techniques have been proven to be effective in many cases. An evidence of this can be found in the application of reversed-phase high-performance liquid chromatography (RP-HPLC) [23], size exclusion chromatography (SEC) [24] and ion-exchange chromatography (IEC) [25] in a larger number of studies involving separation and purification of peptides.

Chromatographic separations by different principles can be used successfully in function of the strong hydrophobicity often observed in ACE-inhibitor peptides. This evidence has been reflected by the studies through the adoption of a combination of operations to achieve the separation of peptides of interest [20,23,25]. However, it is reasonable to believe that the use of alternative chromatographic columns can be more effective to do so, with a reduced number of steps. In that view, one can propose the use of a chromatographic separation column (C4) whose composition differs from the traditional C18 columns widely used in most of literature report found. This new proposal is expected to effectively promote the separation of AH peptides with similar hydrophobicity. Thus, the present study intended to obtain casein hydrolysates using reaction conditions previously optimized by our team [26] and, as a new step ahead, to separate peptides are responsible for ACE-inhibition *in vitro* displayed by these hydrolysates, via RP-HPLC using a C4 column. Furthermore, spectrometric assays using matrix assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-

TOF/TOF) were undertaken to identify the amino acid sequence of the most promising peptides in terms of ACE-inhibitory potential.

2. MATERIALS AND METHODS

2.1. Enzymes and substrates

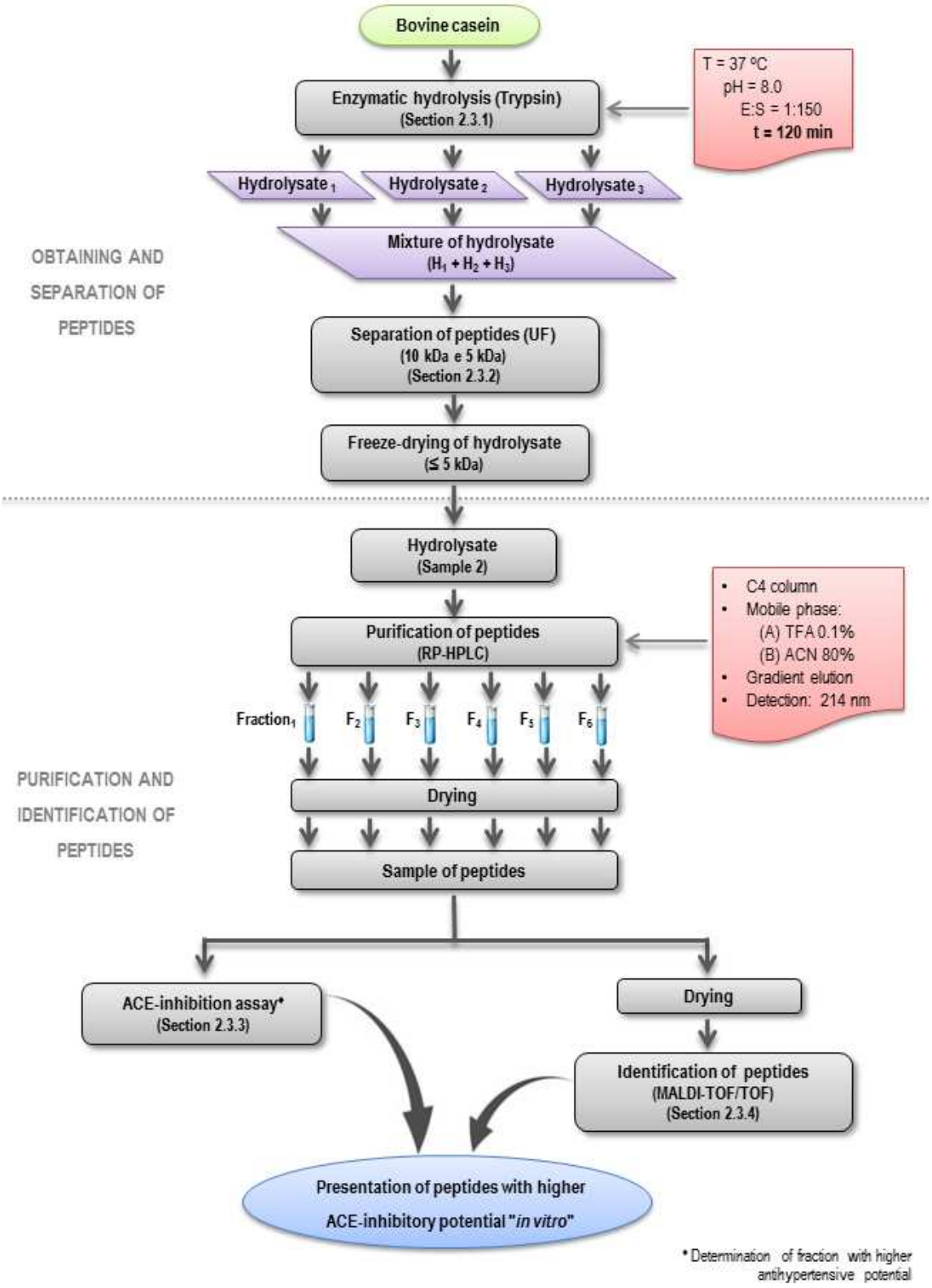
The enzymes used were: trypsin (E.C.: 3.4.21.4; bovine pancreas; 10.000 U/mg; SIGMA, USA) and angiotensin I converting enzyme (ECA from rabbit lung; E.C.: 3.4.15.1; 0.25 U/mg; SIGMA, USA). The respective substrates were: bovine casein (SYNTH, Brazil) and N-hippuryl-His-Leu hydrate (HHL) (SIGMA, USA; purity ≥ 98%).

2.2. Other chemicals and solvents

In the casein hydrolysis step and ACE-inhibitory assays the following chemicals were used: glycerin (SIGMA, USA), phosphate buffer (phosphate sodium dibasic (VETEC, Brazil, purity ≥ 99%), phosphate sodium monobasic (VETEC, Brazil, purity ≥ 98%), potassium phosphate (VETEC, Brazil, purity ≥ 98%), citric acid (VETEC, Brazil, purity ≥ 99.5%), sodium chloride (VETEC, Brazil; purity ≥ 99%), hydrochloric acid (HCl) (VETEC, Brazil; purity ≥ 37%) and ethyl acetate (VETEC, Brazil; purity ≥ 99.5%). For purification and identification of peptides, the following chemicals were used: acetonitrile (ACN) (MERCK, Germany; purity ≥ 99.9%), trifluoroacetic acid (TFA) (VETEC, Brazil; purity ≥ 99.8%), both HPLC grade, and MALDI matrix α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany). Highly purified water (Millipore Inc, Milli-Q, USA; electrical resistivity ≈ 18.2 MΩ·cm at 25 ºC) was used for preparation of all buffers and solutions.

2.3. Experimental

The steps of the experimental study are schematically represented in Figure 1 and detailed in the subsections thereafter.



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138 **Figure 1**

139 Schematic representation of the steps for obtaining, separating and identifying peptides with
140 antihypertensive potential in casein hydrolysates.

2.3.1. Preparation of bovine casein hydrolysates (BCHs)

The casein peptides samples were produced using the method as reported by Souza, Coimbra, Oliveira, & Bonomo [27], with some adaptations proposed by Silva [26]. Briefly, a solution of bovine casein (20 $mg \cdot mL^{-1}$) was prepared in buffer sodium phosphate (0.2 $mol \cdot L^{-1}$)/citric acid (0.1 $mol \cdot L^{-1}$), at pH 8.0. A solution of trypsin (20 $mg \cdot mL^{-1}$) was prepared using this same buffer. To start the reaction, both solutions were mixed so that the final E:S ratio of 1:150 (w/w). The hydrolysis was carried out at controlled temperature of 37 °C, in a B.O.D incubator (SPLabor, SP-500, Brazil), under stirring with a magnetic stirrer (Tecnal, TE–0851, Brazil), for 120 minutes. After that, trypsin was inactivated by heating the reaction medium at 95 °C for 10 minutes in a thermostatic bath (LUCADEMA, LUCA-155/22, Brazil). Then, the medium was centrifuged (Eppendorf, 5430, Germany) at 5000*g* for 10 minutes, and the supernatant of each hydrolysate was collected. This reaction was carried out three times, separately, and the three corresponding hydrolysates (namely H_1, H_2 and H_3) were mixed and stored at 4 °C (Consul, CRD41ABANA, Brazil), to be used in the subsequently steps.

Two control experiments were simultaneously performed under the same reaction conditions: (1) without trypsin (system containing only buffer and casein) and (2) without casein (system containing only buffer and trypsin). The casein control (1) intended to ascertain whether the casein not hydrolyzed by trypsin action or possible casein fragments produced independently on the hydrolysis process may have some ACE-inhibitory activity. On the other hand, the trypsin control (2) aimed at verifying if peptides maybe formed from trypsin self-hydrolysis may have ACE-inhibitory activity.

2.1.1. Fractioning of peptides in BCHs

The casein hydrolysates obtained in three independent reactions of hydrolysis were polled, and subjected to ultrafiltration (UF) followed by RP-HPLC, as detailed thereafter. The same procedure was adopted for casein control and trypsin control.

The supernatant of the samples were subjected to UF through a stirred UF cell (Millipore, Amicon model 8400, USA) under compressed air (50 psi) and refrigeration (ice bath). In this process, were sequentially used polyethersulfone membranes, PBGC07610 and PBCC07610 (Millipore, Amicon Bioseparations, USA) with molecular mass cut-offs of 10 and 5 kDa nominal molecular weight limit (NMWL), respectively.

Prior to use, both membranes were activated by spinning 100 mL of distilled water and kept under this condition overnight. The permeate (containing compounds of molecular mass ≤5 kDa) was freeze-dried (Terroni, LS 3000, Brazil), at – 42 °C, under vaccum, and the powder obtained was stored at – 20 °C for further use.

The freeze-dried sample (125 mg) was suspended in 1 mL of Milli-Q water (125 mg/mL). Due to its high salt concentration, this powder was subjected to two stages of dilution, being thus prepared the sample 1 and sample 2, as follows:

- Sample 1: 50% of sample (125 mg/mL) and 50% of TFA (0.2%, v/v);
- Sample 2: 10% of sample 1, 45% of TFA (0.2%, v/v) and 45% of Milli-Q water.

The same process was applied to casein control, trypsin control and blank sample containing only buffer. Then each system was filtered through a 0.22 µm filter (PVDF, Millipore Corporation, Bedford, MA, USA) before purification by RP-HPLC. Volumes of 100 µL of sample 2 were loaded into a YMC-Pack C4 analytical column (250 mm length x 4.6 mm internal diameter, 5 µm particle size, 30 nm porosity; Waters, Massachusetts, USA), attached to a C4 guard column (Delta-Pak™ C4 100 Å, Waters). Both columns arranged in sequence were connected to a HPLC system (Waters, USA) equipped with a gradient pump (Binary HPLC Pump, model 25E), a photodiode array detector (Dual ʎ Absorbance, model E05487482M) and a fraction collector (model WFC, Waters, Japan). Solvent A was 0.1% (v/v) TFA in Milli-Q water, and solvent B was 80% (v/v) ACN in Milli-Q. Both mobile phases were filtered through a Whatman no. 40 filter and degassed in ultrasonic bath (Unique, USC-1400A, Brazil) simultaneously with vacuum pump (FANEM, 089-CAL, Brazil) for 5 minutes. The column was equilibrated with solution A and the peptides were eluted at 1 $mL \cdot min^{-1}$ using a linear gradient of 100% solvent A for 15 minutes and with the following increasing solvent B concentration: 0-15 minutes, 0%; 15-17 minutes, 0-30%; 17-30 minutes, 30%; 30-35 minutes, 30-100%; 35-45 minutes, 100%; 45-47 minutes, 100-0% and 47-55 minutes, 0%. The column temperature was maintained at 23 °C and detection was monitored at 214 nm. The RP-HPLC profiles were analysed by Breeze software (Version 3.30 SPA, 2002, Waters Corporation). At the end of this process, nine chromatographic analysis were held, and each fraction was collected according to the elution peaks. The samples were concentrated under vacuum using a SpeedVac concentrator (Thermo Electrom, Integrated Speedvac SPD1010-115, Asheville, NC, USA) and stored at – 20

°C for further analyses. For ACE-inhibition assays, each fraction was re-suspended in Milli-Q water (150 µL).

2.1.2. Quantification of the ACE-inhibitory activity of peptides fractions

The assessment of ACE-inhibitory activity *in vitro* of chromatographic fractions was measured according to modified version of the methodology firstly proposed by Cushman & Cheung [28]. The method is based on the hydrolytic action of ACE on the HHL substrate, releasing hippuric acid and the dipeptide His-Leu. This hippuric acid is spectrophotometrically quantified according to the intensity of absorbance at 228 nm. In ACE-inhibitor presence the formation rate of hippuric acid decreases, thus decreasing the absorbance at this wavelength.

The reaction was carried out as follows: 10 µL of sample of the reaction medium (hydrolysate) and 25 µL of HHL solution (5 mmol·L^{-1} in buffer potassium phosphate (0.1 mol·L^{-1}) / sodium chloride (0.3 mol·L^{-1}), at pH 8.3) were mixed and incubated at 37 °C in a thermostatic bath (SCHOTT, SCHOTT GERATE GMBH, Germany) for 5 minutes Then, 5 µL of ACE solution (50% w/w) in glycerin was added and the mixture incubated at 37 °C for 30 minutes. After this time, 37.5 µL of HCl solution (1 mol·L^{-1}) was added to inactivate enzyme by drastic reduction of pH. Hippuric acid liberated by ACE was extracted by adding 250 µL of ethyl acetate to the system, which was subsequently submitted to by vigorous agitation for 30 seconds, utilizing a vortex mixer (Vortex, AP56, Brazil), and centrifuged at 3000*g* for 15 minutes, at 25 °C. The colorless supernatant (organic phase) containing hippuric acid formed was collected (190 µL) and transferred to a clean tube and dried by heating to 95 °C for 5 minutes in thermostatic bath. The hippuric acid was re-suspended in 200 µL of Milli-Q water, which was agitated in vortex mixing. The samples was deposited in wells of ELISA plate (180 µL) and absorbance was measured at 228 nm using UV-spectrophotometer (Thermo Scientific, Multiskan GO 1510, Vantaa, Finland). Each reaction was carried out in triplicate.

In order to express quantitatively (%) the ACE-inhibitory activity ($\%I_{ACE}$), the average value from three determinations of hippuric acid content was used for purposes of calculation as follows:

$$\%\mathrm{I}_{\mathrm{ACE}} = \left[\frac{(\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{sample}})}{(\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{white}})}\right] \times 100\% \qquad (\mathrm{Eq.}\,2)$$

Where:

- $A_{control}$: Absorbance of the solution containing HHL + ACE + water
- A_{sample} : Absorbance of the solution containing HHL + ACE + hydrolysate
- A_{white} : Absorbance of the solution containing HHL + HCl + ACE + hydrolysate

2.1.3. Identification of amino acid sequence of peptides in the fractions

All fractions of peptides obtained by RP-HPLC were vacuum concentrated before mass spectrometry (MS) analyses. The structural analysis of peptides was carried out on MALDI-TOF/TOF mass spectrometer model Ultraflex III (Bruker Daltonics GmbH, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix (10 mg·mL^{-1} in 50% (v/v) ACN and 0.1% (v/v) TFA) in a ratio of 1:1 (v/v) (sample:matrix). Samples were applied onto an polished steel target (MTP 384 SP, Bruker Daltonics). The calibration of the method was conducted using the Peptide Calibration Standard II (Bruker Daltonics, Germany). Laser energy and the number of shots per segment spectrum were manually adjusted. The peaks most intense ions (MS) were subjected to fragmentation (MS/MS).The MS1 spectra were acquired in positive ion reflector mode, recording ions of 500 up to 3500 Da. For the realization of MS2, using the LIFT in the positive mode were selected the ions with intensity greater than 3000 and mass charge ratio (m/z) of more than 700 Da. The equipment operates with the FlexControl Software (Version 3.3, Bruker Daltonics, Germany), being the resulting spectra of MS/MS analyses processed with the aid of the FlexAnalysis software. The peak lists of MS/MS were generated in *Generic Mascot Format* (MGF) by BioTools software (Version 3.2, Bruker Daltonics, Germany).

Peptides identification was confirmed using the MASCOT software (Version 2.4.0, Matrix Science, London, UK) against a protein database for the BOVIDAE family obtained as a subset of the UNIPROT database (Download on 10/18/2016, with 89,937 entries). The database search parameters included no restrictions on the protein molecular mass, maximum of one tryptic missed cleavage, fixed modifications for carbamidomethylation of cysteine residues, and variable modifications for oxidation of methionine residues. The peptide mass tolerance was 0.2 Da for the precursor ions in MS spectra and 0.5 Da for the fragment ions in MS/MS spectra. Significant hits for MASCOT were defined as P-value < 0.05 for peptides showing matches using MS/MS ion search. The statistical validation of peptides was carried out for score

values of P > 90% in Scaffold software (Version 3.6.4, Proteome Software Inc., Portland, OR, EUA).

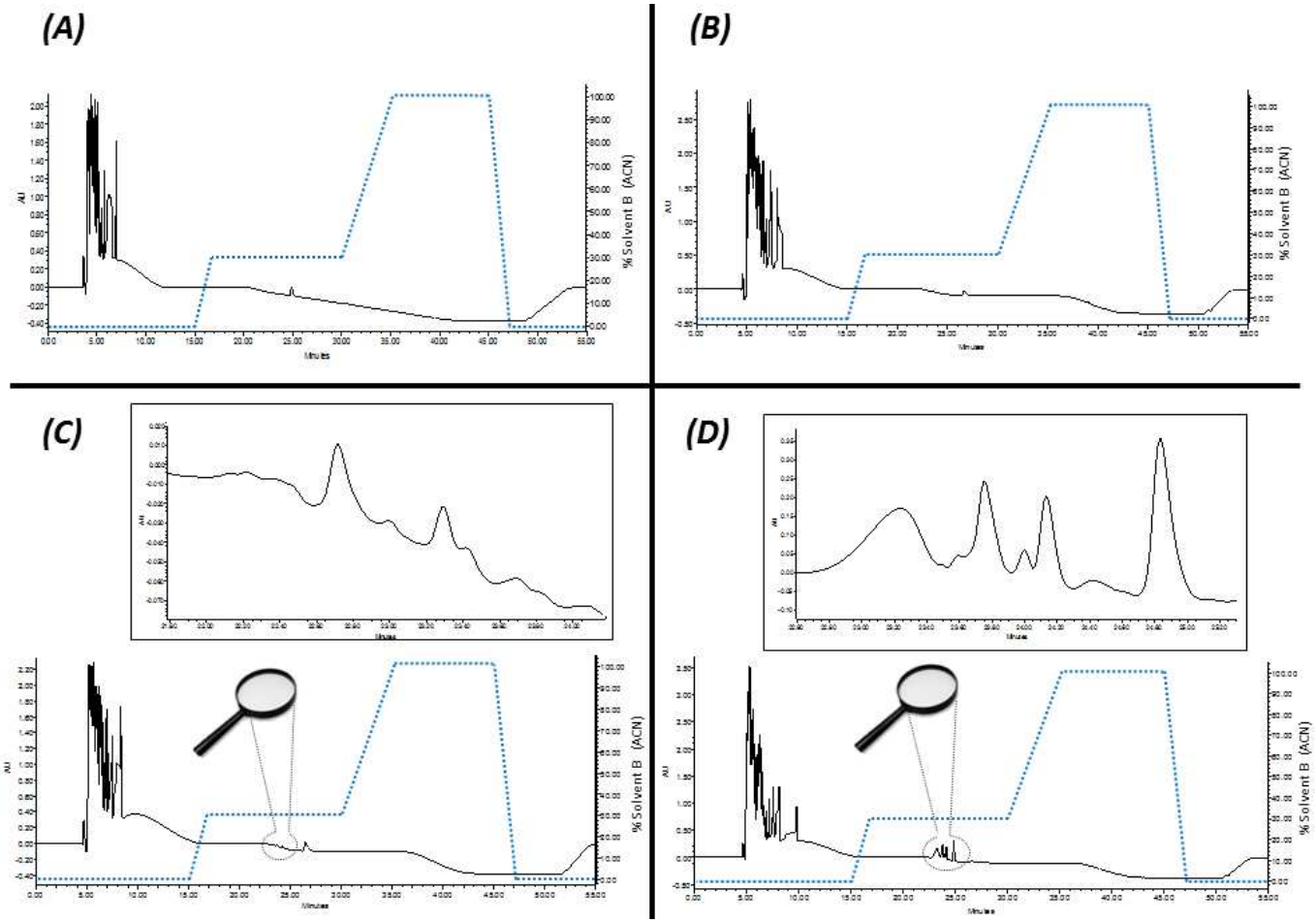
3. RESULTS AND DISCUSSION

3.1. Fractioning of peptides from BCHs

In a previous step of this project, fractions of BCHs were collected at 0, 30, 60, 90, 120, 150 and 180 minutes after hydrolysis beginning and theirs ACE-inhibitory activity were evaluated [26]. The highest ACE-inhibitory activity was observed for hydrolysates produced after 120 minutes (89.7 ± 4.2% of inhibition). Starting from these results, this trypsinolysis of bovine casein was reproduced during 120 minutes, at 37 °C, pH 8.0, and E:S 1:150 (w/w).

The fractionation process of hydrolysates was applied, firstly considering the peptides molecular dimensions, by UF. In this step, three fractions were obtained (≥10 kDa, from 10-5 kDa, and ≤5 kDa). The use of 10 kDa NMWL membrane aimed to retain the trypsin and remaining casein molecules. The 5 kDa NMWL membrane was used to obtain peptides with low molecular weight (≤5 kDa) in the permeate. Among the specificities that characterize peptides with high ACE-inhibitory activity, such small peptides have been standing out as potential candidates [29–31]. For this reason only, the permeate obtained with the 5 kDa cut-off membrane was collected for further investigations.

Peptides from casein hydrolysate with molecular mass ≤5 kDa were properly prepared and submitted to RP-HPLC analyses. The same procedure was to applied for trypsin control, casein control and blank of the reaction (medium containing only buffer). In this process, the samples were loaded on a YMC-Pack C4 analytical column and eluted with a linear gradient of ACN (80%) and TFA (0.1%) for 55 minutes. The RP-HPLC profiles of samples are presented in Figure 2.



297

298 **Figure 2**

299 Separation chromatographic profile (214 nm) of samples using RP-HPLC with C4 analytical column: (A) Blank sample (contained only buffer), (B)
300 Trypsin control, (C) Casein control and (D) Hydrolysate from bovine casein.

In Figure 2 there are similarities of signals exhibited in the beginning of the chromatographic analyses for all samples. It can be attributed to step cleaning, for instance, a desalination process. The trypsin control chromatogram (Figure 2B) did not show any peak when compared with chromatogram of blank sample (Figure 2A). This result points out the non-occurrence of trypsin self-hydrolysis under the conditions evaluated. Casein control chromatogram displayed some peaks with reduced amplitude and low resolution (Figure 2C) when compared with casein hydrolysate (Figure 2D). These peaks were pooled in three fractions, named $F_{cc}1$, $F_{cc}2$ and $F_{cc}3$ (see Figure 1 on Supplementary Material), to which were collected, concentrated and evaluated in relation ACE-inhibition. The values of the analyzed activity for the fractions $F_{cc}1$, $F_{cc}2$ and $F_{cc}3$ were 1.8%, 17.9% and 5.9%, respectively. These values can be considered negligible, when compared to those regarding the ACE-inhibitory activities of casein hydrolysate, confirming that ACE-inhibition observed came from the peptides generated by trypsinolysis of bovine casein (see discussion below).

The chromatogram presented in Figure 2D shows that peptides elution for casein hydrolysate occurred between 22.60 and 25.20 minutes, with 70% solvent A (0.1% TFA) and, 30% solvent B (80% ACN) in a hydrophobic column. As shown in Figure 3A, there were six peaks for casein hydrolysate sample, named F1-F6, all of which were collected separately, concentrated and evaluated regarding their ACE-inhibitory activity (Figure 3B).

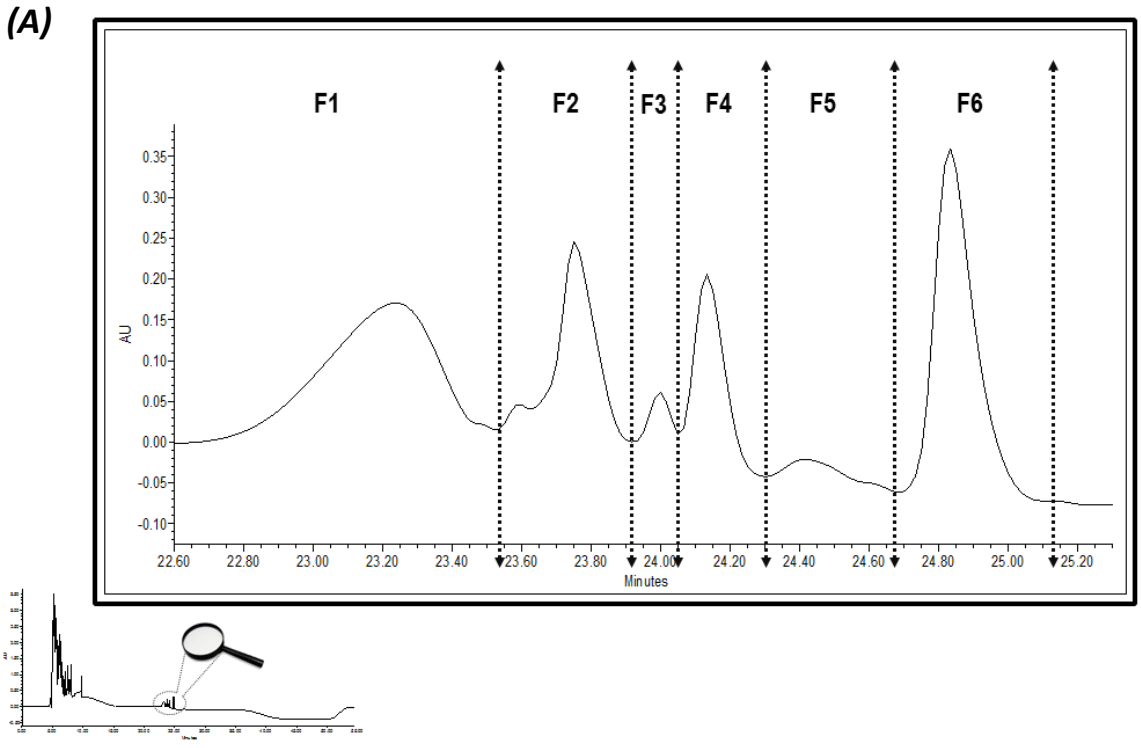


Figure 3

Separation chromatographic profile (214 nm) of hydrolysate from bovine casein by RP-HPLC with C4 analytical column (A); ACE-inhibitory activity of each collected fraction and theirs respective estimate of peptide content (B).

Some studies have reported that peptides fractions with high ACE-inhibitory activity separated by RP-HPLC are usually those at the middle or end of chromatogram, corresponding to peptides predominantly hydrophobic [12,31–33]. This is in agreement with our results, given the large time for elution of peptides in the hydrophobic chromatography column (22.60 and 25.20 minutes). This suggests that AH peptides from bovine casein obtained in this research work possess strong hydrophobic properties.

The separation of casein hydrolysate was performed based on the differences of polarity of the peptides within the sample. The first fraction (F1), corresponding to the peak at time of 23.25 minutes, suggested that it contained a group of lower hydrophobicity peptides. The sixth peak (F6) was eluted at approximately 24.90 minutes, suggesting that it contained peptides formed by more hydrophobic amino acids. As shown in Figure 3B, all of six fractions contained peptides with some ACE-inhibitory activity. Such results are consistent with previous reports on low molecular weight peptides with AH capacity [33,34], once the peptides analyzed in this study were obtained from the 5 kDa permeated in UF step. The relative amount of peptides was estimated by integrating the area under the peak for each fraction using Breeze software. These data, along with the ACE-inhibitory activity values for each fraction, are presented in Table 1.

Table 1

The ACE inhibiting ratio, estimate of the purified peptide content of each fraction collected by RP-HPLC and ratio between both.

Fractions	***ACE-inhibition (%)***	***Peak area (%)***	**** I_{ACE}:A ratio***
F1	80.7	33.9	2.4
F2	79.0	21.2	3.7
F3	49.2	4.4	11.2
F4	62.4	12.4	5.0
F5	16.3	3.2	5.1
F6	47.8	25.0	1.9

* I_{ACE}:A ratio - percentage of ACE-inhibition / percentage of peak area ratio

ACE-inhibitory activity was measured based on the spectrophotometric quantification of hippuric acid released from the ACE-catalyzed hydrolysis of the HHL

355 substrate. Fractions F1 e F2 had the higher values of ACE-inhibition activity (80.7%,
356 and 79.0%, respectively) and fraction F5 showed the lower analysed activity (16.3%).
357 However, when assessing the standardized data, that is, the values obtained of the
358 ratio between percentage of ACE-inhibition and percentage of peak area (I_{ACE}:A), is
359 possible to conclude that the fraction F3 presented the higher result (I_{ACE}:A = 11.15),
360 being therefore the fraction of greater interest in this study. On the other hand, the
361 smaller I_{ACE}:A ratio was verified in fraction F6 (1.91), thus indicating that for a large
362 amount of peptides estimated, the ACE-inhibitory activity was relatively low.

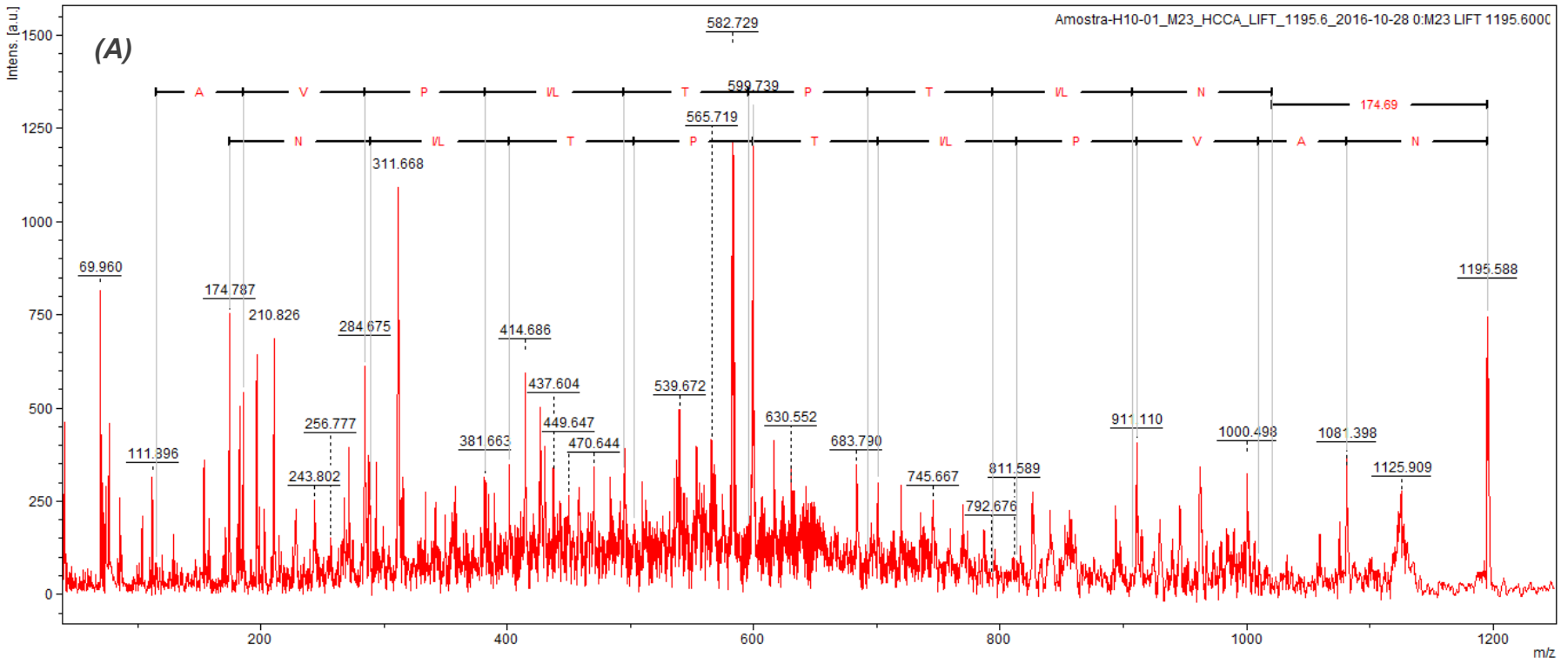
363 RP-HPLC separation process has been shown highly efficient in separating and
364 conserving biological activity of peptides [10,25,35]. The usage of low hydrophobicity
365 column (YMC-Pack C4 analytical column) was defined in terms of similar feature of
366 peptides identified by Silva [26]. For this reason, the good separation efficiency
367 achieved in the present study would have not been be possible by using a column of
368 high hydrophobicity, as for instance, a C8 or C18 column. The strong similarity of the
369 peptides identified would probably result in a grouping of peptides in a smaller number
370 of fractions, i.e., there would be fewer peaks in the chromatogram and these would be
371 composed of more peptides.

372 ### 3.2. Identification of amino acid sequence of the peptides in the
373 fractions

374 The six chromatographic fractions obtained by RP-HPLC were individually
375 submitted to MALDI-TOF/TOF analyses, in order to gain detailed information about the
376 amino acid composition of the peptides wherein (see Figure 3 on Supplementary
377 Material and Figure 4).

378

379


(A)
Amostra-H10-01_M23_HCCA_LIFT_1195.6_2016-10-28 0:M23 LIFT 1195.6000
Intens. [a.u.]
m/z
A V P I/L T P T I/L N
N I/L T P T I/L P V A N
174.69
582.729
599.739
565.719
311.668
69.960
174.787
210.826
284.675
414.686
437.604
539.672
256.777
449.647
470.644
381.663
630.552
683.790
911.110
1000.498
1081.398
1125.909
745.667
811.589
792.676
111.396
243.802
1195.588

380

381

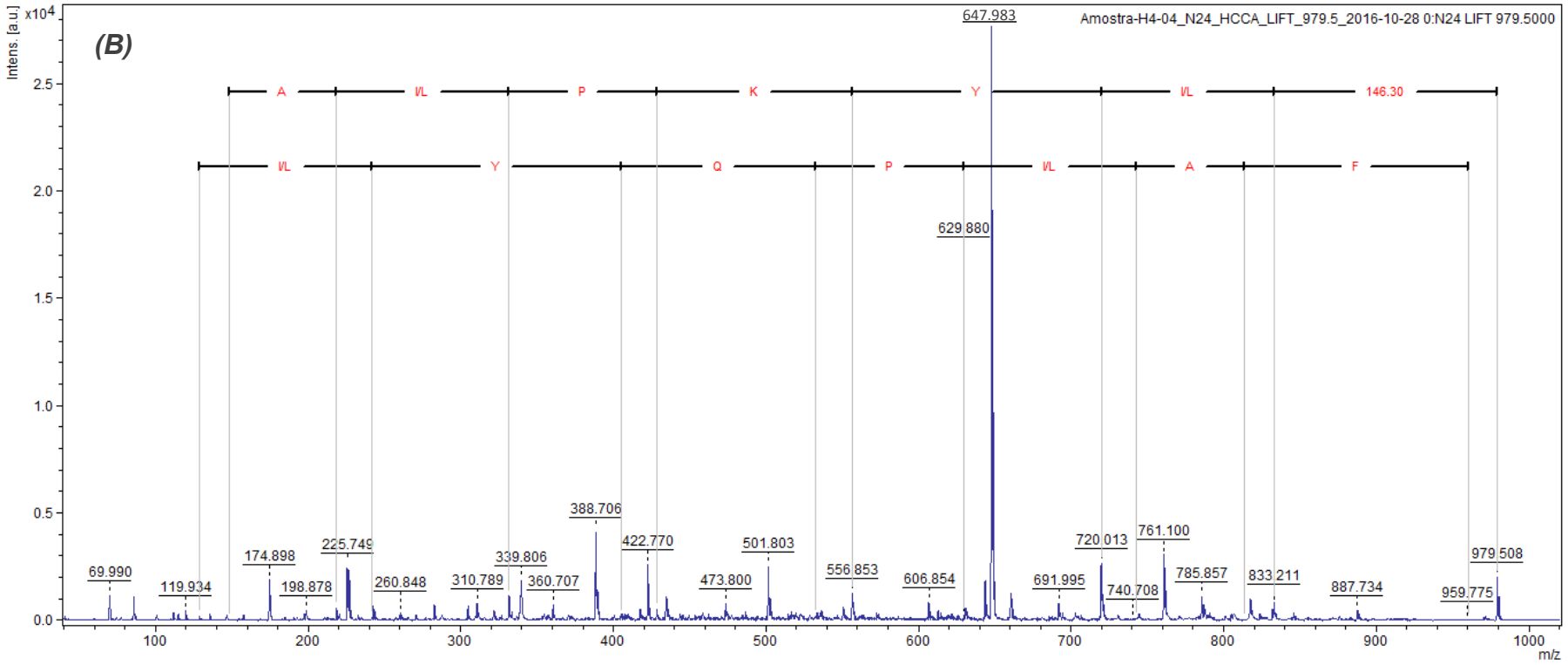


Figure 4

The mass spectra (MS/MS) referents of the amino acids residues of the peptides whose validation was carried out by *de novo* sequencing in manual mode. (*A*) Fraction F1, NAVPITPTLNR peptide (m/z 1195.6) and (*B*) Fraction F4, FALPQYLK peptide (m/z 979.5).

Note: In "*b*" series validation, for FALPQYLK peptide (B), was found the amino acid K (128.05 u) instead of Q (128.09 u) This is due to the high proximity of their mass values.

The statistical validation of peptides contained in each fraction was carried out by Scaffold software. However, for the peptides NAVPITPTLNR (Fraction F1) and FALPQYLK (Fraction F4), identified in MASCOT, the MS/MS data were evaluated by the manual *de novo* sequencing (Figure 4). As presented on Table 2 all fractions were composed by more than one peptide with the exception of fraction F2.

Table 2

Identification of amino acid composition and characterization of peptides present in fractions from the casein hydrolysate by trypsin.

Fractions	***◊ Protein fragment***	***Sequence***	***Observed mass (m/z)***	***♦ Hydropathicity (GRAVY)***
F1	α_{S2}-casein	NAVPITPTLNR**	1195.678	-0.164
	α_{S2}-casein	ALNEINQFYQK*	1367.720	-0.891
F2	α_{S2}-casein	FALPQYLK*	979.578	0.237
F3	α_{S1}-casein	HQGLPQEVLNENLLR*	1759.931	-0.753
	α_{S1}-casein	HPIKHQGLPQEVLNENLLR*	2235.229	-0.816
F4	α_{S2}-casein	FALPQYLK**	979.500	0.237
	α_{S1}-casein	HQGLPQEVLNENLLR*	1759.957	-0.753
F5	k-casein	YIPIQYVLSR*	1251.670	0.400
	α_{S1}-casein	HQGLPQEVLNENLLR*	1759.912	-0.753
F6	α_{S1}-casein	PFPEVFGK*	920.442	-0.150
	α_{S1}-casein	YLGYLEQLLR*	1267.678	0.070
	α_{S1}-casein	FFVAPFPEVFGK*	1384.716	0.867

(◊) Usin the mass spectrometry search engines MASCOT
(*) Sequence valided by Scaffold
(**) Sequence validated by manual "*de novo*" method
(♦) Data obtained in ProtParam by link: http://web.expasy.org/protparam/
GRAVY: Grand average of hydropathicity

A total of 9 peptides were identified in all fractions. Among them, it is possible to observe in fraction F3 the existence of missed cleavage (HPIKHQGLPQEVLNENLLR), which may be due to a reaction time not long enough to promote this cleavage, concentration of enzyme and substrate, among others factors. Furthermore, fraction F6 showed a derivative peptide of non-specific cleavage (PFPEVFGK). This peptide may have been formed by a protein fragmentation not originated from the regioselective action of trypsin, but because of other hydrolysis conditions (e.g., agitation, temperature and pH). For all of other identified peptides, the hydrolysis of caseins

412 occurred at positions of the casein' sequences which were expected, according to the
413 known catalytic specificity of trypsin [36].

414 Fractions composed by more than one peptide (F1, F3, F4, F5, and F6), may
415 suggest that they did not concentrate during the separation by RP-HPLC. Thus, it is
416 reasonable to believe that this mixture occurred due to a similarity of hydrophobicity
417 between the peptides. Moreover, the isocratic condition at the time of elution of the
418 compounds of interest may also have favored these results. Therefore, the application
419 of a gradient elution condition of peptide or re-chromatograph the fractions represents
420 an interesting proposal for obtaining fractions with a single composition.

421 It is noteworthy that the majority of generated peptides were derived from αs_1-
422 casein (5 peptides), αs_2-casein (3 peptides) and, only one peptide formed by k-casein
423 fraction. Moreover, there was not any peptide from β-casein. Such evidences may be
424 related to high content of lysine (Lys) in αs_1- and αs_2-casein in relation to β- and k-
425 casein fractions (see Table 1 on Supplementary Material). Besides this, large peptides
426 (≥5 kDa) may have been formed and retained in membrane filtration step and possible
427 small peptides formed may have agglomerated with the retentate and consequently
428 have been retained in UF. This hypothesis is strongly supported by the distribution of
429 residues of Lys and Arg along the amino acid chain of β-casein, which indeed points
430 out the trend of β-casein for generating relatively large peptides when undergoing
431 trypsinolysis (e.g., peptides ≥15 amino acids) or small (e.g., peptides ≤5 amino acids).
432 Another hypothesis about this discussion is referent the peptides that may not have
433 been detected by identification technique adopted. By virtue of this, The possibility of
434 using other identification methods, such as ESI-Q TOF, could enrich the work with the
435 identification of other peptides.

436 Since the proportion of the main constituents of bovine casein, normally follows
437 the approximate ratio of 3:1:3:1 for αs_1, αs_2, β- and k-casein [37], respectively, it could
438 be reasonable to expect greater amounts of peptides generated from αs_1-casein and β-
439 casein. However, in addition to the facts discussed above, the physicochemical
440 characteristics of the β-casein fraction in the casein structure may have hampered the
441 access of trypsin, because it is relatively localized in the most interior region of casein.

442 The amino acid sequence of all peptides identified (Table 2) was compared with
443 those fragments predicted in a *in silico* test (see Table 2 on Supplementary Material).

444 Such fragments were generated by simulating the hydrolysis of casein catalyzed by
445 bovine pancreas trypsin. In fact, such procedure confirmed that the 7 peptides, among
446 9 identified in this study, were compatible with the known regioselectivity of trypsin
447 hydrolysis.

448 The relationship between structural properties of peptides and their ACE-
449 inhibitory activity was not yet fully elucidated [38]. However, it has been often
450 suggested that two features are linked to this inhibition property: i) short peptide length
451 (2 to 12 amino acids residues), and ii) high content of hydrophobic amino acid residues
452 (Pro, Tyr, Phe and Leu) [9,12,23,33,38,39] . More specifically, the C-terminal tripeptide
453 sequence when composed by hydrophobic amino acid residues such as proline (Pro),
454 tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) has been related to inhibitors
455 with high binding capacity in the active site of ACE [9,28,38,40–42]. Among these, the
456 Tyr comprises an aromatic amino acid and can interact with its receptor (ACE) through
457 both hydrogen bonds and/or π-π interactions [23].

458 The characteristics of peptides identified in the current study are in agreement
459 with these previous findings. A possible evidence that confirms this can be detected by
460 a similarity of amino acid sequence of the peptides that composed the fractions with
461 higher activity. In other words, the greater percentages of ACE-inhibition were 80.7%
462 (F1), 79.0% (F2) and, 62.4% (F3) which containing in their composition, among others,
463 the peptides ALNEINQF**Y**QK, FALPQ**Y**LK and FALPQ**Y**LK, respectively, that have in
464 common the presence of Tyr in the third position from the C-terminal residue.
465 Therefore, the high percentages of ACE-inhibition measured in presence of the
466 chromatographic peptides fractions F1, F2 and F3 is likely to be due to these peptides.
467 The two peptides mentioned above have already been cited in the literature for display
468 AH activity [43,44].

469 The hydropathy index of an amino acid is defined as a value representing
470 the hydrophobic or hydrophilic properties of its side chain. It was originally coined by
471 Kyte & Doolittle [45] in an article describing an algorithm that identifies helical trans
472 membrane. The larger number of hydropathy positive values means that more
473 hydrophobic is the amino acid (non-polar) and vice-versa. The GRAVY (Grand Average
474 of Hydropathy) value for a peptide or protein is calculated as the sum of hydropathy
475 values of all the amino acids, divided by the number of residues in the sequence. It is
476 used for the visualization of hydrophobicity over the length of a peptide sequence. As

shown in Table 2, the GRAVY values did not follow a defined sequence between the peptide hydrophobicity ratio and ACE-inhibitory activity, i.e., fractions containing more hydrophobic peptides did not necessarily present higher ACE-inhibition percentages, or vice-versa. This finding indicates that peptide binding with in the active site of ACE may be more related to the C-terminal tripeptide sequence rather than to the overall peptide composition itself.

An attempt was made to identify the peptides contained in the chromatographic fractions for the control of casein ($F_{cc}1$, $F_{cc}2$ and $F_{cc}3$) by MALDI-TOF/TOF. However, when confronted such results with the MASCOT database (UNIPROT / BOVIDAE), no matches could be found. Ions present in the spectra of these samples indicated only the presence of matrix (α-cyano-4-hydroxycinnamic acid). Hence, for the method applied in this work, no relevant peptide was found when casein was not hydrolysed by the action of trypsin.

4. CONCLUSIONS

Bovine casein was successfully hydrolysed by using pancreatic trypsin as biocatalyst, in buffered aqueous medium. The RP-HPLC protocol of separation applied was highly effective in fractioning the peptides formed and preserving their ACE-inhibitory activity, being thus enabled a new proposal of separation of AH peptides with the use of column C4. The structural analysis (MALDI-TOF/TOF) of peptides contained in the chromatographic fractions has confirmed the association between low molecular mass peptides with high AH capacity. In addition, results outlined in this study suggest that the C-terminal tripeptide sequence may have a more significant influence on ACE-inhibition effect than the overall hydrophobicity of the peptide itself. The peptides HQGLPQEVLNENLLR and, HPIKHQGLPQEVLNENLLR found in the fraction with greater relative ACE-inhibition (F3) should now be synthetized and individually tested in new *in vitro* assays. The most promising among them may be the subjected of *in vivo* investigations afterwards.

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SUPPLEMENTARY MATERIAL (SM) FOR

Combined RP-HPLC and MALDI-TOF/TOF analysis for separating and identifying casein-derived peptides with antihypertensive potential

Mara Rose de Oliveira[a], Thaís Jordânia da Silva[a],

Edvaldo Barros[b], Maria Cristina Baracat Pereira[c], Valéria Monteze Guimarães[c],

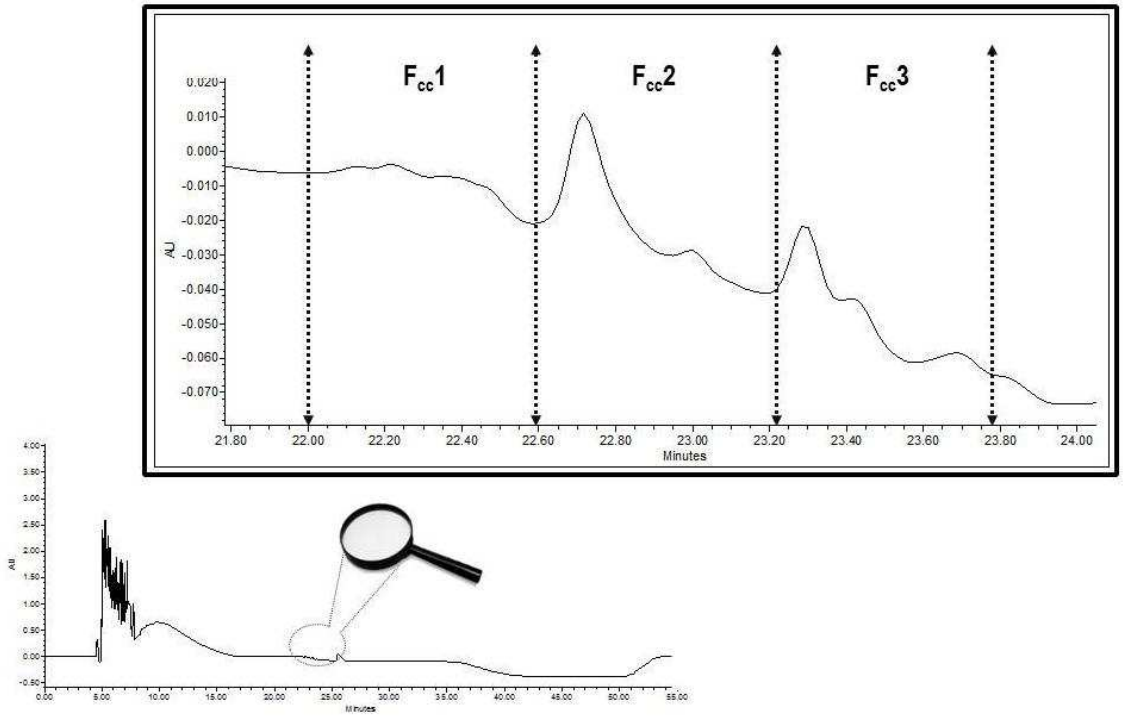
Monique Renon Eller[a], Jane Sélia dos Reis Coimbra[a], Eduardo Basílio de Oliveira[a] ✉

[a] *Departamento de Tecnologia de Alimentos (DTA), Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

[b] Núcleo de Análise de Biomoléculas (NuBioMol)*, Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

[c] Departamento de Bioquímica e Biologia Molecular (DBB)*, Universidade Federal de Viçosa (UFV), Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil.*

✉ Corresponding author: eduardo.basilio@ufv.br



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681 **Figure 1:** Chromatogram of casein control and collected fractions.

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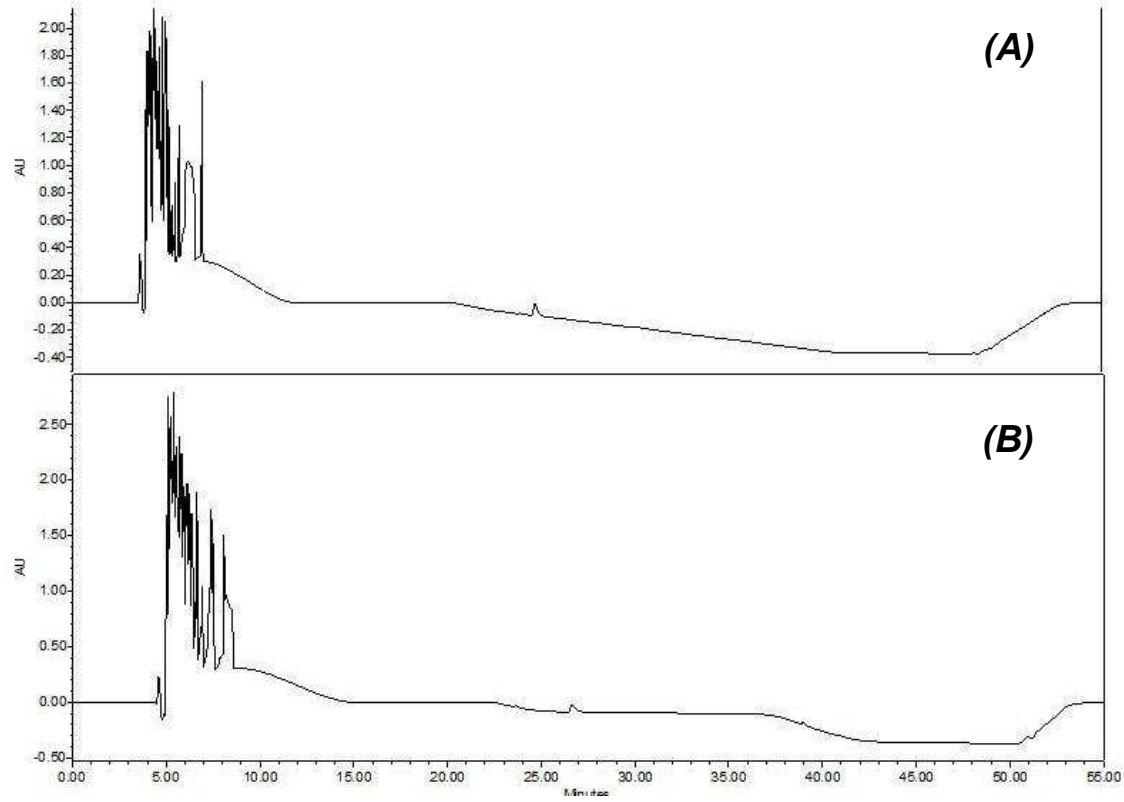

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684 **Figure 2:** Chromatogram of trypsin control (A), and chromatogram of blank (B).

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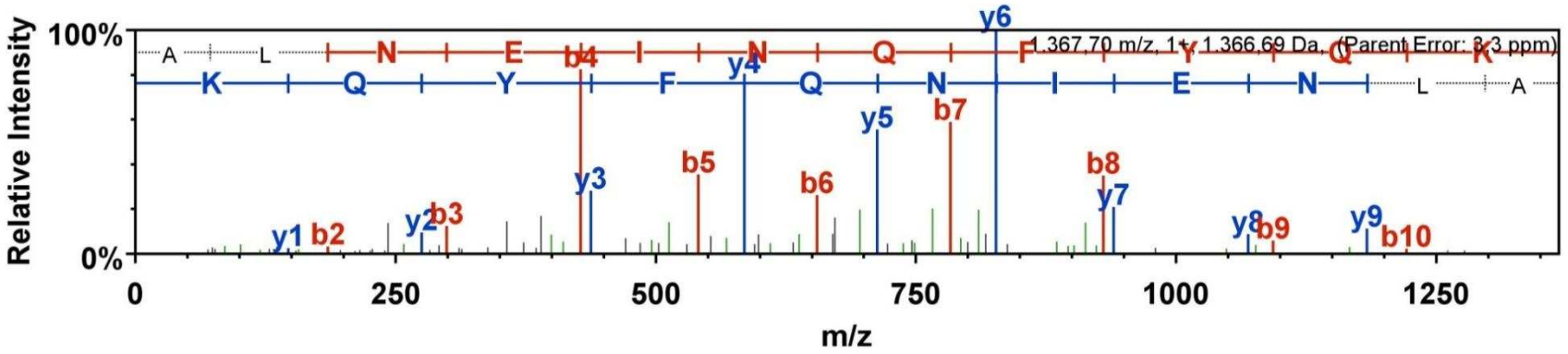
(A)



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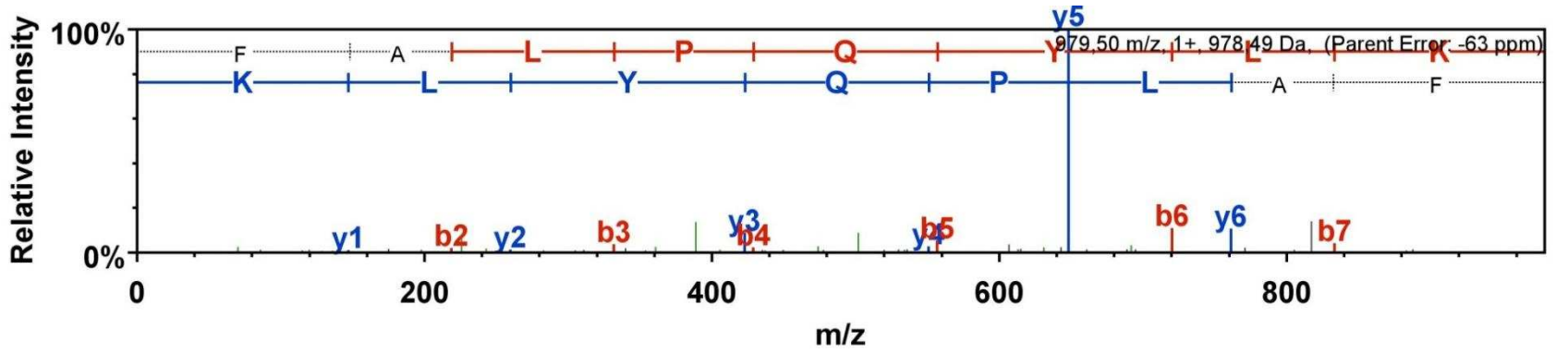

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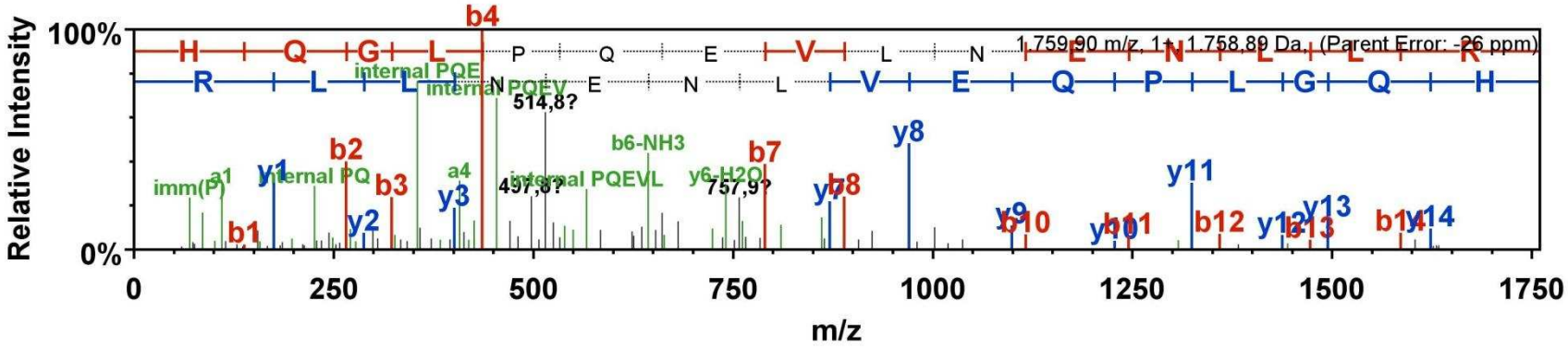

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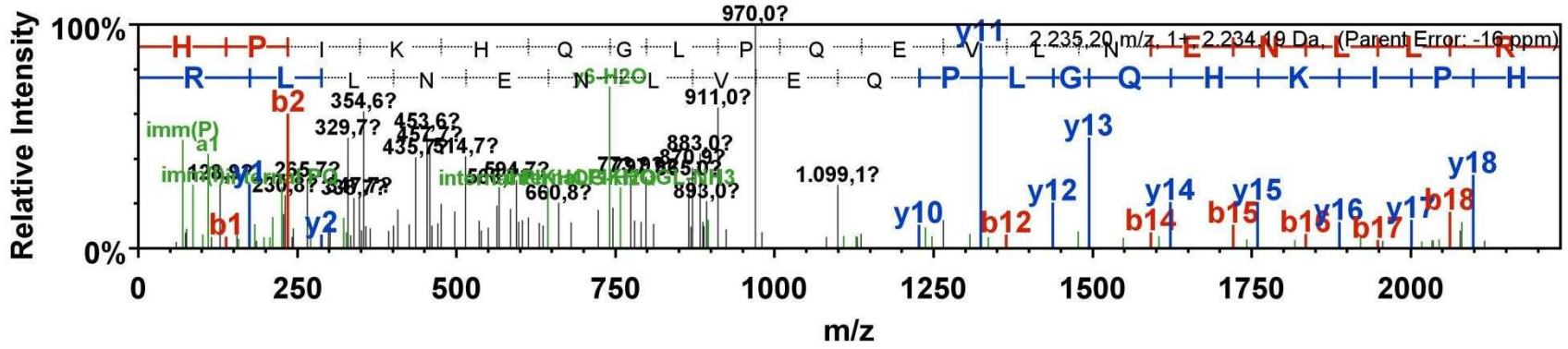

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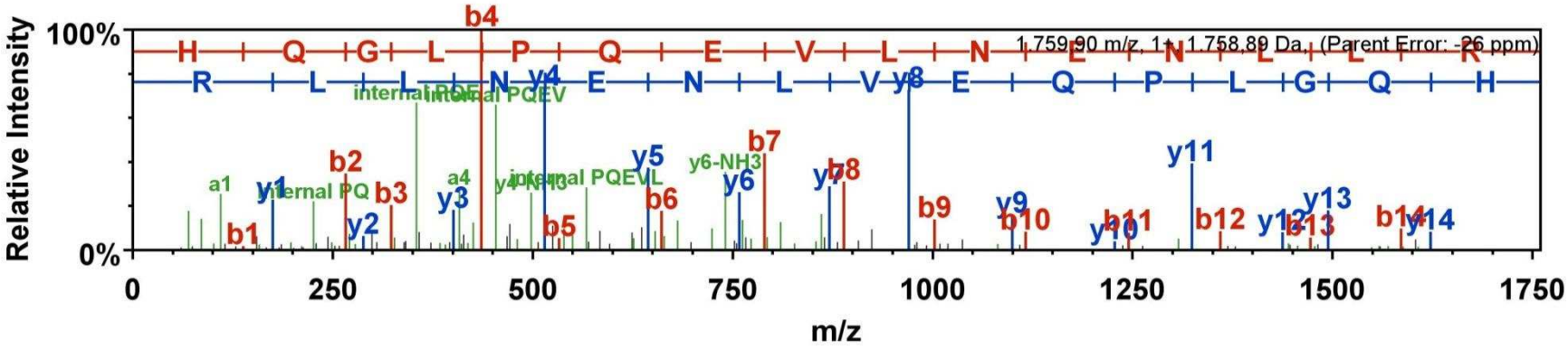

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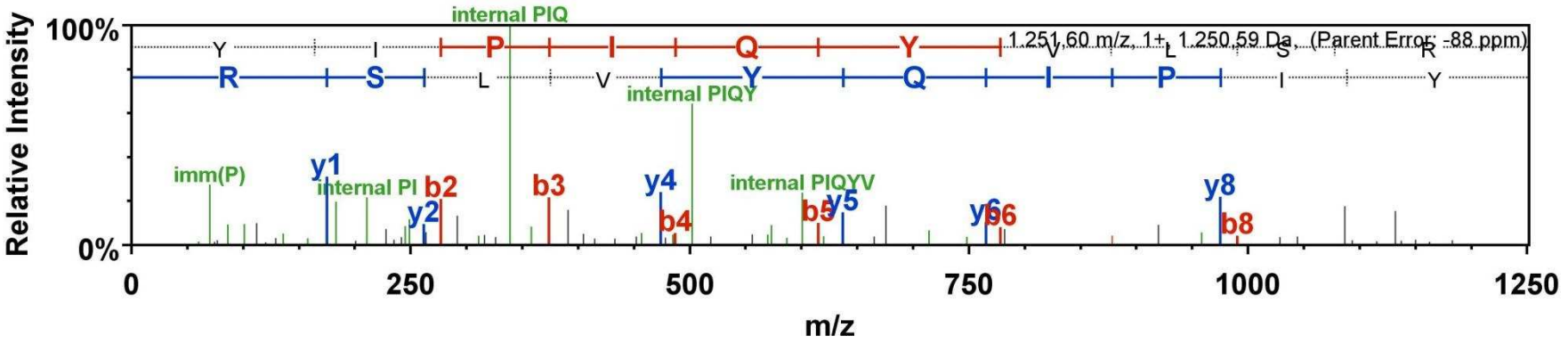

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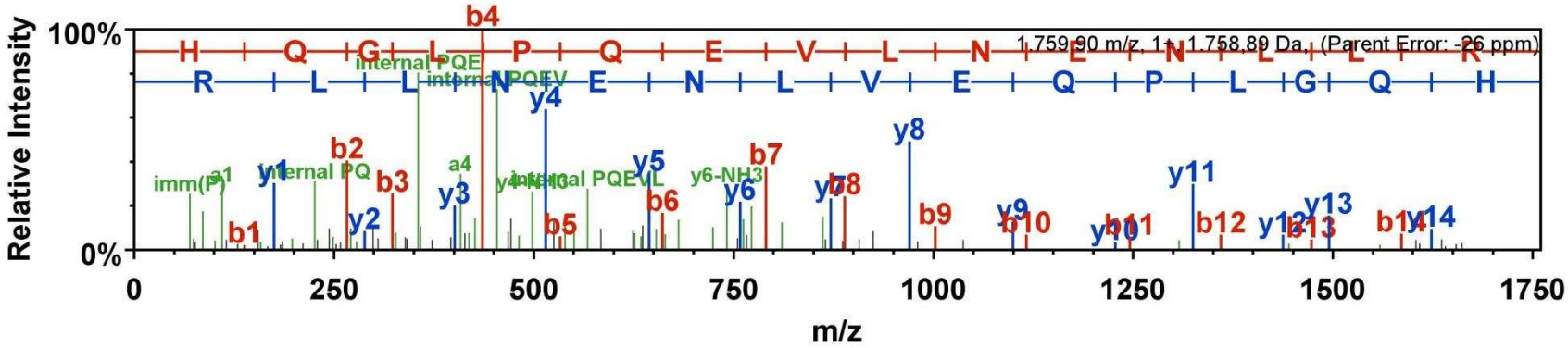
700 **(G)**



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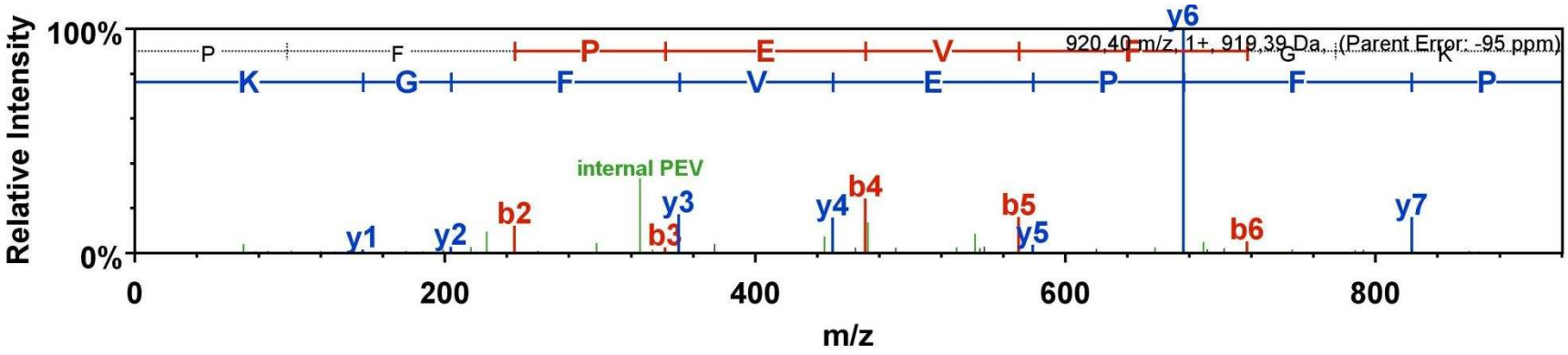
702 **(H)**



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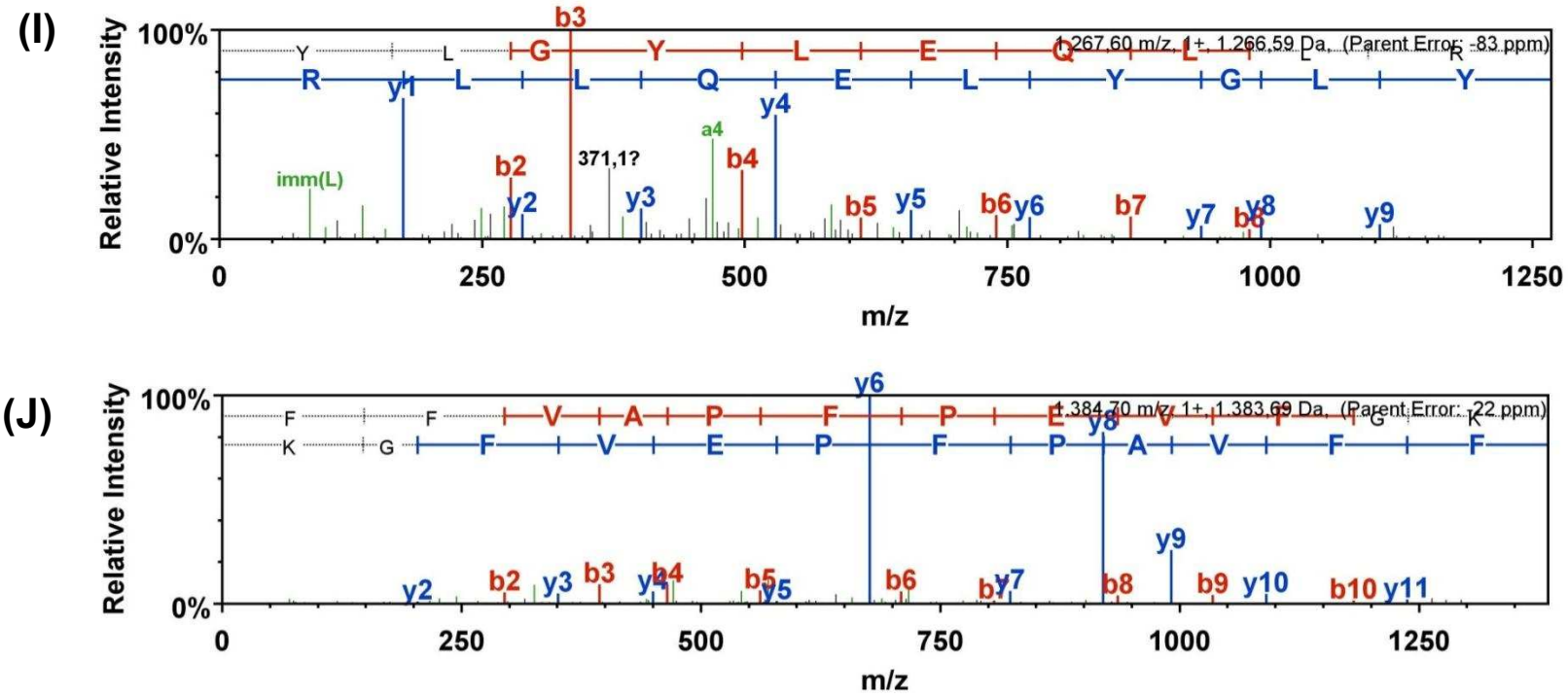

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706 **Figure 3:** Mass fragmentation spectra for the derived-casein peptides, generated by Scaffold: (A) ALNEINQFYQK (Fraction 1), (B) FALPQYLK
707 (Fraction 2), (C) HQGLPQEVLNENLLR (Fraction 3), (D) HPIKHQGLPQEVLNENLLR (Fraction 3), (E) HQGLPQEVLNENLLR (Fraction 4), (F)
708 YIPIQYVLSR (Fraction 5), (G) HQGLPQEVLNENLLR (Fraction 5), (H) PFPEVFGK (Fraction 6), (I) YLGYLEQLLR (Fraction 6), (J) FFVAPFPEVFGK
709 (Fraction 6). The “y” axis represents the relative intensity of the ion precursor and each of fragment ions. The “x” axis shows the m/z, in which fragments
710 ions were detected.

Table 1. Primary sequence of caseins α_{s1}, α_{s2}, β e κ.

α_{s1}-casein	M**K**LLILTCLVAVALA**R**P**K**HPI**K**HQGLPQEVLNENLL**R**FFVAPFPEVFG**K**E**K**VNELS**K** DIGSESTEDQAMEDI**K**QMEAESISSSEEIVPNSVEQ**K**HIQ**K**EDVPSE**R**YLGYLEQLL **R**L**KK**Y**K**VPQLEIVPNSAEE**R**LHSM**K**EGIHAQQ**K**EPMIGVNQELAYFYPELF**R**QFYQ LDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSE**K**TTMPLW
α_{s2}-casein	M**K**FFIFTCLLAVALA**K**NTMEHVSSSEESIISQETY**K**QE**K**NMAINPS**K**ENLCSTFC**K**E VV**R**NANEEEYSIGSSSEESAEVATEEV**K**ITVDD**K**HYQ**K**ALNEINQFYQ**K**FPQYLQY LYQGPIVLNPWDQV**KR**NAVPITPTLN**R**EQLSTSEENS**KK**TVDMESTEVFT**KK**T**K**LT EEE**K**N**R**LNFL**KK**ISQ**R**YQ**K**FALPQYL**K**TVYQHQ**K**AMKPWIQP**K**T**K**VIPYV**R**YL
β-casein	**R**ELEELNVPGEIVESLSSSEESIT**R**IN**KK**IE**K**FQSEEQQQTEDELQD**K**IHPFAQTQS LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVS**K**V**K**EAMAP**K**H**K**EMPFP**K** YPVEPFTE**R**QSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQS **K**VLPVPQ**K**AVPYPQ**R**DMPIQAFLLYQEPVLGPV**R**GPFPIIV
κ-casein	MM**K**SFFLVVTILALTLPFLGAQEQNQEQPI**R**CE**K**DE**R**FFSD**K**IA**K**YIPIQYVLS**R**YPS YGLNYYQQ**K**PVALINNQFLPYPYYA**K**PAAV**R**SPAQILQWQVLSNTVPA**K**SCQAQP TTMA**R**HPHPHLSFMAIPP**KK**NQD**K**TEIPTINTIASGEPTSTPTTEAVESTVATLEDS PEVIESPPEINTVQVTSTAV

Fonte: http://www.uwm.edu.pl/biochemia/index.php/en/biopep

Note: The amino acids shown in underlined and bold are those susceptible to cleavage by the action of the trypsin.

717 **Table 2**. Possible fragments (predicted *in silico*) generated by hydrolysis of casein
718 catalyzed by bovine pancreas trypsin. Peptides shown in underlined were equivalent to
719 those identified by mass spectrometry on the research work of Silva (2016) and
720 peptides shown in bold were identified in this research work.

Fragment	Molecular mass (Da)	Amino acid sequence*
α_{s1}-casein		
1-2	277.39	MK
3-16	1,468.89	LLILTCLVAVALAR
17-18	243.3	PK
19-22	493.6	HPIK
23-37	1,759.96	**HQGLPQEVLNENLLR**
38-49	1,384.62	**FFVAPFPEVFGK**
50-51	275.3	EK
52-57	688.77	VNELSK
58-73	1,767.82	DIGSESTEDQAMEDIK
74-94	2,321.47	QMEAESISSSEEIVPNSVEQK
95-98	524.61	HIQK
99-105	830.84	EDVPSER
106-115	1,267.47	**YLGYLEQLLR**
116-117	259.35	LK
118-118	146.19	K
119-120	309.36	YK
121-134	1,580.74	VPQLEIVPNSAEER
135-139	614.76	LHSMK
140-147	909.99	EGIHAQQK
148-166	2,316.63	EPMIGVNQELAYFYPELFR
167-208	4,718.01	QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK
209-214	747.9	TTMPLW
α_{s2}-casein		
1-2	277.39	MK
3-16	1,556.95	FFIFTCLLAVALAK
17-36	2,299.43	NTMEHVSSSEESIISQETYK
37-39	403.43	QEK
40-47	874.0160	NMAINPSK
48-56	1,044.21	ENLCSTFCK
57-60	501.58	EVVR

Fragment	Molecular mass (Da)	Amino acid sequence*
61-85	2,688.67	NANEEEYSIGSSSEESAEVATEEVK
86-91	689.75	ITVDDK
92-95	574.63	HYQK
96-106	1,367.51	**ALNEINQFYQK**
107-128	2,710.09	FPQYLQYLYQGPIVLNPWDQVK
129-129	174.2	R
130-140	1,195.37	**NAVPITPTLNR**
141-151	1,251.26	EQLSTSEENSK
152-152	146.19	K
153-164	1,386.53	TVDMESTEVFTK
165-165	146.19	K
166-167	247.29	TK
168-173	747.79	LTEEEK
174-175	288.3	NR
176-180	633.78	LNFLK
181-181	146.19	K
182-185	502.57	ISQR
186-188	437.49	YQK
189-196	979.17	**FALPQYLK**
197-203	902.99	TVYQHQK
204-206	348.46	AMK
207-212	767.91	PWIQPK
213-214	247.29	TK
215-220	745.91	VIPYVR
221-222	294.35	YL
β-casein		
1-1	174.2	R
2-25	2,646.81	ELEELNVPGEIVESLSSSEESITR
26-28	373.45	INK
29-29	146.19	K
30-32	388.46	IEK
33-48	1,981.98	FQSEEQQQTEDELQDK
49-97	5,319.18	IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK
98-99	245.32	VK
100-105	645.77	EAMAPK

Fragment	Molecular mass (Da)	Amino acid sequence*
106-107	283.33	HK
108-113	747.9	EMPFPK
114-122	1,137.24	YPVEPFTER
123-169	5312.13	QSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
170-176	779.97	VLPVPQK
177-183	829.94	AVPYPQR
184-202	2,186.57	DMPIQAFLLYQEPVLGPVR
203-209	741. 92	GPFPIIV
κ-casein		
1-3	408.58	MMK
4-31	3,173.66	SFFLVVTILALTLPFLGAQEQNQEQPIR
32-34	378.45	CEK
35-37	418.4	DER
38-42	642.7	FFSDK
43-45	330.42	IAK
46-55	1,251.47	**YIPIQYVLSR**
56-67	1,523.64	YPSYGLNYYQQK
68-84	2,011.32	PVALINNQFLPYPYYAK
85-89	512.6	PAAVR
90-107	1,980.27	SPAQILQWQVLSNTVPAK
108-118	1,193.36	SCQAQPTTMAR
119-132	1,608.91	HPHPHLSFMAIPPK
133-133	146.19	K
134-137	503.51	NQDK
138-190	5,455.84	TEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV

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723 **Table 3:** Absorbance of samples and their respective percentage of inhibition-ACE
724 activity.

Peaks	Mean absorbance (228 nm)	% I_{ACE}
Hydrolysate		
Peak 1	0.199	80.68
Peak 2	0.202	79.00
Peak 3	0.215	49.19
Peak 4	0.212	62.44
Peak 5	0.243	16.30
Peak 6	0.215	47.81
Blank (hydrolysate + HCl)		
Peak 1	0.186	-
Peak 2	0.188	-
Peak 3	0.173	-
Peak 4	0.187	-
Peak 5	0.179	-
Peak 6	0.171	-
Control (water)		
Control	0.255	-
Casein Control		
Peak 1	0.254	1.78
Peak 2	0.244	17.88
Peak 3	0.251	5.86
Blank (casein control + HCl)		
Peak 1	0.180	-
Peak 2	0.195	-
Peak 3	0.181	-
Control (water)		
Control	0.255	-

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CAPÍTULO 4

Conclusão Geral

A análise da literatura sobre um assunto a ser pesquisado, bem como a riqueza de informações compiladas em artigos do tipo *Review*, são recursos de imensa utilidade para o desenvolvimento de pesquisas cujos resultados agreguem de fato inovações, ainda que modestas, ao campo técnico-científico em estudo. No estudo bibliográfico – que, mesmo não sendo exaustivo, foi abrangente – foi avaliada e discutida a literatura recente no tocante às diversas variáveis relacionadas à geração de peptídeos com potencial anti-hipertensivo, tais como: tipo de substrato e enzima, as condições reacionais (pH, temperatura e tempo), e o mecanismo de obtenção dos peptídeos (fermentação ou hidrólise enzimática). Além disso, tendências relacionadas ao estudo de peptídeos bioativos foram apresentadas e se destacam principalmente com relação ao desenvolvimento de processos de purificação dos referidos compostos, devido ao alto custo e baixos rendimentos ainda prevalentes. A aplicação destes peptídeos em fórmulas alimentícias representa a outra vertente que demanda mais estudos, sejam esses referentes à estabilidade dos compostos bioativos ao longo da cadeia de produção e estocagem de alimentos, bem como a garantia de biodisponibilidade e eficiência de atuação no organismo humano.

Em termos experimentais, os resultados demonstraram a viabilidade do emprego de colunas C4 para separação cromatográfica de peptídeos inibidores da enzima conversora de angiotensina I (ECA) derivados da tripsinólise de caseínas bovinas. Com base na sequência aminoacídica dos peptídeos identificados *via* espectrometria de massas, foi possível apresentar algumas características que demonstram alta relevância para o desenvolvimento estrutural de peptídeos com potencial anti-hipertensivo, dentre as quais se destaca a composição tripeptídica na região C-terminal.

Ao final desta dissertação, propostas promissoras de pesquisas futuras podem ser apresentadas. Uma ideia que se apresenta interessante para a continuidade dos trabalhos, seria a determinação do peptídeo com maior atividade anti-hipertensiva *in vitro*, dentre os candidatos apresentados, para posterior avaliação *in vivo*. Além disso, a perspectiva de emprego de outras enzimas, como pepsina e quimotripsina, na etapa de hidrólise da caseína, bem como a utilização de um novo gradiente de eluição cromatográfica, representam abordagens de relevante interesse para futuras investigações do presente estudo.