UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

LUÍSA OZORIO

OBTAINMENT OF BIOFUNCTIONAL INGREDIENTS FROM WHEY PROTEIN CONCENTRATES

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Luísa Ozorio

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Doctoral thesis presented to the Postgraduate Program in Food Science, Institute of Chemistry, Federal University of Rio de Janeiro, as part of the requirements to obtain the degree of Doctor in Science.

Supervisors: Dr. Lourdes Maria Correa Cabral
Dr. Caroline Mellinger Silva
Dr. Didier Dupont

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Approved by:

Prof. Lourdes Maria Correa Cabral
Embrapa Agroindústria de Alimentos – EMBRAPA

Prof. Maria Gabriela Koblitz
Universidade Federal do Estado do Rio de Janeiro – UNIRIO

Prof. Adriano Gomes da Cruz
Instituto Federal do Rio de Janeiro – IFRJ

Prof. Carlos Adam Conte Junior
Universidade Federal do Rio de Janeiro - UFRJ

Prof. Daniel Perrone Moreira
Universidade Federal do Rio de Janeiro - UFRJ

To my dear mother, Maria Elizabeth Ozorio

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ABSTRACT

Whey is a dilute nutrient stream which needs to be concentrated and purified in order to obtain the high added value byproducts named “whey proteins”. Implementing the technologies required to produce them may be costly, especially for small industries, but it is becoming very profitable with the increasing demand of this market sector. Whey proteins present high nutritional value and technological properties enabling their used into a large variety of products. These properties may be enhanced by enzymatic processes, which also are able to release bioactive peptides from the native proteins. Bioactive peptides have been subject of several studies over the years, as they may play important roles in different systems of the body, as opioid, anticancer, antidiabetic, antioxidant and antihypertensive molecules. In this sense, the present study aimed to develop three biofunctional ingredients from different whey protein concentrates for its future utilization by food and nutraceutical industries. The first ingredient, a bioactive whey protein hydrolysate, presented interesting technological properties as high oil absorption capacity and solubility in a large pH range, besides presenting great vasorelaxant potential, including in pilot scale. The acceptability of this ingredient was also high, and the vascular relaxation was enhanced after a simulated gastrointestinal digestion. The *in vitro* absorption assays showed that oligopeptides could be absorbed through the intestinal epithelium of piglets, including antihypertensive, as well as other bioactive molecules. Concerning the 2nd ingredient, the results of the chemical assays showed that microwave 600 W and autoclave 120°C treatments were able to modify β-lactoglobulin’s structure, allowing its complete hydrolysis by pepsin. These results were very promising for the future development of a hypoallergenic whey hydrolysate. The third ingredient was developed to add value to the most commercialized whey protein in Brazil, the WPC 34%. The enzymatic processes were able to enhance the emulsifying and antioxidant capacities of the ingredient, especially the fraction of 120 min of the tryptic hydrolysate which may be used in several applications by food industries. The results altogether brought the possibility of developing three whey hydrolysates to supply different market demands of food and nutraceutical industries: the first one, the niche of bioactive ingredients for health benefits; the second, the increasing market of hypoallergenic products; and the third one, aiming to achieve the national market with an ingredient with double functionality.

Keywords: Whey proteins, bioactive peptides, hydrolysates, gastrointestinal digestion, absorption, technological properties, vasorelaxant potential, antioxidant activity.

RESUMO

O soro de leite é uma solução de nutrientes diluída que necessita de ser concentrada e purificada para a obtenção dos co-produtos de alto valor agregado conhecidos como *whey proteins*. A implementação das tecnologias necessárias para sua produção pode ser dispendiosa, especialmente para pequenas indústrias, mas têm se tornado bastante lucrativa devido a crescente demanda deste setor de mercado. As proteínas do soro apresentam alto valor nutricional e propriedades tecnológicas que permitem seu uso em uma ampla variedade de produtos. Essas propriedades podem ser melhoradas por processos enzimáticos, que também são capazes de liberar peptídeos bioativos das estruturas das proteínas nativas. Peptídeos bioativos têm sido tema de diversos estudos ao longo dos anos, devido ao seu potencial em desempenhar papéis importantes em diferentes sistemas do organismo, atuando como moléculas opióides, anticâncer, antidiabéticas, antioxidantes e anti-hipertensivas. Nesse sentido, o presente estudo teve como objetivo desenvolver três ingredientes biofuncionais a partir de diferentes concentrados proteicos de soro de leite visando sua futura utilização pelas indústrias alimentícia e nutracêutica. O primeiro ingrediente, um hidrolisado de soro de leite bioativo, apresentou propriedades tecnológicas interessantes como alta capacidade de absorção de óleo e solubilidade em uma ampla faixa de pH, além de apresentar grande potencial vasorelaxante, inclusive em escala piloto. Este ingrediente apresentou alta aceitabilidade e os relaxamentos vasculares observados foram aumentados após a digestão gastrointestinal simulada. Os ensaios de absorção *in vitro* mostraram que oligopeptídeos puderam ser absorvidos através do epitélio intestinal de leitões, incluindo moléculas anti-hipertensivas, bem como outros peptídeos bioativos. Com relação ao ingrediente 2, os resultados dos ensaios químicos mostraram que os tratamentos de microondas 600 W e, autoclave 120°C foram capazes de modificar a estrutura da β-lactoglobulina, permitindo sua completa hidrólise pela pepsina. Os resultados obtidos até o momento se mostram bastante promissores para o futuro desenvolvimento de um hidrolisado de soro hipoalergênico. O terceiro ingrediente foi desenvolvido para agregar valor ao soro de leite concentrado mais comercializado no Brasil, o WPC 34%. Os processos enzimáticos utilizados foram capazes de melhorar a capacidade emulsificante e antioxidante do ingrediente, especialmente a fração de 120 min do hidrolisado tríptico, que pode vir a ser utilizado em diferentes formulações pelas indústrias alimentícias. Os resultados em conjunto trouxeram a possibilidade de desenvolver três hidrolisados de soro de leite, a fim de suprir diferentes demandas das indústrias alimentícias e nutracêuticas: o primeiro, voltado para o nicho de ingredientes bioativos com benefícios à saúde; o segundo, o visando

attender a crescente demanda de mercado por produtos hipoalergênicos; e o terceiro, visando attender ao mercado nacional com um ingrediente com dupla funcionalidade.

Palavras-chave: Proteínas de soro, peptídeos bioativos, hidrolisados, digestão gastrointestinal, absorção, propriedades tecnológicas, potencial vasorelaxante, atividade antioxidante.

LIST OF FIGURES

Section **Page**

Thesis Structure

Figure 1. Diagram of thesis structure 20

Literature Review

Figure 1. (A) Primary structure of bovine β-lactoglobulin A; (B) Tertiary structure of β-lactoglobulin as a monomer. 37

Figure 2. (A) Primary structure of bovine α-lactalbumin A; (B) Tertiary structure of α-lactalbumin as a monomer. 38

Figure 3. Chemical structure of amino acids. 41

Figure 4. Formation of a peptide bond. 43

Figure 5. Blood pressure regulation through angiotensin-I converting enzyme inhibitors in the renin-angiotensin system. 45

Figure 6. Bioactive peptides pathways involved in blood pressure control. 46

Figure 7. Diagram representing processes and whey-based products. 50

Chapter 1

Figure 1. RP-HPLC chromatograms of unhydrolyzed WPC88 (A) and WPC88 hydrolyzed by pepsin solutions of (B) 0.48 %, (C) 0.95 %, (D) 1.43 % and (E) 1.91 % (w/w). 62

Figure 2. Vascular relaxation induced by pepsin hydrolysate from whey protein concentrate. Typical tracings (A) and data (B) showing the effect of the cumulative addition of 1, 3, 5 and 10 $mg.mL^{-1}$ of the hydrolyzed fraction (PC3) in endothelium of rat aortic rings previously contracted by phenylephrine. 65

Figure 3. Frequency of notes distribution of global acceptance (A) and intention to purchase (B) assigned by consumers to dairy desserts without (D1) and with the addition of the hydrolyzed whey (D2). 68

Figure A.$_1$. Hydrolysis curves of whey protein isolate treated with commercial pepsin 0.48%, 0.95%, 1.43% and 1.91% (w/w), measured as soluble protein content. 70

Figure A.$_2$. Particle size profile distribution of PC3 whey hydrolysate fraction. Isopropyl alcohol was used as dispersing agent. 70

Chapter 2

Figure 1. Flow diagram of a proposed industrial plant for whey protein hydrolysate production. 77

Figure 2. Peptide profiles of whey hydrolysates assessing different enzyme inactivation conditions and drying Technologies. 79

Figure 3. Vascular relaxation induced by whey hydrolysates in rat aortic rings precontracted by phenylephrine. 80

Chapter 3

Figure 1. Free amino acids release during whey hydrolysate *in vitro* gastrointestinal digestion. 91

Figure 2. Electrophoretic profile of undigested and digested whey hydrolysate samples. 92

Figure 3. RP-HPLC peptide profiles of (A) control sample – WPC88; (B) undigested whey hydrolysate; (C) intestinal digested whey hydrolysate. 93

Figure 4. Nano-RSLC – MS/MS heat map built with the frequency of appearance, in percentage, that each amino acid was identified as part of a peptide sequence from β-Lactoglobulin (β-lg), α-Lactalbumin (α-la), β-Casein (β-cas) and κ-Casein (κ-cas) after whey hydrolysate (WPH) *in vitro* gastrointestinal digestion. 94

Figure 5. Whey hydrolysates-induced vascular relaxation. Trace records of the vascular relaxation induced by undigested and digested whey hydrolysates in endothelium-denuded (A) and endothelium-intact (B) rat aortic rings. 97

Chapter 4

Figure 1. Average molecular weight and total number of peptides identified for undigested (WPH) and digested (ID) whey hydrolysate during Ussing Chamber absorption assay in apical compartments. 110

Figure 2. Average molecular weight and total number of peptides identified for undigested (WPH) and digested (ID) whey hydrolysate during Ussing Chamber absorption assay in basolateral compartments. 112

Figure 3. Nano-RSLC–MS/MS heatmap built with all the peptides identified along the Ussing chamber assays from undigested (WPH) and previously digested (ID) whey protein hydrolysates grouped in 7 clusters by similarity. 114

Figure 4: Undigested (WPH) and previously digested (ID) whey protein hydrolysate peptides identified by Nano-RSLC-MS/MS at the basolateral compartment of the Ussing chambers from β-lactoglobulin (β-lg) and β-casein (β-cas). 115

Chapter 5

Figure 1. Peptide profiles of the (A) whey protein concentrate – control sample; (B) whey protein hydrolysate; whey protein hydrolysates pre-treated for 5 min with (C) ultrasound 100 W, (D) microwave 600 W, (E) autoclave 120°C. 125

Chapter 6

Figure 1. Degree of hydrolysis of WPC by the use of commercial proteases. 134

Figure 2. Chromatographic peptide profiles of WPC hydrolysates. 135

Figure 3. *In vitro* antioxidant activity of WPC hydrolysates. 137

Figure 4. Emulsifying capacity of WPC hydrolysates. 138

LIST OF TABLES

Section **Page**

Literature Review

Table 1. Composition of sweet and acid whey 35

Table 2. Comparison between the recommended dietary intake of essential amino acids for adults and the essential amino acids content in whey proteins concentrate (WPC 88%) and isolates (WPI). 42

Table 3. Commercial products containing milk-derived bioactive peptides evaluated in clinical studies concerning their effects in systolic blood pressure. 44

Chapter 1

Table 1. MALDI-TOF/TOF MS analysis of the peptides obtained from whey protein concentrate treated with commercial pepsin for 3h. 63

Table 2. Solubility and foaming capacity of peptic whey hydrolysate and whey protein concentrate. 66

Chapter 4

Table 1. Undigested (WPH) and digested (ID) whey protein hydrolysate absorbed peptides identified in the Ussing chamber assays by Nano-RSLC-MS/MS with bioactive potential reported in the literature. 116

Chapter 5

Table 1. WPC 88 hydrolysis pre-treatments using ultrasound, autoclave and microwave technologies. 123

Chapter 6

Table 1. Hydrolysis conditions of WPC 35 by the use of different enzymes 132

LIST OF ABREVIATIONS

AA%, percentage of antioxidant activity
AACC, American Association for Clinical Chemistry
Abs, absorbance
ACE, angiotensin-I converting enzyme
ACN, acetonitrile
AG-I, angiotensin I
AG-II, angiotensin II
ANOVA, analysis of variance
AT_1, angiotensin 1 receptors
BHA, butylated hydroxyanisol
BHT, butylated hydroxytoluene
BP, blood pressure
BSA, bovine serum albumin
Chy, chymotrypsin
CI, chemically inactivate
CMP, caseinomacropeptide
Cys, Cysteine
D1, control dessert
D2, dessert added with peptides
DF, dilution factor
DH, degree of hydrolysis
DIAAS, digestible dietary indispensable amino acid
DNA, deoxyribonucleic acid
DPP-IV, Dipeptidyl-peptidase-IV
DTT, DL-Dithiothreitol
EAI, emulsifying activity index
EC, emulsifying capacity
ECE, endothelin-converting-enzyme
EDTA, Ethylenediamine tetraacetic acid
ELISA, enzyme-linked immunosorbent assay
eNOS, endothelial nitric-oxide synthase
ESI, emulsifying stability index
Et_a/Et_b endothelin cell receptors
FAO, Food and Agriculture Organization
FE, foam expansion
FS, foam stability
G1/G2, groups 1 and 2
Glu, glutamine
He, emulsified phase height
Ht, solution height
ID, intestinal digested whey protein hydrolysate
IGs, immunoglobulins
KNOS, kinin-nitric oxide system
mA, milliamperes
MALDI/ TOF-TOF, matrix-assisted laser desorption/ionization tandem time-of-flight
Met, methionine
MH, mild hypertension
MS, mass spectrometry

NH, high-normal blood pressure
OPA, orthophtaldialdehyde
PBS, phosphate-buffered saline
PC3, 3-hour aliquot of the whey protein hydrolysate
PepT1, proton dependent H^+/peptide
PG, propyl gallate
pH, potential of hydrogen
Phe, phenylalanine
pI, isoelectric point
PPC, commercial pepsin
PSS, physiological saline solution
Ptn, protein
ROS, reactive oxygen species
RP-HPLC, reversed phase high performance liquid chromatography
RSLC, rapid separation liquid chromatography
SBP, systolic blood pressure
SDS-PAGE, dodecyl sulfate polyacrylamide gel electrophoresis
SEC, size exclusion chromatography
SOPT1/SOPT2, sodium dependent oligopeptide transporters
T0, T1, T2, T3, T4, T5, time of aliquot: 0, 1, 60, 120, 180, 240 e 300 minutes of reaction
TBHQ, tertiary butylhydroxyquinone
TI, thermally inactivated
Try, trypsin
U, units
USDEC, United States Dairy Export Council
Val, valine
WHO, World Health Organization
WPC, whey protein concentrate
WPC34, whey protein concentrate containing 34% of proteins
WPC88, whey protein concentrate containing 88% of proteins
WPH, whey protein hydrolysate
WPI, whey protein isolate
α-La, alphalactalbumin
β-cas, beta-casein
β-Lg, betalactoglobulin
κ-cas, kappa-casein

Thesis Structure

THESIS STRUCTURE

This document was divided as follows: general introduction, objectives, literature review and other five chapters, in which the experimental results were joined into scientific papers.

Chapters 1 to 5, as well as the text in Appendix handle with the development of three whey-based ingredients, as presented in Figure 1. The first is a whey hydrolysate with biofunctional potential, focusing on its vasorelaxant capacity (Chapters 1 – 4); the second, is a whey ingredient which aims to achieve the increasing market of the hypoallergenic formulas (Chapter 5) and the third one is a whey product with dual functionality, developed to add value to the main whey protein concentrate produced in Brazil, the WPC 34% (Chapter 6).

The first chapter focused on the different steps for the development of a biofunctional ingredient to food industries, ranging from production to consumer's evaluation. The enzyme concentration and the time of hydrolysis were analyzed as variables for the obtainment of the vasorelaxant peptides. The most promising condition, decided through chemical assays, was then dried and the powder obtained was physically and technologically characterized. Moreover, different peptides were sequenced and the vasorelaxant activity of the hydrolysate was measured. Furthermore, the whey hydrolyzed powder was sensory evaluated. This chapter was published in the Food Research International (2019) as a research paper (OZORIO, Luísa et al. Whey hydrolysate-based ingredient with dual functionality: From production to consumer's evaluation. Food Research International, v. 122, p. 123–128, 2019).

Aiming to bring the production of the whey hydrolysate to a commercial scenario, in order to verify if its production in large scale was viable, the second chapter approached the development of the vasorelaxant whey hydrolysate in semi-pilot scale. This part of the study focused on the limiting points of the process which were the enzyme inactivation and the drying technology used. This chapter was published in the International Dairy Journal (2019) as a research paper (OZORIO, Luísa et al. Enzyme inactivation and drying technologies influencing the vasorelaxant activity of a whey protein hydrolysate in semi-pilot scale. International Dairy Journal, v. 93, p. 11–14, 2019).

The third chapter brought attention to the biological events that happen after ingesting the whey hydrolysate, evaluating the molecular alterations generated during the gastrointestinal digestion of the whey hydrolysate and how they influence its vasorelaxant activity. The results of this

part of the study were very interesting and were submitted to Food Research International journal as a research paper that is under revision.

The promising results obtained in the chapter 3 lead us to deepen the studies in this theme and Chapter 4 accomplished the molecular alterations generated in the small intestine, after the gastrointestinal digestion of the whey hydrolysate. In this sense, the digested whey was applied in a system that simulates absorption using part of the small intestine of piglets, named "Ussing Chamber". The effect of the brush border enzymes was evaluated, as well as the absorption of peptides and amino acids. This chapter was structured according to Food Chemistry guidelines aiming its submission in a near future.

Aiming to achieve the increasing market of hypoallergenic products, a second ingredient obtained from the whey protein concentrate 88% is under development and the partial results obtained are presented in Chapter 5. The whey protein concentrate 88% was submitted to different pre-treatments with microwave, autoclave and ultrasound, followed by pepsin enzymatic hydrolysis, in order to reduce its allergenicity. The most promising treatments were selected and are being evaluated by ELISA immunoassays. Since the study is in final stage of conclusion, it will be soon submitted as a short communication to a scientific journal.

In the Chapter 6 a third ingredient was developed, aiming to achieve national market demands. Different from the previous one, this ingredient was obtained from the whey protein concentrate 34%, which is the whey protein concentrate mainly produced in Brazil. In this sense, aiming to valorize this byproduct produced by the national industry, different enzymes were evaluated to the development of an ingredient with antioxidant and emulsifying capacities. The results obtained in this chapter were published in the Food Science and Technology as a research paper (ROSA, Luísa O. L et al., A comparison of dual-functional whey hydrolysates by the use of commercial proteases. Food Science and Technology, v. 38, p. 31-36, 2018).

In the end of this document the final considerations of the study are presented, and the references used to produce it are listed.

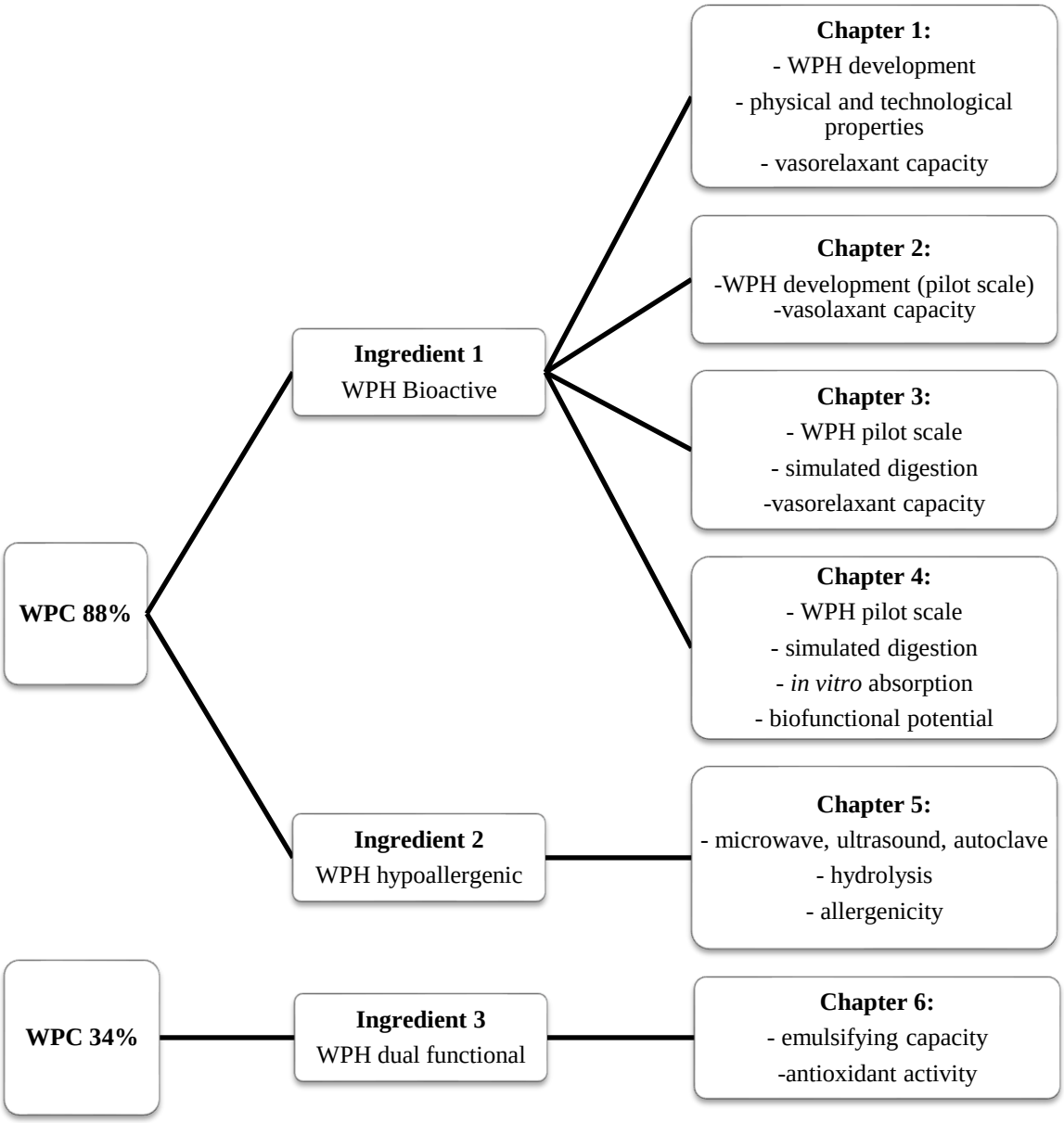

Figure 1. Diagram of thesis structure.

Scientific Production

SCIENTIFIC PRODUCTION

During the development of this thesis, the following research papers were published in scientific journals:

- **LUISA OZORIO**; LUCIANO P. SILVA; MAURA V. PRATES; CARLOS BLOCH JR.; CRISTINA Y. TAKEITI; DANILLO MACÊDO GOMES, JOSÉ EDUARDO DA SILVA-SANTOS, ROSIRES DELIZA; ANA IRAIDY S. BRÍGIDA, CAROLINE MELLINGER-SILVA, LOURDES M.C. CABRAL. 2019. Whey hydrolysate-based ingredient with dual functionality: from production to consumer's evaluation. Food Research International, v.122, p.123-128. **(Chapter 1)**

- **OZORIO, L**.; PEREIRA, N. R.; SILVA-SANTOS, J. E.; BRIGIDA, A. I.S.; MELLINGER-SILVA, C.; CABRAL, L. M.C. 2019. Enzyme inactivation and drying technologies influencing the vasorelaxant activity of a whey protein hydrolysate in semi-pilot scale. International Dairy Journal, v. 93, p. 11-14. **(Chapter 2)**

- **ROSA, LUISA O.L**.; SANTANA, M. C.; AZEVEDO, T. L.; BRIGIDA, A. I. S.; GODOY, R.; PACHECO, S.; MELLINGER-SILVA, C.; CABRAL, L. M. C. 2018. A comparison of dual-functional whey hydrolysates by the use of commercial proteases. Food Science and Technology, v. 38, p. 31-36. **(Chapter 6)**

The following research paper was submitted to Food Chemistry journal and is under revision:

- **LUÍSA OZORIO**; NATÁLIA KIMIE MATSUBARA; JOSÉ EDUARDO DA SILVA-SANTOS; GWENAËLE HENRY; YANN LE GOUAR; JULIEN JARDIN; CAROLINE MELLINGER-SILVA; LOURDES M. C. CABRAL; DIDIER DUPONT. Gastrointestinal digestion enhances the endothelium-dependent vasodilation of a whey hydrolysate in rat aortic rings (**Chapter 3**).

The following research papers are under final construction and will be soon submitted to scientific journals:

- **LUÍSA OZORIO**; JULIEN JARDIN; GAELLE BOUDRY; CAROLINE MELLINGER-SILVA; LOURDES M. C. CABRAL; DIDIER DUPONT. Brush border peptidase activity and absorption of oligopeptides from a whey protein hydrolysate through the small intestine of piglets in Ussing chamber *in vitro* assays. Molecular Nutrition & Food Research (**Chapter 4**).

- **OZORIO, L**.; CUNHA, L.C.M.; CONTE-JR, C.A; BRÍGIDA, A.I.S; MELLINGER-SILVA, C..; CABRAL, L.M. C. Emerging technologies assisting whey β-Lactoglobulin hydrolysis aiming the development of hypoallergenic whey-based ingredients (**Chapter 5**).

The following abstracts were published in scientific conferences:

- **OZORIO, L**.; PIMENTA, G.; PEREIRA, A.; MELLINGER-SILVA, C.; CABRAL, L. M. C.; TONON, R. V. Development of a biofunctional ingredient

obtained from Alicante Bouschet grape pomace In: 32nd EFFoST International Conference, 2018, Nantes. Developing innovative food structures and functionalities through process and reformulation to satisfy consumer needs and expectations, 2018.

- **OZORIO, L**.; PEREIRA, N. R.; SILVA-SANTOS, J. E.; MELLINGER-SILVA, C.; CABRAL, L. M. C. Gastrointestinal digestion improving the antihypertensive activity of whey peptides In: 32nd EFFoST International Conference, 2018, Nantes. Developing innovative food structures and functionalities through process and reformulation to satisfy consumer needs and expectations, 2018.

- PEREIRA, N. R.; **OZORIO, L.**; MELLINGER-SILVA, C.; SILVA-SANTOS, J. E. Activation of nitric oxide/guanylate-cyclase pathway accounts for the vasodilatory effect of a gastrointestinal digested whey protein hydrolysate In: 50th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE), 2018, Ribeirão Preto. 50th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE), 2018.

- **OZORIO, L**.; PEREIRA, N. R.; SILVA-SANTOS, J. E.; BRIGIDA, A. I. S.; MELLINGER-SILVA, C.; CABRAL, L. M. C. Optimizing the development of an antihypertensive whey hydrolysate in semi-pilot scale In: 32nd EFFoST International Conference, 2018, Nantes. Developing innovative food structures and functionalities through process and reformulation to satisfy consumer needs and expectations, 2018.

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- **ROSA, LUISA O.L.**; SILVA-SANTOS, J. E.; MELLINGER-SILVA, C.; CABRAL, L. M. C. NEW FINDINGS IN ANTIHYPERTENSIVE WHEY PEPTIDES CONSIDERING STRUCTURE-ACTIVITY RESPONSES In: São Paulo School of Advanced Sciences on Reverse Engineering of Processed Foods, 2017, Campinas, SP. São Paulo School of Advanced Sciences on Reverse Engineering of Processed Foods, 2017.

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- Gastrointestinal digestion enhances the vasorelaxant activity of a whey hydrolysate. IV *Simpósio de Alimentação e Nutrição*, 2019, Rio de Janeiro, Brazil.

Other:

- Winner of the second place in the oral presentations of the *IV Simpósio de Alimentação e Nutrição* (2019), Rio de Janeiro, Brazil.

SUMMARY

General Introduction 29

GENERAL INTRODUCTION 30

Objectives 32

OBJECTIVES 33

1. GENERAL OBJECTIVE 33
2. SPECIFIC OBJECTIVES 33

Literature Review 34

LITERATURE REVIEW 35

1. WHEY 35
2. WHEY PROTEINS 36
3. NUTRITIONAL PROPERTIES OF WHEY PROTEINS 40
4. BIOACTIVE PEPTIDES 42
5. TECHNOLOGICAL PROPERTIES OF WHEY PROTEINS 48
6 WHEY-BASED PRODUCTS 49

Chapter 1 52

ABSTRACT 54

1. INTRODUCTION 55

2. MATERIALS AND METHODS 56

2.1 MATERIALS 56
2.2 ENZYMATIC HYDROLYSIS 56
2.3 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) 57
2.4 MASS SPECTROMETRY 57
2.5 VASCULAR RELAXATION 57
2.6 PHYSICAL AND TECHNOLOGICAL PROPERTIES 58
2.7 SENSORY ANALYSIS 60

3 RESULTS AND DISCUSSION 61

3.1 COMMERCIAL PEPTIC WHEY PROTEIN HYDROLYSATE PROFILES 61
3.2 PEPTIDES IDENTIFICATION 63
3.3 VASCULAR RELAXATION 64
3.4 TECHNOLOGICAL PROPERTIES OF WHEY PROTEIN HYDROLYSATE 65
3.5 SENSORY ANALYSIS 67

4 CONCLUSIONS 68

SUPPLEMENTARY MATERIAL 70

Chapter 2 71

ABSTRACT 73

1 INTRODUCTION 74

2 MATERIALS AND METHODS 74

2.1 MATERIALS 74

2.2 WHEY HYDROLYSATE OBTAINMENT 75
2.3 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) 75
2.4 AORTIC RING PREPARATION AND ANALYSES OF VASCULAR RELAXATION 76

3 RESULTS AND DISCUSSION 76

4 CONCLUSIONS 80

Chapter 3 82

ABSTRACT 84

1 INTRODUCTION 85

2 MATERIALS AND METHODS 86
2.1 MATERIALS 86
2.2 WHEY ENZYMATIC HYDROLYSIS 86
2.3 WHEY HYDROLYSATE *IN VITRO* DIGESTION 86
2.4 DEGREE OF HYDROLYSIS (DH) 87
2.5 PROTEIN ELECTROPHORESIS 87
2.6 REVERSED PHASE – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) 88
2.7 TANDEM MASS SPECTROMETRY 88
2.8 *IN VITRO* VASCULAR RELAXATION 89

3 RESULTS AND DISCUSSION 90
3.1 DEGREE OF HYDROLYSIS AND PROTEIN ELECTROPHORESIS 90
3.2 RP-HPLC PEPTIDE PROFILES 92
3.3 PEPTIDE SEQUENCES 93
3.4 INFLUENCE OF DIGESTION ON WHEY-INDUCED VASCULAR RELAXATION 96

CONCLUSIONS 97

Chapter 4 103

ABSTRACT 105

1. INTRODUCTION 106

2. MATERIALS AND METHODS 107
2.1 MATERIALS 107
2.2 WHEY ENZYMATIC HYDROLYSIS 107
2.3 WHEY HYDROLYSATE *IN VITRO* DIGESTION 107
2.4 *EX-VIVO* BRUSH BORDER HYDROLYSIS AND INTESTINAL TRANSEPITHELIAL PASSAGE ASSAYS - USSING CHAMBER 108
2.5 TANDEM MASS SPECTROMETRY 108

3. RESULTS AND DISCUSSION 109

4. CONCLUSIONS 117

Chapter 5 119

1. INTRODUCTION 121

2. MATERIALS AND METHODS 122
2.1 MATERIALS 122
2.2 PRE-TREATMENTS AND HYDROLYSIS PROCESS 122
2.2 REVERSED PHASE - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) 123

2.3 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) 124

3. PARTIAL RESULTS AND DISCUSSION *124*

3.1 RP-HPLC PEPTIDE PROFILES 124

Chapter 6 127

ABSTRACT *129*

1. INTRODUCTION *130*

2 MATERIALS AND METHODS *131*

2.1 MATERIALS 131

2.2 ENZYMATIC HYDROLYSIS 131

2.3 CHROMATOGRAPHIC ANALYSIS OF PEPTIDES AND PROTEINS 132

2.4 *IN VITRO* ANTIOXIDANT ACTIVITY 132

2.5 EMULSIFYING CAPACITY (EC) 133

3 RESULTS AND DISCUSSION *133*

3.1 HYDROLYSIS OF WPC AND PEPTIDE PROFILE OF THE HYDROLYSATES 133

3.2 *IN VITRO* ANTIOXIDANT ACTIVITY OF WHEY HYDROLYSATES 135

3.3 EMULSIFYING CAPACITY OF WHEY HYDROLYSATES 137

4 CONCLUSIONS *138*

Final Considerations 140

FINAL CONSIDERATIONS *141*

References 142

REFERENCES *143*

General Introduction

GENERAL INTRODUCTION

Whey is a byproduct of dairy industry obtained from the precipitation of milk caseins during the cheese-making process. This precipitation occurs via acid or enzymes addition, obtaining acid whey or sweet whey, respectively. Whey is mainly composed by water (approximately 94%) and several steps are required to obtain the different kinds of whey-based products, which vary in lactose, minerals and proteins content (KILARA; VAGHELA, 2018).

These products contain some of the milk's functional and nutritional properties, such as high essential amino acids concentration, solubility and emulsifying capacity, which are of great interest to food and pharmaceutical industries, justifying its wide use as food supplement and as ingredient in different food products (ADJONU *et al.*, 2014; VILLA *et al.*, 2018).

The large use of whey-based products, though, generates another concern, regarding the β-lactoglobulin's (β-lg) allergenicity. Allergenic reactions to β-lg happens mainly in children under 2 years old, but it may severely occur in adults (STANIC-VUCINIC *et al.*, 2012). The use of pre-treatments, as well as hydrolysis processes assisted by emerging technologies such as microwave, high pressure and ultrasound may alter β-lg's structure, changing its allergenic epitopes and expanding its possible uses (EL MECHERFI *et al.*, 2015; ORIVUORI *et al.*, 2014).

Besides reducing allergenicity, enzymatic hydrolysis is also used to release bioactive peptides from its parent proteins. In the recent years the bioactive properties of whey peptides have been extensively investigated, as there is a growing tendency in replacing allopathic medicines for functional foods (BELTRÁN-BARRIENTOS *et al.*, 2016). Whey peptides have been associated with different health benefits, including immunomodulation, antioxidant and antihypertensive activities. The last one is the most studied biofunctionality which can be attributed to the high prevalence of this chronic degenerative disease that achieves more than 1 billion people worldwide. The commercialization of these bioactive peptides, however, is lacking, highlighting the need to continue the studies in this field.

Despite the great interest in these whey-based products with high added value, the main issue of Brazilian industries is related to the high costs for implementing whey processing plants and the large volume of liquid whey required to justify this processing. In this sense, although the number of processing plants have increased from 2009 on, 24.6 thousand tons of whey are

still imported per year and it is estimated that 40% of the whey national production is discharged into hydric systems without treatment, generating another concern regarding its high chemical and biological demands (DERELI *et al.*, 2019; PITHAN E SILVA; BUENO; SÁ, 2017).

Faced with the need to contribute with the scientific and technological process in this theme, the present study aimed to develop biofunctional ingredients through the enzymatic hydrolysis of different whey protein concentrates for food and nutraceutical industries.

Objectives

OBJECTIVES

1. GENERAL OBJECTIVE

The present doctoral thesis aimed to develop three biofunctional ingredients from the enzymatic hydrolysis of different whey protein concentrates to food and nutraceutical industries.

In order to achieve the general objective, the following specific objectives were proposed:

2. SPECIFIC OBJECTIVES

2.1 Ingredient 1: Bioactive whey protein hydrolysate with potential vasorelaxant activity obtained from a WPC 88

2.1.1 To characterize physically and technologically the whey protein hydrolysate

2.1.2 To evaluate the influence of scaling up the hydrolysis process, as well as the influence of the enzyme inactivation conditions and drying technologies in the vasorelaxant capacity of the whey hydrolysate

2.1.3 To simulate the gastrointestinal digestion of the vasorelaxant whey hydrolysate using the harmonized protocol COST - INFOGEST

2.1.4 To evaluate peptides' *in vitro* absorption by the use of Ussing Chambers

2.1.5 To obtain the molecular profiles and identify digested and absorbed whey peptides through nano-RSLC-MS/MS

2.2 Ingredient 2: Hypoallergenic whey hydrolysate obtained from WPC 88

2.2.1 To verify the influence of ultrasound, microwave and autoclave technologies as pre-treatments to reduce the allergenicity of a whey hydrolysate through the immune assay ELISA

2.3 Ingredient 3: Whey hydrolysate with technological and antioxidant properties obtained from WPC 34

2.3.1 To evaluate the Trypsin, Novo pro D, Alcalase and Flavourzyme capacities in catalyze the whey proteins' hydrolysis to the development of an ingredient with technological and antioxidant capacities, to be used in the formulation of food products, aiming to add value to its ingredient mainly produced in Brazil.

Literature Review

LITERATURE REVIEW

1. WHEY

Whey is the fluid remaining after the acid or rennet-induced coagulation and removal of milk caseins in the cheese-making industry. This white-yellow fluid represents more than 90% of the volume of the milk, contains around 50% of milk's total solids, including all lactose and whey proteins, part of its salts and fat (FOX *et al.,* 2017).

Acid whey is obtained through the precipitation of casein on its isoelectric point (pI 4.6), during the manufacture of cottage cheese and acid casein. The decrease in pH occurs via bacterial fermentation of lactose or by acid addition, generating an acid byproduct of restricted use. Sweet whey, however, has the pH similar to the fresh milk (pH 5.9 – 6.6) and it is more commonly produced and used, resulting from the enzymatic coagulation of the casein micelles during the manufacture of the other types of cheese. (FOX *et al.*, 2017; PA'EE *et al.,* 2015).

Both kinds of whey differ in composition, depending on cow's breed, location, season, type of milk used and type of cheese produced. Differences in composition between acid and sweet whey are presented in Table 1. When compared, acid whey presents higher concentration of calcium and phosphorus, because of the solubilization of the complex calcium-phosphate existing in casein micelles in acid pH. Moreover, bacterial conversion of lactose in lactic acid during cheese-making process explains the lowest concentration of this carbohydrate in the fermented acid whey (TOMMASO *et al.,* 2012).

Table 1. Composition of sweet and acid whey (Adapted from Tommaso et al., 2012).

Composition	**Sweet whey**	**Acid whey**	
		Acid	**Fermentation**
Solids (%)	6.6	5.1	6
pH	6.1	4.7	4
Lactose (%)	4.8	3.7	3.9
Proteins (%)	0.9	0.73	0.72
Ash (%)	0.59	0.6	0.72
Lactic acid (%)	0.13	0	0.6

Fat (%)	0.06	0.05	0.003
Calcium (ppm)	430	1200	1140
Phosphorous (ppm)	440	680	900
Potassium (ppm)	1460	1200	1530
Sodium (ppm)	430	270	400
Chloride (ppm)	970	2600	910

Approximately 2 x 10^8 tons of whey are produced per year worldwide and despite its high nutritional quality, whey is still considered a very relevant wastewater, which may cause serious environmental hazards. Hence, extensive treatment is needed prior to discharge it into hydric systems because of its high biological and chemical oxygen demands. In this sense, whey is often disposed of by the cheapest methods, such as animal fed, spray irrigated onto land or treated as effluent (FOX *et al.*, 2017; PRAZERES; CARVALHO; RIVAS, 2012). When well treated, it is possible to develop different products with high added value from whey, but the technologies required to it are expensive and most of the small to medium industries do not have neither technical know-how, nor economic conditions to invest in them (DERELI *et al.*, 2019).

2. WHEY PROTEINS

Whey is a solution composed by approximately 94% water and 6% total solids, including 4.5% of lactose, 0.7% of minerals and 0.8% proteins. These proteins represent 20 – 25% of milk proteins and are considered of high nutritional value, because of its essential amino acids composition. β-lactoglobulin and α-lactalbumin represent 70% - 80% of whey proteins, which also present lower concentrations of bovine serum albumin, caseinomacropeptides, immunoglobulins and lactoferrin (KILARA; VAGHELA, 2018).

2.1 β-lactoglobulin

β-lactoglobulin (β-lg) is not present in human milk, but it is produced by the mammary glands of ruminants, pigs, horses, cats and dogs and accounts for 10% of total milk proteins and 50 – 60% of whey proteins. This 18.3 kDa protein is composed by 162 amino acids linked in a globular structure, stabilized by two disulfide bridges. The first one is located between Cys66-

Cys160 amino acid residues and the other one between Cys106-Cys119, while the Cys121 residue is a free thiol group that also contributes to the stability of the native protein (LE MAUX *et al.*, 2014).

From the isoelectric point – pH 5.2 – until pH 7.9, β-lg exists as a dimer, while when the pH is below 3.0 or above 8.0 it appears as a monomer. When the pH of the medium is between 3.1 and 5.1, the β-lg structures tend to associate and form octamers (KILARA; VAGHELA, 2018). β-lg presents more than 10 genetic variants, being the variants A and B the most common. They differ from each other in the positions 64 and 118, in which variant B presents a glycine and an alanine residue, while variant A has an aspartic acid and a valine residue in these positions, respectively (LE MAUX *et al.*, 2014). Primary and tertiary structures of β-lg are shown in Figure 1.

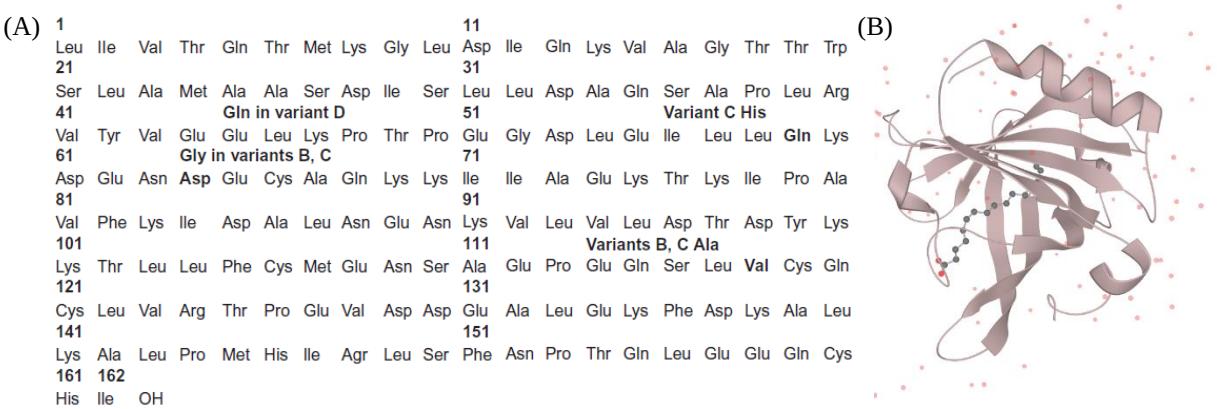

Figure 1. (A) Primary structure of bovine β-lactoglobulin A. The locations of the amino acid substitutions in the genetic variants are indicated in bold letters (Source: Kilara & Vaghela, 2018); (B) Tertiary structure of β-lactoglobulin as a monomer (Source: Uniprot database).

Its globular structure is associated with its resistance to acids and stomach proteases, while the low proline content and the presence of cysteine, cystine and methionine are related to the thermal instability and sensibility to alkaline (pH>8) conditions. The exposure of this protein to temperatures above 70°C leads to structural changes and consequent denaturation, with exposure of hydrophobic amino acid residues, leading to aggregation (ANTUNES, 2003). β-lg is known for its capacity to bind hydrophobic molecules, including fatty acids and vitamins, however, the biological functions of these complexes is not well established and may be related to the increase in the absorption of fatty acids, protection of sensitive ligands against oxidation

or other stresses, modification of the bioaccessibility of the ligands, and when interacting with vitamin A, it may regulate mammary glands (KILARA; VAGHELA, 2018; LE MAUX *et al.*, 2014).

Despite its high allergenic levels, mainly affecting children under 2 years old, β-lg is constantly associated with health benefits related to its bioactive peptides. (STANIC-VUCINIC *et al.*, 2012). These functional molecules may be released from the parent protein during food processing or gastrointestinal digestion and act as immunomodulators, anti-carcinogenic, opioid, antioxidant and anti-hypertensive molecules. Furthermore, its branched chain amino acids are related to protein synthesis and muscular recovery after physical exercising (O'KEEFFE; FITZGERALD, 2018; SHARMA, RANJAN; SHAH, 2010).

2.2 α-lactalbumin

α-lactalbumin (α-la), different from β-lg, is the most prevalent protein in human milk and accounts for approximately 3% of total bovine milk proteins, being the second most prevalent protein in whey. This protein of 123 amino acids organized in a globular structure has a molecular mass of 14.2 KDa and four disulfide bonds, as illustrated in Figure 2 (DELAVARI *et al.*, 2015).

(A)

1 **Arg in variant B**

Glu Gln Leu Thr Lys Csy Glu Val Phe **Gln** Glu Leu Lys Asp Leu Lys Gly Tyr Gly Gly
21 **31**
Val Ser Leu Pro Glu Trp Val Cys Thr Thr Phe His Thr Ser Gly Tyr Asp Thr Glu Ala
41 **51**
Ile Val Glu Asn Asn Gln Ser Thr Asp Tyr Gly Leu Phe Gln Ile Asn Asn Lys Ile Trp
61 **71**
Cys Lys Asn Asp Gln Asp Pro His Ser Ser Asn Ile Cys Asn Ile Ser Cys Asp Lys Thr
81 **91**
Leu Asn Asn Asp Leu Thr Asn Asn Ile Met Cys Var Lys Lys Ile Leu Asp Lys Val Gly
101 **111**
Ile Asn Tyr Trp Leu Ala His Lys Ala Leu Cys Ser Glu Lys Leu Asp Gln Trp Leu Cys
121 **123**
Glu Lys Leu OH

(B)

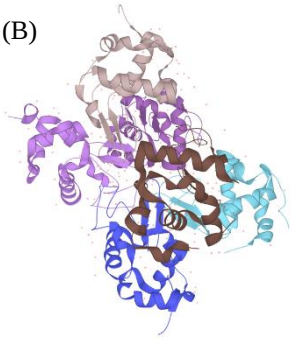

Figure 2. (A) Primary structure of bovine α-lactalbumin A. The location of the amino acid substitution in the genetic variant B is indicated in bold letters (Source: Kilara & Vaghela, 2018); (B) Tertiary structure of β-lactoglobulin as a monomer (Source: Uniprot database).

α-La is involved in the production of lactose in the mammary tissue changing the substrate specificity of the protease galactosyltransferase from N-acetylglucosamine to glucose, binding uridine diphosphate-galactose (UDP) to glucose, forming lactose and UDP in the Golgi

complex during pregnancy or lactation (FOX *et al.*, 2015). α-La is also able to bind hydrophobic ligands such as retinol and oleic acid, moreover, it uses calcium to form intramolecular bonds, which contributes to its molecular stability and thermal resistance (DELAVARI *et al.*, 2015).

α-La is constantly used in infant formulas, as this protein presents high essential amino acids concentration, contributing to the nutrition and growth of neonates and premature children. The presence of cysteine in its structure is related to the improvement of the immune system and wound healing, while the high tryptophan content contributes to a best sleep, humor and cognitive development of infants. Furthermore, it is often used as ingredient in food supplements, because of its high essential and branched-chain amino acids contents (STÃNCIUC; RÂPEANU, 2010).

2.3 Bovine serum albumin

Corresponding to approximately 5% of whey proteins, bovine serum albumin (BSA) presents a molecular mass of 66 KDa and a structure of 582 amino acids, 17 disulfide bonds and one sulfhydryl group in the 34^{th} residue. BSA is capable of biding to hydrophobic molecules, including free fatty acids and other lipids, enabling their transport in bloodstream and preventing oxidation. This protein is also a precursor of glutathione, a tripeptide with antioxidant capacity (JAHANBAN-ESFAHLAN; PANAHI-AZAR, 2016; KILARA; VAGHELA, 2018).

2.4 Immunoglobulins

Immunoglobulins (IGs) are globular proteins, among which three variants are present in whey, IG_A, IG_M, IG_G, the last one corresponding of approximately 80% of total IGs. These proteins present similar structures as monomers or polymers with four chains, two light (approximately 25 KDa) and two heavy molecules (50 – 70 KDa). IGs are not produced in mammary glands and need to be transported to milk, via vesicles or specific receptors (HARAGUCHI; DE ABREU; DE PAULA, 2006).

2.5 Lactoferrin

Lactoferrin is a transferrin with 80 kDa found in different body fluids. This protein is capable of biding iron, which is essential to its antimicrobial activity and accounts for its

antioxidant capacity, as well. Lactoferrin is not only part of the primary system of defense of mammalians against pathological microorganisms, but also modulates host's defense preventing overreactions (LEGRAND, 2016).

2.6 Caseinomacropeptides

Caseinomacropeptides are present exclusively in sweet whey, since they result from the enzymatic cleavage of κ-casein during the cheese-making process. This cleavage occurs between the 105 – 106 amino acid residues, separating the protein in two main fragments, the hydrophobic para-κ-casein (Glu1 – Phe105), which remains in the cheese and the caseinomacropeptide (Met106 – Val169), that dissolves in whey. This 64 amino acids polypeptide is associated to many biological and technological functions that are interesting for food and pharmaceutical industries (HALLÉN *et al.*, 2010; KASTBERG MØLLER *et al.*, 2012).

3. NUTRITIONAL PROPERTIES OF WHEY PROTEINS

According to Damodaran & Parkin (2018) proteins are the main functional and structural component of a cell, playing different roles through the body, as transporters, hormones, enzymes, among other functions essential for life. They are formed by the combination of different amino acids, linked by peptide bonds, which consists in the biochemical reaction of the amino-group of one amino-acid with the carboxyl-group of other amino acid, releasing a water molecule. Among the twenty existing amino acids, eight are not synthesized by the organism but are essential for its maintenance and must be regularly ingested throughout diet: phenylalanine, valine, tryptophan, threonine, lysine, leucine, isoleucine and methionine. All amino acids' structures are illustrated in Figure 3.

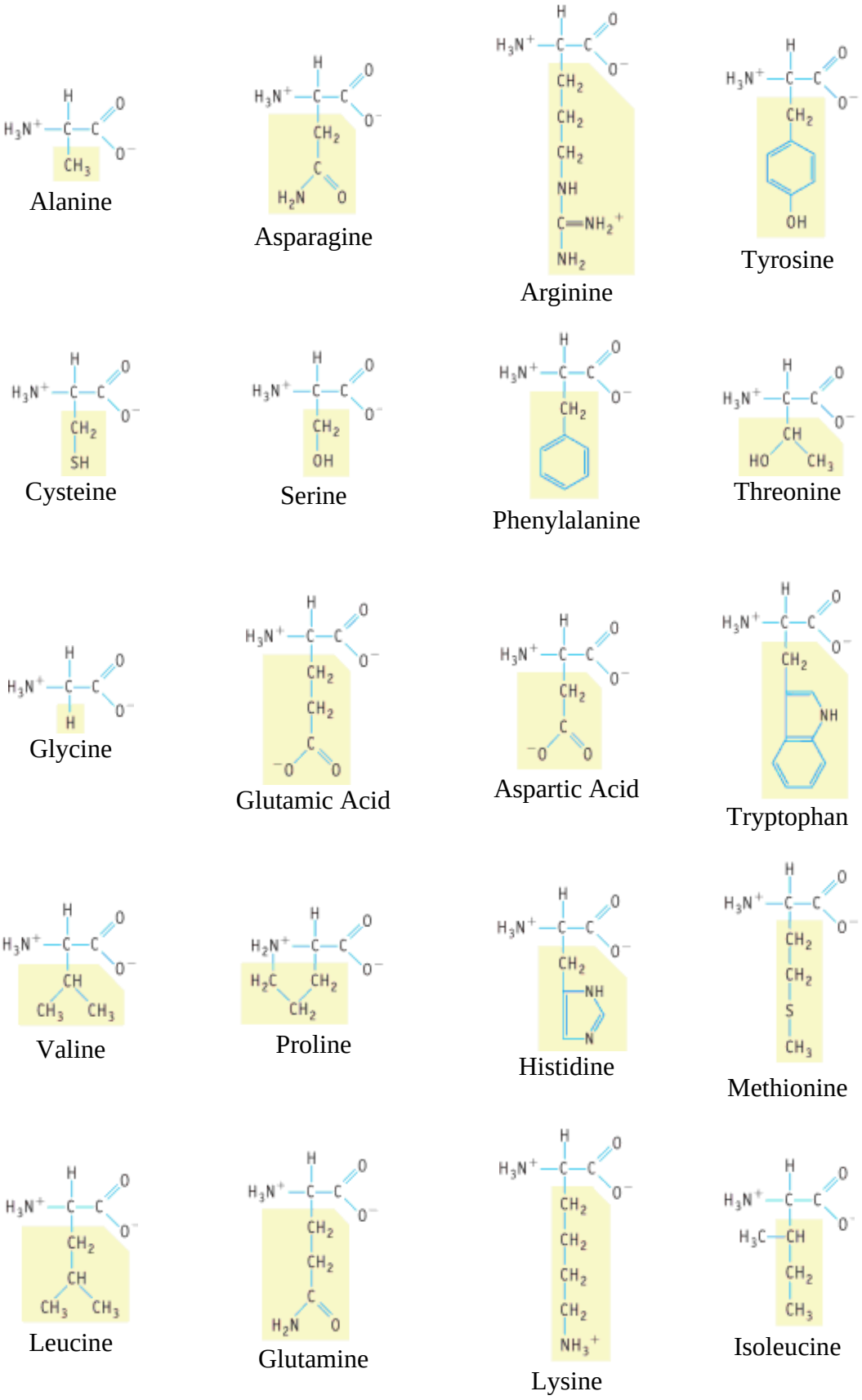

Figure 3. Chemical structure of amino acids. (Source: Kotz, Treichel, & Townsend, 2012).

According to the Report of a FAO Expert Consultation (FAO, 2013), the nutritional quality of a protein should be measured in DIAAS, in which: DIAAS % = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1g of the reference protein).

Whey proteins present all essential amino acids, while the whey protein concentrates above 80% and whey protein isolates exceed the recommendations for adults and can be considered of high nutritional value proteins, as shown in Table 2. These proteins are also of high digestibility and fast absorption, ideal for stress situations in which body needs fast protein reposition (DEVRIES; PHILLIPS, 2015).

Table 2. Comparison between the recommended dietary intake of essential amino acids for adults and the essential amino acids content in whey proteins concentrate (WPC 80%) and isolates (WPI). ptn = protein. Source: adapted from (FAO, 2013; USDEC, 2019).

Amino acid	**Dietary intake adults ($mg.g^{-1}$ ptn)**	**WPC 80% ($mg.g^{-1}$ ptn)**	**WPI ($mg.g^{-1}$ ptn)**
Isoleucine	30.0	48.0	59.0
Leucine	59.0	80.8	130.0
Lysine	45.0	78.4	91.5
Methionine + Cysteine	22.0	43.2	51.5
Phenylalanine +Tyrosine	38.0	47.2	54.5
Threonine	23.0	53.6	62.5
Tryptophan	6.0	12.0	15.0
Valine	39.0	44.6	53.5

Moreover, different bioactivities have been associated with whey proteins and peptides, including immunomodulatory, anti-microbial, anti-carcinogenic, antioxidant and anti-hypertensive (DULLIUS; GOETTERT; DE SOUZA, 2018; NONGONIERMA; FITZGERALD, 2015). The last two will be further discussed in the present document.

4. BIOACTIVE PEPTIDES

Peptides are biomolecules of 2 – 100 amino acid residues linked by peptide bonds, which may be described as a condensation reaction between the α-amino group of one amino

acid with the α-carboxyl group of another amino acid with the loss of one water molecule, as shown in Figure 4 (DAMODARAN; PARKIN, 2018).

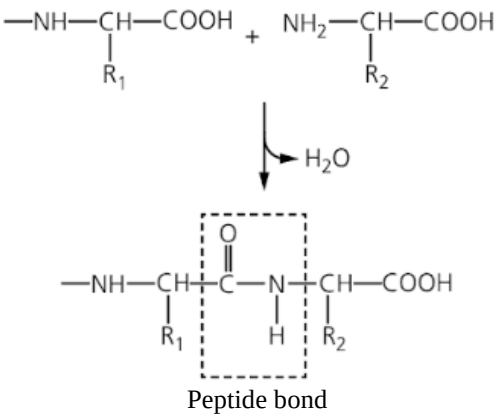

Figure 4. Formation of a peptide bond (Source: Damodaran & Parkin, 2018).

Bioactive peptides are protein fragments that cause positive impact in different body functions and may contribute for health improvement (MANN *et al.*, 2019). Whey proteins present several peptides encrypted within their native structures which become bioactive after being released during gastrointestinal digestion or food processing, and may affect different body systems, including cardiovascular, digestive, endocrine, immune, and nervous systems (DULLIUS; GOETTERT; DE SOUZA, 2018; MANN *et al.*, 2019).

4.1 Antihypertensive peptides

The antihypertensive potential is the most studied class of whey peptides, which can be attributed to the high prevalence of this chronic degenerative disease that affects one fourth of global population and is the main factor for cardiovascular disease, which accounts for 30% of all death causes (O'KEEFFE; FITZGERALD, 2018; TAVARES, TG; MALCATA, 2013). Over the past years, people are becoming more health conscious and interested in replacing allopathic medicines for functional foods, in this sense, food industries have been developing new products aiming to supply this growing demand (BELTRÁN-BARRIENTOS *et al.*, 2016). Some commercial products containing antihypertensive milk-derived peptides were evaluated in clinical studies and are listed in Table 3.

Table 3. Commercial products containing milk-derived bioactive peptides evaluated in clinical studies concerning their effects in systolic blood pressure. High-normal (NH) blood pressure; (MH) mild hypertension. (Adapted from Hernández-Ledesma, Del Mar Contreras, & Recio (2011) and Pins & Kennan (2002).

Product	**Dose**	**Duration of treatment (weeks)**	**Effect in systolic blood pressure (SBP) (mm Hg)**	**Reference**
Calpis/Ameal S® (fermented milk by *L. helveticus* and *S. cerevisiae*)	160 g/day (1.2 mg IPP+2.0 mg VPP)	4	−5.2 mm Hg	(MIZUSHIMA *et al.*, 2004)
Tablets containing powdered fermented milk by L. helveticus CM4	6 tablets/day (total 12 g, 4.7 mg IPP + 8.3 mg VPP)	4	−3.2 mm Hg (NH) * −11.2 mm Hg (MH) **	(AIHARA *et al.*, 2005)
Evolus® (fermented milk by *L. helveticus LBK-16H*)	150 mL/day (2.25 mg IPP + 3 mg VPP)	21	−6.7 mm Hg vs. placebo	(SEPPO *et al.*, 2003)
Milk (enriched in a WPC) fermented by *L. casei* TCM0409 and *S. thermophilus* TCM1543	2×200 mL/day	8	−7 mmHg vs. placebo	(KAWASE *et al.*, 2000)
BioZate® (whey protein hydrolysate)	20 g/day	6	-11.0 mm Hg	PINS & KEENAN, 2002.

Blood pressure (BP) regulation is very complex and involves behavioral and metabolic processes, including physical exercises, dietary intake, administration of diuretics, angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists (TAVARES, TG; MALCATA, 2013). Among food-derived peptides involved in the control of BP, the ACE-inhibitory ones are the most studied.

ACE is involved in two of the main systems of BP regulation, the renin-angiotensin (RAS) and the kinin-nitric oxide (KNOS). In the RAS, the renin produced by the kidneys

cleaves the angiotensinogen bond in the N-terminal position, generating the decapeptide angiotensin I (AG-I). Then, ACE removes two C-terminal amino acids (HL) from AG-I, forming angiotensin II (AG-II). AG-II binds to AT_1 and AT_2 located in peripheral tissues and in the brain, generating vasoconstriction Figure 5. Besides causing vasoconstriction, once activated by AG-II, AT_1 receptors also stimulates aldosterone synthesis and release, leading to sodium retention and consequent blood pressure increasing. Bioactive peptides are able to inhibit AG-I conversion in AG-II by ACE, avoiding these vasoconstrictor pathways. This mechanism, however, is not completely efficient, as in some cells AG-I can be converted in AG-II by the enzyme chymase (NORRIS; J., 2013; O'KEEFFE; FITZGERALD, 2018; TAVARES, TG; MALCATA, 2013).

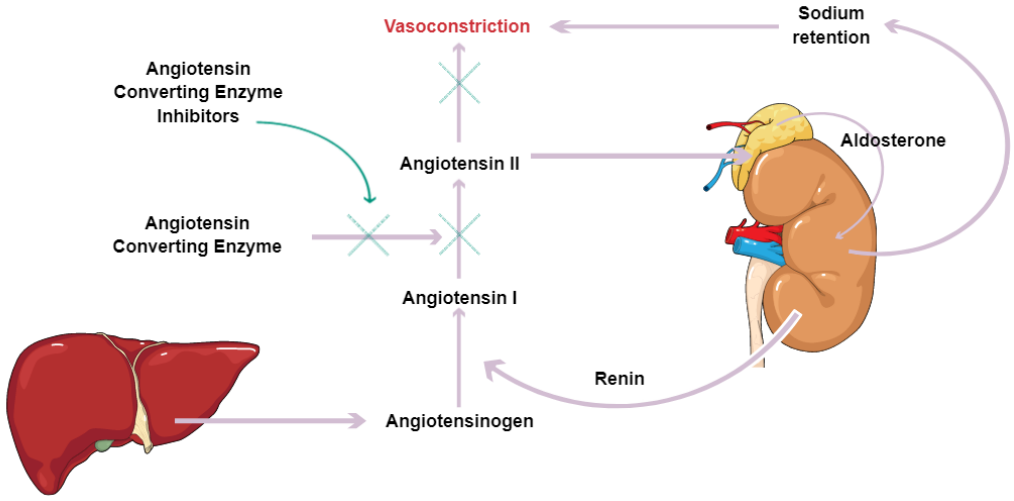

Figure 5. Blood pressure regulation through angiotensin-I converting enzyme inhibitors in the renin-angiotensin system.

In the KNOS, kallikrein synthesizes kallidin from kininogen and its further action on kallidin generates bradykinin and other vasoactive peptides. Bradykinin binds to β-receptors increasing intracellular calcium (Ca^{+2}) concentration, this phenomenon stimulates Ca^{2+}/calmodulin-dependent endothelial nitric-oxide synthase (eNOS) to convert L-arginine in the vasodilator nitric-oxide (NO) and citrulline. NO can also react with homocysteine, forming another vasodilator, the S-nitroso-homocysteine. In this system, ACE hydrolyses bradykinin, preventing NO production (NORRIS; J., 2013; UDENIGWE; MOHAN, 2014). There are some

antihypertensive commercial drugs that act exclusively inhibiting ACE, including captopril and enalapril.

Although the regulatory mechanism of BP through ACE inhibition is the best known, there are other pathways in which peptides may act, as shown in Figure 6. Renin inhibition is an important mechanism in BP regulation, since angiotensinogen is the only known substrate for this renal enzyme, which will not convert this prohormone in AG-I and, consequently, in the vasoconstrictor AG-II (NORRIS; FITZGERALD, 2013a). However, inhibiting renin may possibly not prevent this cascade of events, since prorenin exhibits renin activity if it binds to its cell receptors. In this sense the blockage of the AG-II cell receptors suppresses AG-II induced vasoconstriction. Losartan was the first and is the best known AT_1 receptor suppressor available in the market (UDENIGWE; MOHAN, 2014).

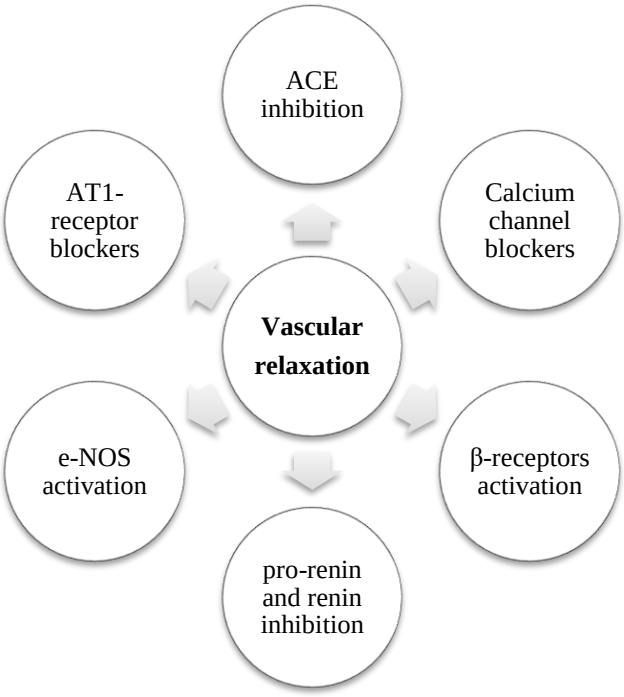

Figure 6. Bioactive peptides pathways involved in blood pressure control.

Food-derived peptides, including the β-lactoglobulin fragment ALPMHIR, may also control BP through endothelin-converting-enzyme (ECE) inhibition, in the endothelin (ET) system. ECE cleaves big endothelin forming ET-1. ET-1 binds to the cell receptors ET_a and ET_b, which mediate smooth muscle contractions. The peptides may also interact with opioid

receptors, which regulate different processes in the body, including blood circulation (NORRIS; FITZGERALD, 2013a; TAVARES, TG; MALCATA, 2013). Furthermore, bioactive peptides may also interact with voltage-dependent calcium channels in the cardiac muscle and blood vessels, reducing Ca^{+2} intracellular influx and preventing vasoconstriction (UDENIGWE; MOHAN, 2014).

4.2 Antioxidant whey peptides

The generation of free radicals from unsaturated fatty acids in the presence of oxygen is named lipid oxidation. This deterioration process affects sensory and nutritional quality of food products and is influenced by different factors that will determine its shelf-life, including fatty acids composition, presence of antioxidant substances, temperature, surface area in contact with oxygen, among others. (SIKORSKI; KOŁAKOWSKA, 2010).

Propyl gallate (PG), tertiary butylhydroxyquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisol (BHA) are synthetic antioxidants commonly used as food preservatives postponing lipid oxidation. These synthetic substances present potential health risks and are being replaced by natural food-derived preserving substances, such as tocopherol, vitamin C and phenolic compounds (PENG; XIONG; KONG, 2009). Penã-Ramos & Xiong (2003) showed that whey hydrolysates may also be used as food preservatives, when they incorporated it in cooked pork patties and successfully suppressed lipid oxidation. Mann et al. (2015) also found a great antioxidant potential in a flavored milk containing whey hydrolysate. According to Kumar, Chauhan, Shinde, Subramanian & Nadanasabapathi (2018), whey is a rich source of antioxidant peptides, which are presented as low molecular weight protein fractions and peptides between 500 – 3000 Da.

Lipid oxidation also occur *in vivo*, as the human body and other aerobic organisms contain reactive oxygen species (ROS), products of different metabolic processes that are essential for cells' survivor (SIKORSKI; KOŁAKOWSKA, 2010). Antioxidant molecules are also present in the cells, producing a homeostatic balance. The alteration of this balance for excess of ROS or low levels of antioxidants is called oxidative stress, in which cells are exposed to high levels of free radicals. When in excess ROS destroy protective enzymes as superoxide dismutase, catalase and peroxidase causing cellular damage by oxidizing membrane lipids, cellular proteins, DNA and enzymes, leading to apoptosis. The oxidative stress is considered a

significant causative factor for different pathologies like cardiovascular, cancer, diabetes and inflammatory diseases (CORROCHANO *et al.*, 2018; MANN *et al.*, 2019).

The ingestion of dietary antioxidants, such as ascorbic acid, α-tocopherol, polyphenols, and carotenoids may help the body to prevent and stop oxidative stress. The antioxidant potential of milk-derived peptides had also been studied and involve lipid peroxidation and scavenging of free radicals (CORROCHANO *et al.*, 2018; SHARMA, SHRIKANT; SINGH; RANA, 2011). In whey proteins, the antioxidant activity is associated to the ROS scavenging via tyrosine and cysteine amino acid residues. Although different studies demonstrate the *in vitro* antioxidant potential of whey proteins and peptides (ATHIRA *et al.*, 2015; DRYÁKOVÁ *et al.*, 2010; MANN *et al.*, 2015), evidences are lacking about their activity *in vivo*. In 2014, Lollo et al. showed the effects of a 12-week intervention with whey protein hydrolysate (WPH) in the performance and body composition of an elite soccer players' team. The WPH supplementation led to a decrease in creatine kinase and lactate dehydrogenase, that are oxidative stress and tissue damage markers.

5. TECHNOLOGICAL PROPERTIES OF WHEY PROTEINS

Besides their nutritional benefits, whey proteins present interesting technological properties, including high solubility, gelling, foaming and emulsifying capacities, which allow their use in a large variety of food products, such as bakery, confectionary, meat, beverage and dairy products.

Solubility is a technological property of primary importance, since it influences in the formation of emulsions, foams and gels. It is a property related to surface interactions, which may be hydrophobic (protein-protein) or hydrophilic (protein-solvent). In food systems interactions are generally between protein and water, being classified as hydrophilic (PELEGRINE; GASPARETTO, 2005).

Pelegrine & Gasparetto (2005) also reported that proteins are generally soluble in acid or alkaline pH, which may be explained by the excess of charges with the same sign, causing repulsion and leading to a high water-protein interaction. In this sense, at the isoelectric point, proteins present lowest solubility, as there is no electrostatic force and no protein-water interaction, favoring aggregation and precipitation. Whey proteins, however, are highly soluble

in a large pH range (from 2 to 10), especially in acidic pH (BATISTA; CAMPOS; SILVESTRE, 2018; ISMAIL; GU, 2010; SOLAK; AKIN, 2012).

In food systems which contain hydrophobic and hydrophilic molecules, whey proteins also play a role reducing interfacial tension, promoting stabilization by surface-active agents and preventing creaming, coalescence, flocculation and oiling off (KUMAR *et al.*, 2018; SOLAK; AKIN, 2012). According to Sinha, Radha, Prakash, & Kaul (2007), heat treatments and enzymatic hydrolysis modify proteins' structures and may reduce their emulsifying capacity.

Foaming capacity and foaming stability are properties related to the reduction of the interfacial tension between air and water by proteins. Whey proteins are able to form and stabilize air bubbles in a liquid (SOLAK; AKIN, 2012). The enzymatic hydrolysis of whey proteins can increase or decrease its foaming capacity, while mild hydrolysis improves this property by the reduction in peptides size, promoting rapid adsorption in the air-water interface. Extensive hydrolysis generates many small peptides, slowing the increase in the interfacial viscosity and reducing foam expansion (IPSEN *et al.*, 2001; ISMAIL; GU, 2010).

6 WHEY-BASED PRODUCTS

As aforementioned, whey is a very diluted nutritive solution which require different treatments to generate high added value products, as shown in Figure 7. The simplest one is the whey powder, obtained after whey drying. Whey powder is often used as ingredient in dairy products, bakery, ice cream, meat products, among others, since it presents interesting technological properties and it is used to emulsify, enhance texture, color and flavor (KRÓLCZYK *et al.*, 2016; KUMAR *et al.*, 2018).

In natura whey can be ultrafiltered to reduce lactose content, generating a reduced lactose whey powder and some whey concentrates. This lactose is also a byproduct with different functionalities, being used as a food ingredient in the ethanol production, as a substrate for yeasts and to more noble uses, being an excipient in the tableting process in the pharmaceutical industry, for example (TOMMASO *et al.*, 2012; ZOPPELLARI; BARDI, 2013).

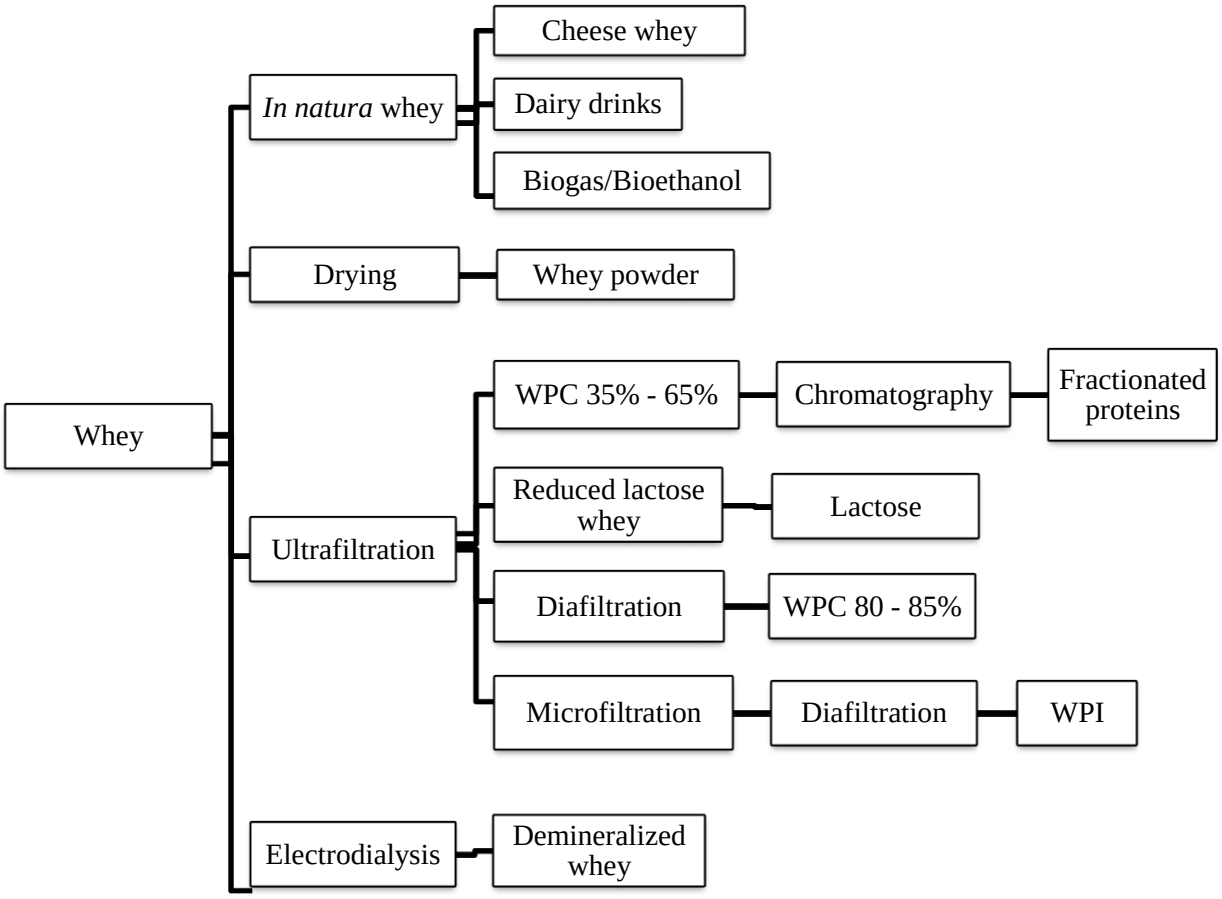

Figure 7. Diagram representing processes and whey-based products.

Whey concentrates present from 25% to 90% of proteins and the methods applied will depend on the product desired. The most popular ones are the whey protein concentrates 35%, 55% and 80%. Proteins' concentration may occur by evaporation process, osmose or membrane ultrafiltration. To the concentrates above 80%, a diafiltration step is included, consisting on water addition to best remove lactose and minerals, generating a pure and functional concentrate (ANTUNES, 2003; KILARA; VAGHELA, 2018). Whey protein isolates (WPI) present above 90% of proteins and do not contain or present very low concentrations of lipids and lactose. To obtain a WPI three different processes are necessary, ultrafiltration, microfiltration and diafiltration (SOLAK; AKIN, 2012).

Whey hydrolysates are a complex mixture of free amino acids and peptides of different size chains. These products are obtained from the cleavage of peptide bonds catalyzed by acids,

alkali or enzymes. The chemical hydrolysis processes are severe, extensive and unspecific, which may result in the formation of toxic compounds and racemic mixtures of amino acids (MANNINEN, 2009; WROLSTAD *et al.*, 2005). In this sense, the use of enzymes to obtain whey hydrolysates is becoming popular, as the enzymatic treatment contribute to enhance physical, chemical, functional, organoleptic and nutritional properties of the protein fragments. Furthermore, it is possible to control the degree of hydrolysis, there is low formation of salts and sub-products, besides the selectivity of the cleavage sites (BIASUTTI *et al.*, 2008).

One disadvantage of the enzymatic treatments states in the bitter taste of the hydrophobic and low molecular mass di- and tripeptides, which needs to be masked before using as a food ingredient (LI-CHAN, 2015). Moreover, as some whey proteins are known for its high allergenicity, low degree of hydrolysis processes may expose these antigenic epitopes, increasing their immunoreactivity. Nevertheless, as the degree of hydrolysis is augmented, the antigenic properties are reduced and peptides in the range of 800 - 1500 Da are considered hypoallergenic (PASUPULETI; DEMAIN, 2010).

In this way, the popularity of whey hydrolysates increases, being constantly used in infant formulas as 0.5% - 10% of the neonates develop some degree of food allergy, mainly related to milk proteins (PASUPULETI; DEMAIN, 2010; VANDENPLAS, 2017). The consumption of whey hydrolysates is also indicated to people with nutritional and/or physiological necessities which may not be filled by conventional nutrition, or which present some specific disease, such as Crohn, ulcerative colitis, syndrome of the small intestine, pancreatitis, among other, being necessary the administration of hydrolyzed protein to facilitate the absorption (HERNÁNDEZ-LEDESMA *et al.*, 2014).

The ingestion of whey concentrates and isolates by athletes is more common than the hydrolysates the hydrolysates, but the last ones are also related to the anabolism of the skeletal muscles in men and women (BROWN; STEVENSON; HOWATSON, 2018; MORO *et al.*, 2019). The consumption of food products which contain whey hydrolysate in the formula, such as different cheeses, yogurts and other dairy products may also improve health because of the biological activities played by its peptides.

Chapter 1

Whey hydrolysate-based ingredient with dual functionality: from production to consumer's evaluation

Luisa Ozorio[a], Luciano P. Silva[b], Maura V. Prates[b], Carlos Bloch Jr.[b]; Cristina Y. Takeiti[d]; Danillo Macêdo Gomes[c], José Eduardo da Silva-Santos[c], Rosires Deliza [d]; Ana Iraidy S. Brígida[d], Caroline Mellinger-Silva[d]*, Lourdes M.C. Cabral[d]

[*]Corresponding author.

Avenida das Américas, 29.501 Guaratiba, Rio de Janeiro, RJ, Brazil.

Tel.: +55 21 3622 9622; Fax: +55 21 3622 9713.

e-mail: caroline.mellinger@embrapa.br

[a] Chemistry Institute, Federal University of Rio de Janeiro, Av. Athos da Silveira Ramos, 149 - Cidade Universitária, Rio de Janeiro - RJ, 21044-020, Brazil.

[b] Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, PqEB, Av. W5 Norte, Brasília – DF, 70770-917, Brazil.

[c] Laboratory of Cardiovascular Biology, Department of Pharmacology, Federal University of Santa Catarina, Campus Reitor João David Ferreira Lima, s/n - Trindade, Florianópolis - SC, 88040-900, Brazil.

[d] Embrapa Agroindústria de Alimentos, Avenida das Américas, 29501 - Rio de Janeiro - RJ, 23020-470, Brazil.

ABSTRACT

The aim of the present study concerns the development, characterization and sensory evaluation of a dual-functional whey hydrolysate. Four concentrations of commercial pepsin (0.48 %, 0.95 %, 1.43 %, 1.91 % *w/w*) were evaluated. The hydrolyses curves and the Reversed-Phase High Performance Liquid Chromatography analyses showed a direct relationship between enzyme concentration and degree of hydrolysis. Through mass spectrometry 21 peptides were identified and 5 of them have never been described in the literature before. The hydrolysate produced (PC3) induced a vascular relaxation of 65.02 % in phenylephrine-contracted rat aortic rings. PC3 powder presented a homogeneous aspect with a mean particle size of 86.39 µm, high water solubility (> 92 %) in a wide pH range (1-12) and an increase of 33 % in oil absorption capacity, when compared to the unhydrolyzed product. Sensory analysis showed a high acceptance (7.6 in a 9-point hedonic scale) of the hydrolysate among 100 consumers. The results brought the possibility of developing a whey hydrolysate with high vasorelaxant activity, great technological properties and sensory appeal, as an interesting dual-functional ingredient to be incorporated into food products.

keywords: whey protein; enzymatic hydrolysis; pepsin; functionality; peptides; hypertension; sensory evaluation; technological properties.

1. INTRODUCTION

Whey is a byproduct of dairy industry well known for its technological and nutritional properties. In 2014 global production of whey amounted 240 million metric tons (MORDOR INTELLIGENCE, 2017b) and investments in technologies for the concentration and purification of proteins provide more diversified whey protein utilities, going beyond the consolidated markets of food supplements and food ingredients, and reaching more valuable markets such as nutraceutical and pharmaceutical industries (ROHLFES *et al.*, 2011; TAVARES, TG; MALCATA, 2013).

Among nutritional properties of whey, its protein content has been extensively studied due to the bioactive properties of its peptides, which are able to cross the intestinal barrier and modulate responses in nervous, immunological, gastrointestinal and cardiovascular systems (LE MAUX *et al.*, 2014; MADUREIRA *et al.*, 2010; TAVARES, TG; MALCATA, 2013). Among cardiovascular diseases, hypertension is the most prevalent affecting one in each five adults worldwide and accounting for 9.4 million deaths every year (CELERMAJER *et al.*, 2012; WHO, 2015). Whey peptides are able to lower blood pressure through different mechanisms, including the well-known inhibition of angiotensin-converting enzyme (ACE), as well as rennin and pro-rennin enzymes, blocking calcium channels and AT1 receptors and stimulation of nitric oxide production, among others (MELLINGER-SILVA *et al.*, 2015; NORRIS; FITZGERALD, 2013b; UDENIGWE; MOHAN, 2014).

As food ingredients, whey-based products are also of great interest for food industries because of its technological properties, including oil and water holding and emulsifying capacity, foam capacity and solubility (ADJONU *et al.*, 2014), enabling its application into different products, such as bakery, ice cream, confectionary, among others. Beyond the desirable properties for food industries, the use of whey concentrates, isolates and hydrolysates as food ingredients may please health-conscious consumers, since it also improves the nutritional and biofunctional value of these products, even though the bitter taste of whey peptides may be a limiting factor for its application (LACROIX *et al.*, 2016).

In this manner, the present study was designed to evaluate the process of obtaining a whey hydrolysate using a commercial pepsin system (PPC). Biological effects on vascular tone, technological properties and consumers' acceptance were accessed aiming its future incorporation as a functional ingredient to be added to food products. This kind of complete study, englobing the process of obtainment, functional characterization, biological assays and sensory evaluation application is a novelty and it is important for the future commercialization and dissemination of this type of biofunctional product.

2. MATERIALS AND METHODS

2.1 MATERIALS

Bovine whey protein concentrate composed by 88 % (WPC88) of protein was gently donated by Alibra Ingredientes Ltda (Campinas, SP, Brazil) and was used as substrate together with a commercial pepsin (E.C. 3.4.23.1, Bela Vista Produtos Enzimáticos Ind. e Com. Ltda, Bela Vista, SC, Brazil) from porcine gastric mucosa (enzymatic activity of 0.28 $\mu U.mL^{-1}$ - Sigma-Aldrich, 2017). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany). A creamy commercial dairy dessert, white chocolate flavor available in Brazil was used to the sensory analysis.

2.2 ENZYMATIC HYDROLYSIS

The WPC88 was suspended in distilled water at a concentration of 1.25% (w/v), the pH was adjusted to 2 (1M HCl) and the temperature was maintained at 37°C during all the experiment. Different pepsin concentrations (0.48 %, 0.95 %, 1.43 % and 1.91 % *w/w*) were evaluated and the hydrolysis processes occurred for 5 hours, then pepsin was thermally inactivated (80 °C/ 5 min). Aliquots were collected (1 min, 1 h, 2 h, 3 h, 4 h and 5 h) along the period (MELLINGER-SILVA *et al.*, 2015). Soluble protein content was measured according to Bradford (1976). Results were submitted to analysis of Student's t-test with a significance level of 5 % ($p < 0.05$). Samples were then spray dried at 0.52 MPa, inlet temperature: 170°C; outlet temperature: 85°C (Buchi Mini Spray Dryer 190, Büchi Labortechnik, Flawil, Switzerland) and kept frozen until further analysis.

2.3 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

Peptides obtained from whey protein hydrolysate were analyzed by RP-HPLC. An analytical HPLC unit from Jasco (Jasco Corporation, Japan) with a Hypersil BDS C18 column (100 x 4.6 mm, particle size 2.4 µm, Thermo Scientific, USA), was used and the analysis was carried out according to Mellinger-Silva et. al. (2015). Briefly, 20 µL of the samples were injected and chromatographic runs were of 40 min with a flow rate of 1.0 $mL.min^{-1}$ at room temperature (approximately 20°C). 0.1 % trifluoroacetic acid (TFA) in ultrapure water (v/v) and 0.1 % TFA in acetonitrile (ACN) (v/v) were used as solvents A and B, respectively. Hydrolysates were eluted as follows: 0-2 min, 5% B; 2-15 min, 5-20% B; 15-20 min, 20-30% B; 20-25 min, 30-40% B; 25-28 min, 40-50% B; 28-32 min, 50-60% B; 32-34 min, 60-70% B; 34-36 min, 70-80% B; 36-38 min, 80-5% B; 38-40 min, 5% B. Peptides were detected at 216 nm.

2.4 MASS SPECTROMETRY

Peptides were identified through matrix assisted laser desorption ionization associated to two time-of-flight mass analyzers *in tandem* (MALDI-TOF/TOF-MS) using a mass spectrometer UtraFlex III (Bruker Daltonics, Germany). Dried samples were resuspended in ultrapure water and added to α-cinamic-4-hidroxicinamic acid matrix. After external calibration, peptides' monoisotopic masses were obtained in a positive reflector mode to the detection range of m/z 700-4,000. The MS/MS spectra were obtained through LIFT™ operation mode. Peptide primary structures were manually obtained and *de novo* interpretations of the structures were compared to Swiss-Prot, Uniprot, Protein Blast e Protein Prospector (MS-Digest) databases using "bovine whey" data.

2.5 VASCULAR RELAXATION

Cumulative concentrations of the hydrolyzed fraction PC3 (1, 3, 5 and 10 $mg.mL^{-1}$) were applied in rat aortic ring preparations (n=6), as described by Mellinger-Silva et al. (2015). The results were expressed as the mean ± SEM of the maximal relaxation induced by PC3. Student's t-test for independent samples was used (GraphPad Software, La Jolla, CA, USA). A

p value less than 0.05 ($p<0.05$) was considered statistically significant. The experiments were approved by of our local Institutional Animal Care and Use Committee (authorization number 5371190815).

2.6 PHYSICAL AND TECHNOLOGICAL PROPERTIES

2.6.1 Moisture content

Approximately 2 g of the samples were kiln-dried for 20 hours (until the samples get a constant weight for 3 consecutive weightings), at 105 °C, then cooled into desiccators and weighed (AACC, 2000). The result was expressed as average of humidity percentage in the samples, according to the following equation 1:

$$RH\ \% = (100 \times N)/W \quad (1)$$

where RH % is the relative humidity expressed in percentage; N is the mass (g) of sample lost; W is the weight (g) of the sample before being dried.

2.6.2 Absolute Density

The absolute density of the powder was considered as the total volume excluding the volume of the open and closed pores. The absolute density was measured according to Webb (2001) by using an automatic gas pycnometer (AccuPyc 1330, Micromeritics, USA).

2.6.3 Particle Size Distribution

The particle size distribution analysis was performed according to AACC (1999). A Microtrac S3500 laser diffraction particle size analyzer (Microtrac Inc., North Largo, FL, USA) coupled with a Microtrac Flex software was used. The relative refractive index value of 1.53 was used for the hydrolysate sample. Isopropyl alcohol was used as dispersing agent and the sample was added under stirring until reaching laser obscuration of 7 %. The volume weighed mean ($d_{4,3}$) of the particle size distribution was taken as the mean particle size of the hydrolysate.

2.6.4 Solubility

Samples were dispersed in ultrapure water (1% w/v) with pH adjusted to 1-12 with NaOH or HCl solutions. The suspensions were centrifuged at 9677g for 15 min and the supernatant was diluted in 50mM EDTA, 8M urea buffer at pH10 (1:10). Solubility was obtained from the absorbance ratio (280 nm, AJX-3002PC Spectrophotometer, Micronal, Brazil) of the supernatant and the suspension before centrifugation (JAMBRAK *et al.*, 2008).

2.6.5 Foam Capacity

The foam capacity of the sample was analyzed through foam expansion (FE) and stability (FS) (KAEWRUANG; BENJAKUL; PRODPRAN, 2013). Samples were dissolved in ultrapure water (0.5 % w/v) and homogenized at 2554 g for 1 min at room temperature (~20°C) in IKA® Ultra-Turrax T25 digital homogenizer (IKA Labortechnik, Germany). The volumes of the solutions were measured after 0, 5, 10, 30 and 60 min by the use of a digital pachymeter (200 mm, IP67, PD202, Vonder, PR, Brazil).

FE and FS were calculated according to the following equations (2) and (3):

$$FE\ (\%) = (V_T/V_0) \times 100 \quad (2)$$

$$FS\ (\%) = (V_t/V_0) \times 100 \quad (3)$$

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for different times (5, 10, 30 and 60 min).

2.6.6 Oil-Holding Capacity

Samples (0.13 mg) were weighed into pre-weighed microtubes following the addition of 1.3 mL of soybean oil. The solutions were dispersed by the use of a vortex mixer for 30 s. Samples were allowed to stand for 30 min at room temperature (20°C) , then centrifuged at

9677 g for 30 min. The oil released on centrifugation was drained and the oil absorption capacity was expressed as grams of oil per gram of sample (WANI *et al.*, 2015).

2.6.7 Emulsifying Properties

Soybean oil (10 mL) and 30 mL of 0.2 % PC3 solution (w/v) (pH 3, 5 and 7) were mechanically homogenized at 1422 g by the use of a IKA® Ultra-Turrax T25 digital homogenizer (IKA Labortechnik, Germany) for 1 min. 50 µL of the bottom of the emulsion were added to 5 mL of 0.1 % sodium dodecyl sulphate (SDS) solution (w/v) at 0 and 10 min after homogenization. Absorbances were measured at 500 nm (AJX-3002PC Spectrophotometer, Micronal, Brazil) immediately (A_0) and 10 min (A_{10}) after emulsion formation. Emulsifying activity index (EAI) and the emulsion stability index (ESI) were calculated according to the following equations (4) (5):

$$EAI\ (m^2/g) = 2 \times 2.303 \times A_0 \times DF / c \times \varphi \times \theta \times 10{,}000 \quad (4)$$

$$ESI\ (min) = A_0 / (A_0 - A_{10} \times 10) \quad (5)$$

Where DF is the dilution factor (100), c is the initial concentration of protein solution ($g.mL^{-1}$), φ is the optical path (10), θ is the oil volume fraction of the emulsion (0.25), A_0 and A_{10} are the absorbances of the emulsion at 0 and 10 min, respectively (WANI *et al.*, 2015).

2.7 SENSORY ANALYSIS

Spray-dried hydrolysate was added to a commercial dairy dessert. The control dessert (D1) and the dessert added with peptides PC3 (2 % w/w) (D2) were offered (8g each) to 100 participants at 8- 10 °C, following a balanced order of presentation. Subjects were separated in two groups: group 1 (G1), the first 50 consumers – individuals were not informed of the peptides addition in one of the desserts; group 2 (G2), the remaining 50 participants – they were informed of the addition. The information was presented as a leaflet and contained the benefits of the peptides. The leaflet was specially prepared for the study and the purpose was to investigate whether the provision of information about the functional appeal of the product

would influence consumers' opinions. All participants evaluated the acceptance and purchase intention of the two samples. For global acceptance a hedonic scale of 9 points was used, ranging from 1 (extremely dislike) to 9 (extremely like); while for intention to purchase the scale ranged from 1 (certainly not buy) to 3 (certainly buy) (MEILGAARD; CIVILLE; CARR, 1999). Results were submitted to analysis of variance (ANOVA) by the use of LSTAT software (Addinsoft, 2014). A p value less than 0.05 ($p<0.05$) was considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 COMMERCIAL PEPTIC WHEY PROTEIN HYDROLYSATE PROFILES

According to the spectrophotometric analyses a dose-dependent relation between enzyme and protein concentrations was observed and the treatment using 1.91 % of PPC (w/w) achieved a decrease of 86 % in soluble protein content after 5h of hydrolysis (Fig. A_1 - supplementary material), according to Bradford (1976) method.

The RP-HPLC analyses corroborated with the spectrophotometric data (Fig. 1). Treatments using 0.48 % or 0.95 % of PPC showed less varied peptide profiles, with only a partial α-lactalbumin (α-La) hydrolysis. The 1.91 % (w/w) - PPC treatment showed that α-La was intensely hydrolyzed in less than 2 h of reaction, while β-lactoglobulin (β-Lg) remained mostly resistant. An intense protease activity was observed in the first hours of hydrolysis, generating a rich molecular profile. From 3 h to 5 h the peptide profiles seemed to stabilize and no significant changes were found. The susceptibility of α-La to pepsin's action is related to its structure and to its instability in acid pH. On the other hand, the resistance of β-Lg to the action of pepsin can be explained by its stable globular configuration which prevents the access of pepsin to hydrophobic residues (NIK; WRIGHT; CORREDIG, 2010).

As the most favorable hydrolysis condition was achieved by the use of 1.91 % (w/w) of PPC for 3 h, the following analyses were performed only in this condition, which was named PC3.

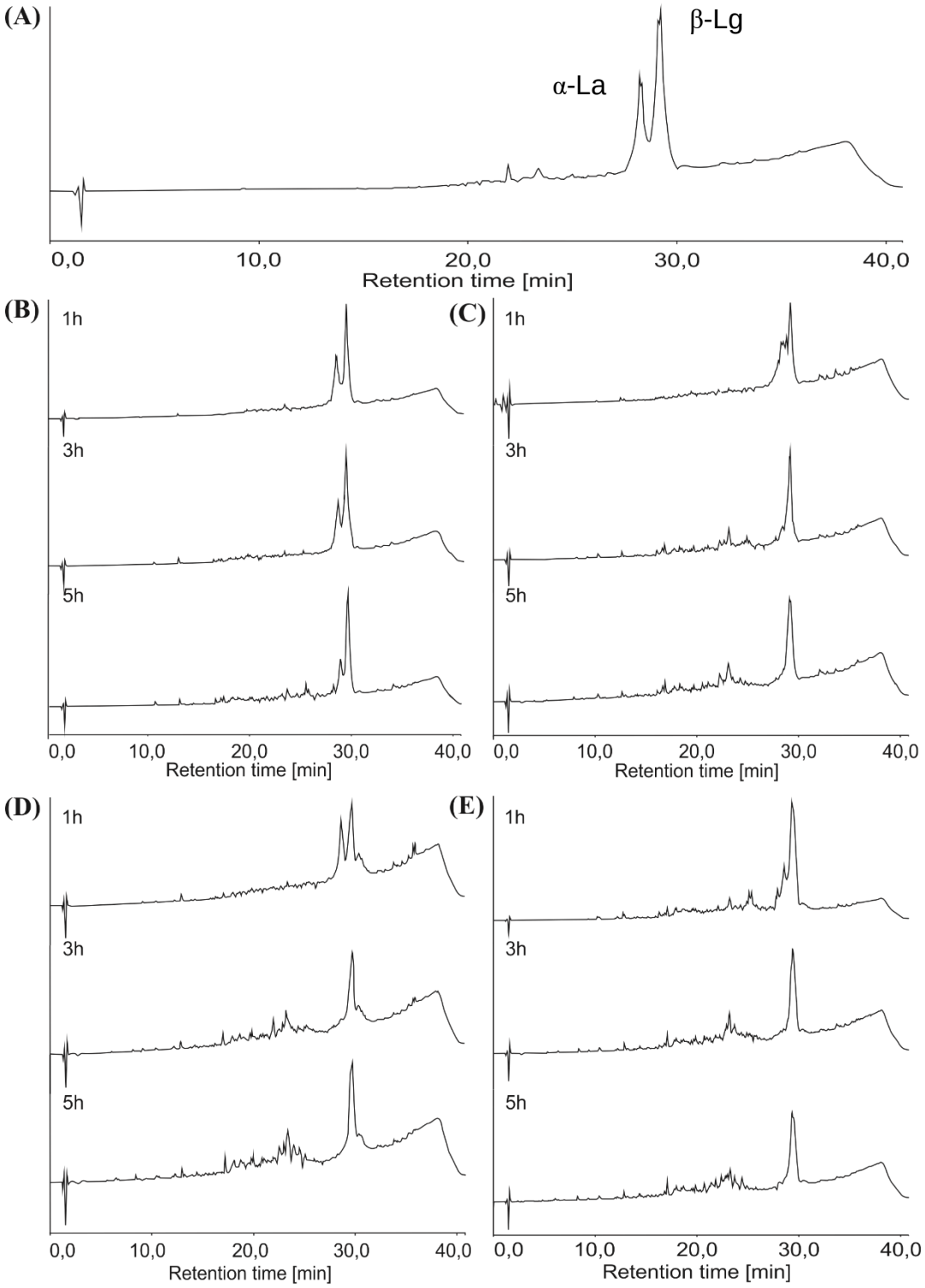

Figure 1. RP-HPLC chromatograms of unhydrolyzed WPC88 (A) and WPC88 hydrolyzed by pepsin solutions of (B) 0.48 %, (C) 0.95 %, (D) 1.43 % and (E) 1.91 % (*w/w*). Aliquots collected in 1, 3 and 5 hours of reaction. α-La – alpha-lactalbumin; β-Lg – beta-lactoglobulin.

3.2 PEPTIDES IDENTIFICATION

In the present study 21 peptides were identified through MALDI-TOF/TOF MS and 16 have already been described in the literature, as shown in Table 1. Among the identified peptides, two β-casein fragments have already been described in the literature as potent antihypertensive agents: VYPFPGPIHNSLPQNIPPLTQT (Recio et al., 2010) and PVVVPPFLQPEVM (Hu et al., 2013). Although the amino acid sequence EKFDKALKALPMHIRL from β-Lg (fragment 134-149) have not been reported as an antihypertensive peptide, Maes et al. (2004) identified antihypertensive activity in the internal sequence ALPMHIR contained in the fragment 134-149 of β-Lg, which may possibly indicate the antihypertensive potential of this peptide.

Table 1. MALDI-TOF/TOF MS analysis of the peptides obtained from whey protein concentrate treated with commercial pepsin for 3h.

Protein	**Fragment**	**Peptide Sequence**	***m/z***	**Reference**
K-casein	106-119	MAIPPKKNQDKTEI	2139.22	Not reported
β-casein	47-58	DKIHPFAQTQSL	1512.82	Not reported
β-Lg	42-54	YVEELKPTPEGDL	1489.82	Not reported
K-casein	109-128	PPKKNQDKTEIPTINTIASG	2155.20	Not reported
β-Lg	95-105	DTDYKKYLLVC	1382.99	Not reported
β-casein	59-80	VYPFPGPIHNSLPQNIPPLTQT	2430.34	Recio et al., 2010
β-Lg	81-93	PVVVPPFLQPEVM	1451.95	Hu et al., 2013
β-Lg	133-149	LEKFDKALKALPMHIRL	2023.35	Singh et al. 2014
α-La	34-40	HTSGYDTQA	979.51	Singh et al. 2014
β-Lg	95-103	DTDYKKYLL	1158.00	Singh et al. 2014
β-Lg	134-139	EKFDKALKALPMHIRL	1910.07	Maes et al., 2004
β-Lg	118-133	VRTPEVDDEAL	1243.00	Lacroix et al., 2016
β-Lg	12-19	IQKVAGTW	902.55	Lacroix & Li-Chan, 2014
β-Lg	25-41	AASDISLLDAQSAPLRV	1727.06	Lacroix & Li-Chan, 2014
α-La	104-117	WLAHKALCSEKLDQ	1641.91	Lacroix & Li-Chan, 2014
β-casein	63-73	KIHPFAQTQSL	1269.75	Kumar & Wong

β-casein	4-14	EELNVPGEIVE	1625.84	Boutrou et al., 2013
β-casein	94-105	GVSKVKEAMAPK	1244.70	Chabance et al., 1998
Lactoferrin	560-572	WAKNLNREDFRL	1561.97	Tonda et al., 2017
β-Lg	1-19	LIVTQTMKGLDIQKVAGTW.Na	2124.26	Jiang et al., 2017
β-Lg	32-42	LDAQSAPLRVY	1232.71	Jiang et al., 2017

β-Lg – beta-lactoglobulin; α-La – alpha-lactalbumin. *m/z*: mass-to-charge ratio of the precursor ions. The sequences correspond to C-terminal peptides. Peptide sequences were manually obtained and a *de novo* interpretation was realized in Swiss-Prot Database, Uniprot, Protein Blast e Protein Prospector (MS-Digest) databases using bovine whey data. Peptides in bold letter have already been described as antihypertensive molecules.

Five fragments identified in the present work have not been described in the literature yet, which are: YVEELKPTPEGDL and DTDYKKYLLVC (β-Lg fragments); MAIPPKKNQDKTEI and PPKKNQDKTEIPTINTIASG (κ-casein fragments); DKIHPFAQTQSL (β-casein fragment). According to the literature, peptides which contain aromatic and/or branched-chain amino-acid residues in the carboxy-terminal or amino-terminal end present greater potential to exhibit antihypertensive activity (COSTA; GONTIJO; NETTO, 2007; NORRIS; FITZGERALD, 2013b). In this sense, although only two peptides have been described as antihypertensive agents, all the fragments identified show potential to display anti-hypertensive activity.

3.3 VASCULAR RELAXATION

As illustrated in the typical tracings presented in Fig. 2, PC3 was able to induce a concentration-dependent relaxation in endothelium-intact rat aortic rings, reaching maximal relaxation of 65.02 ± 4.2% when the highest concentration was added into the preparations (10 $mg \cdot mL^{-1}$). Thus, our study not only reinforces the potentially beneficial effects of whey peptides obtained by pepsin on the vascular function, but also demonstrates that the hydrolysis may be conducted with the affordable commercially available pepsin. As vascular relaxation was observed in aortic vessel instead of an *in vitro* experiment, many mechanisms of action can be involved on the promotion of the action, going further from the well-known ACE mediated

effects, as widely reported in the literature ((MELLINGER-SILVA *et al.*, 2015; NORRIS; J., 2013; UDENIGWE; MOHAN, 2014)

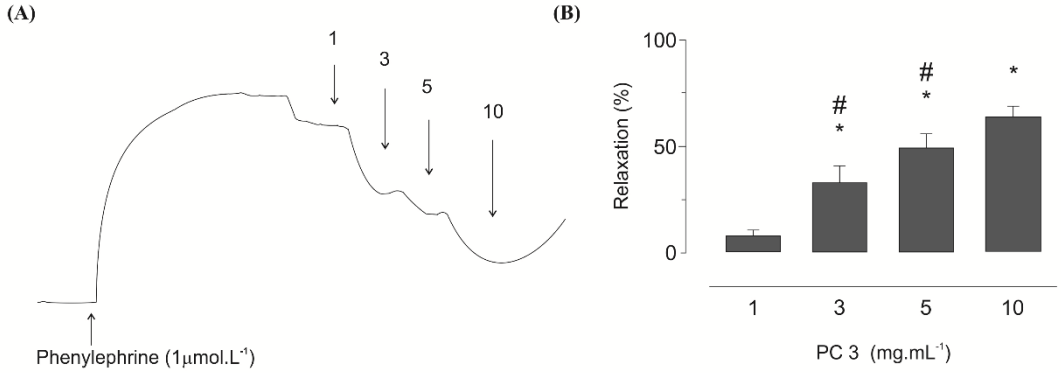

Figure 2. Vascular relaxation induced by pepsin hydrolysate from whey protein concentrate. Typical tracings (A) and data (B) showing the effect of the cumulative addition of 1, 3, 5 and 10 mg.mL^{-1} of the hydrolyzed fraction (PC3) in endothelium of rat aortic rings previously contracted by phenylephrine. The results (panel B) show the mean ± SEM of six preparations per group. * indicates significant relaxation ($p < 0.05$) compared to the maximum contraction obtained before hydrolysate's addition. # indicates $p < 0.05$ when compared with the concentration previously added.

3.4 TECHNOLOGICAL PROPERTIES OF WHEY PROTEIN HYDROLYSATE

PC3 powder presented 2.89 % (± 0.25) of moisture content and an absolute density of 1.3169 g.cm^{-3} (± 0.0031). The mean particle size ($d_{4,3}$) of pepsin hydrolysate was 86.39 µm (±7.73); 10 % of the particles presented up to 28.80 µm (± 12.74), 50 % showed 85.28 µm (± 6.99) and 90 % showed up to 139.60 µm (± 7.3) of diameter (Fig. A_2 - supplementary material). Creusot and Gruppen (2007) attributed the high particle size of whey peptides to agglomerates that are formed during hydrolysis, because of the exposure of hydrophobic groups, which may create a three-dimensional network and form a gel structure. The exposure of hydrophobic groups may also be related to the increase in oil absorption capacity of PC3 (33 %) when compared to the unhydrolyzed sample (control – WPC 88). This property plays an important role since it represents the ability of proteins and peptides to interact with lipidic compounds, improving mouthfeel and flavor retention (WANI *et al.*, 2015; YUST *et al.*, 2010).

Solubility data for integrated and hydrolyzed proteins are shown on Table 2. Both PC3 and WPC88 samples presented solubility above 90 % for all pHs evaluated, which may be very interesting since it allows PC3's incorporation as an ingredient into a large variety of food products. The results obtained for the WPC88 are similar to the ones showed by Jambrak and co-workers (2008), however, the enzymatic hydrolysis performed by them reduced this functional property in different treatments, which was observed in only one of the conditions evaluated in the present study.

WPC88 presented greater foam expansion (FE) and stability (FS) results when compared to PC3, as shown in Table 2. Foaming properties are intrinsically related to degree of hydrolysis (DH). Limited DH may improve foaming properties of whey protein preparations, since it may lead to a more rapid increase in the viscoelastic properties of air-water interfacial films and a concomitant increase in foam expansion compared to the native protein. However, more severe hydrolysis result in a much slower initial increase in interfacial viscosity and lower maximum value of foam expansion, explaining the lowest results found for PC3 samples (IPSEN *et al.*, 2001).

Table 2. Solubility and foaming capacity of peptic whey hydrolysate and whey protein concentrate.

	Solubility (%)			**Foaming Capacity (%)**	
pH	**PC3**	**WPC**		**PC3**	**WPC**
1	94.8 ± 4.4	96.11 ± 4.2	**FE**	19.38 ± 2.40*	49.73 ± 1.88*
2	99.6 ± 0.8*	92.59 ± 2.2	**FS 5 min**	18.06 ± 2.10*	35.05 ± 3.22*
3	96.6 ± 1.2	96.25 ± 3.8	**FS 10 min**	13.42 ± 3.50	25.66 ± 2.53
4	95.6 ± 4.1	98.50 ± 1.5	**FS 30 min**	7.15 ± 0.79	20.19 ± 2.24
5	94.8 ± 3.2	95.71 ± 7.2	**FS 60 min**	3.43 ± 0.79	16.29 ± 1.84
6	95.8 ± 3.0	95.95 ± 6.6			
7	95.7 ± 1.5	100 ± 3.9			
8	93.9 ± 1.2*	99.24 ± 1.0			
9	98.4 ± 5.0	95.80 ± 3.0			
10	95.1 ± 3.5	94.86 ± 4.4			
11	92.3 ±	95.27 ± 5.6			
12	97.2 ±	98.42 ± 1.6			

PC3 – 3h pepsin whey hydrolysate; WPC – whey protein concentrate 88 %; FE – foam expansion; FS – foam stability; (*) – indicates Test-t statistical significant difference between PC3 and WPC samples ($p < 0.05$).

Emulsifying activity index of PC3 hydrolyzed samples varied from 14.01 to 6.74 m^2g^{-1}, for pH 3 and 7, respectively, achieving the lowest value, 3.29 m^2g^{-1}, at pH 5, which may be

attributed to its nearness to isoelectric point where the net charge of proteins is zero and repulsive interactions of proteins are low (LAM; NICKERSON, 2013). However, the same was not observed for WPC88 samples, which presented increasing EAI values according to the pH, achieving 5.90, 6.84 and 8.22 $m^2 g^{-1}$ for pH 3, 5 and 7, respectively. WPC88 and PC3 samples presented low ESI.

3.5 SENSORY ANALYSIS

To evaluate the sensory acceptance of the developed ingredient, PC3 was incorporated into a dairy dessert and evaluated by 100 consumers. The dessert added with PC3 (D2) received higher average score (7.6 points in a 9 points hedonic scale) for global acceptance while the mean of 7.1 was attributed to the control dessert (D1) ($p < 0.005$), indicating that the traditional bitter taste of the whey peptides was masked after the incorporation into a dairy dessert matrix (Fig. 3A). Furthermore, the provided information on the benefits of the peptide had no effect on consumer product evaluation.

Similar results were observed when the intention to purchase test was applied. The dessert added with peptides reached higher averages in both groups of participants (with and without information), with means of 2.8 and 2.7 for G1 and G2 groups, respectively ($p > 0.005$), in a 3-point hedonic scale; while the control dessert received average of 2.6 from both groups (Fig. 3B), reinforcing that the information did not affect consumer´s opinion. The presented results suggest that the incorporation of PC3 into a dairy dessert may hide undesirable peptides bitterness and offer a biofunctional option to consumers who are concerned about the food they eat.

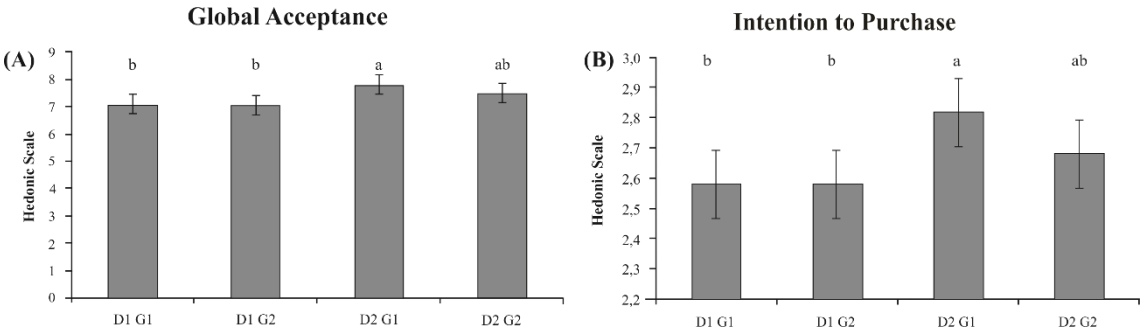

Figure 3. Frequency of notes distribution of global acceptance (A) and intention to purchase (B) assigned by consumers to dairy desserts without (D1) and with the addition of the hydrolyzed whey (D2). G1 – group 1 (not informed of the PC3 addition); G2 – group 2 (informed of the PC3 addition). a and b indicate significant difference ($p<0.05$).

4 CONCLUSIONS

Among the four conditions evaluated, the process conducted during 3 h with 1.91 % *w/w* of pepsin was the most favorable for the obtainment of the vasorelaxant peptides. These results may be related to any of the 21 peptides identified, since all of them possess the main characteristics reported in the literature to induce vascular relaxation, and two of them have their effectiveness already proven. Besides its bioactivity, PC3 powder also presented technological properties of great interest for food industries, such as high solubility in a wide pH range and oil absorption capacity, which are valuable for products' development. Moreover, high averages of global acceptance and intention to purchase were achieved for the dessert incorporated with PC3 than the control sample, showing that the bitterness of whey peptides was successfully hidden. These results altogether indicate that it is possible to generate a whey peptic hydrolysate with high vasorelaxant potential and interesting technological properties which may become a dual-functional ingredient to be added to food products.

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Declarations of interest: none

Author Contributions

Luisa Ozorio executed all the experiments, drafted and interpreted the manuscript. Luciano P. Silva and Carlos Bloch Jr. performed the mass spectrometry analysis. Maura V. Prates was responsible for the RP-HPLC analyses. Cristina Y. Takeiti and Ana Iraidy S. Brígida developed the analyses of the technological properties. Danillo Macêdo Gomes and José Eduardo da Silva-Santos performed the anti-hypertensive activity assays. Rosires Deliza was responsible for the sensory analysis. Caroline Mellinger-Silva designed he study and interpreted the results. Lourdes M.C. Cabral supervised the study. All authors have approved the final article.

SUPPLEMENTARY MATERIAL

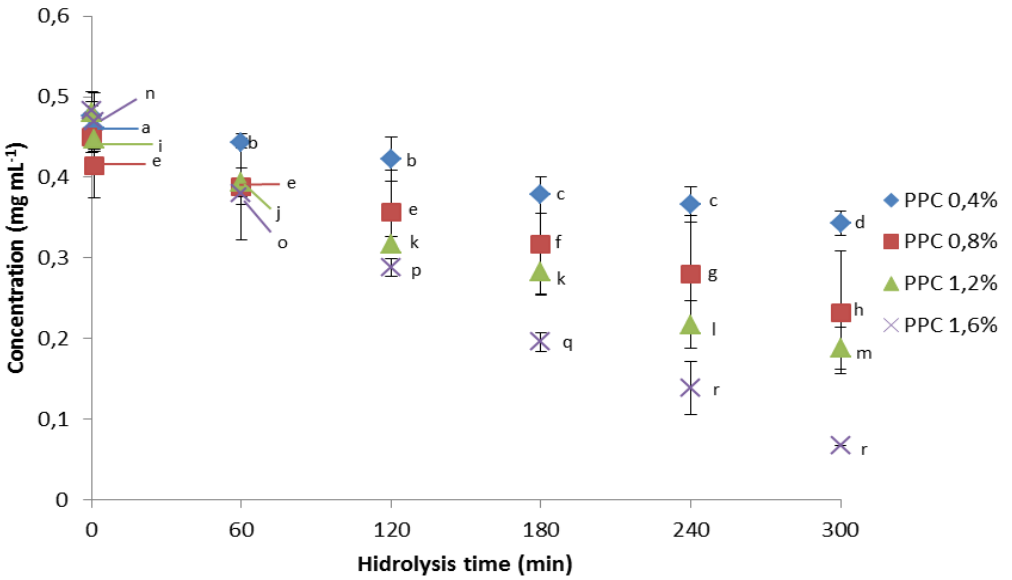

Figure A.$_1$. Hydrolysis curves of whey protein isolate treated with commercial pepsin 0.48%, 0.95%, 1.43% and 1.91% (w/w), measured as soluble protein content. The letters represent statistical significance for t-test among collected samples (0, 1, 60, 120, 180, 240, 300 min).

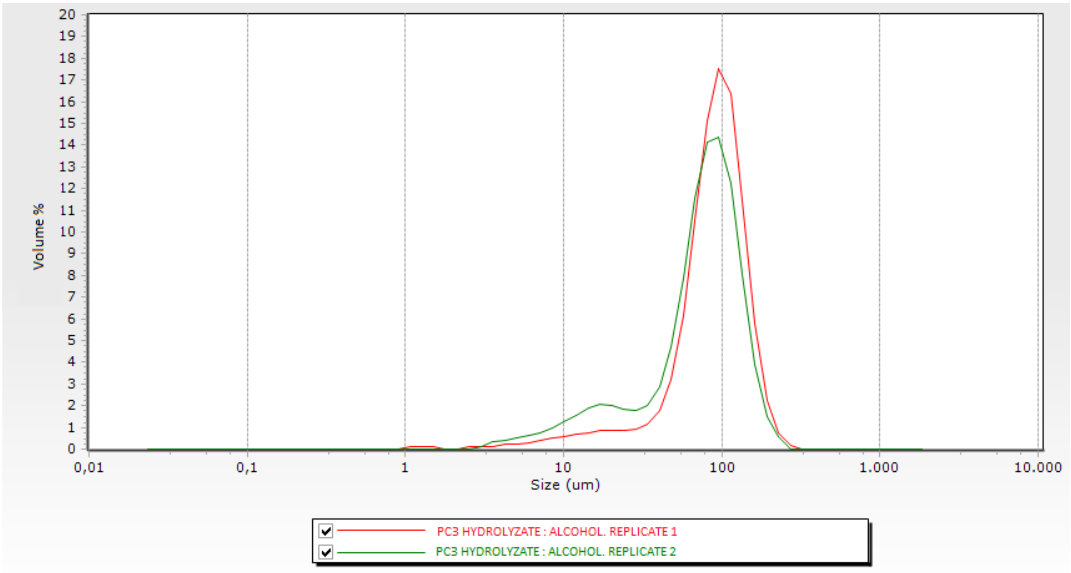

Figure A.$_2$. Particle size profile distribution of PC3 whey hydrolysate fraction. Isopropyl alcohol was used as dispersing agent.

Chapter 2

Enzyme inactivation and drying technologies influencing the vasorelaxant activity of a whey protein hydrolysate in semi-pilot scale

Ozorio, L.[a], Pereira, N. R.[b], José E. da Silva-Santos[b], Ana I. S. Brígida[c], Caroline Mellinger-Silva[c]*, Lourdes M.C. Cabral[c]

[a] Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[b]Laboratório de Biologia Cardiovascular, Departamento de Farmacologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil

[c] Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil

[*]Corresponding author.
Avenida das Américas, 29.501 Guaratiba, Rio de Janeiro, RJ, Brazil.
Tel.: +55 21 3622 9622; Fax: +55 21 3622 9713.

e-mail: caroline.mellinger@embrapa.br

ABSTRACT

The present study aimed to generate a vasorelaxant whey hydrolysate in semi-pilot scale, assessing the influence between two different enzyme inactivation conditions (pH and temperature) and drying technologies (freeze- and spray-drying). Hydrolyses were performed with a 1.25% (w/v) whey protein concentrate solution and 1.91% (w/w) of pepsin (pH 2, 3h, 37°C). Reactions were stopped either by increasing the pH up to 7 (5 M NaOH) or by heating the solution at 80°C for 5 min. Thermally inactivated samples induced a vascular relaxation *ex vivo* above 70 %, while the chemically inactivated ones reached only 20 %. Both drying technologies were effective in maintaining the bioactivity of the hydrolysate. These results brought the possibility of developing a whey-based product with high vasorelaxant potential in semi-pilot scale; thermal treatment followed by spray drying seemed to be the more affordable alternatives to food and nutraceutical industries generate this bioactive product.

1 INTRODUCTION

The use of whey proteins and whey peptides in sports nutrition, bakery and infant formulas is well consolidated (ALLIED MARKET RESEARCH, 2017). Despite its diversified use, large part of the whey produced remains underutilized, representing a serious environmental concern, because of its high chemical and biochemical demands of oxygen (DERELI *et al.*, 2019). This factor, together with the need to create new market niches and value old products, makes industries seek for new alternatives to their residues (YADAV *et al.*, 2015).

Among other functionalities, whey proteins and whey peptides are associated with modulation of hypertension (BELTRÁN-BARRIENTOS *et al.*, 2016; O'KEEFFE; CONESA; FITZGERALD, 2017), a chronic disease with high prevalence, which its complications account for 9.4 million deaths every year (WHO, 2015). Despite the lack of molecular studies, it is generally described that whey peptides may induce vascular relaxation and putatively reduce the blood pressure through different mechanisms, including inhibition of the angiotensin converting Enzyme (ACE) and renin, stimulation of nitric oxide production, and blockage of calcium channels, among others (UDENIGWE; MOHAN, 2014). Besides all well-known health benefits, there are only a few commercial products available on the market based on the antihypertensive activity of whey peptides (BELTRÁN-BARRIENTOS *et al.*, 2016), highlighting the need of more studies in this field.

The main challenge of food and nutraceutical industries consists of high costs of production of the antihypertensive peptides since different advanced technologies are required. Therefore, the present study aimed at producing a whey protein hydrolysate with vasorelaxant effect by the use of commercial pepsin in a semi-pilot scale, assessing the influence of two different enzyme inactivation conditions and drying technologies.

2 MATERIALS AND METHODS

2.1 MATERIALS

Bovine whey protein concentrate (WPC), containing 88% of proteins, 2% of carbohydrates, 1.6% of lipids and 8.4% of cholesterol, vitamins and minerals was provided by Alibra Ingredientes Ltda (Campinas, SP, Brazil). Porcine pepsin (enzyme activity: 0.28 μU.mL^{-}

[1]) was provided by Bela Vista Produtos Enzimáticos Ind. e Com. Ltda (Bela Vista, SC, Brazil). Phenylephrine hydrochloride, acetylcholine chloride, and all salt used to prepare the physiological saline solution (PSS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other reagents were of analytical grade.

2.2 WHEY HYDROLYSATE OBTAINMENT

Hydrolyses were performed according to Mellinger-Silva et al. (2015). Briefly, in a semi-pilot scale reactor of 20 liters, WPC was suspended in distilled water at a concentration of 1.25% (w/v) and the pH was adjusted to 2 with 1M HCl. Pepsin (4.2 g) was used to prepare 10.5 L of enzyme solution (0.4% w/v), corresponding to 1.91 g of pepsin per gram of protein (1.91% w/w). Pepsin solution was further added to the WPC solution and the reaction occurred during 3 hours at 37°C. Temperature and pH were monitored during all the process. Pepsin was either thermally (80°C for 5 min) (TI) or chemically inactivated (CI) by increasing pH up to 7 with 5 M NaOH. Samples were either freeze-dried at -52 °C, 67.73 Pa (Liotop, São Paulo, Brazil) or spray-dried at 0.52 MPa (inlet temperature: 170°C; outlet temperature: 85°C (Buchi Labortechnik, Flawil, Switzerland).

2.3 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

RP-HPLC was performed according to Mellinger-Silva et al. (2015). A HPLC (Jasco, São Paulo, Brazil) coupled with a BDS Hypersil C18 (100 x 4.6 mm, particle size 2.4 μm, Thermo Scientific, USA) was used. Trifluoracetic acid (TFA) in ultrapure water (0.1 %, v/v) and 0.1% TFA in acetonitrile (v/v) were used as solvents A and B, respectively. Hydrolysates were eluted as follows: 0-2 min, 5% B; 2-15 min, 5-20% B; 15-20 min, 20-30% B; 20-25 min, 30-40% B; 25-28 min, 40-50% B; 28-32 min, 50-60% B; 32-34 min, 60-70% B; 34-36 min, 70-80% B; 36-38 min, 80-5% B; 38-40 min, 5% B. Runs occurred at a flow rate of 1.0 $mL.min^{-1}$ for 40 min at ambient temperature (approximately 20°C). The detection wavelength used was 216 nm. Bovine α-Lactalbumin (α-La) and β-Lactoglobulin (β-Lg) from bovine milk (Sigma-Aldrich, Saint Louis, MO, USA) were used as standards with retention times of 29.84 and 30.70 min, respectively.

2.4 AORTIC RING PREPARATION AND ANALYSES OF VASCULAR RELAXATION

Six male Wistar rats (250-300 g) provided by *Universidade Federal de Santa Catarina* (Florianópolis, Brazil) were used in the experiments, which were approved by our local Institutional Animal Care and Use Committee (authorization number 5371190815). Animals were maintained in standard laboratory conditions under controlled temperature (22 ± 2°C) with free access to water and diet *ad libitum*. For these experiments, animals were killed by anesthetic overdose (ketamine and xylazine, 140 and 40 mg/kg, i.p.) and the preparation of endothelium-intact rat aortic rings was performed as previously described (MELLINGER-SILVA, 2015). Briefly, the aortic rings were mounted in organ baths and kept in PSS (composition in mM: NaCl 131.3, KCl 4.7, KH_2PO_4 1.18, $MgSO_47H_2O$ 1.17, $NaHCO_3$ 14.9, D-glucose 5.5, $CaCl_22H_2O$ 1.6, EDTA 0.08, pH 7.4) at 37 °C and continuously bubbled with 95% O_2/5% CO_2. To evaluate the presence of a functional endothelium, acetylcholine (1µmol.L^{-1}) was used to induce relaxation in phenylephrine (1µmol.L^{1}) contracted preparations. Only the rings that achieved 80% of relaxation to acetylcholine were considered with functional endothelium. The changes in vascular tone were recorded by isometric force transducers, coupled to a recording system PowerLab® and its application software LabChart v. 7.4 (both from ADInstruments, Castle Hill, Australia). After stabilization, the vessels were contracted by 1 µmol.L^{-1} phenylephrine and cumulative concentrations of the hydrolyzed fractions (1, 3, 5 and 10 mg.mL^{-1}) were added. The results were expressed as the mean ± SEM of 6 aortic rings from different animals per group. Each preparation was tested with only one of the hydrolyzed fractions. The results were analyzed using two-way analyses of variance (ANOVA) followed by Tukey's post hoc analysis. A value of $p < 0.05$ was considered statistically significant. Graphs and statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, CA, USA).

3 RESULTS AND DISCUSSION

Scaling up the process is essential when it is aiming to develop an ingredient for food industries and processes changes must be well controlled to avoid alterations in the final product. The integrative process for the development of whey-based products with high economic value includes several steps, such as the reception of the *in natura* whey, membrane concentration, hydrolysis, drying and packing (Figure 1) (O'KEEFFE; CONESA; FITZGERALD, 2017; TAVARES, TÂNIA G. *et al.*, 2012).

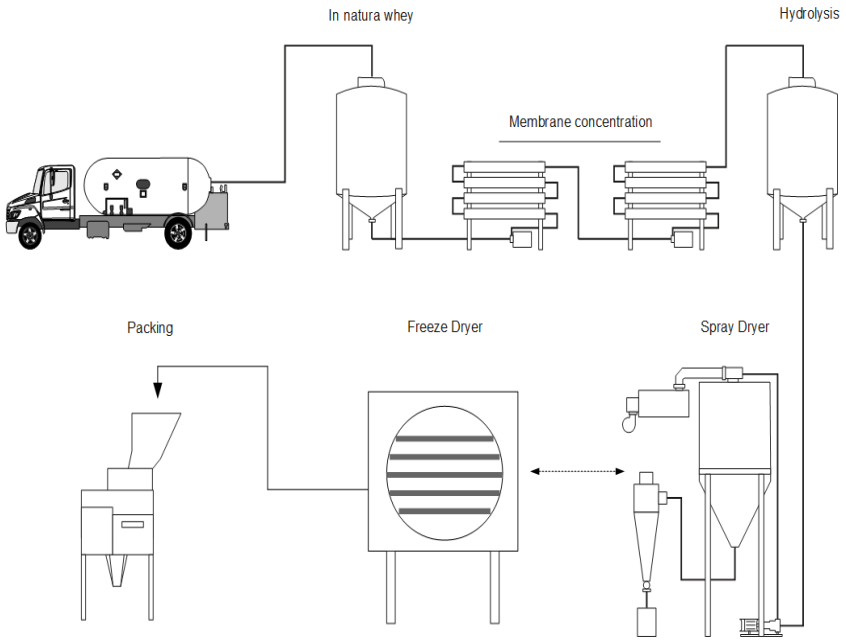

Figure 1. Flow diagram of a proposed industrial plant for whey protein hydrolysate production.

Enzymes are extensively used to generate biofunctional products (LE MAUX *et al.*, 2016). Pepsin presents high affinity for cleaving peptide bonds between hydrophobic and preferably aromatic amino acids (BANNIGAN *et al.*, 2018). The presence of these amino acid residues at each of the C-terminal tripeptide positions seems to be related to the angiotensin I-converting enzyme (ACE) inhibition, which may be considered a good physiological target for the clinical treatment of hypertension, as it is involved in the control of blood pressure through two different systems, the renin-angiotensin and the kinin-nitric oxide systems (KO *et al.*, 2015; NORRIS; FITZGERALD, 2013a). Pepsin is also found in the human gastrointestinal tract, in this manner, after a previous peptic hydrolysis of the WPC, any *in vivo* gastric digestion is expected further, possibly allowing a controlled formation of the vasorelaxant peptides. More studies are being carried out to evaluate the influence of the gastrointestinal digestion in the release of the bioactive peptides.

Besides the choice of the enzyme, its inactivation and the method used to dry the solution are also parameters that may influence the composition of the hydrolysate and its bioactivity. Among the drying methods, lyophilization is well-known for preserving quality and the integrity of bioactive compounds in food matrices, however, it requires high energy and

presents operational costs about six-times higher than spray-drying (HAQUE; ADHIKARI, 2015; SCHULZE; HUBBERMANN; SCHWARZ, 2014). The spray-dryer, more commonly used in the food industries, applies high working temperatures, possibly causing heat degradation and affecting the stability of thermally labile compounds (DROSOU; KROKIDA; BILIADERIS, 2016).

Figures 2B and 2C reveal that the drying method may influence the peptide formation of the CI samples, as the freeze-dried hydrolysate presented a more varied peptide composition than the spray-dried one. This may be related to the heat and pressure applied during spray-drying, causing sample degradation with generation of free amino acids (DROSOU; KROKIDA; BILIADERIS, 2016). The same was not observed for the TI samples, as both lyophilized (Figure 2D) and spray-dried (Figure 2E) samples presented similar peptide profiles, including hydrophobic and hydrophilic molecules. Comparing CI and TI samples, it is possible to observe a higher β-Lactoglobulin hydrolysis on the TI ones than on the CI samples, which may be a result of the thermal denaturation of this protein during the thermal inactivation process (Figure 2).

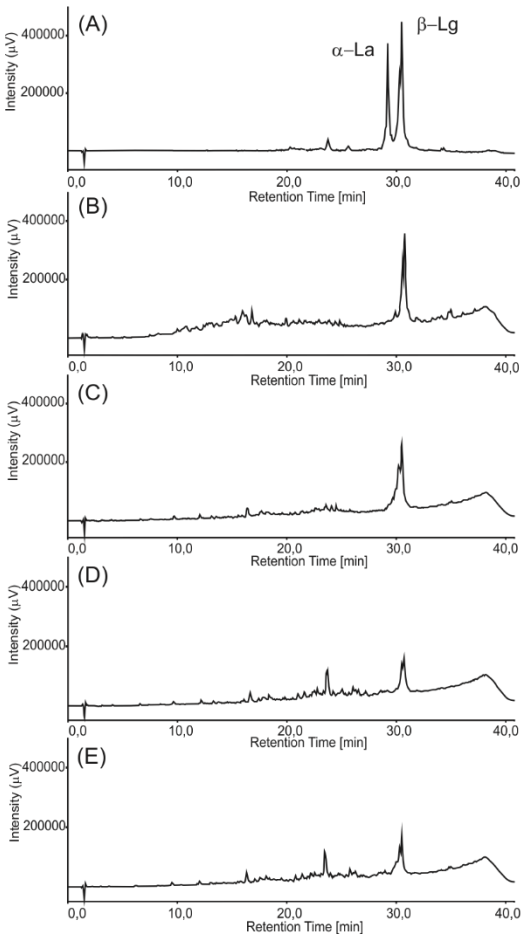

Figure 2. Peptide profiles of whey hydrolysates assessing different enzyme inactivation conditions and drying technologies. (A) control sample, whey protein concentrate 88%; α-La: α-Lactalbumin; β-Lg: β-Lactoglobulin; (B) CI (adjusted to pH 7) Lyophilized and (C) CI Spray-dried samples; (D) TI (80°C x 5 min) Lyophilized and (E) TI Spray-dried samples.

As shown in Figure 3, the enzyme inactivation method directly influenced the biofunctionality of the hydrolysates, as TI samples were able to induce greater vasorelaxant effect when compared to CI samples (Figure 3A). Notably, CI spray-dried hydrolysate completely missed its capability to induce vascular relaxation, compared with TI sample (Figures 3B and 3C). Furthermore, the TI hydrolysate induced significant levels of vascular relaxation in pre-contracted aortic rings, achieving 72.1 ± 11.4% and 81.0 ± 14.3%, for the freeze- and spray-dried samples, respectively.

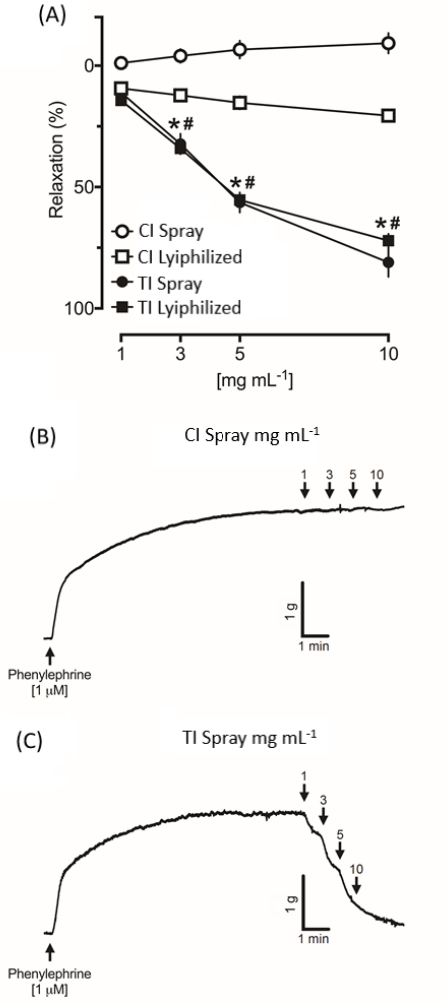

Figure 3. Vascular relaxation induced by whey hydrolysates in rat aortic rings pre-contracted by phenylephrine. (A) Cumulative concentrations of 1, 3, 5, 10 mg.mL^{-1} of whey hydrolysates CI (adjusted pH to 7) and TI (80°C x 5 min) lyophilized or spray-dried were added. (B) CI spray-dried hydrolysate; (C) TI spray-dried hydrolysate. * indicates significant vascular relaxation ($p<005$) for both TI samples; # indicates $p<005$ compared with the previous concentration of used in TI samples (two-way ANOVA followed by Tukey's post hoc test. The results were expressed as the mean ± SEM of 6 aortic rings from different animals per group

4 CONCLUSIONS

The present study revealed that the enzyme inactivation was an important step in the process of generation of a whey hydrolysate with vasorelaxant activity. The hydrolysates heat inactivated presented higher vascular relaxant potential than the chemically inactivated ones. Despite applying high temperatures and pressure, the spray-drying technology presented a tendency to be more efficient in maintaining vascular relaxation capacity of the hydrolysate, besides presenting lower working costs and reduced time of process than freeze-drying, being a more feasible technology to food industries. In this manner, thermal inactivation of pepsin

followed by spray-drying the hydrolysate might be recommended for the generation of an antihypertensive whey hydrolysate in semi-pilot scale.

Acknowledgments

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Declarations of interest: none

Chapter 3

Gastrointestinal digestion enhances the endothelium-dependent vasodilation of a whey hydrolysate in rat aortic rings

Luísa Ozorio[1]; Natália k. Matsubara[2]; José Eduardo da Silva-Santos[2]; Gwenaële Henry[3]; Yann Le Gouar[3]; Julien Jardin[3]; Caroline Mellinger-Silva[4]*; Lourdes M. C. Cabral[4]; Didier Dupont[3]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil;

[2]Laboratório de Biologia Cardiovascular, Departamento de Farmacologia, Universidade Federal de Santa Catarina, Florianópolis, Brasil;

[3]STLO, INRA Agrocampus Ouest, Rennes, France;

[4]EMBRAPA Agroindústria de Alimentos, Rio de Janeiro, Brasil.

*Corresponding author.

Avenida das Américas, 29.501 Guaratiba, Rio de Janeiro, RJ, Brasil.

Tel.: +55 21 3622 9622; Fax: +55 21 3622 9713.

e-mail: caroline.mellinger@embrapa.br

Declarations of interest: none

ABSTRACT

Whey proteins present encrypted biofunctional peptides that need to be released from the native protein to exert their biological activity. Antihypertensive whey peptides are the most studied ones, which can be explained by high prevalence of this chronic degenerative disease. The present study investigated whether the molecular changes occurred during the gastrointestinal digestion of a whey protein hydrolysate could modulate its vasorelaxant potential in rat aortic rings. Spectrophotometric data and SDS-PAGE gel showed a small degree of hydrolysis during the gastric phase and intense intestinal proteolysis. RP-HPLC revealed the formation of a large peptide profile. During the simulated digestion, 198 were generated and identified and, left-shifted the concentration-response curve of the endothelium-dependent vasorelaxation, as recorded for the digested hydrolysates. In conclusion, gastrointestinal digestion of the whey hydrolysate leads to the generation of bioactive peptides with enhanced vasodilatory potency, reinforcing the relevance of whey-derived products in blood pressure regulation.

Keywords: whey protein; peptides; hydrolysis; digestion; vascular relaxation; hypertension.

1 INTRODUCTION

Digestion is a complex process in which foods, after ingestion, pass through different mechanical and enzymatic transformations to micro and macronutrients that can be used by the body for numerous functions, such as growth, energy, cells maintenance and replacement (Guerra et al., 2012). Among macronutrients, proteins present encrypted fragments with biological activity, named bioactive peptides. These peptides are not active within the parent protein and need to be released during food processing and/or gastrointestinal digestion to be further absorbed and reach the target organs (Nongonierma & Fitzgerald, 2015).

Whey proteins present several of these encrypted peptides, being associated with different health benefits, including antihypertensive, antithrombotic, anticarcinogenic and immunomodulatory effects (Bulut Solak & Akin, 2012; Nongonierma & Fitzgerald, 2015; O'Keeffe & FitzGerald, 2018). Antihypertensive whey peptides are subject of various studies, which is justified by the high prevalence of this chronic degenerative disease. Moreover, along the years people are becoming more health conscious and tendentious to replace allopathic medicines for natural treatments and functional foods (Beltrán-Barrientos, Hernández-Mendoza, Torres-Llanez, González-Córdova, & Vallejo-Córdoba, 2016). Whey peptides may lower blood pressure (BP) through different mechanisms, such as inhibition of angiotensin-I converting enzyme (ACE) , renin and pro-renin, blockage of calcium channels and AT1 receptors, and stimulation of nitric oxide production (Udenigwe & Mohan, 2014). These byproducts of the dairy industry, however, are rarely commercialized as bioactive products, highlighting the need to continue the studies in this field.

One subject that deserves attention concerns the effects of food processing and digestion on the release of the antihypertensive peptides. Indeed, bioactive peptides may be released from the parent protein through chemical or enzymatic hydrolysis but their profile may completely change after gastrointestinal digestion (Phelan & Kerins, 2011; Sinha, Radha, Prakash, & Kaul, 2007). Studying the release of bioactive peptides during gastrointestinal digestion directly in humans would be the more effective and relevant approach. However, studying multistage processes in vivo is very complex, costly and involves several restrictions for ethical reasons (Guerra et al., 2012; Ménard et al., 2014). The INFOGEST network on food digestion proposed an international consensus for in vitro digestion (Minekus et al., 2014) that is being currently validated with other in vitro and in vivo data (Egger, Ménard, Delgado-Andrade, Alvito,

Assunção, Balance, Barberá, Brodkorb, Cattenoz, Clemente, Comi, Dupont, Garcia-Llatas, Lagarda, et al., 2016; Sanchón et al., 2018) and may provide us some immediate answers about the modifications that occur with these peptides during digestion. In this sense, the present study aimed to follow the biochemical transformations occurring in a vasorelaxant whey protein hydrolysate during in vitro gastrointestinal digestion and its influence on the regulation of vascular function. Our results disclosed a new piece of evidence regarding the antihypertensive potential of whey peptides.

2 MATERIALS AND METHODS

2.1 MATERIALS

Bovine whey protein concentrate 88% of protein (WPC88) was donated by Alibra Ingredientes Ltda (Campinas, SP, Brazil) and was used as substrate. Commercial pepsin (E.C. 3.4.23.1) from porcine gastric mucosa was donated by Bela Vista Produtos Enzimáticos Ind. e Com. Ltda. (Bela Vista, SC, Brazil). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany).

2.2 WHEY ENZYMATIC HYDROLYSIS

The enzymatic hydrolysis of the WPC88 was performed in pilot scale (100 L) according to Ozorio et al. (2019b). Briefly, the WPC88 was suspended at a concentration of 1.25% (w/v), and the pH was adjusted to 2 with 1M HCl. Pepsin (5.34 $\mu U.g^{-1}$ of protein) was added and the hydrolysis occurred at constant stirring for 3 h at 37°C. The pH and the temperature were monitored during the entire experiment. The reaction was stopped by increasing the temperature to 80°C for 5 min. Whey hydrolysate was then spray dried (Niro atomizer, Copenhagen, Denmark; inlet temperature: 170°C; outlet temperature: 90°C) and the hydrolyzed powder was maintained frozen (-20°C) until further analysis.

2.3 WHEY HYDROLYSATE *IN VITRO* DIGESTION

The *in vitro* gastrointestinal digestion of the whey hydrolysate was performed according to the harmonized INFOGEST protocol (Minekus et al., 2014) with slight modifications. Briefly, a whey hydrolysate solution (8% w/v) was prepared and added to a simulated gastric fluid containing pepsin from porcine gastricz mucosa (2000 $U.mL^{-1}$, EC 3.4.23.1, Sigma-

Aldrich, St. Louis, MO, USA) at a ratio of 50:50 (v/v). The gastric digestion occurred for 2 h in a water bath (Grant, OLS 200, Cambridgeshire, England) under constant stirring (100 rpm). Samples were collected at 1, 10, 30, 60 and 120 min and added to microtubes containing 0.5 $mg.mL^{-1}$ of the protease inhibitor pepstatine A (P5318, Sigma-Aldrich, St. Louis, MO, USA). After 120 min, the pH was adjusted to 7 with 2 M NaOH to perform the intestinal digestion and the solution was mixed to a simulated intestinal fluid (50:50 v/v) containing trypsin (100 $U.mL^{-1}$ of final mixture), chymotrypsin (25 $U.mL^{-1}$ of final mixture) and bile (10 $mmol.mL^{-1}$ of final mixture). Samples were withdrawn at 1, 10, 30, 60 and 120 min and added to microtubes containing the protease inhibitor pefabloc (0.1M, 76307, Sigma-Aldrich, St. Louis, MO, USA). All samples were maintained at -20°C until further analysis. As the samples investigated in this study contain high protein concentration, authors chose not to perform the oral phase of the in vitro digestion.

2.4 DEGREE OF HYDROLYSIS (DH)

The release of free amino groups (NH2) was measured according to the OPA (orthophtaldialdehyde) method described by Darrouzet-Nardi, Ladd, & Weintraub (2013) with slight modifications. The OPA reagent was prepared with ethanol and 700 µL of 1M DTT (DL-Dithiothreitol, D0632, Sigma) instead of methanol and β-mercaptoethanol, respectively. The assay was carried out by adding 100 µL of the OPA/DTT reagent to 50 µL of the samples which were previously diluted (1:40 v/v). The OPA when in contact with free amino groups and with an SH-compound form a compound that absorbs light at 340 nm. The 96-well plate was measured after 10 min of incubation (Multiscan Go, Thermo Scientific). A standard curve was prepared using 0.2; 0.4; 0.6; 0.8; 1 and 2 mM methionine standard solutions (Sigma-Aldrich) and the degree of hydrolysis was expressed as mg $NH2.mL^{-1}$ of sample. Results were submitted to a one-way analysis of variance (ANOVA) followed by t-test, using the XLSTAT software (Addinsoft, 2019). A p value < 0.05 ($p < 0.05$) was considered statistically significant.

2.5 PROTEIN ELECTROPHORESIS

The SDS-PAGE protein electrophoresis was performed according to Laemmli (1970). Samples were diluted (1:50 v/v) in Invitrogen NuPAGE LDS sample buffer 4x (Thermo Fisher Scientific, California, USA) and applied to the NuPAGETM 4-12% Bis-TRIS gels. Besides the whey hydrolysate digested and undigested samples, two control samples were used: pepsin in

gastric simulated fluid; trypsin and chymotrypsin in intestinal simulated fluid, both fluids prepared as described by Minekus et al. (2014). The electrophoretic run occurred throughout 90 min at 50 mA and 100 V using a Bio-Rad Mini PROT'EAN® Tetra Cell (California, USA). Gels were fixed (fixing buffer: 40% ethanol; 10% acetic acid) for 30 min, then stained overnight (BioRad Bio-Safe Coomassie G-250). After discoloration, gels were scanned using G:BOX by Syngene (Cambridge, UK).

2.6 REVERSED PHASE – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

The WPC88, the whey hydrolysate and the digested whey sample solutions were analyzed using an analytical HPLC unit from Jasco (Jasco Corporation, Japan) coupled with a Hypersil BDS C18 column (100×4.6 mm, particle size 2.4 µm, Thermo Scientific, USA), according to Ozorio et al. (2019a). Samples were injected in a total volume of 20 µL and the chromatographic runs were of 40 min with a flow rate of 1.0 mL.min−1 at room temperature (approximately 20°C). Solutions of 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v) and 0.1% TFA in acetonitrile (ACN) (v/v) were used as solvents A and B, respectively. Hydrolysates were eluted as follows: 0–2 min, 5% B; 2–15 min, 5–20% B; 15–20 min, 20–30% B; 20–25 min, 30–40% B; 25–28 min, 40–50% B; 28–32 min, 50–60% B; 32–34 min, 60–70% B; 34–36 min, 70–80% B; 36–38 min, 80–5% B; 38–40 min, 5% B. Peptides were detected at 216 nm.

2.7 TANDEM MASS SPECTROMETRY

Mass spectrometry (MS) analysis was conducted as previously described by Deglaire et al. (2016). A nano-RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source was used. Digested samples were diluted 1000 times in the injection buffer and filtered (0.45µm cut-off) before concentration on a µ-precolumn pepMap100 (C18 column, 300 µm i.d. × 5 mm length, 5 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) and separation on a PepMap RSLC column (C18 column, 75 µm i.d. x 150 mm length, 3 µm particle size, 100 Å pore size; Dionex).

Peptide separation was performed at a flow rate of 0.3 µL.min^{-1} using solvents A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA] and B [95% (v/v) acetonitrile,

0.08% (v/v) formic acid and 0.01% (v/v) TFA]. The elution gradient first rose from 5 to 35% solvent B over 40 min, then up to 85% solvent B over 3 min before column re-equilibration. The mass spectra were recorded in positive mode using the m/z range 250-2000. The resolution of the mass analyzer for m/z of 200 amu (atomic mass unit) was set in the acquisition method to 70000 for MS and 17 500 for MS/MS. For each MS scan, the ten most intense ions were selected for MS/MS fragmentation and excluded from fragmentation for 20 s.

Peptides were identified from the MS/MS spectra using the X!TandemPipeline software (https://pubs.acs.org/doi/abs/10.1021/acs.jproteome.6b00632J. Proteome Res. 16, 2, 494-503) against a homemade database composed of major milk proteins to which was added the common Repository of Adventitious Protein (http://thegpm.org/crap). The possible post-translational modifications were serine or threonine phosphorylation, methionine oxidation. Peptides identified with an e-value < 0.05 were automatically validated. The peptide false discovery rate was less than 0.6%. Peptides with angiotensin-I-converting enzyme inhibitory activity, were identified using the Biopep database.

2.8 *IN VITRO* VASCULAR RELAXATION

The procedures described in this study were approved by the Institutional Animal Care and Use Committee of Universidade Federal de Santa Catarina (Florianópolis, SC, Brazil; authorization number 5371190815). We used male Wistar rats (300-350 g), provided by our Central Vivarium, housing in ventilated cages under controlled temperature (22 ± 2°C) and light/dark cycle (12/12 h), with water and chow *ad libitum*.

For these experiments, the animals were euthanized by anesthetic overdose using ketamine and xylazine (140 and 40 mg/kg) administered by intraperitoneal route and the thoracic aorta was immediately removed and placed in Petri dishes containing cold (~ 4 °C) physiological saline solution (PSS, composition in mM: 131.3 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4 \cdot 7H_2O$, 14.9 $NaHCO_3$, 5.5 D-glucose, 1.6 $CaCl_2 \cdot 2H_2O$, 0.08 EDTA, pH 7.4). The arteries were cleaned from adipose and connective tissues, cut in rings with 3-4 mm length, and placed in organ baths contained warmed PSS (37°C) continuously bubbled with 95% O_2/5% CO_2. The vascular tone was detected by isometric transducers and recorded by a computer-coupled data acquisition hardware and software (PowerLab® and LabChart v. 7.4, respectively, both from AD Instruments, Castle Hill, Australia).

The aortic rings were allowed to stabilize for 60 min under a basal tone of 3 g, and the contractility responses to 120 mM modified PSS (composition, in mM: 14.4 NaCl, 119.9 KCl, 1.6 $CaCl_2 \cdot 2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$, .5 D-glucose, 1.6 $CaCl_2 \cdot 2H_2O$, 0.08 EDTA, pH 7.4) were assessed to verify the integrity of the preparations. A new interval was allowed, and the vasodilatory effect of acetylcholine (1 $\mu mol.L^{-1}$) under the tonic contraction induced by phenylephrine (1 $\mu mol.L^{-1}$) was evaluated. The experiments were performed in both endothelium-intact and endothelium-denuded vessels, which had the endothelial function disrupted by rubbing a small needle around the lumen. The effectiveness of endothelial removal was confirmed by the complete absence of vasodilatory responses to acetylcholine, and only vessels that reached a relaxation greater than 90% were used as endothelium-intact preparations. Following evaluation of endothelial function, the baths were washed for removal of vasoactive agents. After 60 min of rest, cumulative concentrations of the undigested (1, 3, 5 and 10 $mg.mL^{-1}$) and digested (1, 3, 10, 30, 50 and 100 $\mu g.mL^{-1}$) whey protein hydrolysates were added in phenylephrine-contracted preparations. The results were expressed as the mean ± standard error of the percentage of maximal relaxation induced by the hydrolysates in at least six aortic rings obtained from different animals. The data were subjected to two-way analyses of variance (ANOVA) followed by Tukey's post hoc analyses. A value of $p < 0.05$ was accepted as statistically significant. Graphs and statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA).

3 RESULTS AND DISCUSSION

3.1 DEGREE OF HYDROLYSIS AND PROTEIN ELECTROPHORESIS

The proteolytic activity during gastrointestinal digestion of the whey hydrolysate was estimated by the release of free amino groups. The results illustrated in Figure 1 show an increase in the concentration of amino groups after gastrointestinal digestion, which ranged from 12.4 $mg.g^{-1}$ to 24.2 $mg.g^{-1}$. A small degree of hydrolysis was observed during the gastric phase, which was already expected, since pepsin had been previously used to hydrolyze the WPC88 and a more prominent digestion occurred during the intestinal phase. Even so, the release of these molecules was not very high, which can be an indicative of the formation of several peptides, instead of free amino acids. These results were confirmed by the SDS-PAGE gel (Figure 2), which showed only a slight decrease in the β-lactoglobulin (β-lg) band that remained partially resistant to pepsin digestion. The resistance of β-lg to gastric digestion was

previously reported by Madalena et al. (2016); Nik, Wright & Corredig (2010) and Sawyer (2013), who attributed this finding to the β-lg stable globular configuration at low pH, as target amino acid residues are buried in the protein core. In contrast, an intense proteolytic activity was observed during the intestinal phase, leading to an intense β-lactoglobulin hydrolysis in less than 10 min of intestinal digestion, which is possibly related to a conformational transition at pH above 7, exposing tyrosine residues that are sensitive to trypsin and chymotrypsin hydrolysis (Diarrassouba et al., 2013; Sanchón et al., 2018).

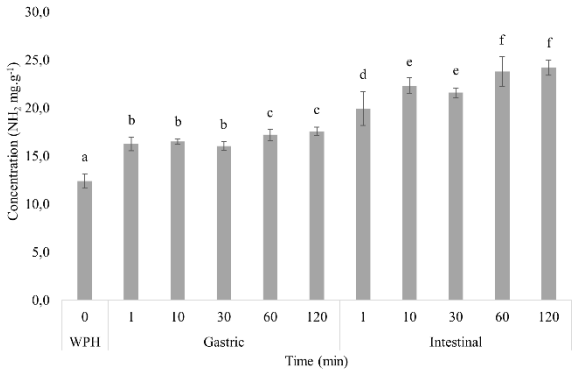

Figure 1. Free amino acids release during whey hydrolysate in vitro gastrointestinal digestion. WPH – control sample, undigested whey hydrolysate. Letters indicate significant difference ($p < 0.05$).

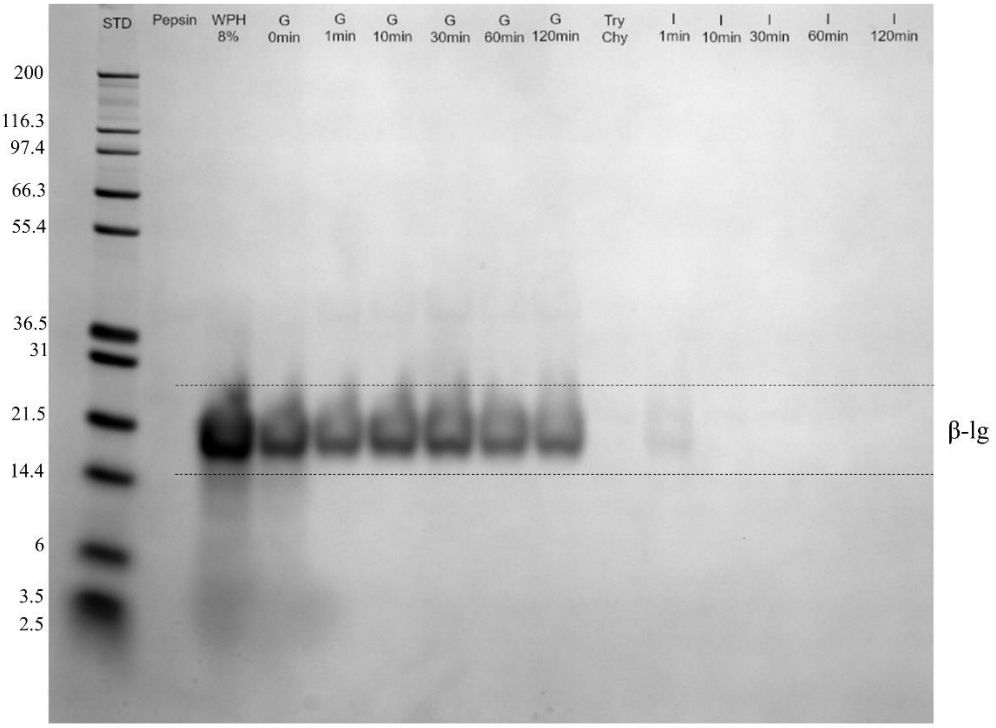

Figure 2. Electrophoretic profile of undigested and digested whey hydrolysate samples. STD: standard solution (molecular weight in Daltons); Pepsin: pepsin diluted in gastric simulated fluid; Try, Chy: Trypsin and chymotrypsin diluted in gastrointestinal fluid; WPH 8%: whey protein hydrolysate 8% (w/v); G: Gastric phase; I: Intestinal phase; β-Lg: β-lactoglobulin; 0 – 120 min: time of digestion.

3.2 RP-HPLC PEPTIDE PROFILES

The peptide profiles confirm what was aforementioned, showing the effects of processing and simulated gastrointestinal digestion in the hydrolysis of major whey proteins α-lactalbumin (α-la) and β-lg. Different from β-lg, α-la was extensively hydrolyzed during the hydrolysis process before the in vitro gastrointestinal digestion. The acid pH used in the reaction possibly altered the α-la globular structure, exposing its hydrophobic interior and increasing its susceptibility to proteolysis by pepsin, with subsequent generation of several peptides (Kamau, Cheison, Chen, Liu, & Lu, 2010; Nik et al., 2010). After the intestinal phase, β-lg was also hydrolyzed, and a wide variety of peptides was obtained, including by hydrophobic and hydrophilic molecules, as shown in Figure 3C.

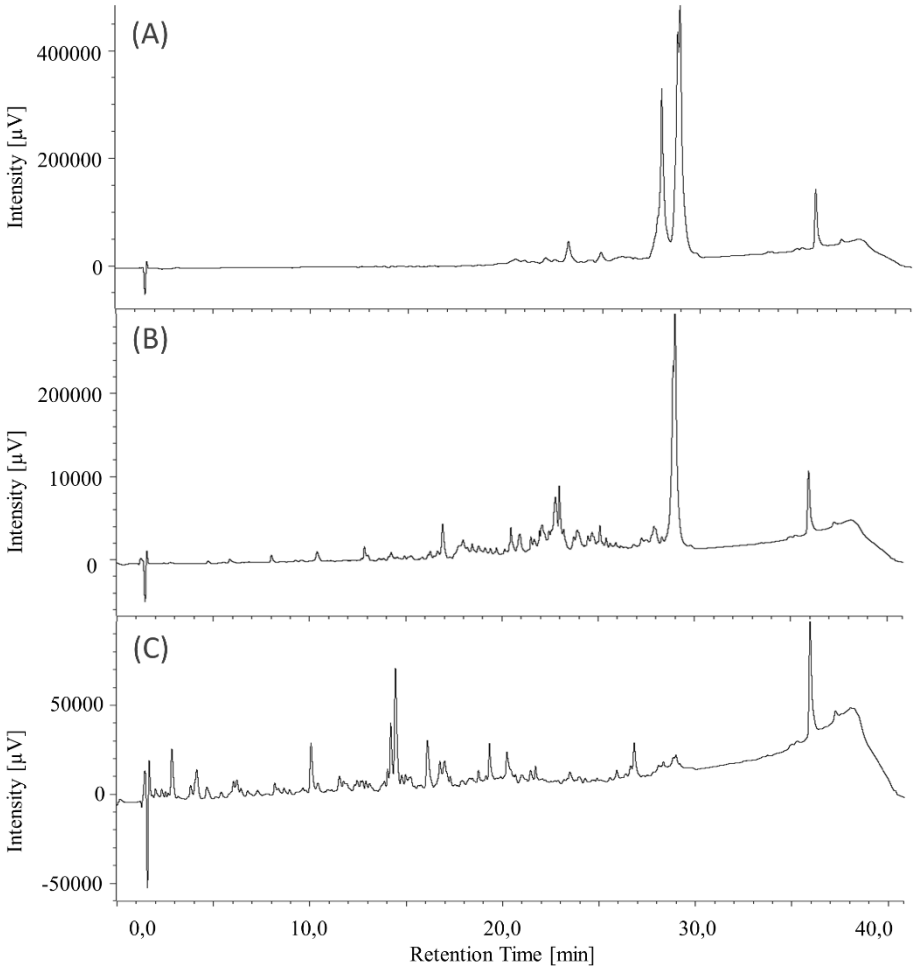

Figure 3. RP-HPLC peptide profiles of (A) control sample – WPC88; (B) undigested whey hydrolysate; (C) intestinal digested whey hydrolysate. α-la: α-lactalbumin; β-lg: β-lactoglobulin.

3.3 PEPTIDE SEQUENCES

Several new peptides generated during the in vitro gastrointestinal digestion process were identified by MS analysis. Figure 4 presents a heat map comparing peptide profiles of the undigested samples with gastric and intestinal whey digested samples, presenting the frequency, in percentage, that each amino acid appears as part of a peptide sequenced from a determined protein. β-lg and α-la were chosen to be part of the graph because of the large number of peptides identified from these proteins, as well as κ-casein and β-casein, remained from the cheese-making process. All other peptides sequenced are listed in the Supplementary Table S1.

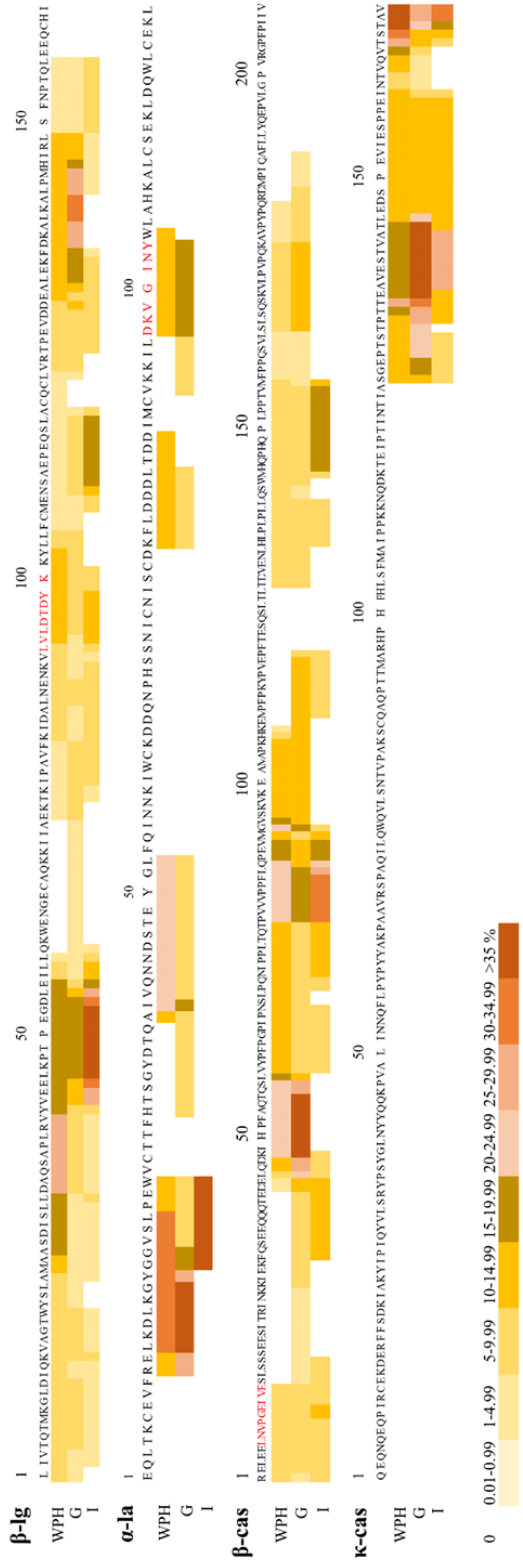

Figure 4. Nano-RSLC – MS/MS heat map built with the frequency of appearance, in percentage, that each amino acid was identified as part of a peptide sequence from β-Lactoglobulin (β-lg), α-Lactalbumin (α-la), β-Casein (β-cas) and κ-Casein (κ-cas) after whey hydrolysate (WPH) *in vitro* gastrointestinal digestion. G: gastric phase; I: intestinal phase. White color represents no frequency and dark orange high frequency. Letters in red represent ACE inhibitory peptide sequences identified in the samples through the Biopep database.

The partial resistance of β-lactoglobulin to enzymatic hydrolysis was also confirmed by the MS analysis, once peptides from different portions of the protein were sequenced in the three fractions analyzed, showing that only part has been hydrolyzed to small peptides and free amino acids undetectable by the method used. Notably, the peptide VLDTDYK, which was previously described as an in vitro inhibitor of ACE (Hernández-Ledesma, Recio, & Amigo, 2008; Mann, Athira, Sharma, Kumar, & Sarkar, 2019; Tavares & Malcata, 2013), was found among the 56 peptides generated during the intestinal phase. As previously discussed, α-lactalbumin is very susceptible to acidic conditions and was totally cleaved during the hydrolysis process performed with pepsin (Nik et al., 2010). The MS analysis confirmed this susceptibility, once only 17 peptides could be sequenced from the different fractions of the whey analyzed, showing that the protein was cleaved in small fragments and free amino acids. Among the identified peptides, DKVGINY, also previously described as an in vitro inhibitor of ACE (Tavares & Malcata, 2013) (Egger, Ménard, Delgado-Andrade, Alvito, Assunção, Balance, Barberá, Brodkorb, Cattenoz, Clemente, Comi, Dupont, Garcia-Llatas, Jesús Lagarda, et al., 2016) was found in the gastric fluid. Nevertheless, DKVGINY was probably hydrolyzed by intestinal enzymes, once it could not be identified after enteric digestion.

Whey is obtained during the cheese-making process when κ-casein is cleaved between the Phe105-Met106 amino acids, being separated into two main fragments, the hydrophobic para-κ-casein (Glu1−Phe105) that forms the cheese curd and the hydrophilic caseinomacropeptide (CMP) (Met106−Val169), which is dissolved in whey. During this process, the casein micelle is broken, and part of the caseins may get lost in the whey (Hallén, Lundén, Allmere, & Andrén, 2010; Kastberg Møller, Rattray, Sørensen, & Ardö, 2012), which justifies the identification of β-casein peptides in the different whey fractions analyzed. The evolution of the β-casein digestion can also be observed in the heat map (Figure 4), by the increasing blanc spaces and by the reduction and enhancement of the intensity of the colors in different areas. Even so, LNVPGEIVE, another peptide with inhibitory effect on ACE in in vitro assays was found in the undigested and gastric digested samples, and both VVVPPF and VYPFPGPI were identified encrypted within the structure of other β-casein peptides from the intestinal fraction. Concerning κ-casein, as revealed in Figure 4, no peptide was identified from the para-κ-casein portion (Glu1−Phe105) of the protein, and from the hydrophilic CMP, 45 peptides were sequenced among which eight present ACE-inhibitory peptides encrypted. Although this study has not been designed to explore the relationship between these peptides

and their effectiveness as ACE inhibitors, it is reasonable to suggest that their presence in the digested hydrolysate reinforce the putative beneficial effects of whey against cardiovascular diseases.

3.4 INFLUENCE OF DIGESTION ON WHEY-INDUCED VASCULAR RELAXATION

The experiments performed to evaluate the vascular effects of both undigested and intestinal digested whey hydrolysates revealed that both presented vasodilatory activity, as illustrated in the typical records showed in Figure 5A, which shows the concentration-dependent relaxation induced by undigested and digested hydrolysates in endothelium-intact arteries previously stimulated by the □1-adrenergic receptor agonist phenylephrine. As shown in Figures 5B and 5C, the vasodilatory effect induced by the undigested hydrolysate does not depend on the endothelial function, since there was no difference between the vascular relaxation obtained in endothelium-intact (maximal effect of 94.6 ± 1.2%) and endothelium-denuded (maximal effect of 97.2 ± 1.6%) arteries. Nonetheless, the gastrointestinal digested hydrolysate resulted in a maximal relaxation of 84.2 ± 1.6% in endothelium-intact preparations, but it was completely ineffective in arteries without endothelial function (Figures 5C and 5D). Importantly, if in one hand the gastrointestinal digestion resulted in a loss of activity in endothelium-denuded arteries, on the other hand, the concentrations of the digested hydrolysate required to induce vasodilation in endothelium-intact preparations were significantly reduced, compared with the undigested hydrolysate. This is important to understand the mechanisms involved in the vasodilatory effect, as well as possible adverse symptoms of the consumption of the peptides, which will be further investigated. Indeed, the half maximal effective concentration for the vasodilatory effect, a parameter often used to compare the potency of different drugs was reduced from 5017 (4740-5270) $\mu g.mL^{-1}$ for undigested to 1.07 (0.75-1.51) $\mu g.mL^{-1}$ for the digested hydrolysate. Thus, the molecular changes occurred during gastrointestinal digestion, including the generation of 198 identified peptides in gastric and intestinal phases, resulted in a significant increase in the potency of the vasodilatory effect of the whey hydrolysate, as well inactivation of the peptides associated with the endothelium-independent effect.

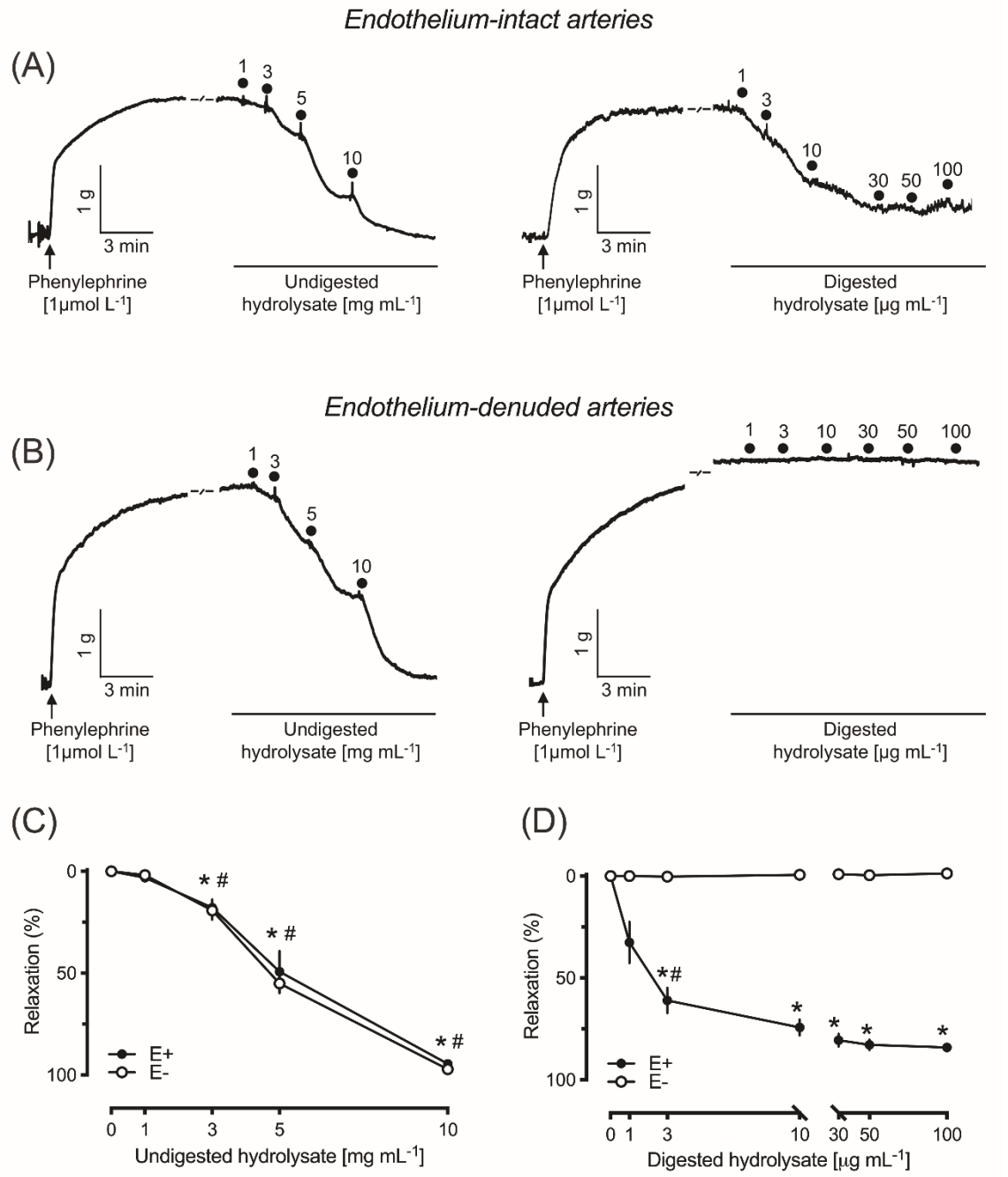

Figure 5. Whey hydrolysates-induced vascular relaxation. Trace records of the vascular relaxation induced by undigested and digested whey hydrolysates in endothelium-denuded (A) and endothelium-intact (B) rat aortic rings. The mean ± SEM of relaxation of arteries from 6 different animals after incubation of the undigested and digested hydrolysates are presented in panels C and D, respectively. * indicates $p < 0.05$ compared with the maximal vascular tone before de addition of the hydrolysate. # indicates $p < 0.05$ compared with the previous concentration.

CONCLUSIONS

The gastrointestinal digestion of the whey hydrolysate generated a diverse molecular profile, composed by hydrophobic and hydrophilic peptides. Among these molecules, 198

peptides were sequenced from the gastric and intestinal digested samples, some of them previously described as ACE inhibitors. The molecular changes occurred during gastrointestinal digestion potentiated the endothelium-dependent vasorelaxant activity of the whey hydrolysate. The results obtained in the present study are very promising, demonstrating that the digestion process enhanced the potential effects of the WPH on the cardiovascular system. New research is being carried out to best evaluate the bioaccessibility and mechanisms involved in the vascular relaxation promoted by the whey peptides, so as it can be further used as a biofunctional ingredient by food and pharmaceutical industries.

Acknowledgments

Authors would like to thank Alibra Ingredientes Ltda for supplying WPC, and Bela Vista Produtos Enzimáticos for supplying the commercial pepsin.

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SUPPLEMENTARY MATERIAL

List of peptides identified from all digested and undigested samples:

AASDISLLDAQSAPLRV
AASDISLLDAQSAPLRVY
AASDISLLDAQSAPLRVYVE
AASDISLLDAQSAPLRVYVEELKPTPEGDLEIL
AASDISLLDAQSAPLRVYVEELKPTPEGNLEIL
AASDISLLDAQSAPLRVYVEQLKPTPEGDLEIL
YVEELKPTPEGDLEILL
QSLVYPFPGPIPNSLPQNIPPLTQT
QSWMHQPHQPLPPTVM
RELEELNVPGEIVE
SDISLLDAQSAPLRV
TDVENLHLPLPLLQS
TIKYLKSLFSHA
STVATLEDSPEVIESPPEINT
TLEELHYSPEGSLHTRG
SLSQSKVLPVPQKAVPYPQRDMPIQ
AIVQNNDSTEYGL
ALNENKVLVL
ALNENKVLVLDTDYKKYLL
AMAASDISLLDAQSAPLRV
AQSAPLRVYVEELKPTPEGDLEIL
ASDISLLDAQSAPLRV
AVESTVATL
DAQSAPLRVYVEELKPTPEGDL
DAQSAPLRVYVEELKPTPEGDLE
GVSKVKEAMAP
FVAPFPEVF
LIVTQTMKGLDIQKVAG
LIVTQTMKGLDIQKVAGTW
LIVTQTMKGLDIQKVAGTWYSLA
LIVTQTMKGLDIQKVAGTWYSLAM
LKDLKGYGGVSL
FFVAPFPEVF
LVYPFPGPIHNSLPQNIPPLTQT
LVYPFPGPIPNSLPQNIPPLTQT
LYYANKYNGVF
MAASDISLLDAQSAPLRV
MAASDISLLDAQSAPLRVY
MAASDISLLDAQSAPLRVYVEELKPTPEGDLE
IL
MAASDISLLDAQSAPLRVYVEQLKPTPEGDLE
IL
GVSKVKEAMAPKHK
MELGHKIMRNLENTVKETIKY
KFLDDDLTDD
KIDALNENKV
KIDALNENKVLVLD
KIDALNENKVLVLDTDYKKYLL
KIDALNENKVLVLDTDYKKYLLF
KIDALNENKVLVLDTDYKKYLLFCMENSAEP
EQSLACQCL
KIDALNENKVLVLDTDYKKYLLFCMENSAEP
EQSLVCQCL
RELKDLKGYGGVSL
TTEAVESTVATLEDSPEVIEGPPEINT
VAKMLGVPVNRIL
VELLKHKPKATEEQLKT
VFEAGRDPYKLRPVA
VLDTDYKKYLLF
VRTPEVDDEALEKFD
VRTPEVDDEALEKFDKALKALPMHIRL
VRTPEVDDEALEKFDKALKALPMHIRLSFNPT
QL
VVVPPFLQPEVM
YSLAMAASD
YSLAMAASDISL
YSLAMAASDISLL
YSLAMAASDISLLDAQSAPLRV
YVEELKPTPEG
DAQSAPLRVYVEELKPTPEGDLEIL
DEALEKFDKALKALPMHIRL
DKLKHLVDEPQNL
DKVGINYW
DTDYKKY
DTDYKKYLLF
EALEKFDKALKALPMHIRL
IIVTQTMKG
IIVTQTMKGLD
IIVTQTMKGLDIQKVAGTW
ISLLDAQSAPLRVY
IVTQTMKGLDIQKVAGTW
LISKEQIVIR
LIVTQTMKG
LDAQSAPLRVYVEELKPTPEGDLE
LDAQSAPLRVYVEELKPTPEGDLEIL
LDIQKVAGTW
PFDEHVKL
PVVVPPFLQPEVMGV
PVVVPPFLQPEVMGVSKVKEAMAPK
PVVVPPFLQPEVMGVSKVKEAMAPKH
QDKIHPFAQTQ
QINNKIWCKDDQNPHSSNICNISCDKFLDDDL
TDDIMCVKKILDKVGINY
ALEKFDKALKALPMHIRL
DAQSAPLRV
DAQSAPLRVY
GVSKVKEAMAPK
GVSKVKEAMAPKH
DKALKALPMHIRL
DELQDKIHPFAQTQSL
DKTEIPTINT
DLISKEQIVIR
DTDYKKYLL
IRNLQISNEDL
IQKVAGTW
ISLLDAQSAPLRV

IVQNNDSTEYGL
IVTQTMKGLD
KADEKKFWGKY
KALKALPMHIRL
EKFDKALKALPMHIRL
EKTKIPAVF
MAIPPKKNQD
MAIPPKKNQDKTEIPTINT
MELGHKIMRNLE
MELGHKIMRNLEN
LEKFDKALKALPMHIRL
LIVTQTMKGLD
LKDLKGY
LKSLFSHA
LNVPGEIVE
LPMHIRL
LQDKIHPFAQTQSL
LRIPSKVDSAL
VRTPEVDDE
VRTPEVDDEA
VRTPEVDDEAL
VRTPEVDDEALE
VYPFPGPIHNSL
VYPFPGPIHNSLPQNIPPLTQT
VYPFPGPIPNSL
VYPFPGPIPNSLPQNIPPLTQT
WMHQPHQPLPPTVM
QSAPLRV
FDKALKALPMHIRL
FPPQSVL
KIDALNENKVLVL
KIHPFAQTQS
YVEELKPTPEGDLE
YVEELKPTPEGDLEIL
KIHPFAQTQSL
KTKIPAVF
LDAQSAPLRV
LDAQSAPLRVY
KFDKALKALPMHIRL
QDKIHPFAQTQS
QDKIHPFAQTQSL
QDKTEIPTINT
RELKDLKGY
SSRQPQSQNPKLPLSIL
TVKETIKYLKSL
VATLEDSPEVIESPPEINT
VKETIKY
SLSQSKVLPVPQK
SLSQSKVLPVPQKAVPYPQ
SQKFPKAEF
TDVENLHLPLPLL
VLDTDYKKYLL
NAVPITPTL
ISKEQIVIR
IESPPEINT
ILNKPEDETHL
EASPEVIESPPEINT
EDSPEVIEGPPEINT
EDSPEVIESPPEINT
ELKPTPEGDL
ELKPTPEGDLE
LEDSPEVIESPPEINT
LEDSPEVIEGPPEINT
GGVSLPEW
ATLEASPEVTESPPEINT
ATLEDSPEVIESPPEINT
IASGEPTSTPTIE
IASGEPTSTPTIEA
IASGEPTSTPTTE
IASGEPTSTPTTEA
EAQPTDASAQF
PQNIPPLTQT
PVVVPPFL
PVVVPPFLQ
PVVVPPFLQPEV
PVVVPPFLQPEVM
PVVVPPFLQPEVMG
VQVTSTAV
RELEELNVPGE
YVEELKPTPEGD
YVEELKPTPEGDL
VEELKPTPEGD
VEELKPTPEGDL
VEELKPTPEGDLE
SFNPTQL
SPEVIEGPPEINT
SPEVIESPPEINT
TLEDSPEVIEGPPEINT
TLEDSPEVIESPPEINT
VEELKPTPEGDLEIL
AILNKPEDETHL
AIPPKKNQDKTEIPTINT
ALEKFDKALKA
ALEKFDKALKAL
ALEKFDKALKALPM
ALEKFDKALKALPMH
AQKKIIAE
AQKKIIAEKTKIPAVF
HTSGYDTQAI
DEALEKFDKALKA
DELQDKIHPFAQT
DELQDKIHPFAQTQS
DIQKVAGTW
DKALKALPM
DKALKALPMH
DKVGINY
DTDYKKYL
DTHKSEIAHRFKDLGEEHFKG
EKFDKALKA
EKFDKALKALPM
EKFDKALKALPMH
EKTKIPAV
ELKPTPEGDLEI
DVENLHLPLPLL
EALEKFDKALKA
ESAPLRVY

ETTEHTPSDASTTEGKL
FDKALKALPMH
FKIDALNE
FDKLKHLVDEPQNL
FRELKDLKGY
GVSKVKEAMAPKHKEMPFPKY
GVSKVKEAMAPKHKEMPFPKYPVE
IESPPEIN
KFLDDDL
KIEKFQSEEQQQTEDE
KIEKFQSEEQQQTEDEL
KIHPFAQT
KSLFSHA
KTEIPTINT
KTEIPTINTI
KTKIPAV
IHPFAQTQSL
IRNLQISNE
IRNLQISNED
ISKEQIVIRS
IVTQTMKGL
KALKALPMH
KALPMH
KALPMHIRL
KFDKALKA
KFDKALKALPM
KFDKALKALPMH
KIDALNE
HKEMPFPKYPVE
KVKEAMAPKHK
LDTDYKKYL
LDTDYKKYLL
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LEDSPEVIESPPEIN
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LEKFDKALKAL
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LQDKIHPF
LQDKIHPFAQT
LQDKIHPFAQTQS
LQKWEND
LRLKKYKVPQL
LKPTPEGDLE
LKALPM
LKALPMH
LKALPMHIRL
LNENKVL
LVRTPEVDD
LVRTPEVDDE
LVRTPEVDDEA
LVRTPEVDDEAL
MELGHKIMRNLENT
MAIPPKKNQDKTEIPTINTI
MAIPPKKNQDKTEIPTIN
PVVVPPFLQPEIM
PPKKNQDKTEIPTINTIA
PVVVPPFLQPEVMGVS
QDKIHPFAQT
QKVAGTW
RELKDLKGYGG
RELKDLKG
QSWMHQPHQPLPPTV
QSAPLRVY
RLKKYKVPQL
RNAVPITPTL
RPKHPIKHQGLPQE
NKVLVLDTDYKKYLL
SDIPNPIGSENSEKTTM
SKEPSISREDLISKEQIVIR
SLSQSKVLPVPEKAVPYPQRDMPIQ
SLSQSKVLPVPQ
SLSQSKVLPVPQKAVPYPQRD
SLSQSKVLPVPQKAVPYPQRDMPIQA
SPEVIESPPEIN
SQKFPKAE
SRQPQSQNPKLPLSI
SRQPQSQNPKLPLSIL
SSRQPQSQNPKLPLSI
TDVENLHLPLP
TDYKKYLL
TIASGEPTSTPTTE
TIKYLKSL
TINGVQYPLSPSA
TQTMKGLD
TRKVPQVSTPTL
TSTPTTEAVESTVATLEDSPEVIESPPEINT
TTEHTPSDASTTEGKL
TVKETIKY
VEKDAIPENLPPLTA
VEKDAIPENLPPLTAD
VKITVDDKHYQ
VKITVDDKHYQK
VKKILDKVGINY
VEELKPTPEGDLEI
VRTPEVDD
WLKPDPSQKQTF
YANKYNGVF
YQKFPQY
VTQTMKGLD
VEELKPTPEG
SAEPEQSL
NQDKTEIPTINT
NSAEPEQSL
NSAEPEQSLA
PEVIESPPEINT
MENSAEPEQSL
MENSAEPEQSLA
EASPEVIESPPEIN
LPMHIR
LKPTPEGDL
KVAGTW
EELKPTPEGDL
EDSPEVIESPPEIN
ENSAEPEQSL

DAIPENLPPLTA
DAIPENLPPLTAD
DTVQVGGISDTNQIF
DQGLVSQDLF
DTGSSNLW
EIVPNSAEER
ELKPTPEGD
FQSEEQQQTEDE
FQSEEQQQTEDELQDK
FTSHIESEEMHDAPK
GLDIQK
GLSETEPGSF
GTIGIGTPAQDF
HLPLPLL
HQGLPQEVL
HQPHQPLPPTV
HQPHQPLPPTVM
IDALNENK
IGDEPLENY
AMAASDISLL
IGDEPLENYLDTEY
IITHPNFNGNTLDNDIM
IPAVFK
IVTQTMK
KIDALNENK
LDAQSAPLR
LGEHNIDVLEGNEQF
LSFNPTQL
LSSNDDSGSVVL
LVLDTDYK
MAIPPK
MHQPHQPLPPTVM
NIPPLTQT
NKPEDETHL
NQDKTEIPTIN
PFPGPIHN
PVVVPPF
PVVVPPFLQPEIMG
SSGSSYPSLL
TEIPTIN
TEIPTINT
TKIPAVF
TPEVDDEAL
TPEVDDEALEK
TPEVDDEALEKF
TSHIESEEMHDAPK
VAPFPEV
VEELKPTPEGDLEILL
VEELKPTPEGDLEILLQ
VLDTDY
VLDTDYK
VLVLDTDYK
VPQVSTPTL
VYPFPGPIHN
VYPFPGPIPN
VYVEELKPTPEGDLEIL

Chapter 4

Brush border peptidase activity and intestinal transepithelial passage of oligopeptides from a whey protein hydrolysate in Ussing Chamber ex-vivo assays

Luísa Ozorio[1]; Caroline Mellinger-Silva[2]; Lourdes M. C. Cabral[2]; Julien Jardin[3]; Gaelle Boudry4; Didier Dupont[3]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil;

[2]EMBRAPA Agroindústria de Alimentos, Rio de Janeiro, Brasil;

[3]STLO, INRA Agrocampus Ouest, Rennes, France.

[4]Institut Numecan, INRA, INSERM, Univ Rennes, Saint-Gilles, France

Declarations of interest: none

ABSTRACT

For many years it was believed that only amino acids, dipeptides and tripeptides could reach the bloodstream. Nowadays, the bioavailability of oligopeptides is also being considered, requiring further research. Hence, the activity of the brush border enzymes on undigested (WPH) and digested (ID) whey hydrolysates and the absorption of the resultant peptides through the small intestine of piglets was investigated. Among all samples, 884 oligopeptides were sequenced. The brush border peptidase activity was intense in the first 10 min of the transepithelial passage assays, generating several new peptides. Among the oligopeptides absorbed, 85 resisted to digestion and absorption assays, more than 50% were from β-lactoglobulin, 20% were β-casein fragments and 37 have been previously reported as bioactive peptides. This study is a novel evidence of the proteolytic activity of the brush border enzymes on oligopeptides and an important step to confirm the bioavailability of these peptides with biofunctional potential.

Keywords: whey protein hydrolysate; Ussing Chamber; intestine; absorption; brush border peptidases

1. INTRODUCTION

Whey proteins present high nutritional value and bioactive peptides within their structures which may be released either via food processing or during digestion. In the gastrointestinal tract numerous scattered enzymes release these peptides from the native proteins, beginning in the stomach by the activity of pepsin (Miner-Williams, Stevens, & Moughan, 2014; Plaisancié et al., 2013). Pepsin, however, is only able to hydrolyze 10-15% of dietary proteins and the pancreatic proteases trypsin, chymotrypsin, elastase and carboxypeptidases continue the digestion process in the intestine, cleaving large molecules into free amino acids (~30%) and oligopeptides (~70%) (Goodman, 2010).

In the small intestine, brush border enzymes, including aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases, represent the final stage of peptide digestion prior to their absorption into enterocytes, reducing most poly- and oligopeptides into their monomer constituents (Hooton, Lentle, Monro, Wickham, & Simpson, 2015). Some dairy peptides are known for exerting their bioactivity prior to absorption directly in the intestinal lumen, by stimulating mucus production, modulating mineral absorption, satiety, inflammation and diabetes (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014).

Before absorption, peptides may also undergo intracellular digestion by cytosolic, lysosomal and microsomal enzymes, and the indigestible ones are mostly excreted in the urine or bile (Hooton et al., 2015; Vij, Reddi, Kapila, & Kapila, 2016). Peptides able to achieve the bloodstream are yet susceptible to plasma enzymes and the resistant ones may reach target organs and play different roles as antioxidant, antihypertensive, opioid, anticancer and antidiabetic molecules (Choi, Sabikhi, Hassan, & Anand, 2012; Fernández-Musoles et al., 2013; Hernández-Ledesma, Del Mar Contreras, & Recio, 2011; O'Keeffe & FitzGerald, 2018).

Different pathways are involved in the absorption of amino acids and peptides. Amino acids, di- and tripeptides uptake generally occur via passive diffusion, tight junctions, endocytosis or transcellular carrier-mediated transport. There are also some transmembrane co-transporters such as the proton dependent H^+/peptide PepT1 and the sodium dependent oligopeptide transporters SOPT1 and SOPT2. For many years it was believed that only free amino acids, di- and tripeptides could be absorbed through the brush border membrane (Miner-Williams et al., 2014) and the evidences on the absorption of oligopeptides, as well as the

mechanisms involved in this process are still limited in knowledge, requiring further investigation.

In this sense, the present study aimed to investigate the molecular alterations generated by the intestinal brush border peptidases on undigested and digested whey hydrolysates, as well as the absorption of these peptides by the small intestine of piglets using the Ussing chamber (UC) ex-vivo absorption model, as another step towards understanding their absorption, bioavailability and possible bioactivity.

2. MATERIALS AND METHODS

2.1 MATERIALS

Bovine whey protein concentrate 88% (WPC88) and commercial pepsin (E.C. 3.4.23.1) from porcine gastric mucosa were gently donated by Alibra Ingredientes Ltda (Campinas, SP, Brazil) and Bela Vista Produtos Enzimáticos Ind. e Com. Ltda. (Bela Vista, SC, Brazil), respectively.

2.2 WHEY ENZYMATIC HYDROLYSIS

The WPC88 was hydrolyzed in pilot scale (100L) according to Ozorio et al. (2019). Briefly, a 1.25% (w/v) solution of WPC88 was prepared and the pH was adjusted to 2 (1M HCl). Pepsin was added (5.34 $\mu U.g^{-1}$ of protein) and the hydrolysis lasted for 3h at 37°C under constant stirring. pH and temperature were controlled during the whole experiment. Pepsin was inactivated by increasing the temperature to 80°C for 5 min. The hydrolyzed whey solution was then spray dried (Niro atomizer, Copenhagen, Denmark. Inlet temperature: 170°C; outlet temperature: 90°C) and the powder was maintained frozen until further use and analysis.

2.3 WHEY HYDROLYSATE *IN VITRO* DIGESTION

The in vitro gastrointestinal digestion of the whey protein hydrolysate obtained (WPH) followed the harmonized protocol for in vitro digestion INFOGEST (Minekus et al., 2014). As samples present high protein/peptides concentration, the oral phase of the in vitro digestion was not performed. The WPH was suspended (8% w/v) in distilled water and mixed to a simulated gastric fluid containing pepsin from porcine gastric mucosa (2000 $U.mL^{-1}$, Sigma-Aldrich, St.

Louis, MO, USA) at a ratio of 50:50 (v/v). After 120 min, pepsin was inactivated by increasing the pH to 7 with 2 M NaOH and the simulated intestinal fluid (50:50 v/v) containing trypsin (100 $U.mL^{-1}$ of final mixture), chymotrypsin (25 $U.mL^{-1}$ of final mixture) and bile (10 $mmol.mL^{-1}$ of final mixture) was added. The digestion was carried out in a water bath (Grant, OLS 200, Cambridgeshire, England) at 37°C under constant orbital stirring at 100 rpm. The WPH was suspended in the same fluids, except for the addition of enzymes to perform the same analysis, as a control sample. Samples were kept frozen (-20°C) until further use or analysis.

2.4 *EX-VIVO* BRUSH BORDER HYDROLYSIS AND INTESTINAL TRANSEPITHELIAL PASSAGE ASSAYS - USSING CHAMBER

The ex-vivo absorption assays of WPH and ID were conducted under the guidelines of the French Ministry of Agriculture for animal research. An 6-8 weeks-old piglet, mixed race (Large white x Landrace x Duroc) was euthanized with ketamine/T61. After laparotomy, a 30 cm segment of the proximal jejunum, beginning at the ligament of Treitz was removed and placed in ice-cold Krebs (6.2 g NaCl, 0.32g KCl, 0.15g KH_2PO_4, 0.54g $MgSO_4$, 1.86g $NaHCO_3$, 0.01 g $CaCl_2$). Tissue was dissected to remove the muscle layers and it was cut in 12 sections to be used in the Ussing Chambers (Physiologic Instrument, San Diego, USA). 1-cm^2 tissue sections were exposed to 2.5 mL Krebs-mannitol (10 mM) and 2.5 mL Krebs-glucose (10 mM) solutions on the mucosal and serosal sides, respectively. Solutions were maintained at 39°C, constantly stirred and oxygenated with 95% 0_2 - 5% CO_2. 200 µL of the mucosal compartments were aliquoted and this volume was replaced by WPH and ID solutions in 5 chambers each, the other two chambers were used as controls without sample addition. Samples (200 µL) were withdrawn at 10, 60 and 120 min and were immediately frozen for further analysis. Fresh buffer was added each time to maintain a constant volume.

2.5 TANDEM MASS SPECTROMETRY

Mass spectrometry (MS) analysis was conducted as previously described by Deglaire et al., 2016. Briefly, a nano-RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) coupled to a nanoelectrospray ion source was used.

Ussing Chamber aliquoted solutions were evaluated concerning the release of amino groups along the time of experiment (data not shown) and the 3 highest concentrated samples were selected to perform the MS analysis. Sample solutions were diluted in acidic buffer (0.1%

TFA, 2% ACN) and filtered (0.45µm cut-off) prior to the analysis. Samples were concentrated on a µ-precolumn pepMap100 (C18 column (300 µm i.d. × 5 mm length, 5 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) and separated on a PepMap RSLC column (C18 column, 75 µm i.d. x 150 mm length, 3µm particle size, 100 Å pore size; Dionex). Runs occurred at a flow rate of 0.3 µL.min-1 using solvents A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA] and B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA]. Peptides were eluted as follows: 5 - 35% B over 40 min; 85% B for 3 min, followed by column re-equilibration. The mass spectra were recorded in positive mode using the m/z range 250-2000. The resolution of the mass analyzer for m/z of 200 amu (atomic mass unit) was set in the acquisition method to 70000 for MS and 17500 for MS/MS. For each MS scan, the ten most intense ions were selected for MS/MS fragmentation and excluded from fragmentation for 20 s.

X!TandemPipeline software (https://pubs.acs.org/doi/abs/10.1021 /acs.jproteome.6b00632J. Proteome Res. 16, 2, 494-503) was used to identify peptides from the MS/MS spectra against a homemade database composed of major milk proteins to which was added the common Repository of Adventitious Protein (http://thegpm.org/crap). The possible post-translational modifications were serine or threonine phosphorylation, methionine oxidation. Peptides identified with an e-value < 0.05 were automatically validated. The peptide false discovery rate was less than 0.6%. Dairy peptides found in the control chambers were considered carryovers and were excluded from the other samples.

3. RESULTS AND DISCUSSION

Numerous peptides can be released from their respective native proteins during food processing and may be further hydrolyzed through gastrointestinal digestion, resulting in a pool of peptides with different sequences and lengths (Toldrá, Reig, Aristoy, & Mora, 2018). In the present study, 884 dairy peptides were sequenced in total, among which 619 were found in WPH solution and presented an average molecular weight (MW) of 1527 Da. After in vitro digestion 442 dairy peptides with an average MW of 1452 Da were found in the ID solution, as can be observed in Figure 1. Among these 442 ID dairy peptides, 360 had resisted to the digestion process, since they were also present in the WPH starting material, while the other 82 peptides resulted from the proteolytic activity of gastric and pancreatic enzymes. Both WPH and ID

samples were then exposed to a segment of a piglet's small intestine on an assay to simulate peptides brush border hydrolysis and mucosal absorption.

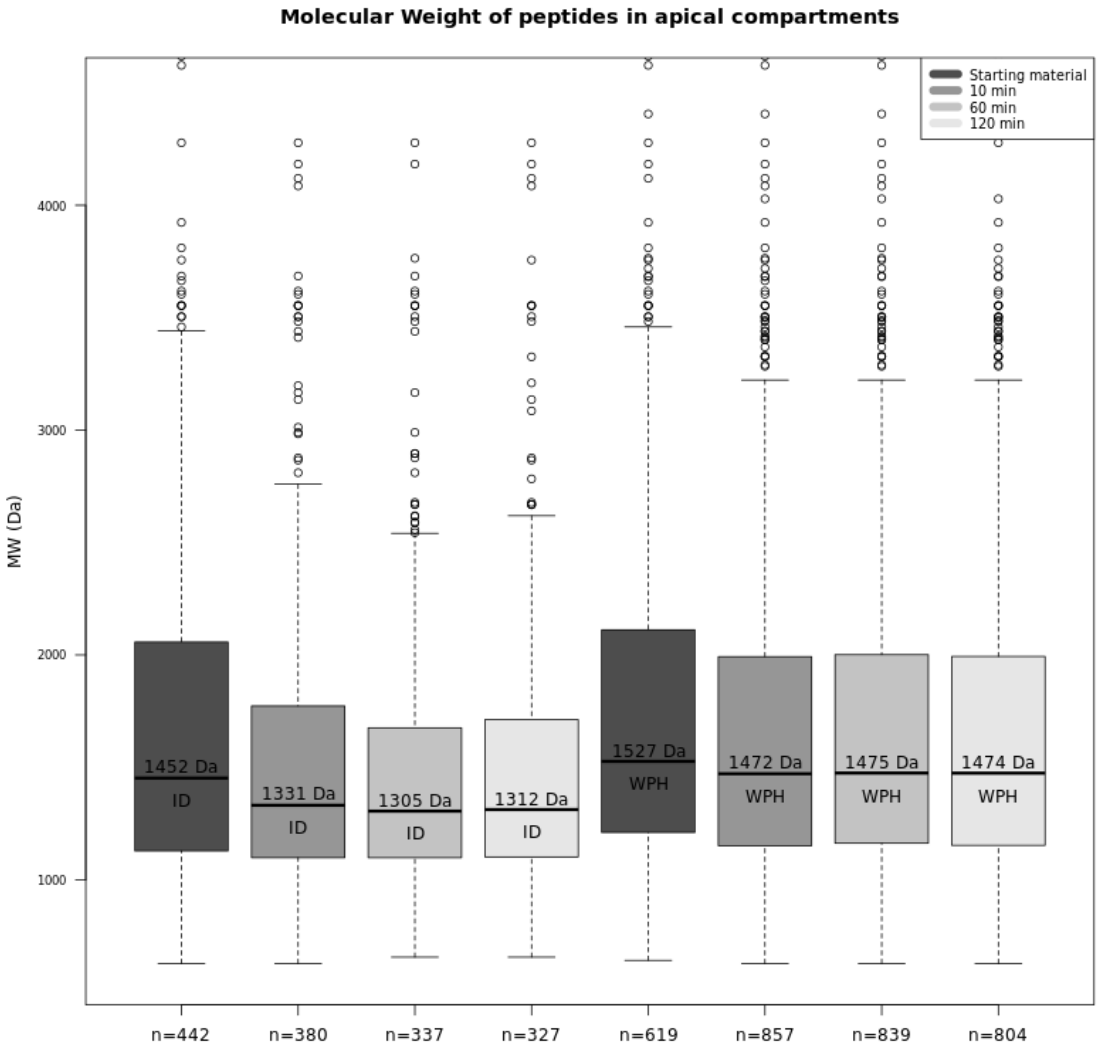

Figure 1. Average molecular weight and total number of peptides identified for undigested (WPH) and digested (ID) whey hydrolysate during Ussing Chamber absorption assay in apical compartments.

The small intestine presents several brush border and intracellular peptidases with affinity for a large number of potential substrates that will be further absorbed to exert different functions in the body (Miner-Williams et al., 2014; Pauletti et al., 1996). In the present study, it was identified a brush border peptidase activity during the first 10 min of experiment, as there was a decrease in the average MW of dairy peptides in the mucosal compartment for both WPH and ID samples, which remained stable after that. Moreover, the number of dairy peptides identified decreased from 442 to 380 after 10 min in the mucosal compartment for ID samples, while for

the WPH ones, it increased from 619 to 857. The MS method used identifies only peptides with more than 6 amino acid residues, in this sense, the activity of the enteropeptidases on the ID solution may have generated small peptides and free amino acids which could not be sequenced, while for WPH samples, initial dairy peptides identified presented a higher average MW, and were probably hydrolyzed to new ones able to be identified, explaining the different results observed for both type of samples. Along the rest of the experiment, the decrease in the number of peptides observed may be attributed to their dilution in the Ussing Chambers, since the volumes withdrawn from the compartments were replaced by the respective Krebs-mannitol (10 mM) and Krebs-glucose (10 mM) physiological solutions on the mucosal and serosal sides.

Before absorption, dairy peptides and amino acids may play an important role in gut health promotion, stimulating mucous formation, preventing gastrointestinal barrier-related disorders and strengthening the barrier function of the intestine against toxins, bacteria, self-digestion and physical abrasions (Fernández-Tomé et al., 2017; Schaafsma, 2007). Fernández-Tomé et al. (2017) and Martínez-Maqueda et al. (2013) showed that β-lactoglobulin (β-lg) and β-casein (β-cas) hydrolysates were able to stimulate mucins' secretion in vitro and in vivo, and some of the peptides identified by them were also found in the mucosal compartment of the chambers containing both WPH and ID samples (β-lg: LIVTQTMK, GLDIQK, SLAMAASDISLL, VYVEELKPTPEGDLEIL, VEELKPTPEGDLEIL, VEELKPTPEGDLEILLQK, KIDALNENK, IDALNENK, VLVLDTDYK, VLDTDYK, TPEVDDEALEK, EVDDEALEKFDK, EVDDEALEK, LSFNPTQLEEQ, LSFNPTQL, LSFNPTQLEEQCHI, SFNPTQLEEQCHI; β-cas: QDKIHPFAQTQS, RELEELNVPGE, VYPFPGPIPNSL, PVVVPPFLQPEV, PVVVPPFLQPEVM, GVSKVKEAMAPK, TDVENLHLPLPLL, LTDVENLHLPLPLL, VENLHLPLPLL, WMHQPHQPLPPTVM, FPPQSVL; αs1-casein: RPKHPIKHQGLPQEVL, FVAPFPEVFGKEKVNE, LRLKKYKVPQL; κ-casein: VQVTSTAV). Thus, it can be suggested a possible influence of WPH and ID dairy peptides in the formation of the mucus gel layer cover, which needs to be further investigated.

The selectivity of the intestinal barrier, as well as the proteolytic activity of the brush border enzymes was confirmed in the present study, as 360 and 286 dairy peptides from the WPH and ID samples, respectively, were found in the serosal compartment of the chamber after the 120 minutes assay (Figure 2). Interestingly, the average MW of the peptides found in the serosal compartment was higher than that of the WPH and ID samples before the absorption assays,

showing that oligopeptides can also be absorbed. The absorption of small peptides, however, cannot be disregarded, since the method used cannot identify peptides with less than 6 amino acid residues. Little is known about the absorption of large peptides, and according to Horner, Drummond, & Brennan (2016) small peptides can be absorbed by specific transporters such as PepT1, while oligopeptides are absorbed via transcytosis and paracellular pathways, this last one being the main mechanism for the transport of intact peptides.

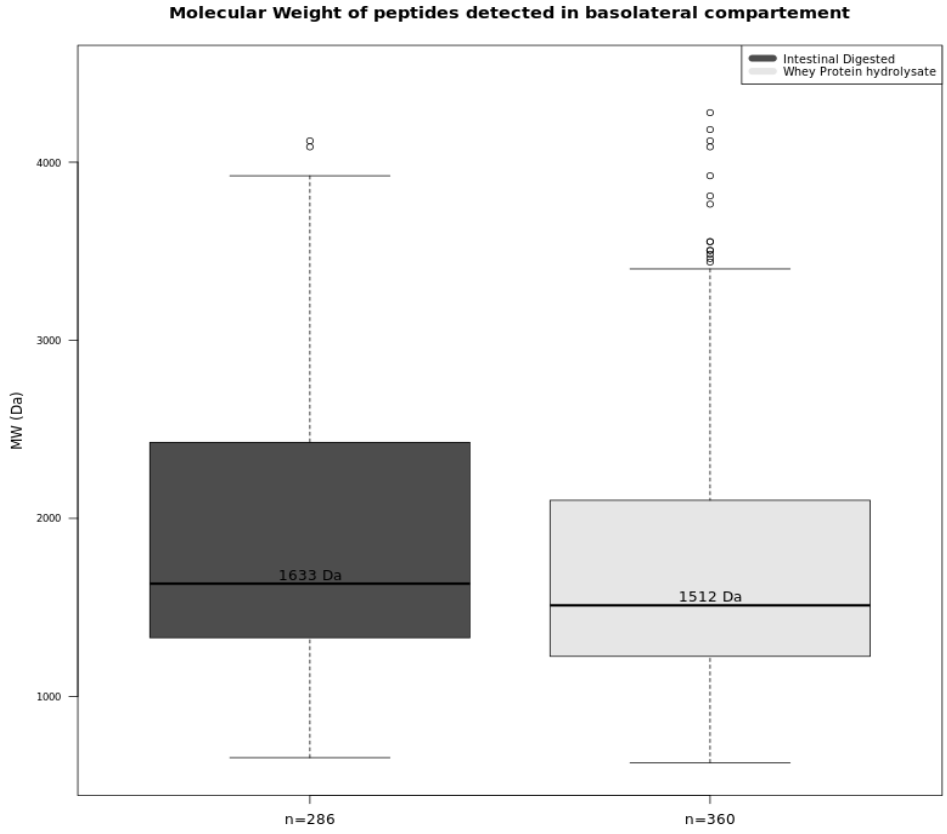

Figure 2. Average molecular weight and total number of peptides identified for undigested (WPH) and digested (ID) whey hydrolysate during Ussing Chamber absorption assay in basolateral compartments.

Regazzo et al. (2010) showed that the absorption of the β-cas 17-amino acid residues peptide YQEPVLGPVRGPFPIIV through Caco-2 cells occurred mainly through transcytosis via internalized vesicles, although the paracellular transport via tight-junctions could not be excluded. For the first time, Caira et al. (2016) confirmed the bioavailability of 15 casein-derived oligopeptides in human plasma after supplementation with Parmigiano Reggiano cheese for one week, but the authors did not reveal the mechanisms involved in the absorption of these peptides. In this sense, although some studies started to prove the absorption of

oligopeptides, the mechanism involved in this process, as well as the concentrations required to observe biological effects need to be explored. Vij, Reddi, Kapila & Kapila (2016) also investigated the mechanisms involved in the absorption of the ACE-inhibitory peptide VLPVPQK and suggested that it may have occurred via SOPT2 or PepT1 like transporter. This peptide was also found in the present study among the peptides present in the mucosal side of the WPH samples, but it was probably hydrolyzed by the brush border or intracellular peptidases, as it could no longer be observed in the basolateral portion.

Figure 3 presents a heatmap of all the 884 milk-derived peptides identified classified in 7 clusters by similarity. As aforementioned, the activity of the brush border peptidases was more intense in the first 10 min of the experiment, with 250 new peptides found for the WPH samples. Most of them (68%) belonged to the cluster 5, distinguished by the presence of charged, basic and polar amino acids. For the ID samples, 91 peptides were not present in the starting material and were generated after 10 min of the absorption assays. Among these peptides, 69% were from cluster 1, which contains peptides different from the others by the presence of aromatic amino acids.

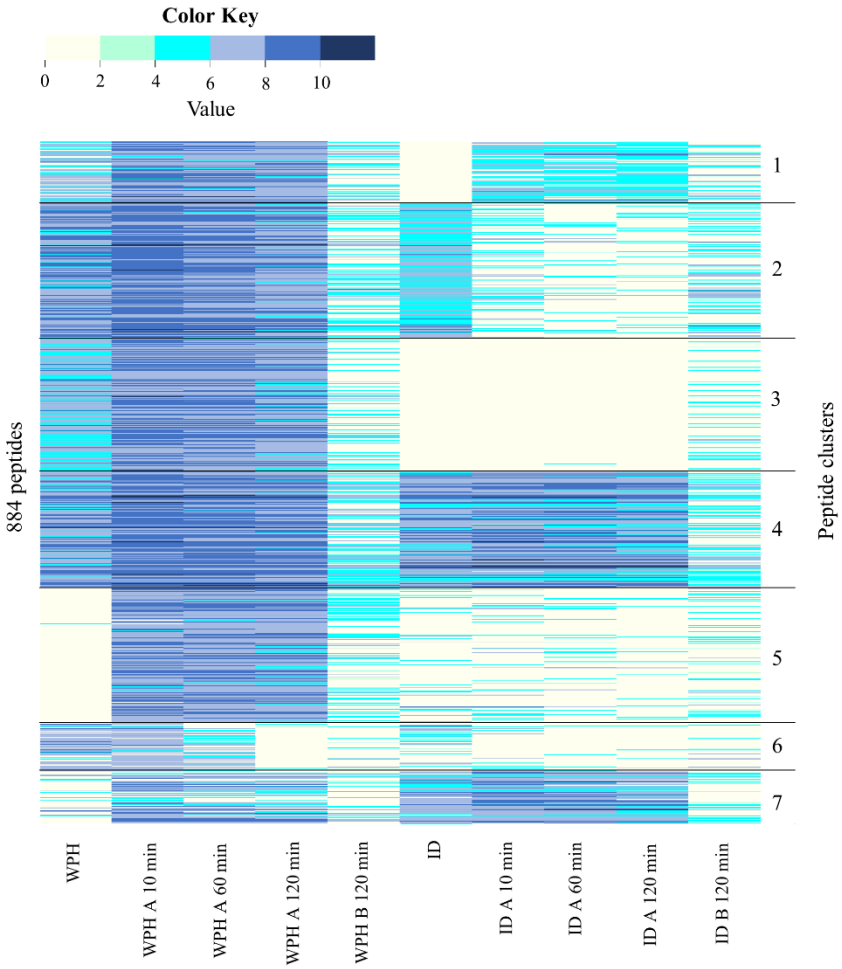

Figure 3. Nano-RSLC–MS/MS heatmap built with all the peptides identified along the Ussing chamber assays from undigested (WPH) and previously digested (ID) whey protein hydrolysates grouped in 7 clusters by similarity. Each line represents a peptide sequenced. A: apical compartment; B: basolateral compartment. 10 - 120: time (min) of aliquot.

Among the 286 peptides absorbed from ID samples, 85 were also present in the WPH starting material, resisted to the digestion process and were absorbed. 96% of them were classified in cluster 4, which contains peptides with acidic amino acids and also hydrophobic, aliphatic and aromatic amino acids. In general, peptides absorbed from WPH and ID samples were mostly present in clusters 4 and 2. Peptides from cluster 2 present aliphatic, hydrophobic and nonpolar amino acids in their sequences. According to Horner et al. (2016) and Sultan, Huma, Butt, Aleem, & Abbas (2017), molecular size, weight distribution and other characteristics such as hydrophobicity and charge are the key factors to determine the transport routes for peptides absorption. Chabancea et al. (1998) identified two casein peptides in the blood of volunteers and suggested that they present common transport pathways through the intestinal tissue, as

they result from two major chymosin cleavage sites, are highly hydrophilic and are located on the surface of milk micelles.

Boutrou et al. (2013) detected and sequenced 146 peptides in the jejunal effluents of individuals following whey ingestion. Most of them presented a large size (nine to fifteen amino acid residues) and derived from □-lg. In the present study, among the absorbed peptides identified from both WPH and ID samples, most of them were from the main whey protein β-lg and from β-cas. They covered almost all the extension of these proteins, demonstrating the high efficiency of the digestion process and both WPH and ID aliquoted solutions presented similar profiles, as shown in Figure 4.

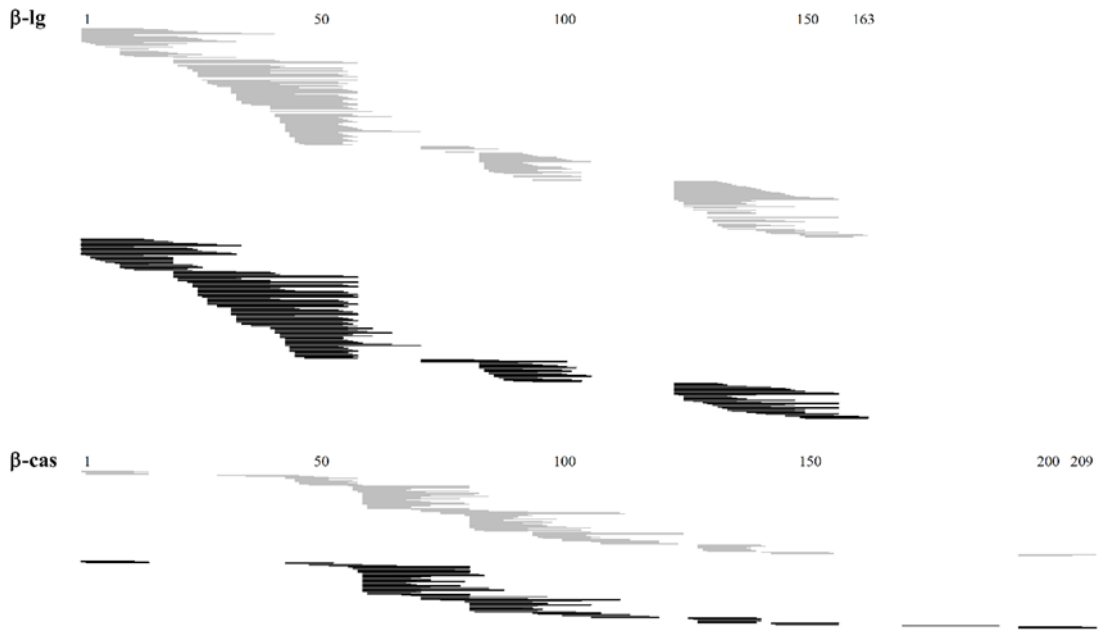

Figure 4: Undigested (WPH) and previously digested (ID) whey protein hydrolysate peptides identified by Nano-RSLC-MS/MS at the basolateral compartment of the Ussing chambers from β-lactoglobulin (β-lg) and β-casein (β-cas). Peptides found in the WPH and ID samples are represented by grey and black continuous lines, respectively. Numbers represent the amino acid chain of each protein.

Many studies attributed different bioactivities to dairy peptides. In the present study, among the absorbed peptides identified, 10 β-cas fragments have previously been reported for different bioactivities. 26 peptides derived from β-lg were also absorbed and are potential candidates to exert antihypertensive, antioxidant, antimicrobial and antidiabetic activities, as well as to improve memory function, as reported by several authors and presented in Table 1.

Table 1. Undigested (WPH) and digested (ID) whey protein hydrolysate absorbed peptides identified in the Ussing chamber assays by Nano-RSLC-MS/MS with bioactive potential reported in the literature. β-lg – betalactoglobulin; α-la – alphalactalbumin; β-cas – beta-casein; κ-cas – kappa-casein; α-casS1 – alpha-casein S1.

Protein	Peptide	WPH	ID	Bioactivity	Reference
β-lg	KGLDIQKVAGTW	X	X	ACE-Inhibitory	US20100056458A1, 2010
	GLDIQK	X	X		Pihlanto-Leppälä et al., 2000
	VLDTDYK		X		
	IVTQTMKG		X		Ortiz-Chao et al., 2009
	VLDTDYKK		X		
	KTKIPAV	X	X		
	VEELKPTPEGDLE	X			
	LEKFDK	X			
	LDIQKVAGTW	X			Nielsen et al., 2017
	RELKDLKGYGG	X	X	Anti-diabetic	Lacroix & Li-Chan, 2014
	LIVTQTMKGLD		X		
	IVTQTMKGLD	X			
	IVTQTMKGLDIQ		X		
	LKPTPEGDL	X	X	DPP-IV inhibitory	Nielsen et al., 2017
	TPEVDDEALEK	X	X		
	VLVLDTDYK	X	X		
	LVLDTDYK		X	Antimicrobial	Akalın, 2014
	ALKALPMHI	X			Brandelli, Daroit, & Corrêa, 2015
	GLDIQKVAGT	X	X		Nielsen et al., 2017
	TPEVDDEALEK	X	X		
	VLVLDTDYK	X	X		
	IDALNENK	X	X		
	SLAMAASDISLL	X	X		
	KPTPEGDLEI	X		Memory function	Ano et al., 2018
	KTKIPAVF	X		Antioxidant	Mann et al., 2014
	NENKVLVLDTDYKKY	X			Dullius, Goettert, & de Souza, 2018
α-la	GGVSLPEW	X		ACE-Inhibitory	Tavares et al., 2011
	DKVGINYW	X			Nielsen et al., 2017
	LKGYGGVSLPEW		X		
β-cas	LVYPFPGPIPN	X	X		Hernández-Ledesma, Del Mar Contreras, & Recio, 2011
	VYPFPGPIPN	X	X		Nielsen et al., 2017
	YPFPGPIPN	X	X		
	YPFPGPIHNSLPQ	X			
	VENLHLPLPL	X		Anticancer	US007597.904B2, 2007
	VYPFPGPIPN	X	X	Antioxidant	Nielsen et al., 2017
	YPFPGPIPN	X	X	DPP-IV inhibitory	
	VYPFPGPIP	X	X	Prolyl endopeptidase-inhibitory	
	PVVVPPFLQPE	X	X	Antimicrobial	
κ-cas	VQVTSTAV	X		Antimicrobial	
α-cas$_{S1}$	SDIPNPIGSENSEK	X	X		

Despite the controversy about the bioavailability and bioactivity of peptides related to their susceptibility to plasma soluble peptidases and the concentrations required to observe the effects (Fernández-Musoles et al., 2013; Martin et al., 2019), some in vivo studies confirmed the biological impact of dairy peptides on the intestinal barrier (Fernández-Tomé et al., 2017; Plaisancié et al., 2013) and in different systems after being absorbed, as well (Aihara, Kajimoto, Hirata, Takahashi, & Nakamura, 2005; Cadée et al., 2007; Lollo et al., 2014; Mizushima et al., 2004; Mulder, Connellan, Oliver, Morris, & Stevenson, 2008). As mentioned above, the concentration needed to obtain biological effects and the mechanisms involved in the transepithelial absorption process have to be further investigated. In this sense, the present study constitutes a new evidence of the absorption of oligopeptides and is also a starting point for a better understanding of dairy peptides bioavailability and bioactivity.

4. CONCLUSIONS

This study demonstrated the fast action of brush border enteropeptidases on large peptide fragments from undigested and digested whey protein hydrolysates. Some of the peptides identified in the apical portion of the chambers presented potential bioactivity on the intestinal epithelium, requiring further investigation. Interestingly, several oligopeptides were absorbed through the intestinal epithelium, most of them presenting hydrophobicity, aromatic, acidic and aliphatic amino acids, and some of them have a potential bioactivity. The results found contradicts the outdated consensus about the exclusive absorption of amino acids, dipeptides and tripeptides, and reinforces the new evidences about bioavailability of dairy oligopeptides and indicates their probable bioactivity. Further research needs to be carried out in order to deepen the understanding of the presented data, as well as to investigate the mechanisms involved in the intestinal transepithelial passage of these peptides.

Acknowledgments

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Chapter 5

Emerging technologies assisting whey β-Lactoglobulin hydrolysis aiming the development of hypoallergenic whey-based ingredients

Ozorio[1], L.; Cunha[2], L.C.M.; Conte-Jr[1,2], C.A; Brígida[3,4], A.I.S; Mellinger-Silva[4*], C..; Cabral[4], L.M. C.

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[2]Universidade Federal Fluminense, Rio de Janeiro, Brazil

[3]Embrapa Agroindústria Tropical, Fortaleza, Brazil

[4]Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil

*Corresponding author.

Avenida das Américas, 29.501 Guaratiba, Rio de Janeiro, RJ, Brasil.

Tel.: +55 21 3622 9622; Fax: +55 21 3622 9713.

e-mail: caroline.mellinger@embrapa.br

1. INTRODUCTION

Cow's milk is a member of the "Big-8" group of the major food allergens, which also includes egg, soy, wheat, peanuts, tree nuts, fish and shellfish. Cow's milk allergy (CMA) affects up to 8% of children under 3 years old and in adulthood it is less prevalent (0.1-0.5%) but it can be more severe, generating symptoms as gut inflammations, asthma and atopic dermatitis (ORIVUORI *et al.*, 2014; STANIC-VUCINIC *et al.*, 2012; VANDENPLAS, 2017). Milk allergy usually causes fast reactions mediated by IgE, although the number of non-IgE-mediated hypersensitivity (late reactions) diagnoses is also increasing (CAIRA *et al.*, 2012; ORIVUORI *et al.*, 2014).

CMA patients react to different protein fractions and mainly to whey proteins. Whey proteins are the soluble fraction of milk proteins, being a great source of essential and branched chain amino acids, besides presenting interesting technological properties, that allow their use into a large variety of products, including bakery, infant formulas and dairy products (OZORIO *et al.*, 2019). Their extensive use, however, gives rise to a new concern related to their high immunoreactivity, mainly associated with β-lactoglobulin (β-lg) (ROTH-WALTER *et al.*, 2014; VILLA *et al.*, 2018).

Although β-Lg is the major whey protein present in cow's milk, it is missing in human milk, which may be the cause of sensitivity reactions after consumption (ROTH-WALTER *et al.*, 2014). For many years, studies focused on heat treatments to reduce CMA, but with the increasing number of cases, new research are being developed focusing on finding alternative technologies to produce safer food products (EL MECHERFI *et al.*, 2015)

Enzymatic hydrolysis is one alternative that can been used to reduce β-Lg immunoreactivity, but it may be not completely efficient as β-Lg presents a globular conformation that hides allergenic epitopes, preventing enzymes' access to the target sites. In this sense, the combination of enzymatic hydrolysis with thermal and non-thermal technologies on reducing β-Lg allergenicity has been evaluated. High pressure, pulsed electric filed, microwaves and ultrasound are known for altering β-Lg conformation, exposing cleavage sites, increasing protein hydrolysis and reducing allergenicity (MECHERFI *et al.*, 2011; PASUPULETI; DEMAIN, 2010).

In microwave treatments, microwaves penetrate the food leading to the oscilation of ionic componentes, generating heat by friction. This oscilatory migration modifies the struture of the food contituents, including proteins (EL MECHERFI *et al.*, 2015). Microwave heating also improves enzymatic hydrolysis processes by reducing reaction time, when compared to conventional heating. The promoted unfolding facilitates the proteases access to target peptide bonds, releasing bioative peptides and also reducing allergenic epitopes (KETNAWA; LICEAGA, 2017). Ultrasound is also an emerging technology that has been used for different purposes, such as homogenization, tenderization and accelerate dehydration (RAHAMAN; VASILJEVIC; RAMCHANDRAN, 2016). The high energy mechanical waves of the ultrasound generates cyclic movements and collaps of sonication bubbles (cavitation). This collapsed cavities generates conformational changes in proteins' structures, modifying allergenic epitopes, as well as exposing target sites to proteases (NAYAK *et al.*, 2017).

Hence, this study represents a great novelty comparing the use of the technologies of microwave, ultrasound and autoclave as pre-treatments for the hydrolysis of a whey protein concentrate, aiming to develop a hypoallergenic whey protein hydrolysate.

2. MATERIALS AND METHODS

2.1 MATERIALS

The bovine whey protein concentrate 88 % (WPC88) and the commercial pepsin (E.C. 3.4.23.1) from porcine gastric mucosa were gently donated by Alibra Ingredientes Ltda (Campinas, SP, Brazil) and Bela Vista Produtos Enzimáticos Ind. e Com. Ltda (Bela Vista, SC, Brazil), respectively. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany).

2.2 PRE-TREATMENTS AND HYDROLYSIS PROCESS

Whey protein concentrate 88% (WPC 88) solutions (1.25% w/v, pH 2, 1 M HCl) were prepared and submitted to different pre-treatments using microwave (DGT 100 Plus, Provecto Analítica, São Paulo, Brazil), ultrasound (UIP1000hdT, sonotrode BS2d18, head area of 2.5 cm^2, booster B4.18, Hielscher Ultrasonics, Teltow, Germany) and autoclave (CS, Prismatec, São Paulo, Brazil) technologies, as shown in Table 1. Treated solutions were immediately cooled in ice bath under constant stirring until reaching 37°C.

After achieving 37°C, pepsin (5.34 μU.g^{-1} of initial protein) was added and the hydrolysis process was conducted for 3h. pH and temperature were monitored during all the experiment, aliquots were withdrawn at 1, 60, 120 and 180 min of hydrolysis and pepsin was thermally inactivated in water bath at 80°C/5min. Samples were cooled in ice bath, freeze-dried and kept at -20 °C until further analyses. The same WPC 88 was also hydrolyzed by pepsin without previous treatment and submitted to the same posterior analyses (OZORIO *et al.*, 2019).

Table 1. WPC 88 hydrolysis pre-treatments using ultrasound, autoclave and microwave technologies.

Technology	Pre-treatment conditions	Time (min)
Ultrasound	50 W 100 W 200 W 300 W	5
Autoclave	100°C – 0.00 kPa; 110°C – 49.03 kPa; 120°C – 98,07 kPa	10
	120°C – 98,07 kPa	5
Microwave	100 W 300 W 400 W 1 600	5

2.2 REVERSED PHASE - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

The RP-HPLC analysis followed the method described by Ozorio et al. (2019). Briefly, the pre-treated whey hydrolysates were suspended at 2 mg.mL^{-1} and analyzed in an analytical HPLC unit from Jasco (Jasco Corporation, Japan) coupled to a Hypersil BDS C18 column (100×4.6 mm, particle size 2.4 μm, Thermo Scientific, USA). Samples were injected (20 μL) and the chromatographic runs occurred during 40 min at a flow rate of 1.0 mL.min^{-1} at room temperature (approximately 20°C). The gradient of elution was programed as follows: 0–2 min, 5% B; 2–15 min, 5–20% B; 15–20 min, 20–30% B; 20–25 min, 30–40% B; 25–28 min, 40–50% B; 28–32 min, 50–60% B; 32–34 min, 60–70% B; 34–36 min, 70–80% B; 36–38 min, 80–5% B; 38–40 min, 5% B. 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v) was used as solvent B and 0.1% TFA in acetonitrile (ACN) (v/v), as solvents A. Peptides were detected at 216 nm.

2.3 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Samples selected after the RP-HPLC analysis were submitted to the immunoassays using an ELISA sandwich kit (RIDASCREN R-Biopharm, Darmstadt, Germany) to evaluate the allergenicity related to β-lactoglobulin. The complex enzyme-substrate was detected at 450 nm.

3. PARTIAL RESULTS AND DISCUSSION

3.1 RP-HPLC PEPTIDE PROFILES

Enzymatic processes may play ambiguous roles in food allergens, either reducing it or exposing the epitopes and increasing allergenicity. In case of β-lactoglobulin (β-lg), the use of enzymatic treatment is not sufficient to decrease immunoreactivity, as its globular stable conformation may hide the allergenic epitopes (MECHERFI *et al.*, 2011; PASUPULETI; DEMAIN, 2010). In the present study, the enzymatic hydrolysis was able to reduce in 45% β-lg concentration and led to the formation of a varied peptide profile (Figure 1).

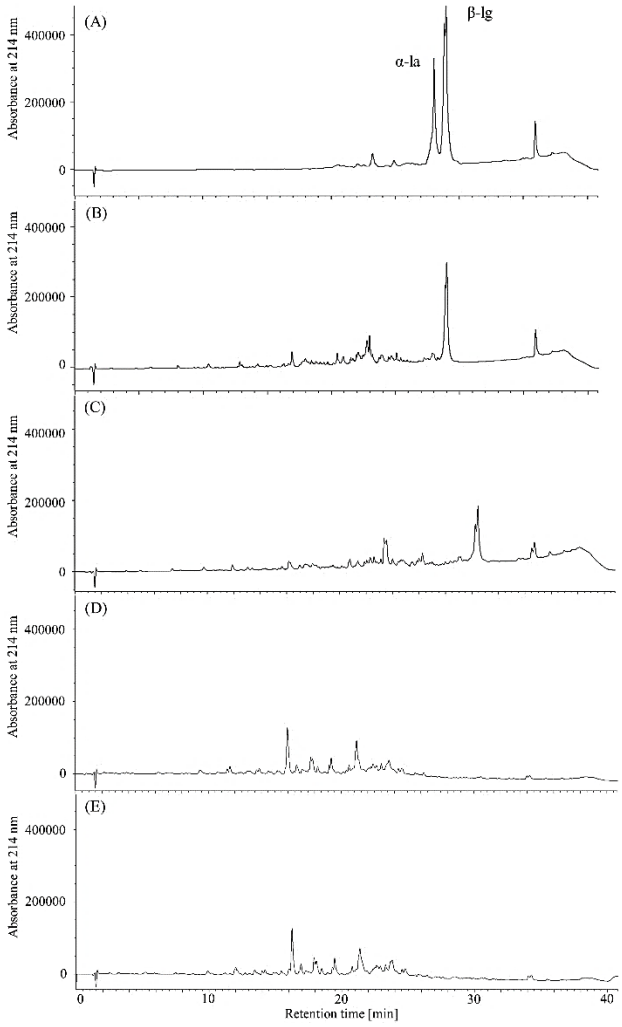

Figure 1. Peptide profiles of the (A) whey protein concentrate – control sample; (B) whey protein hydrolysate; whey protein hydrolysates pre-treated for 5 min with (C) ultrasound 100 W, (D) microwave 600 W, (E) autoclave 120°C. α-la – alpha-lactalbumin; β-lg – beta-lactoglobulin.

In the recent years, different studies have been dedicated to study the use thermal and non-thermal technologies to produce hypoallergenic hydrolysates (BONOMI *et al.*, 2003; EL MECHERFI *et al.*, 2015; STANIC-VUCINIC *et al.*, 2012; ZHONG *et al.*, 2014). β-lactoglobulin is the main whey protein and it is considered the most important cow's milk allergen (ROTH-WALTER *et al.*, 2014). Among the technologies evaluated as pre-treatments to increase β-lg hydrolysis and decrease its allergenicity, ultrasound was more efficient when compared to the enzymatic treatment alone, but less effective than the other pre-treatments used, as it was able to only partially decrease β-lg concentration. Interestingly, the most intense reduction was observed for the mildest condition used, 100W (69%), which was probably

related to the formation of β-Lg aggregates, as the increase in power induces heating, protein's unfolding and aggregation (STANIC-VUCINIC *et al.*, 2012).

In contrast, considering microwave, experiments β-Lg hydrolysis was directly proportional to the power used, with complete hydrolysis being achieved in the treatment applying microwave irradiation at 600W. In this treatment, proteins hydrolysis lead to the generation of a varied peptide profile, with peptides manly eluting between 16 and 26 min of retention time, as shown in Figure 1. In a study evaluating the peptic hydrolysis of β-Lg assisted by microwave, Mecherfi et al. (2011) obtained similar results, showing that the digestion of β-Lg was enhanced with the increase in potency, achieving 52% when applied a power of 200W during 3 min of hydrolysis. They attributed this to the intensity of the electromagnetic field, which produced molecular vibrations inducing molecular and structural changes in the molecules.

After the heat treatments using autoclave followed by pepsin hydrolysis, a reduction of 71% in β-Lg concentration was achieved when applied 100°C for 10min, while when applying 120°C for 10 and 5 min, β-Lg was totally hydrolyzed. For the last one, a varied peptide profile was formed and very similar to the one obtained after the microwave treatment. To Bonomi et al. (2003), high temperature heat denaturation is often used to prepare extensive hydrolysates with low immunoreactivity, as the process itself leads to the removal of conformational epitopes and also allows enzymes' access to the target cleavage sites.

In this sense, the most promising conditions to reduce β-Lg immunoreactivity, ultrasound 100W/5min, microwave 600W/5min and autoclave 120°C/5min were selected (Figure 1) and are being evaluated concerning their immunoreactivity by ELISA immune assays.

Chapter 6

A comparison of dual-functional whey hydrolysates by the use of commercial proteases

Dual functional peptides from whey hydrolysates

Luísa O.L. Rosa[a], Marcelo C. Santana[b], Tatiana L. Avezedo[c], Ana I.S. Brígida[c], Ronoel Godoy[c], Sidney Pacheco[c], Caroline Mellinger-Silva[c*], Lourdes M.C. Cabral[c]

[a] Instituto de Química, Universidade Federal do Rio de Janeiro. Av. Athos da Silveira Ramos, 149 - Cidade Universitária, Rio de Janeiro - RJ, 21044-020, Brazil. Tel.: +55 21 3938 8213

[b] Departamento de Farmácia, Universidade Federal do Rio de Janeiro. Av. Aluizio da Silva Gomes, 50 - Novo Cavaleiros, Macaé - RJ, 27930-560, Brazil. Tel.: +55 22 2141 4012

[c*] EMBRAPA Agroindústria de Alimentos. Avenida das Américas, 29501 - Guaratiba, Rio de Janeiro - RJ, 23020-470, Brazil. Tel.: +55 21 3622 9600

Author's full names and e-mail addresses:

Luísa Ozorio Lopes da Rosa; ROSA, L. O. L.: luisa.ozorio@gmail.com
Marcelo Cerutti Santana; SANTANA, M. C.: cerutti.santana@gmail.com
Tatiana Lima Azevedo; AZEVEDO, T. L.: tatiana.azevedo@embrapa.br
Ana Iraidy Santa Brígida, BRÍGIDA, A. I. S.: ana.iraidy@embrapa.br
Ronoel Godoy, GODOY, R.: ronoel.godoy@embrapa.br
Sidney Pacheco, PACHECO, S.: sidney.pacheco@embrapa.br
Caroline Mellinger Silva, MELLINGER-SILVA, C.: caroline.mellinger@embrapa.br*
Lourdes Maria Correa Cabral; CABRAL, L. M. C.: lourdes.cabral@embrapa.br

ABSTRACT

Whey is well-known for the functional and bioactive properties of its proteins and peptides, which are of great interest to food and nutraceutical industries. The aim of this study was to investigate the effect of four different commercial proteases (Novo ProD® (NPD), Alcalase® (ALC), Pancreas Trypsin® (TRY), and Flavourzyme® (FLA)) in the generation of hydrolysates with emulsifying and antioxidant activities. Hydrolysis processes were carried out for 5 h, reaching maximum degrees of 18.5, 15.5, 9.2, and 8.7% for NPD, ALC, TRY, and FLA, respectively. All tested enzymes generated very diverse, but conservative peptide profiles when comparing the treatments along the time, with the main enzymatic actions up to 120 min-reaction. An increase in the *in vitro* antioxidant activity was found for all treatments, achieving 46%, 40%, 40% and 22% for ALC, TRY, NDP and FLA, respectively. TRY hydrolysate maintained or slightly increased its emulsifying capacity along the time, however, a decrease in emulsifying capacity was found for ALC and NPD hydrolysates when compared to the non-hydrolyzed whey protein concentrate (WPC). All tested enzymes generated hydrolysates with enhanced antioxidant and/or emulsifying activities, which may be used as food ingredients and the choice of the enzyme will depend on the need.

Practical Application: Four proteases were evaluated for the generation of dual functional whey hydrolysates.

Keywords: whey protein; hydrolysis; functionality; emulsification; antioxidant activity.

1. INTRODUCTION

Whey proteins are well known and widely consumed for being a high quality protein source especially incorporated into sports and nutrition products (WEST *et al.*, 2017). Different commercial ingredients and products are available in the market, including whey powders (WP), whey protein concentrates (WPC) and whey protein isolates (WPI) which are classified according to the protein content, varying from less than 30% for WP to 30-90% for WPC and above 90% for WPI (WHIPPLE; ECKHARDT, 2016). In general, higher protein contents will result into better-valued products, mainly to attend nobler uses, as in the market of infant formulas and nutraceutical products.

Annual whey generation is estimated in 240 million metric tons (MORDOR INTELLIGENCE, 2017a). Besides its nutritional and technological properties, whey presents elevated organic matter content, generating high biological and chemical oxygen demands which cause environmental concerns (SULTANA *et al.*, 2016). In this sense, industrial interest in the use of whey has been growing, aiming to concentrate and dry whey proteins into different ingredients mainly to attend food industries.

Whey proteins are also known for the bioactivity of its peptides, which can be liberated through hydrolysis processes catalyzed by chemical or enzymatic agents. The use of enzymes has gained popularity since their specificity for some active-sites, results into ingredients and products with enhanced specialized properties, particularly in terms of biological functionalities (LE MAUX *et al.*, 2016; OJHA *et al.*, 2016).

Among the main investigated biological properties from whey peptides, which comprise beneficial results for the cardiovascular, nervous, gastrointestinal, and immune systems, antioxidant activity is of relevance (CÁNOVAS *et al.*, 2017). The ability of peptides to interact with radical species or inhibit oxidative reactions is important for the body to prevent a number of age-specific diseases and neurodegenerative disorders caused by the oxidative stress. In food, oxidative reactions may shorten shelf life through deterioration of food quality worsening sensorial and nutritional aspects (DRYÁKOVÁ *et al.*, 2010; POWER; JAKEMAN; FITZGERALD, 2013). Thus, the possibility of using a natural antioxidant from a dietary source can be interesting and may even replace noxious, synthetic compounds.

In addition to the bio-functionalities, whey peptides may also enhance technological properties of food formulations, such as water binding, solubility, gelation, and emulsification(MADADLOU; ABBASPOURRAD, 2018), and the possibility of joining both bio and technological functionalities is also of interest for the food industry sector. In this way, the objective of this study was to compare WPC hydrolyses of four commercial proteases, considering both the antioxidant activity and emulsifying capacity of the hydrolysates.

2 MATERIALS AND METHODS

2.1 MATERIALS

A spray-dried whey protein concentrate (WPC 35) from bovine milk, of 34% (w/w) protein, was gently donated by Alibra Ingredientes Ltda (Paraná, Brazil), and was used as substrate. Enzymes were gently donated from Novozymes Latin America Ltda (Paraná, Brazil) and were used as recommended by the manufacturer in terms of enzyme concentration, optimal temperature, pH and the conditions for enzyme inactivation. Reagents were of high purity and purchased from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany).

2.2 ENZYMATIC HYDROLYSIS

WPC hydrolysates were prepared by suspending the powder in ultrapure water at a concentration of 24% (*w/v*), corresponding to 8.2g of protein in 100 mL of suspended whey. The suspension was then continually stirred (350 rpm) and the hydrolysis conditions were kept as indicated in Table 1. Prior the reaction, the systems were allowed to rehydrate and solubilize for 30 min. The suspension was then adjusted to the adequate pH using aq. NaOH (1.0 M). At this point, an aliquot (control – C) was collected. The enzymes were then added to each system as recommended by the manufacturer. Values of pH and temperature were monitored along the reaction. Aliquots were collected after 1 min (T0), 60 min (T1), 120 min (T2), 180 min (T3), 240 min (T4), and 300 min (T5) of hydrolysis. All hydrolyzed samples were heated at 85 ºC for 15 min to inactivate the enzymes, and then cooled down under running tap water (18 °C). Samples were freeze-dried (Liotop, São Paulo, Brazil) then kept at -20 °C for further analyses. Degree of hydrolysis (DH %) was determined by the volume of base consumed, according to the pH-stat method (ADLER-NISSEN, 1986).

Table 1. Hydrolysis conditions of WPC 35 by the use of different enzymes.

Hydrolysis Conditions[a]				
Enzymes[b]	[E/S][c]	pH	T (°C)	EIC[d]
ALC	2% (*w/v*)	8.0	65.0	85 °C/15’
NPD	7% (w/*v*)	9.0	50.0	80 °C/15’
TRY	1% (w/*v*)	8.5	47.0	80 °C/15’
FLA	3% (w/*v*)	7.0	50.0	80 °C/10’

(a) hydrolysis conditions were set according to the manufacturer recommendation; (b) ALC= Alcalase 2.4L FG®; NPD= Novo-Pro D®; TRY= Pancreas Trypsin 6.0S®; FLA= Flavourzyme1.000L®. (c) E/S= enzyme: substrate concentration; (d) EIC= enzyme inactivation condition; T (°C) = temperature in Celsius degrees.

2.3 CHROMATOGRAPHIC ANALYSIS OF PEPTIDES AND PROTEINS

RP-HPLC was used to analyze the peptide profiles generated from the hydrolysis processes. An analytical HPLC unit (Waters – Alliance, separation module 2695, São Paulo, Brazil) with a C18 column, held at 30°C, was used. Gradient elution was carried out by the use of two solvents. Solvent A: 0.1 % trifluoracetic acid (TFA) in ultrapure water (v/v), and Solvent B: 0.1% TFA in acetonitrile (ACN) (v/v). Hydrolysates were eluted as follows: 0-2 min, 95% A; 2-15 min, 95-80% A; 15- 20 min, 80-70% A; 20-25 min, 70-60% A; 25-28 min, 60-50% A; 28-32 min, 50-40% A; 32-34 min, 40-30 % A; 34-36 min, 30-20% A; 36-38 min, 20-95% A; 38-40 min, 95%A. The flow rate was 1.0 mL min^{-1}, and the detection was at 216 nm (Waters PDA Detector, 2996). Total running time was 40 min. For the chromatographic analysis, freeze-dried samples were suspended into ultrapure water (2 mg mL^{-1}) and 20 µL was injected. Bovine α-lactalbumin (α-la) and β-lactoglobulin (β–lg) were used as standards, and the retention times were 28.20 and 31.85 min, respectively.

2.4 *IN VITRO* ANTIOXIDANT ACTIVITY

The *in vitro* antioxidant activity of hydrolysates were measure by scavenging of the ABTS radical (ABTS•+) (2,2’-azinobis (3-ethylbenzothiazoline-6-solfonic acid)) using a decolorization assay (RE *et al.*, 1999). 0.1M aq. phosphate-buffered saline (PBS) was used in pH 7.4. Spectrophotometric (Micronal, São Paulo, Brazil) reading was done 6 min after the addition of ABTS•+, in triplicate (DRYÁKOVÁ *et al.*, 2010). The antioxidant activity was calculated as antioxidant activity % (AA %), following the equation (1):

$$AA\ \% = [(Abs\ blank - Abs\ sample) / Abs\ blank) * 100 \quad (1)$$

2.5 EMULSIFYING CAPACITY (EC)

Hydrolysates EC were evaluated by the emulsifying index of the sample in hexadecane according to Mellinger-Silva et al. (2015), right after hydrolyses processes, as soon as the hydrolysates cooled down from the enzyme inactivation. 1 mL of each hydrolysate was added of 1 mL of hexadecane in a tube test, and the tube was submitted to vigorous and non-interrupted stirring for 3 min. Then, tubes reposed for 24h. The emulsifying capacity (EC%) was calculated by using digital pachymetric measures (200 mm, IP67, PD202, Vonder, PR, Brazil) of the total solution height (Ht) and the emulsified phase height (He), as shown by the equation (2):

$$EC\ (\%) = (He/Ht)*100 \quad (2)$$

3 RESULTS AND DISCUSSION

3.1 HYDROLYSIS OF WPC AND PEPTIDE PROFILE OF THE HYDROLYSATES

The degree of hydrolysis (DH) of WPC differed significantly when comparing the results among the tested enzymes (Figure 1). NPD and ALC presented higher DH%: 18.5 and 15.5, respectively. In both curves it was possible to observe a rapid and intense enzymatic activity until 90 min-reaction, heading to a plateau after 120 min reaction. NPD and ALC were both obtained from *Bacillus licheniformis* and present endopeptidase activity, with a broad possibility of cleavage. The peptide profiles of NPD and ALC (Figure 2) were similar, with intense signals from 4 to 22 min of the chromatograms. HPLC analyses corroborated with the data from DH, showing that the main peaks were generated from 1 to 120 min and maintained up to 300 min-reaction. Both enzymes rapidly degraded α-la, while β-lg was just partially hydrolyzed along the reaction. β-lg is a globular protein which exists as a dimer at physiological pH and temperature, and two disulfide bonds are responsible for its globular stable structure. However, the exposure of β-lg to high temperatures or basic pH (pH>8) generate certain instability to protein's structure, causing dimer's dissociation, which is probably related to the greatest degrees found for the WPC hydrolyses with NPD (pH 9.0) and ALC (pH 8.0) (CHEISON *et al.*, 2010; HERNÁNDEZ-LEDESMA, B.; RECIO; AMIGO, 2008).

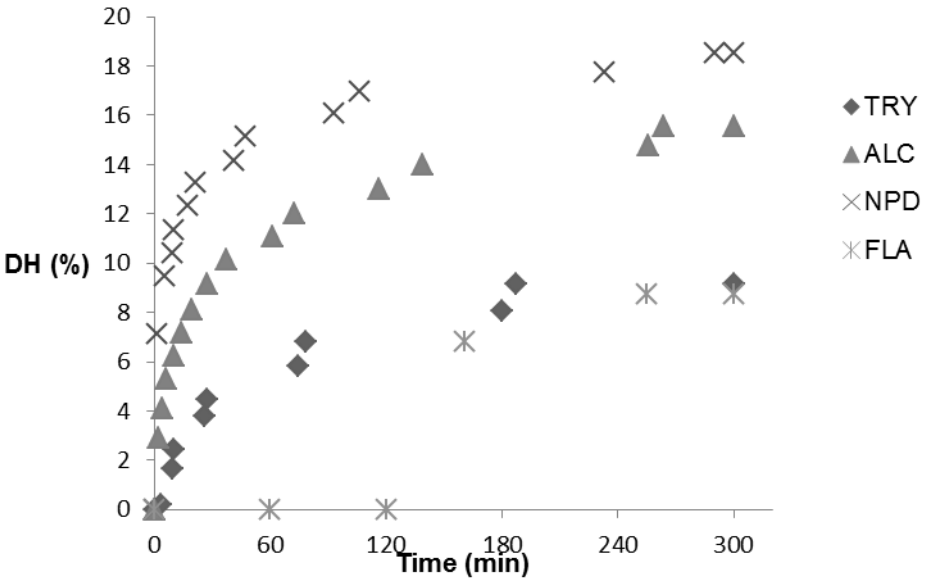

Figure 1. Degree of hydrolysis of WPC by the use of commercial proteases. DH% was determined by the volume of base consumed, according to the pH-stat method. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme.

TRY and FLA showed lower DH% of 9.2 and 8.7%, respectively. Hydrolytic curves (Figure 1) were similar among themselves, but different from the previous ones. Intense hydrolyses were observed up to 180 min reactions for the treatments with both enzymes. TRY is an endoprotease from animal origin that hydrolyses the carboxyl-side of lysine and arginine, and such amino acid specificity may explain a lower DH. Complementing this result, the peptide profile of TRY (Figure 2) was not so varied, but showed an intense peak at 17.17 min that was maintained from the first hour of hydrolysis on.

On the other hand, FLA, a crude fungal enzyme formula from *Aspergillus oryzae*, declared by the manufacturer to present both endo- and exo-peptidase activities, showed the lowest DH and the poorest peptide profile, with only partial cleavage of α-la and no action on the β-lg structure. The stability of the β-lg structure varies according the pH, while in solutions with pH>8 it shows certain instability, in pH<8 it tends to form molecular aggregates, preventing enzyme's access to the amino acid residues that has affinity, which may explain the lowest degree of hydrolysis presented by FLA. (CHEISON *et al.*, 2010).

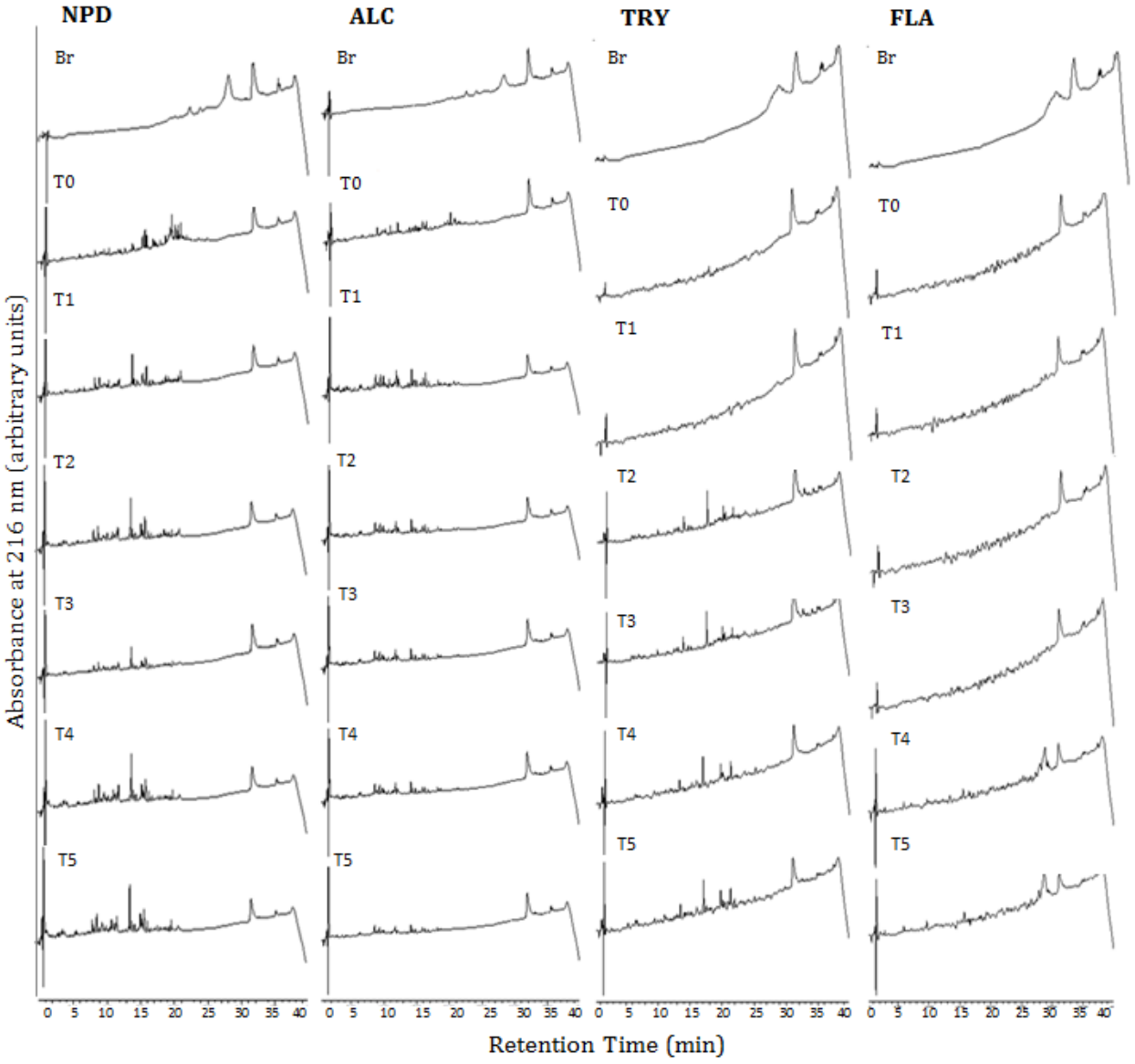

Figure 2. Chromatographic peptide profiles of WPC hydrolysates. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme. (a) α-lactoalbumin; (b) β-lactoglobulin, C = control sample; T0, T1, T2, T3, T4, T5 = aliquots collected in 1, 60, 120, 180, 240 and 300 min-reaction.

3.2 *IN VITRO* ANTIOXIDANT ACTIVITY OF WHEY HYDROLYSATES

All hydrolysates showed an increase in the *in vitro* antioxidant activity (AA) when measured by the ABTS assay, which is a colorimetric method widely used for food matrices and is applicable to water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts. It is based on the reduction of the radical 2,2´-azinobis (3-etilbenzotiazolin-6-sulfonic acid - ABTS.+), which can be generated through chemical, electrochemical or enzymatic

reaction. The quantity of radical consumed by the antioxidant substance is expressed as TEAC - trolox equivalent antioxidant capacity (DRYÁKOVÁ *et al.*, 2010; POWER; JAKEMAN; FITZGERALD, 2013; ZULUETA; ESTEVE; FRÍGOLA, 2009).

All control samples were submitted to the same conditions of other samples with pH and temperature variations, which may impact the AA, justifying the differences in the non-hydrolyzed samples. ALC presented the highest AA, followed by TRY, NPD and FLA. ALC achieved 46% after 300 min-reaction, while TRY and NPD presented similar profiles, ranging from 25% and 20% to 40% in both, respectively, as shown in Figure 3. FLA showed the lowest AA, with 22% after 300 min of reaction. Corroborating with the degree of hydrolysis and peptide profiles, samples presented a tendency to stabilize after 120 min-reaction.

Comparing hydrolysis processes with different enzymes, Dryáková et al. (2010) also found alcalase hydrolysates as the most effective in scavenging the ABTS+ radical, ranging from 19.8% to 54.2%, for non-hydrolyzed to 180 min hydrolyzed samples, respectively. Although the degree of hydrolysis impacts the antioxidant activity, there is also a structure-activity relationship between both, justifying the lower DH of ALC when compared to NPD, and the greater antioxidant activity of the further (DRYÁKOVÁ *et al.*, 2010; MADUREIRA *et al.*, 2010; POWER; JAKEMAN; FITZGERALD, 2013). In this manner, according to the results presented, the hydrolysate obtained with alcalase may be considered as the most efficient when the aim is to produce a whey hydrolysate with high antioxidant capacity.

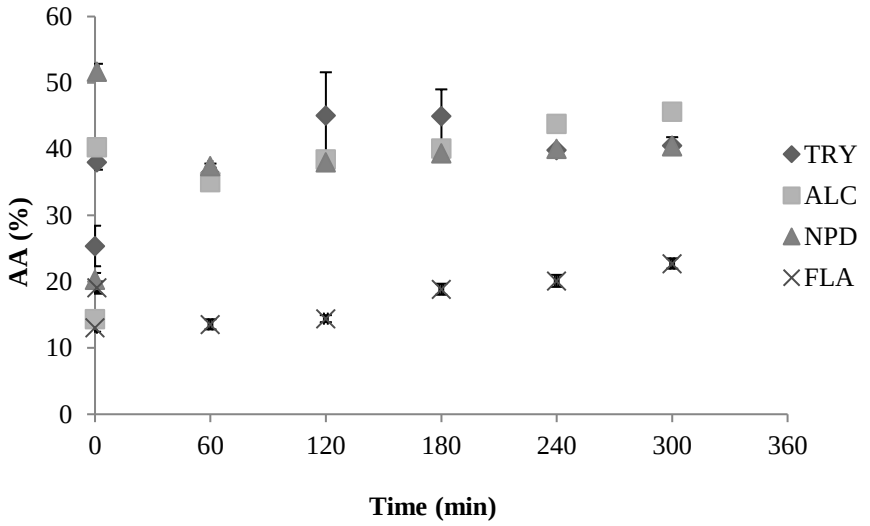

Figure 3. *In vitro* antioxidant activity of WPC hydrolysates. (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin, and (FLA) flavourzyme. Measured by scavenge of the ABTS radical (ABTS•+). The antioxidant activity was calculated as AA%.

3.3 EMULSIFYING CAPACITY OF WHEY HYDROLYSATES

The emulsifying capacity of proteins and peptides is related to their capacity to lower interfacial tension between hydrophobic and hydrophilic components into a food system (A. BOS; VAN VLIET, 2001; LAM; NICKERSON, 2013).

According to the results presented above, as FLA showed the lowest DH and AA, this sample was not included into the EC activity. ALC, NPD and TRY had their EC% assessed until 180 min-reaction (Figure 4), since after this time all the hydrolysates produced presented a tendency to stabilize their DH and AA, as shown in Figures 1 and 3. TRY hydrolysate showed a stable emulsifying capacity, which may be related with the low DH observed that promoted polypeptides generation. Larger size peptides are more likely to have both hydrophobic and hydrophilic residues on the same molecule, in which the hydrophobic residues interact with the oil droplets, while the hydrophilic ones will favor the aqueous phase and stabilize the droplets through steric effects. Adjonu et al. (2014) also related trypsin hydrolysates with high interfacial adsorption, emulsifying capacity and good storage stability. NPD presented an intense decrease on EC after 60 min, going from 85 to 26%, and after 120-180 min-hydrolysis the EC increased, and reached 50% within 120 minutes. ALC emulsifying capacity assays also presented certain instability, with a high decrease in 60-min hydrolysis, reaching 9%, followed by a significant increase at 180 min, rising from 9% to 42% EC.

Different protein characteristics may affect its emulsifying activity, including pH, temperature and protein hydrolysis. In general, low DH retain or increases this functionality, whereas intense hydrolysis diminishes it since peptides are too short to act as effective emulsifiers, saturating the continuous phase rather than adhering to the water–oil interface (LAM; NICKERSON, 2013; WANI *et al.*, 2015). Dual-functional peptides are of great interest for food industries, since it aggregates both technological and biological functions, increasing the added value of a product. Among the hydrolysates obtained, the fraction hydrolyzed with trypsin presented a stable emulsifying capacity along the experiment and a tendency to stabilize its antioxidant activity after 2h-reaction. In this sense, the 120 min tryptic hydrolysate may be considered the best condition for obtaining peptides with both antioxidant and emulsifying capacities.

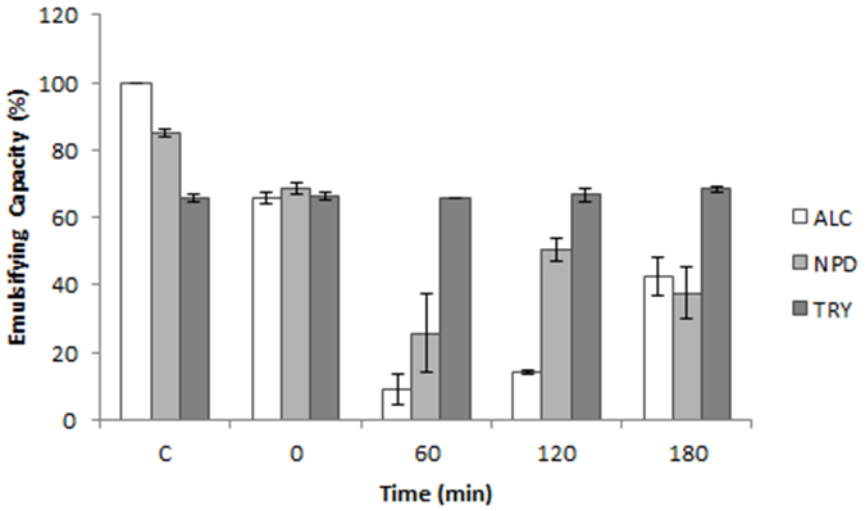

Figure 4. Emulsifying capacity of WPC hydrolysates. (C) control sample, (ALC) alcalase, (NPD) novo pro D, (TRY) pancreas trypsin. 0 – 180: time of hydrolysis (min).

4 CONCLUSIONS

This study dealt with a comparison of WPC hydrolysates by the use of commercial proteases. Alcalase and Novo pro-D presented the highest hydrolysis degrees, corroborating with their peptide profiles, which showed some similarities. According the RP-HPLC chromatograms, all enzyme treatments presented a tendency to stabilize after 120 min-reaction. The same tendency was observed for the antioxidant activity analyses, which revealed ALC

hydrolysate as the most capable of scavenging the radical ABTS+, followed by TRY, NPD and FLA hydrolysates. Pancreas Trypsin showed an average emulsifying activity, while Novo Pro-D and Alcalase showed satisfactory results towards this property. In this sense, among tested enzyme, ALC, TRY and NPD could be used for producing dual-functional hydrolysates, especially the 120 min fraction of TRY hydrolysate, which presented high antioxidant activity and emulsifying capacity.

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Final Considerations

FINAL CONSIDERATIONS

Waste management is a clever strategy not only for solving environmental hazards but also for expanding profits in different areas. From the point of view of dairy industries, investments in valorizing whey continue to increase over the years as it seems to be a promising market. Following this tendency, in this study three ingredients were developed aiming to add value to whey protein concentrates.

The first ingredient was a whey protein hydrolysate with vasorelaxant potential aiming its future use in the prevention and treatment of hypertension, the most important risk factor for cardiovascular diseases worldwide. The development of this ingredient ranged from the production to consumers' evaluation, meaning that it was produced, it was physically and technologically characterized, as well as sensory evaluated. The development of this ingredient was also evaluated in semi-pilot scale. Assays of digestion and absorption were very interesting, as in the first, the results showed that its vasorelaxant potential was enhanced after a simulated gastrointestinal digestion and in the last, several oligopeptides could be absorbed, including not only the antihypertensive ones, but also with other related bioactivities.

Dealing with the second ingredient, the results of the chemical analysis showed that microwave and autoclave treatments were able to modify β-lactoglobulin's structure prior its complete hydrolysis by pepsin. Although the ELISA immune assays were not finished yet, the results obtained until this moment are very promising and this is the first time that a study compares the use of different emerging technologies in the obtainment of a hypoallergenic whey hydrolysate.

Concerning the last ingredient, its development aimed at valorizing the major whey ingredient produced in Brazil and the treatments applied were able to enhance its antioxidant capacity and emulsifying properties, adding value to this ingredient that may be used in the formulation of several food products.

In conclusion, the ingredients were successfully developed and can be used in the formulation of different products by food and nutraceutical industries.

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