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NATALIA CALDEIRA DE CARVALHO

IMPACTO DAS ALTERAÇÕES FÍSICO-QUÍMICAS DO ISOLADO PROTEICO DO SORO DE LEITE POR PROTEÓLISE E TRATAMENTO COM TRANSGLUTAMINASE NO GOSTO AMARGO E NA CAPACIDADE DE SENSIBILIZAÇÃO *IN VIVO*

IMPACT OF PHYSICOCHEMICAL CHANGES IN WHEY PROTEIN ISOLATE BY PROTEOLYSIS AND TRANSGLUTAMINASE TREATMENT IN BITTERNESS AND SENSITIZING CAPACITY *IN VIVO*

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Alimentos em Nutrição, na área de Nutrição Experimental e aplicada à Tecnologia de Alimentos.

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Orientadora: Profª. Drª. Flavia Maria Netto

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

"If you want to go fast, go alone.

If you want to go far, go together."

Provérbio Africano

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RESUMO

A hidrólise enzimática ainda é o método mais utilizado para destruir epítopos, os quais consistem numa porção da proteína capaz de induzir a produção de anticorpos específicos e se ligar a eles provocando resposta alérgica. Os epítopos conformacionais são facilmente destruídos durante a hidrólise. No entanto, epítopos lineares podem ainda sobreviver ao processo, o que resulta em antigenicidade residual em hidrolisados proteicos. Além disso, a hidrólise de proteínas extensiva leva à liberação de peptídeos com gosto amargo. A combinação de hidrólise enzimática e tratamento com transglutaminase (TG) é uma estratégia promissora para diminuir a antigenicidade de proteínas do leite e o gosto amargo normalmente presente nos hidrolisados. Este estudo avaliou as alterações físico-químicas em isolado proteico do soro de leite (IPS) induzidas pela hidrólise com Alcalase seguida por tratamento com TG e se investigou o impacto no gosto amargo e na antigenicidade dos peptídeos. As hidrólises foram realizadas sob condição sem controle de pH a fim de simular a condição empregada na indústria. Para isto, o impacto da hidrólise conduzida sob condições de pH controlada ou não, utilizando diferentes concentrações de substrato ($S_{\%}$, 3 ou 7% de proteína) e relações enzima:substrato (E/S, 50 ou 100 U g^{-1}), na antigenicidade dos hidrolisados foi investigado. O pH diminuiu de 8,5 para 7,0 nos 15 primeiros minutos das hidrólises sem controle de pH e se manteve constante até o final da reação. A redução no pH não alterou significativamente o grau de hidrólise (GH) dos hidrolisados obtidos com e sem controle de pH, utilizando as mesmas $S_{\%}$ e E/S. O GH diminuiu com o aumento do $S_{\%}$, enquanto o aumento da E/S elevou ligeiramente o GH dos hidrolisados. As diferentes condições de pH e $S_{\%}$ resultaram em distintos perfis de hidrofilicidade (CLAE-FR) e distribuição de massa molecular (MM) entre os hidrolisados, indicando mudanças no padrão de hidrólise da Alcalase em função desses parâmetros. Essas diferenças refletiram no comportamento de agregação e conformação espacial dos peptídeos, e nas concentrações de β-Lg e α-La, detectadas por kits comerciais de ELISA. Os resultados de ELISA e *immunoblotting* indicaram que a hidrólise diminuiu a capacidade dos hidrolisados de se ligarem às IgE e IgG anti-α-La e anti-β-Lg, não havendo diferenças significativas entre aqueles obtidos com e sem controle de pH. Todavia, os hidrolisados obtidos sem controle de pH apresentaram maior capacidade de ligação às IgE e IgG anti-BSA, devido à presença de BSA intacta e/ou a liberação de epítopos pelo processo de hidrólise. Diante disso, o estudo prosseguiu usando a hidrólise sem controle de pH, e ultrafiltração usando membrana de corte de 5,0 kDa para a separação da BSA intacta e parcialmente hidrolisada. Os filtrados obtidos foram tratados com TG, sendo avaliado o gosto amargo e antigenicidade. Após o tratamento com TG, houve aumento de 1,5 e 3,0% na proporção de peptídeos entre 1,4 e 3,5 kDa, alterações no perfil cromatográficos, em especial na intensidade dos picos na região de alta e média hidrofilicidade e desaparecimento de um pico na região de baixa hidrofilicidade (CLAE-FR) e na conformação espacial dos peptídeos, confirmada pelos espectros de fluorescência. As alterações no perfil de peptídeos dos filtrados tratados com TG também foram observadas através dos espectros de massas (MALDI-MS), nos quais não foram mais detectados íons com *m/z* entre 1600 e 3000. Apesar dessas alterações, não ocorreu redução na intensidade e/ou persistência do gosto amargo dos filtrados tratados com TG. A hidrólise seguida pela ultrafiltração, associada ou não à TG, foi capaz de diminuir significativamente a capacidade do IPS de se ligar às IgE específicas. O HF7, tratado ou não com TG, não induziu a produção de IgE e IgG1 específicas em camundongos sensibilizados. A TG não exerceu efeito adicional na redução da capacidade de ligação às IgE específicas ou de sensibilização dos filtrados. Portanto, a hidrólise do IPS com Alcalase

seguida pela ultrafiltração mostra-se uma alternativa em potencial para a produção de hidrolisados hipoalergênicos, de acordo com os critérios estabelecidos pela *American Academy of Pediatrics* para a avaliação da hipoalergenicidade de novas fórmulas.

ABSTRACT

Enzymatic hydrolysis is widely used method to destroy epitopes, a part of protein capable to induce the production of specific antibodies and to bind to them eliciting allergic response. Conformational epitopes collapse during hydrolysis, while linear epitopes may survive the process, leading to residual antigenicity in protein hydrolysates. Moreover the extensive hydrolysis of proteins releases bitter peptides. The combination of enzymatic hydrolysis and transglutaminase (TG) treatment is a promising strategy to reduce the protein antigenicity and bitter taste often associated with protein hydrolysates. This study evaluated the physicochemical changes in whey protein isolate (WPI) caused by hydrolysis using Alcalase and TG-treatment and on bitter taste and protein antigenicity, including IgE-binding response and ability to sensitize animal model. The hydrolysis reactions were performed under uncontrolled pH condition in order to simulate conditions used in food industry. For that, the impact of hydrolysis performed with or without pH control, using different substrate concentration ($S_{\%}$, 3 or 7% of protein) and enzyme:substrate ratio (E/S, 50 or 100 U g^{-1}), on antigenicity of the hydrolysates was investigated. The pH dropped from 8.5 to 7.0 within the first 15 min of hydrolysis without pH control, and remained constant until the end of reaction. The decrease in pH did not significantly affect the degree of hydrolysis (DH) of the hydrolysates obtained with and without pH control, under the same $S_{\%}$ and E/S parameters conditions. The DH decreased with increasing $S_{\%}$, whereas the increase in E/S slightly raised the DH of the hydrolysates. The different condition of pH and $S_{\%}$ resulted in different hydrophilicity profiles (RP-HPLC) and molecular mass (MM) distribution among hydrolysates, indicating changes in the cleavage pattern of Alcalase. These differences resulted in different aggregation behavior and spatial conformation of the peptides, and different concentrations of β-Lg and α-La detected by commercial ELISA kits. The ELISA and immunoblotting results indicated that the hydrolysis with Alcalase reduced the binding capacity of hydrolysates to anti-β-Lg and anti-α-La IgE and IgG and no differences were observed among hydrolysates obtained with and without pH control. However, the hydrolysates obtained uncontrolled pH showed increase reactivity to anti-BSA IgE and IgG, due to the presence of intact BSA and/or releasing of epitopes. Therefore the study continued with the hydrolysates obtained under uncontrolled pH, which were ultrafiltered, using a 5.0 kDa cutoff membrane, in order to separate unhydrolysed and partially hydrolyzed BSA. The filtrates were treated with TG and bitter taste and antigenicity were assessed. After TG-treatment, the filtrates showed 1.5 and 3.0% increasing in the amount of peptides between 1.4 and 3.5 kDa, changes in chromatographic profile, especially in peaks intensity in the regions of high and medium hydrophilicity and disappearance of a peak in the region of low hydrophilicity (PR-HPLC) and spatial conformation of the peptides, confirmed by fluorescence spectra. Changes in peptide profile of the filtrates treated with TG were also observed through mass spectra (MALDI-MS), in which ions at *m/z* in the range of 1600 to 3000 were no longer detected. Despite these changes, no difference was observed in the intensity or perception of bitter taste in the filtrates after TG treatment. The hydrolysis with Alcalase followed by ultrafiltration, associated or not with TG, reduced significantly the binding capacity of WPI to specific IgE. The HF7 treated or not with TG did not generate specific IgE and IgG1 in mouse model of allergy. TG had no further effect in reducing of IgE-binding capacity or ability to induce sensitization of the filtrates. Therefore, hydrolysis of WPI with Alcalase followed by ultrafiltration can be a potential alternative to produce hypoallergenic hydrolysates, according to established criteria by American Academy of Pediatrics to determine hypoallergenicity of new formulas.

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LISTA DE ABREVIATURAS E SIGLAS

ALV	Alergia ao leite de vaca
ANOVA	*Analysis of variance*
ANS	*1-anililo-naphthalene-8-sulfonate*
BSA	Albumina sérica bovina; *Bovine serum albumin*
CHCA	*α-cyano-4-hydroxycinnamic acid*
CLAE-FR	Cromatografia líquida de alta eficiência de fase reversa
CMA	*Cow's milk allergy*
Cys	*Cysteine*
DAD	*Diode-array detector*
DH	*Degree of Hydrolysis*
DPP-IV	*Dipeptidyl peptidase IV*
ELISA	*Enzyme-linked immunosorbent assay*
E/S	Relação enzima:substrato; *Enzyme:substrate ratio*
GH	Grau de hidrólise
Glu	*Glutamine*
H3	Hidrolisado obtido com 3% de proteína
H7	Hidrolisado obtido com 7% de proteína
HC3-50	Hidrolisado obtido com controle de pH, 3% de proteína e 50 U g^{-1} de Alcalase
HC3-100	Hidrolisado obtido com controle de pH, 3% de proteína e 100 U g^{-1} de Alcalase
HC7-50	Hidrolisado obtido com controle de pH, 7% de proteína e 50 U g^{-1} de Alcalase
HC7-100	Hidrolisado obtido com controle de pH, 7% de proteína e 100 U g^{-1} de Alcalase
His	*Histine*
HF3	Fração filtrado do hidrolisado obtido com 3% de proteína
HF7	Fração filtrado do hidrolisado obtido com 7% de proteína
HF3-TG	Fração filtrado do hidrolisado obtido com 3% de proteína e tratada com TG
HF7-TG	Fração filtrado do hidrolisado obtido com 7% de proteína e tratada com TG
HPLC	H*igh performance liquid chromatography*
HR3	Fração retentado do hidrolisado obtido com 3% de proteína
HR7	Fração retentado do hidrolisado obtido com 7% de proteína
HUn3-50	Hidrolisado obtido sem controle de pH, 3% de proteína e 50 U g^{-1} de Alcalase
HUn3-100	Hidrolisado obtido sem controle de pH, 3% de proteína e 100 U g^{-1} de Alcalase
HUn7-50	Hidrolisado obtido sem controle de pH, 7% de proteína e 50 U g^{-1} de Alcalase
HUn7-100	Hidrolisado obtido sem controle de pH, 7% de proteína e 100 U g^{-1} de Alcalase
IFN-γ	Interferon γ
IgE	Imunoglobulina E; *Imunoglobulin E*
IgG	Imunoglobulina G; *Imunoglobulin G*
IgG1	Imunoglobulina G1; *Imunoglobulin G1*
IgG2a	Imunoglobulina G2a; *Imunoglobulin G2a*
IgG4	Imunoglobulina G4

IL-2	Interleucina 2
IL-4	Interleucina 4
IL-5	Interleucina 5
IL-6	Interleucina 6
IL-12	Interleucina 12
IL-13	Interleucina 13
IPS	Isolado proteico do soro de leite
Lys	*Lysine*
MM	Massa molecular; *Molecular mass*
m/z	Razão massa:carga; *Mass:charge ratio*
OPA	*O-phthaldialdehyde*
PBS	*Phosphate buffered saline*
PITIC	*Phenylisothiocyanate*
RFI	*Relative fluorescence intensity*
RP-HPLC	*Reverse phase high performance liquid chromatography*
S_0	*Surface hydrophobicity*
SAS	*Statistical Analysis System*
SDS	*Sodium dodecylsulphate*
SDS-PAGE	*Sodium dodecyl sulphate–polyacrylamide gel electrophoresis*
SE-HPLC	S*ize-exclusion high performance liquid chromatography*
$S_{\%}$	Concentração de substrato; Substrate concentration
TCA	*Trichloroacetic acid*
TFA	*Trifluoroacetic acid*
TG	Transglutaminase
Th1	Linfócito T auxiliar do tipo 1; *Lymphocyte T helper type 1*
Th2	Linfócito T auxiliar do tipo 2; *Lymphocyte T helper type 2*
TIAFT	*Time-Intensity Analysis of Flavors and Tastes*
TMB	*3,3´,5,5´-tetramethylbenzidine*
Tris	*Tris(hydroxymethyl)aminomethane*
Trp	*Tryptophan*
U	*One unit of enzyme activity*
WPI	*Whey protein isolate*
α-La	α-lactalbumina; *α-lactabumin*
β-Lg	β-lactoglobulina; *β-lactoglobulin*

SUMÁRIO

CAPÍTULO 1. INTRODUÇÃO GERAL

1.1. Introdução

A alergia alimentar é um importante problema de saúde pública mundial que vem aumentando nas duas últimas décadas. As alergias associadas aos alimentos afetam de 1 a 3% da população adulta e de 4 a 6% das crianças (RAHAMAN, VASILJEVIC e RAMCHANDRAN, 2016). O aumento na prevalência de indivíduos alérgicos está relacionado às mudanças no estilo de vida, hábitos alimentares e fatores epigenéticos (SICHERER e LEUNG, 2011).

Mais de 90% das alergias alimentares são causadas por leite de vaca, ovos, peixes, crustáceos, amendoim, castanhas, trigo e soja, os quais são chamados de "*The Big Eight*" (VERHOECKX *et al.*, 2015). O leite de vaca é uma das principais causas de alergia alimentar em crianças menores de três anos de idade, atingindo de 2 a 5% da população infantil (EL-AGAMY, 2007; SACKESEN *et al.*, 2011).

A alergia ao leite de vaca (ALV) é uma reação imunológica adversa mediada principalmente pelas imunoglobulinas E (IgE) o qual se caracteriza por uma resposta T *helper* 2 (Th2) que resulta na produção de anticorpos IgE específicos ao alérgeno (fase de sensibilização). A ligação das IgE aos receptores de alta afinidade FcεRI na superfície de mastócitos e basófilos seguida pela ligação cruzada dos anticorpos IgE ao alérgeno desencadeia a desgranulação e a liberação de mediadores inflamatórios que provocam os sintomas clínicos da alergia (fase efetora) (KUMAR *et al.*, 2012). A ALV é desencadeada pelas principais proteínas do leite: as caseínas, α-lactalbumina (α-La) e β-lactoglobulina (β-Lg) (MONACI *et al.*, 2006). As porções das proteínas que se ligam aos anticorpos IgE são denominadas epítopos e podem ser tanto uma parte da estrutura tridimensional (epítopo conformacional) quanto uma sequência de aminoácidos na molécula (epítopo linear) (FOEGEDING e DAVIS, 2011). Os epítopos lineares parecem ser mais importantes nas alergias alimentares, uma vez que estes são mais estáveis, podendo ser mantidos mesmo após a digestão (MARTORELL-ARAGONÉS *et al.*, 2015).

A hidrólise enzimática ainda é um dos métodos mais utilizados para destruir epítopos e produzir produtos hipoalergênicos destinados aos indivíduos alérgicos às proteínas do leite, especialmente as crianças (ZHENG *et al.*, 2008). A redução da antigenicidade por hidrólise enzimática não depende apenas da especificidade da enzima, mas também dos parâmetros da reação (pH, concentração de substrato e enzima, temperatura) os quais podem alterar o padrão de hidrólise da enzima (seletividade) (BUTRÉ *et al.*, 2014a).

As fórmulas hipoalergênicas, compostas por proteínas extensivamente hidrolisadas, são a alternativa recomendada na alimentação de crianças diagnosticadas com ALV quando o aleitamento materno não é possível (VAN ESCH *et al.*, 2010). Entretanto, a hidrólise extensiva das proteínas do leite libera peptídeos amargos que tornam essas fórmulas pouco palatáveis (NEWMAN *et al.*, 2015). Além disso, embora epítopos conformacionais sejam facilmente destruídos durante a hidrólise, epítopos lineares podem resistir ao processo. Estudos têm mostrado que algumas fórmulas hipoalergênicas ainda apresentam antigenicidade residual associada à presença de epítopos não hidrolisados (GORTLER, URBANEK e FORSTER, 1995; VAN BERESTEIJN, MEIJER e SCHMIDT, 1995b; PUERTA, DIEZ-MASA e DE FRUTOS, 2006; BRAHIM *et al.*, 2012).

Tendo em vista as limitações da hidrólise enzimática na produção de produtos hipoalergênicos, novas estratégias para redução da antigenicidade de proteínas têm sido estudadas. A polimerização com TG tem sido avaliada com o propósito de diminuir a antigenicidade de proteínas (BABIKER *et al.*, 1998; STANIC *et al.*, 2010; VILLAS-BOAS *et al.*, 2010; VILLAS-BOAS *et al.*, 2012). A TG catalisa reações de transferência de acil entre o grupo γ-carboxiamida dos resíduos de glutamina e aminas primárias, incluindo o grupo ε-amina da lisina (AGYARE e DAMODARAN, 2010). A formação de ligações covalentes inter- e intramoleculares em proteínas/peptídeos pode alterar e/ou mascarar epítopos lineares diminuindo a ligação da proteína aos anticorpos IgE (BABIKER *et al.*, 1998; DAMODARAN e LI, 2017).

A combinação da hidrólise enzimática das proteínas do soro de leite seguida pelo tratamento com TG mostrou-se uma estratégia promissora para a obtenção de produtos hipoalergênicos (WRÓBLEWSKA *et al.*, 2008). Estudos anteriores em nosso laboratório não observaram um efeito adicional do tratamento da β-Lg hidrolisada com TG na redução da antigenicidade dessa proteína (SABADIN *et al.*, 2012; VILLAS-BOAS *et al.*, 2015). No entanto, após a digestão gástrica, a β-Lg hidrolisada e tratada com TG apresentou menor capacidade de ligação à IgE específica em comparação à β-Lg apenas hidrolisada, sugerindo que o tratamento com TG atenuou a resposta antigênica (VILLAS-BOAS *et al.*, 2015).

O tratamento com TG ainda pode ser uma estratégia para diminuir o gosto amargo presente nos hidrolisados proteicos (BABIKER *et al.*, 1996; SONG *et al.*, 2013). A liberação de peptídeos de baixa massa molecular (MM) e com alto conteúdo de resíduos hidrofóbicos são os responsáveis por conferir gosto amargo aos hidrolisados proteicos (NEWMAN *et al.*, 2015). O tamanho dos peptídeos e a presença de resíduos de aminoácidos hidrofóbicos são características determinantes na ligação dos peptídeos aos receptores de gosto amargo nas papilas gustativas (ISHIBASHI *et al.*, 1988a). O tratamento com TG pode diminuir o gosto

amargo dos hidrolisados tanto pelo aumento da MM quanto pela redução da exposição dos resíduos hidrofóbicos (BABIKER *et al.*, 1996).

Os resultados divergentes da combinação de hidrólise enzimática e tratamento com TG no potencial antigênico das proteínas observadas entre os estudos (WRÓBLEWSKA *et al.*, 2008; SABADIN *et al.*, 2012; VILLAS-BOAS *et al.*, 2015; DAMODARAN e LI, 2017) ressaltam a importância da realização de testes pré-clínicos adequados para avaliar a antigenicidade das proteínas a cada alteração nos métodos e/ou condições dos tratamentos realizados (VAN ESCH *et al.*, 2011). Os testes pré-clínicos, preconizados pela *American Academy of Pediatrics* para a avaliação da hipoalergenicidade, incluem a quantificação de material imunorreativo residual e a determinação da capacidade da fórmula de sensibilizar e provocar reações em modelos animais de alergia (AAP, 2000). No entanto, o impacto dos tratamentos na capacidade das proteínas de induzir a produção de anticorpos IgE específicos ainda é muito pouco investigado (VERHOECKX *et al.*, 2015).

Este trabalho estudou o impacto da combinação da hidrólise do isolado proteico do soro de leite (IPS) com Alcalase seguida pelo tratamento com TG nas características físico-químicas, no gosto amargo dos peptídeos liberados pela hidrólise e antigenicidade das proteínas. As propriedades físico-químicas do IPS após os tratamentos enzimáticos foram avaliadas a fim de verificar como alterações nas características das proteínas poderiam influenciar na antigenicidade e gosto amargo. A antigenicidade residual do IPS após os tratamentos foi avaliada (1) *in vitro* pela medida da sua capacidade de se ligar a anticorpos específicos do soro de camundongos sensibilizados e (2) *in vivo* pela sua capacidade de induzir sensibilização em um modelo animal de ALV.

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CAPÍTULO 2. REVISÃO BIBLIOGRÁFICA

2.1. O leite de vaca

O leite de vaca e seus derivados são alimentos tradicionais na dieta humana (HAUG, HOSTMARK e HARSTAD, 2007). O leite de vaca é uma excelente fonte de proteínas, gordura, minerais e vitaminas, sendo um produto de grande importância comercial e industrial. A composição química do leite varia em função da espécie do mamífero, genética do animal e seu estado nutricional, do estágio de lactação e das condições ambientais (PEREIRA, 2014).O leite de vaca contém 87,3% de água, de 3,3 a 3,5% de proteínas, de 3,5 a 3,8% de gordura, 4,9% de lactose, 0,7% de minerais, e 0,1% de vitaminas (SGARBIERI, 2005).

A fração lipídica do leite é composta por 98% de triacilglicerol, 2% de diacilglicerol, menos de 0,5% colesterol, 1% de fosfolipídeos e 0,1% de ácidos graxos livres. Em média, 70% da fração lipídica correspondem a ácidos graxos saturados e 30% a ácidos graxos insaturados. O leite é considerado uma fonte importante de cálcio na dieta humana, mas contém outros importantes minerais para nutrição e funcionamento adequados do organismo, tais como fósforo, magnésio, zinco e selênio. O leite ainda é composto pelas vitaminas lipossolúveis A, D e E, e pelas vitaminas do complexo B tais como tiamina e riboflavina (HAUG, HOSTMARK e HARSTAD, 2007; PEREIRA, 2014).

O leite também é considerado fonte importante de proteína na dieta humana. As proteínas do leite são divididas em duas frações: as caseínas e as proteínas do soro, as quais correspondem a 80% e 20% da proteína total, respectivamente (MONACI *et al.*, 2006). Ambas as frações proteicas são classificadas como proteínas de alto valor biológico, isto é, contêm todos os aminoácidos essenciais, e apresentam alta digestibilidade e biodisponibilidade. As caseínas são especialmente ricas em histidina, metionina e fenilalanina, enquanto as proteínas do soro possuem maior proporção de aminoácidos de cadeia lateral ramificada (leucina, isoleucina e valina) e de lisina (PEREIRA, 2014).

As caseínas são as proteínas mais abundantes no leite de vaca. Elas são compostas por quatro proteínas independentes codificadas por diferentes genes presentes no mesmo cromossomo: α_{S1}-, α_{S2}-, β- e κ-caseínas, além das três γ-caseínas (γ1, γ2 e γ3-caseínas) derivadas da hidrólise da β-caseína, essas proteínas representam 30%, 9%, 28%, 10% e 2% da quantidade total de caseína, respectivamente (MICIŃSKI *et al.*, 2013; TSABOURI, DOUROS e PRIFTIS, 2014). Além de estarem presentes em diferentes concentrações, as frações de caseína diferem quanto ao conteúdo de fósforo, composição de aminoácidos, MM e ponto isoelétrico (MICIŃSKI *et al.*, 2013). A principal função das

caseínas está associada à sua capacidade de se ligar a minerais e de transportá-los pelo organismo, especialmente no caso do cálcio e fósforo (PEREIRA, 2014).

A β-Lg (55-60%) e α-La (15-20%) são as principais proteínas do soro de leite, mas também há outras proteínas presentes em menores quantidades, tais como BSA, imunoglobulinas, lactoferrina, fosfolipoproteínas, lactoperoxidase, lisozima, transferrina, protease-peptonas (IZQUIERDO *et al.*, 2008; MICIŃSKI *et al.*, 2013). As proteínas do soro apresentam quase todos os aminoácidos essenciais em excesso às recomendações nutricionais, exceto os aminoácidos aromáticos os quais não estão em excesso, mas atendem às recomendações para todas as faixas etárias (SGARBIERI, 2004; 2005).

A β-Lg é uma proteína globular com MM de 18,3 kDa com 162 resíduos de aminoácidos e pI (ponto isoelétrico) de 5,1-5,2. A β-Lg ocorre naturalmente na forma de um dímero (36 kDa) não covalente, em equilíbrio no leite, com monômero da β-Lg. Essa proteína do soro apresenta um importante polimorfismo genético com, no mínimo, sete variantes genéticas, sendo as principais variantes a β-Lg A e B. Essas variantes genéticas diferem-se nos resíduos 64 e 118, respectivamente: ácido aspártico e valina na β-Lg A e glicina e alanina na β-Lg B (WAL, 2001; GAUCHE *et al.*, 2008). A molécula de β-Lg possui duas pontes dissulfeto intramoleculares e um grupo tiol livre. Essa estrutura está relacionada às principais características físico-químicas e à interação com a caseína durante tratamentos térmicos (WAL, 2001).

A α-La é uma proteína globular monomérica de 123 resíduos de aminoácido, com quatro ligações dissulfeto, estrutura compacta e MM de 14,4 kDa, e pI entre 4,0-5,0 (GAUCHE, BARRETO e BORDIGNON-LUIZ, 2010). Essa proteína participa da regulação do sistema enzimático da galactosil transferase responsável pela síntese de lactose (SGARBIERI, 2004). A α-La possui alta afinidade de ligação pelo cálcio, o que estabiliza sua estrutura terceária (WAL, 2001).

A BSA corresponde aproximadamente a 5% das proteínas do soro de leite. A BSA é uma proteína globular composta de 580 resíduos de aminoácidos com MM de 66,4 kDa e pI entre 4,7-4,8. Sua estrutura secundária é formada por 54% de α-hélice, 40% de estruturas *β-sheet* e *β-turn* com três domínios específicos para ligação de íons metálicos, de lipídeos e de nucleotídeos (SGARBIERI, 2005). Esta proteína está organizada em três domínios homólogos e consiste de nove loops ligados por 17 ligações dissulfeto. Muitas dessas ligações dissulfeto localizam-se no núcleo da proteína o que dificulta seu acesso. Isto pode explicar a grande estabilidade da sua estrutura terciária mesmo sob condições de desnaturação (MONACI *et al.*, 2006).

A lactoferrina é uma glicoproteína de MM de 76,1 kDa e a pH 8,0 que se liga fortemente ao íon Fe^{+3}. Essa proteína é formada por duas cadeias polipeptídicas homólogas e 691 resíduos de aminoácidos, cada uma com um sítio de ligação ao íon Fe^{+3} e um sítio de glicolisação. Os dois lobos da cadeia polipeptídica da lactoferrina são conectados por uma hélice de três voltas (SGARBIERI, 2005).

As imunoglobulinas são monômeros ou polímeros formados por unidades de quatro cadeias polipeptídicas: duas curtas (~20 kDa) e duas longas (50-70 kDa), ligadas por pontes dissulfeto (SGARBIERI, 2005). A fração proteose-peptona corresponde a 1,1% do total das proteínas do leite e é derivada principalmente da proteólise da β-caseína, e sua concentração no leite pode aumentar com o tempo devido à atividade enzimática da plasmina (MONACI *et al*., 2006).

2.2. Alergia ao leite de vaca (ALV)

O leite humano é o alimento mais natural e completo nutricionalmente para a alimentação de recém-nascidos e crianças. No entanto, quando o aleitamento materno não é possível, o leite de vaca é o substituto mais comum ao leite humano (EL-AGAMY, 2007). O leite humano contém menor concentração de caseínas e maior de proteínas do soro em comparação ao leite de vaca, o que o torna o leite humano mais nutritivo para recém-nascidos. O coágulo formado pelo leite humano após a ingestão resulta em maior digestibilidade e absorção das proteínas, enquanto o leite de vaca forma um coágulo mais difícil de digerir (BARŁOWSKA *et al*., 2011). A substituição do leite humano pelo leite de vaca na alimentação de lactentes não causa somente consequências nutricionais, mas também pode provocar alterações imunológicas, tal como a alergia às proteínas do leite (EL-AGAMY, 2007).

O leite humano não contém α_{S1}-caseína e β-Lg, as quais estão entre as principais proteínas envolvidas na alergia ao leite. Muitos estudos demonstraram que a maioria das crianças com ALV possuem anticorpos predominantemente contra α-caseína e β-Lg, sendo, respectivamente, 60 e 80% desses pacientes sensíveis a essas proteínas. O leite de vaca apresenta alta concentração dessas duas proteínas (BARŁOWSKA *et al*., 2011).

2.2.1. Conceito e epidemiologia

As reações adversas a alimentos são aquelas que ocorrem após a ingestão de um alimento, incluindo tanto aquelas mediadas pelo sistema imunológico (alergias alimentares,

doença celíaca) quanto àquelas não imuno-mediadas (intolerâncias alimentares, reações farmacológicas e mediadas por toxinas microbianas) (ASBAI, 2012).

Há duas principais reações adversas associadas à ingestão de leite de vaca. Uma delas é a intolerância à lactose a qual é provocada por uma diminuição da atividade da β-galactosidase, enzima responsável pela hidrólise da lactose em monossacarídeos durante a digestão. A intolerância à lactose provoca vários sintomas gastrointestinais decorrentes da fermentação deste açúcar no cólon (PEREIRA, 2014). A outra reação adversa consiste na alergia às proteínas do leite. A ALV é uma resposta imunológica de hipersensibilidade que pode ser mediada pela IgE ou não medida pela IgE e desencadeada por uma ou mais proteínas do leite (MONACI *et al.*, 2006; KUMAR *et al.*, 2012). As reações mediadas pela IgE são responsáveis por aproximadamente 60% das reações adversas provocadas pelo leite de vaca (HOCHWALLNER *et al.*, 2014). As reações mediadas pela IgE (ou reações do tipo I) são também conhecidas como hipersensibilidade do tipo imediata pois ocorrem dentro de minutos a horas após a exposição ao alérgeno (KUMAR *et al.*, 2012).

O leite de vaca, juntamente com o ovo e o amendoim, é considerado a causa mais comum de alergia alimentar. A prevalência da ALV tem aumentando nas últimas duas décadas o que pode ser explicado pela diminuição no aleitamento materno associada ao aumento do consumo de fórmulas infantis contendo leite bovino (HOCHWALLNER *et al.*, 2014). No entanto, a real dimensão da ALV na população geral é ainda desconhecida. Os dados sobre a prevalência da ALV são muito variados e refletem as diferenças das populações avaliadas (faixa etária, diferentes fenótipos, fatores geográficos, entre outros) assim como a falta de critérios para diagnósticos universalmente aceitos. No entanto, a prevalência de ALV em crianças parece variar entre 0,1% e 4,2% (SACKESEN *et al.*, 2011; MARTORELL-ARAGONÉS *et al.*, 2015).

Dados a respeito da prevalência de ALV e a severidade da doença para a América Latina ainda são escassos (SACKESEN *et al.*, 2011). No Brasil, um estudo observacional conduzido entre pediatras gastroenterologistas revelou prevalência de 5,4% (513 em 9.478) e incidência de 2,2% (211 em 9.478) de ALV diagnosticada ou suspeita na população estudada (VIEIRA *et al.*, 2010). As estimativas da prevalência de ALV são importantes para o controle da doença e o desenvolvimento de legislações e recomendações que possam melhorar a qualidade de vida dos indivíduos alérgicos.

2.2.2. Mecanismos imunológicos envolvidos, sinais e sintomas

A patogênese e o desenvolvimento da ALV provavelmente envolvem disfunção ou perda da tolerância imunológica durante o início da vida, combinada à permeabilidade

intestinal aumentada (WANG e SAMPSON, 2011). A tolerância é a redução da resposta imune à ingestão de leite ou outros alimentos. As razões pelas quais uma pequena parte da população apresenta resposta de hipersensibilidade aos componentes dos alimentos (basicamente proteínas), ainda não estão claras. Embora a predisposição genética exerça influência, a expressão fenotípica da alergia parece ser mediada por interações complexas entre fatores ambientais e genéticos. Esses fatores ambientais incluem condições higiênico-sanitárias, variações na ingestão de ácidos graxos, antioxidantes ou vitamina D, exposição ao alérgeno e o consumo de alimentos processados (MARTORELL-ARAGONÉS *et al.*, 2015).

As células T CD4+ *naive* dividem-se em duas grandes classes Th1 e Th2, classificadas basicamente de acordo com as citocinas que produzem (KUMAR et al., 2012), como mostra a Figura 2.1. A ALV corresponde a uma resposta imune inapropriada caracterizada pelo desequilíbrio do balanço Th1/Th2. Um perfil de resposta imune predominantemente Th2 determina as reações alérgicas, enquanto citocinas Th1 tendem a suprimir essas reações (LI *et al.*, 2013).

A ALV é causada principalmente por reações de hipersensibilidade mediadas pelas IgE. Na fase da sensibilização, os antígenos são processados pelas células apresentadoras de antígeno e apresentadas às células T. As células T CD4+ *naive* diferenciam-se principalmente em células Th2 na presença de quantidades adequadas de IL-4. As células Th2 ativas secretam as citocinas IL-4, IL-5, IL-6 e IL-13, as quais estimulam a diferenciação de células B e a produção de IgE específicas. As IgE ligam-se aos receptores de alta afinidade FcεRI na superfície de mastócitos e basófilos. A re-exposição ao mesmo alérgeno resulta na ligação cruzada deste aos anticorpos de IgE ligados ao FcεRI, o que desencadeia a desgranulação dos mastócitos e a liberação de mediadores inflamatórios incluindo histaminas, as quais são responsáveis pelos sintomas clínicos (KUMAR *et al.*, 2012; KIEWIET *et al.*, 2015). Na presença de IL-12 e IFN-γ, as células T CD4+ *naive* diferenciam-se principalmente em células Th1. As células Th1 efetoras produzem grandes quantidades de IL-2 e IFN-γ que induzem a ativação dos macrófagos (HALSTENSEN, 1997). Essas citocinas IFN-γ produzidas pelas células Th1 inibem a proliferação de células Th2 (KUMAR et al., 2012).

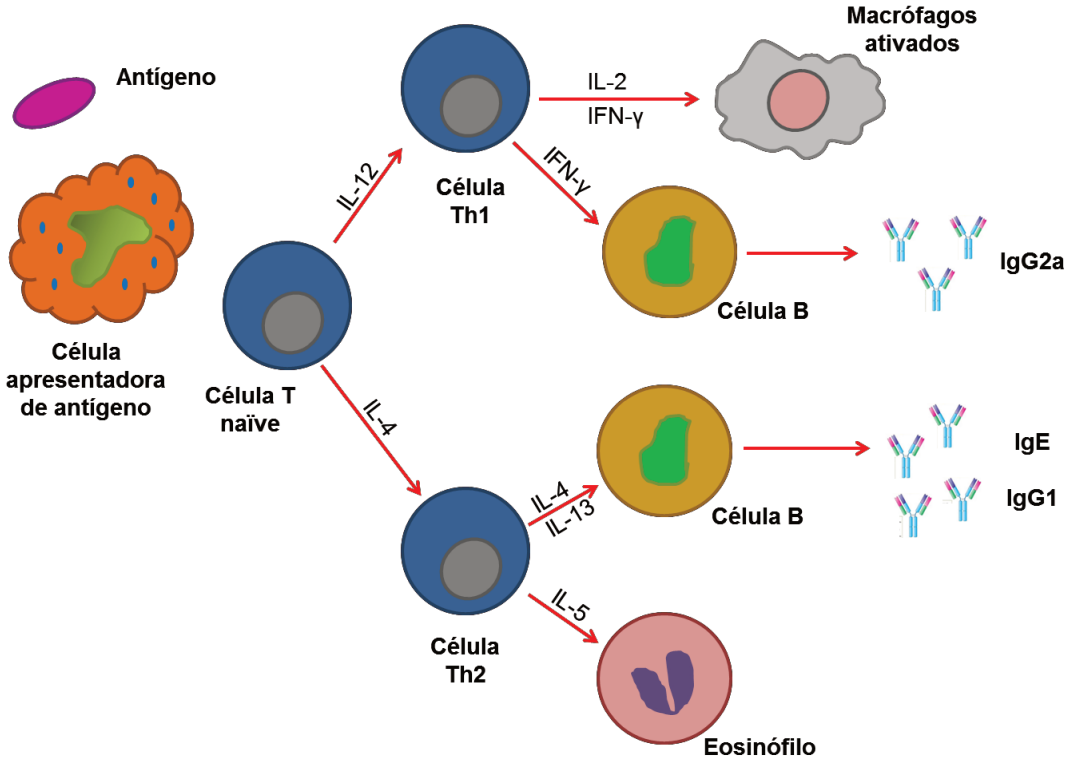

Figure 2.1: Respostas imume do tipo 1 (Th1) e do tipo 2 (Th2).

As reações alérgicas a alimentos não são limitadas apenas a reações mediadas por IgE, podendo também ser induzidas por respostas imune independentes de IgE. As reações alérgicas ao leite de vaca em crianças podem ser mediadas por IgG (HØST, 1997). Em camundongos, a resposta Th2 resulta na produção de IgE e IgG1, enquanto a resposta Th1 resulta na produção de IgG2a (Figura 2.1). Portanto, os isotipos IgG2a e IgG1 também podem ser usados como marcadores na resposta humoral de camundongos após imunização, refletindo a orientação Th1 ou Th2 da resposta, respectivamente (ADEL-PATIENT *et al.*, 2000).

A ALV mediada pela IgE caracteriza-se pelo aparecimento rápido dos sintomas, geralmente até duas horas após a exposição ao alérgeno. Entre as manifestações cutâneas, a urticária e o angiodema são os mais comuns. A hipersensibilidade gastrointestinal imediata compreende dor abdominal seguida de náuseas, vômito e diarreia. Os sintomas respiratórios compreendem congestão nasal, broncoespasmo, rinite, asma, tosse e podem vir associados a sintomas oculares, tais como hiperemia, prurido e lacrimejamento. A manifestação mais grave da ALV é a anafilaxia, a qual consiste em uma reação de hipersensibilidade grave, súbita e potencialmente fatal. Seus sintomas e sinais podem acometer um único órgão ou envolver mais de um sistema, sendo o sistema respiratório o

principal envolvido no choque anafilático (MONACI *et al.*, 2006; ASBAI, 2012; BURKS *et al.*, 2012). A ALV é a terceira causa mais comum de choque anafilático, depois da alergia ao amendoim e nozes, e representa de 10 a 19% dos casos de anafilaxia causados por alimentos (HOCHWALLNER *et al.*, 2014).

Na maioria das crianças, a ALV é controlada até os quatro anos de vida, entretanto algumas permanecem alérgicas para o resto da vida (EL-AGAMY, 2007). Os mecanismos envolvidos no desenvolvimento da tolerância ainda não estão completamente elucidados. Estes mecanismos podem incluir uma redução de anticorpos IgE específicos devido à exclusão do leite da dieta, a troca do isotipo para IgG4 devido à ingestão regular de leite e/ou a presença de anticorpos principalmente contra epítopos conformacionais e não contra epítopos lineares (HOCHWALLNER *et al.*, 2014).

2.2.3. Alérgenos do leite de vaca

A resposta dos indivíduos às proteínas do leite de vaca é caracterizada por uma grande variabilidade, não sendo possível identificar um único alérgeno ou estrutura particular responsável pela alergenicidade do leite. A sensibilidade a várias proteínas é observada em aproximadamente 75% do pacientes com ALV, com grande variabilidade na especificidade e intensidade da resposta de IgE (HOCHWALLNER *et al.*, 2014). Estudos populacionais mostram que mais de 50% dos indivíduos alérgicos sensíveis às caseínas, β-Lg e α-La (TSABOURI, DOUROS e PRIFTIS, 2014). Todavia, proteínas presentes em menores quantidades no leite de vaca, tais como a BSA, lactoferrina e imunoglobulinas, também podem provocar uma resposta alérgica (WAL, 2001).

As porções da proteína que se ligam à IgE são denominadas epítopos. Os epítopos podem ser: (1) lineares, os quais correspondem a uma determinada sequência linear de aminoácidos na molécula proteica; ou (2) conformacionais, ao quais correspondem a uma porção da estrutura tridimensional da proteína (FOEGEDING e DAVIS, 2011). Os epítopos lineares parecem ser mais importantes nas alergias alimentares, uma vez que estes são mais estáveis, podendo ser mantidos após a digestão. Os epítopos conformacionais perdem sua alergenicidade mais facilmente, pois a estrutura tridimensional do alérgeno pode ser destruída por tratamento térmico. A presença de anticorpos IgE que se ligam a múltiplos epítopos lineares pode ser um indicativo da persistência da alergia ou da severidade das reações em pacientes com ALV (JÄRVINEN *et al.*, 2001; MARTORELL-ARAGONÉS *et al.*, 2015).

Os indivíduos com ALV são normalmente sensíveis às diferentes caseínas. No entanto, a α_{S1}-caseína parece ser o alérgeno mais importante da fração das caseínas.

Vários epítopos lineares encontram-se distribuídos ao longo da estrutura da α_{S1}-caseína (HOCHWALLNER *et al.*, 2014). Devido à sua estrutura não globular e flexível, as frações das caseínas contêm mais epítopos lineares que conformacionais, o que pode explicar sua alergenicidade não ser reduzida após aquecimento (RAHAMAN, VASILJEVIC e RAMCHANDRAN, 2016).

A β-Lg possui epítopos conformacionais e lineares que estão distribuídos ao longo da sua estrutura (WAL, 2001). O potencial alergênico desta proteína tem sido atribuído à sua ausência no leite humano e à sua alta estabilidade e resistência às enzimas digestivas (HOCHWALLNER *et al.*, 2014). Devido ao grande número de epítopos lineares nas caseínas e β-Lg, a presença de altos níveis de anticorpos IgE específicos às essas proteínas também podem ser marcadores da persistência da ALV (KUITUNEN *et al.*, 2015).

A α-La também possui epítopos conformacionais e lineares. Contudo, a resposta de IgE aos epítopos lineares desta proteína é menor que a resposta aos epítopos lineares da β-Lg. Estudos (MAYNARD, JOST e WAL, 1997; JÄRVINEN *et al.*, 2001) mostraram que a maioria dos pacientes com ALV responderam à α-La nativa, sugerindo que os epítopos na α-La reconhecidos pela IgE são predominantemente epítopos conformacionais.

A alergenicidade da BSA é causada tanto por epítopos conformacionais quanto lineares (RESTANI *et al.*, 2004). A BSA é um alérgeno de menor importância no leite. Entretanto, é o principal alérgeno em carne (LICCARDI *et al.*, 2011). Dados da literatura mostram que entre 13 e 20% das crianças com ALV também são alérgicas à carne. A sensibilidade à BSA é um marcador de ALV em crianças alérgicas à carne (MARTELLI *et al.*, 2002).

2.2.4. Tratamento nutricional

O tratamento considerado mais eficaz para a ALV, até o momento, é eliminação do leite e seus derivados da dieta. O aleitamento materno é a melhor maneira para prevenir a sensibilização do sistema imune e alimentar crianças alérgicas, sendo indicado que as mães evitem consumir leites e seus derivados quando a criança, em aleitamento exclusivo, apresenta sintomas da ALV (VAN ESCH *et al.*, 2011). As proteínas do leite de vaca podem ser transmitidas para o leite humano (JÄRVINEN e SUOMALAINEN, 2001). Estudos mostraram a presença de α_{S1}-caseína e β-Lg no leite humano em diferentes períodos de lactação (HOST *et al.*, 1990; SORVA, MAKINEN-KILJUNEN e JUNTUNEN-BACKMAN, 1994; LOVEGROVE, MORGAN e HAMPTOM, 1996; COSCIA *et al.*, 2012; DENIS, LORAS-DUCLAUX e LACHAUX, 2012). A presença de proteínas do leite de vaca no leite humano, mesmo em pequenas quantidades, pode provocar sintomas da alergia em crianças

sensibilizadas (JÄRVINEN e SUOMALAINEN, 2001). A quantidade de β-Lg no leite humano é influenciada por variações intra- e inter-indivíduo (SORVA, MAKINEN-KILJUNEN e JUNTUNEN-BACKMAN, 1994).

Quando o aleitamento materno não é possível, as fórmulas hipoalergênicas compostas por proteínas extensivamente hidrolisadas são a alternativa mais adequada para a alimentação infantil (VAN ESCH *et al.*, 2010), sendo tolerada por 95% das crianças com ALV. Somente em caso de persistência dos sintomas, as fórmulas compostas por aminoácidos devem ser prescritas. As fórmulas extensivamente hidrolisadas são mais baratas que as fórmulas compostas por aminoácidos e apresentam resultados clínicos semelhantes (HOCHWALLNER *et al.*, 2014).

O uso de leite de outros mamíferos, tais como ovelha e cabra, não é uma alternativa apropriada para crianças alérgicas, uma vez que há uma alta homologia entre as proteínas dos leites de vaca, cabra e ovelha (MARTORELL-ARAGONÉS *et al.*, 2015). As bebidas e fórmulas à base de proteínas de soja necessitam ser consumidas com cuidado, visto que a soja pode induzir sintomas de alergia em até 15% das crianças com ALV (HOCHWALLNER *et al.*, 2014) devido às sequências de epítopos homólogos aos do leite de vaca.

A imunoterapia oral é uma nova abordagem para o tratamento da ALV e é aplicada somente em um número limitado de centros altamente especializados. Atualmente a imunoterapia não é recomendada como uma ferramenta terapêutica de rotina devido à ausência de protocolos padronizados, sendo empregada apenas em estudos controlados (SACKESEN *et al.*, 2011). Estes protocolos devem ser seguros e eficazes para diferentes faixas etárias, incluir uma dose ótima, grau de proteção, tempo ideal de duração e precisam apresentar uma projeção da severidade das reações adversas (HOCHWALLNER *et al.*, 2014). Os mecanismos envolvidos na imunoterapia incluem redução dos níveis de IgE específicas às proteínas do leite e da liberação de mediadores de basófilos, e a troca do isotipo para IgG4 (KUITUNEN *et al.*, 2015).

2.3. Fórmulas hipoalergênicas

As fórmulas hipoalergênicas são classificadas em parcial e extensivamente hidrolisadas dependendo do grau de hidrólise (GH) das proteínas e MM dos seus peptídeos. As fórmulas compostas por proteínas parcialmente hidrolisadas são indicadas para crianças com predisposição ao desenvolvimento da ALV, enquanto aquelas compostas por proteínas extensivamente hidrolisadas são recomendadas para crianças diagnosticadas com ALV (VAN ESCH *et al.*, 2011). As fórmulas hipoalergênicas compostas por proteínas

extensivamente hidrolisadas derivadas do leite de vaca geralmente contêm peptídeos menores que 1500 Da e aminoácidos livres (AAP, 2000).

Uma fórmula hipoalergênica ideal deve oferecer as seguintes propriedades: ser segura, facilitar a aquisição de tolerância imune, suprir as necessidades nutricionais de lactantes e crianças, ter boa palatabilidade e baixo custo (MARTORELL-ARAGONÉS *et al.*, 2015). As fórmulas hipoalergênicas normalmente incluem caseínas e proteínas do soro hidrolisadas, uma vez que essas proteínas apresentam alto valor nutricional, disponibilidade comercial e custo acessível (CLEMENTE, 2000). Dentre outros diversos fatores, as propriedades físico-químicas e estrutura das proteínas são características determinantes para induzir a tolerância ao alimento ou para sensibilizar o sistema imune e provocar resposta alérgica (SMIT *et al.*, 2016). A aplicação de diferentes enzimas, condições de hidrólise ou outros métodos para produção de produtos ou ingredientes hipoalergênicos tem efeitos diversos nas características das proteínas, o que pode influenciar na eficácia do tratamento e redução da alergenicidade, fazendo assim necessária a avaliação do potencial alergênico por meio de testes adequados (VAN ESCH *et al.*, 2010).

A *American Academy of Pediatrics* e o Guia Europeu para fórmulas infantis (AAP, 2000) estabelecem que as fórmulas, para serem usadas por crianças diagnosticadas com ALV, precisam passar por testes pré-clínicos e clínicos. Os testes pré-clínicos devem anteceder os testes clínicos em estudos conduzidos com crianças a fim de avaliar sua toxicidade, capacidade de manter um balanço positivo de nitrogênio e a probabilidade de crianças alérgicas reagirem adversamente a elas. Dentre esses testes, estão incluídas a determinação da distribuição de MM dos peptídeos provenientes da hidrólise; quantificação de material presente ainda reconhecido imunologicamente; e avaliação da capacidade de sensibilizar e provocar resposta alérgica em modelos animais de alergia.

A detecção e quantificação da imunorreatividade residual de proteínas modificadas pode ser realizada pela determinação da sua capacidade de se ligar aos anticorpos IgE específicos obtidos do soro de animais sensibilizados ou de humanos diagnosticados com ALV, usando diferentes testes imunoquímicos tais como *Western Blotting*, *Immunoblotting* e *Enzyme-Linked Immunosorbent Assay* (ELISA) (MONACI *et al.*, 2006). A redução na capacidade de ligação a anticorpos específicos é um importante indicativo de que a proteína modificada pode não ser mais capaz de provocar resposta alérgica *in vivo* (VERHOECKX *et al.*, 2015). No entanto, essa avaliação por si não assegura a hipoalergenicidade da fórmula, uma vez que epítopos podem ser mascarados pela agregação dos peptídeos e liberados durante a digestão (VAN ESCH *et al.*, 2011). A determinação de alérgenos residuais também pode ser feita avaliando a capacidade desses hidrolisados em provocar ativação de

mastócitos e basófilos por meio de testes de desgranulação em células *in vitro* (HOUBEN, KNIPPELS e PENNINKS, 1997; VAN ESCH *et al.*, 2011).

A avaliação da habilidade de proteínas modificadas em estimular a produção de anticorpos IgE específicos é fundamental, pois uma vez completada a fase de sensibilização o indivíduo está pronto para reagir ao alérgeno (KUMAR *et al.*, 2012). Os mecanismos envolvidos na fase de sensibilização alérgica são complexos e ainda difíceis de reproduzir em testes *in* vitro, o que torna importante o uso de modelos animais para avaliar a capacidade de proteínas modificadas em prevenir a sensibilização do sistema imune e reduzir os sintomas em indivíduos alérgicos (VAN ESCH *et al.*, 2010). Ao longo dos anos, vários modelos animais baseado em roedores foram desenvolvidos com a finalidade de avaliar a capacidade de sensibilização de alérgenos modificados (SMIT *et al.*, 2016).

Historicamente, os porquinhos-da-índia foram usados para investigar a alergenicidade de proteínas dos alimentos. Esses animais podem ser sensibilizados por via oral sem o uso de adjuvantes (FRITSCHE, 2003). Contudo, a sensibilização de porquinho-da-índia gera anticorpos IgG1a ao invés de IgE o que torna o modelo questionável visando a extrapolação para a realidade em humanos (VAN ESCH *et al.*, 2013b). Modelos usando ratos e camundongos também têm sido muito utilizados para testar a alergenicidade de proteínas modificadas. Entretanto, muitos utilizavam predominantemente sensibilização sistêmica por via intraperitoneal e subcutânea (KNIPPELS *et al.*, 1998), o que poderia induzir uma resposta imunológica diferente devido a diferenças no sistema imunológico mucoso e sistêmico (VAN ESCH *et al.*, 2010). Além disso, como a sensibilização sistêmica não reflete a via de sensibilização a alimentos em humanos (SCHOUTEN *et al.*, 2008), Li e colaboradores (1999) desenvolveram um modelo de ALV de sensibilização por via oral em camundongos usando toxina colérica com adjuvante para quebrar a tolerância oral. Atualmente, os modelos usando camundongos e o protocolo de sensibilização por via oral com toxina colérica tem sido apontados como ferramentas adequadas para avaliação da alergenicidade das proteínas do leite de vaca após processamento (VAN ESCH *et al.*, 2011; VAN ESCH *et al.*, 2013a; VAN ESCH *et al.*, 2013b; SMIT *et al.*, 2016).

Após os testes pré-clínicos, a fórmula ainda precisa ser testada em crianças com hipersensibilidade ao leite de vaca, por meio de testes de desafio oral duplo-cego prospectivo e randomizado controlado por placebo (AAP, 2000). De acordo com as recomendações da *American Academy of Pediatrics*, esses testes devem assegurar que a fórmula é tolerada, no mínimo, por 90% das crianças diagnosticadas com ALV mediada pela IgE (com um intervalo de confiança de 95%). Apesar da *European Commission Directive* 2006/141/EC de 22 de dezembro de 2006 regulamentar que as fórmulas infantis com alegação de alergenicidade reduzida devem conter menos de 1% de proteínas

imunorreativas, mas também recomenda que essas fórmulas sejam submetidas a testes clínicos adequados antes de serem utilizadas na alimentação de crianças diagnosticadas com ALV (EUROPEAN COMMISSION, 2006).

2.4. Métodos empregados para a redução da antigenicidade das proteínas do leite

O leite é uma fonte importante de lipídeos, proteínas e outros nutrientes na alimentação durante a primeira infância e sua eliminação da dieta devido a reações alérgicas pode causar problemas nutricionais (KATTAN, COCCO e JÄRVINEN, 2011). Diversos métodos têm sido estudados e aplicados com a finalidade de diminuir a alergenicidade das proteínas do leite para possibilitar o consumo deste alimento por indivíduos alérgicos. Esses métodos utilizados para redução da alergenicidade de proteínas do leite baseiam-se na modificação e/ou destruição de epítopos e incluem desnaturação térmica (KLEBER *et al.*, 2004), fermentação (EHN *et al.*, 2005), reação de *Maillard* ou glicação (CORZO-MARTÍNEZ *et al.*, 2010), alta pressão (IZQUIERDO *et al.*, 2008), polimerização (WRÓBLEWSKA *et al.*, 2008; VILLAS-BOAS *et al.*, 2010) e hidrólise enzimática (ZHENG *et al.*, 2008; SABADIN *et al.*, 2012; VILLAS-BOAS *et al.*, 2012; PESSATO *et al.*, 2016).

2.4.1. Hidrólise enzimática

A hidrólise enzimática ainda é um dos métodos mais utilizados para a destruição de epítopos e redução da antigenicidade das proteínas do leite (ZHENG *et al.*, 2008). Muitas fórmulas hipoalergênicas disponíveis no mercado são compostas por caseínas ou proteínas do soro de leite extensivamente hidrolisadas. O uso de enzimas oferece muitas vantagens, tais como a capacidade de efetuar modificações sob condições moderadas de pH (6-8) e temperatura (40-50°C) e com alta especificidade e estereoseletividade, minimizando reações colaterais indesejáveis e a formação de produtos tóxicos. Além disso, a modificação enzimática de proteínas é bem aceita por consumidores (STANIC-VUCINIC e VELICKOVIC, 2013). No entanto, estudos mostraram que essas fórmulas hipoalergênicas podem ainda apresentar atividade antigênica decorrente de material não hidrolisado (GORTLER, URBANEK e FORSTER, 1995; VAN BERESTEIJN, MEIJER e SCHMIDT, 1995; PUERTA, DIEZ-MASA e DE FRUTOS, 2006; BRAHIM *et al.*, 2012).

A redução da alergenicidade de proteínas por hidrólise enzimática depende de diferentes fatores associados ao processo: (1) a especificidade da enzima empregada, uma vez que a natureza da enzima influencia a composição final do hidrolisado (SVENNING *et*

al., 2000) e (2) os parâmetros de hidrólise: pH, concentração de substrato e enzima, e temperatura (ZHENG *et al*., 2008; BUTRÉ *et al*., 2014a).

A especificidade enzimática corresponde à ligação peptídica clivada pela enzima após determinados resíduos de aminoácidos, sendo essa ligação denominada sítio de clivagem (BUTRÉ *et al*., 2014a). Enzimas com ampla especificidade podem favorecer a destruição de epítopos, especialmente os lineares. Svenning e colaboradores (2000) observaram diferenças na redução da alergenicidade de hidrolisados de proteínas do soro de leite em função da enzima utilizada, porém não encontram correlação entre o grau de hidrólise dos hidrolisados e alergenicidade residual, o que indica a importância da especificidade da enzima na destruição de epítopos.

Alguns estudos relatam o impacto diverso dos parâmetros de reação na alergenicidade residual das proteínas do leite hidrolisadas enzimaticamente. Zheng e colaboradores (2008) encontraram que a temperatura e pH exerceram o maior efeito sobre a redução da resposta antigênica da α-La e β-Lg em comparação à E/S. Liu, Luo e Li (2012), por outro lado, observaram que a relação E/S exerceu o maior efeito na diminuição da alergenicidade das caseínas que o pH e temperatura. Cada enzima possui uma faixa de valores de pH e uma de temperatura dentro das quais sua atividade catalítica é máxima e abaixo ou acima desses valores ocorre uma queda na atividade da enzima e a velocidade de reação (YUST *et al*., 2010).

Apesar de cada enzima apresentar uma especificidade definida, nem todos os sítios de clivagem são hidrolisados na mesma velocidade. A seletividade descreve a taxa relativa que a enzima hidrolisa cada sítio clivagem dentro da sua especificidade. A seletividade de uma enzima pode ser influenciada por diferentes fatores: a temperatura na qual ocorre a reação; a acessibilidade do substrato e o estado de ionização dos aminoácidos dos sítios ativos e de clivagem, o que afeta a interação entre enzima e substrato (BUTRÉ *et al*., 2014a). Estudos mostraram influência do pH (VOROB'EV *et al*., 2000; BUTRÉ *et al*., 2015) e da concentração de substrato (BUTRÉ *et al*., 2014b) na seletividade de uma enzima ocorre devido a mudanças de estado de ionização dos aminoácidos da enzima e substrato e da acessibilidade do substrato à enzima, respectivamente.

2.4.2. Polimerização com transglutaminase (TG)

A TG (EC 2.3.2.13) é uma enzima encontrada em tecidos e fluidos corporais de animais, peixes, plantas e produzidas por micro-organismos (JAROS *et al*., 2006). No entanto, a TG de origem microbiana é homologicamente diferente das TG encontradas em plantas e animais (ROMEIH e WALKER, 2017). A TG microbiana é uma enzima

monomérica obtida de *Streptoverticillium mobaraense* com 37,863 kDa e 331 aminoácidos e estrutura secundária composta por oito cadeias *β-sheet* cercadas por 11 α-hélices (JAROS *et al.*, 2006). O pI da TG é 8,9 e seu pH ótimo está entre 5 e 8, mas, mesmo no pH 4 ou 9 ainda exibe alguma atividade residual. A temperatura ótima para atividade enzimática é de 50 ºC. Contudo, a TG perde atividade quando aquecida a 70 ºC por alguns minutos (MOTOKI e SEGURO, 1998).

A TG é considerada pela comunidade científica como segura para consumo humano, sendo reconhecida como GRAS (*Generally Recognized As Safe*) pelo *Food and Drug Administration* (FDA) desde 1998 (ROMEIH e WALKER, 2017). PEDERSEN *et al.* (2004) avaliaram a alergenicidade da TG conforme a árvore de decisão da FAO/WHO (2001) e não identificaram problemas de segurança em relação ao potencial alergênico da enzima.

A Figura 2.2 ilustra as reações catalisadas pela TG, as quais podem modificar as proteínas por meio da formação de ligações cruzadas, incorporação de aminas e desaminação (MOTOKI e SEGURO, 1998). A TG catalisa reações de transferência de acil entre um grupo γ-carboxiamida de resíduos de glutamina (doador de acil) e os grupos ε-amino de resíduos de lisina (GUJRAL e ROSELL, 2004), resultando na formação de ligações cruzadas (ligação isopeptídica ε-(γ-glutamil)lisina) inter e intramoleculares (BÁEZ *et al.*, 2011). No caso das ligações cruzadas intermoleculares, que é a via predominante, a reação produz polímeros proteicos de cadeia ramificada (LI e DAMODARAN, 2017). Na ausência de aminas primárias, a TG catalisa a desaminação dos resíduos de glutamina a ácido glutâmico e as moléculas de água agem como aceptores de acil (FLANAGAN e FITZGERALD, 2003).

A TG é amplamente utilizada em indústrias de alimentos. Esta enzima age como um agente texturizante no preparo de alimentos adicionando firmeza, estabilidade térmica, capacidade de retenção de água, além de outras modificações qualitativas no alimento tal como o uso da reação de transferência de acil para introdução de aminoácidos ou peptídeos em proteínas com a finalidade de melhorar o valor nutricional (BÁEZ *et al.*, 2011; ROMEIH e WALKER, 2017). Outra aplicação potencial da polimerização com TG é na redução ou eliminação da alergenicidade de proteínas (PEDERSEN *et al.*, 2004). A formação de ligações cruzadas mediada pela TG pode modificar a alergenicidade de proteínas por meio do mascaramento de epítopos conformacionais e lineares, provocado pelo rearranjo da estrutura da molécula (BABIKER *et al.*, 1998). Além disso, segundo Li e Damodaran (2017), essas proteínas polimerizadas podem permanecer hipoalergênicas mesmo após a digestão gastrointestinal, uma vez que o perfil de peptídeos liberados das proteínas polimerizadas pode ser diferente daqueles liberados das proteínas nativas.

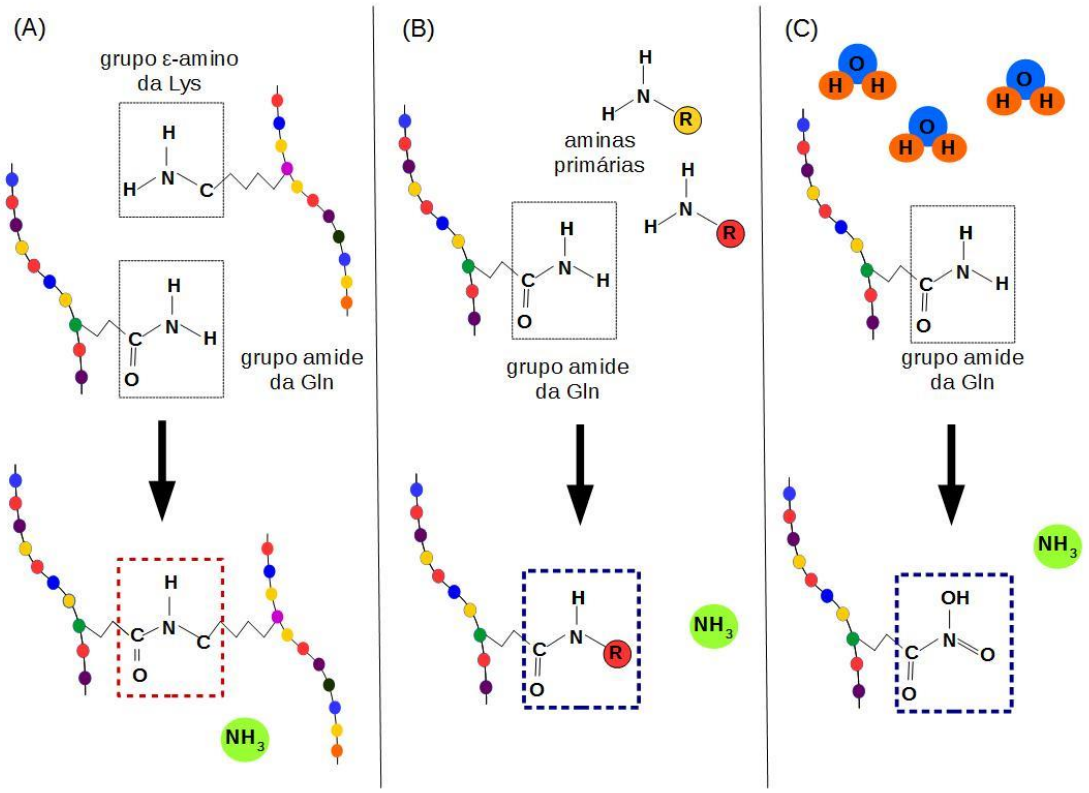

Figure 2.2: Reações catalisadas pela TG: **(A)** Formação de ligações cruzadas; **(B)** Incorporação de aminas; e **(C)** Desaminação.

Stanic e colaboradores (2010) relataram que a polimerização da β-caseína com quatro diferentes enzimas, incluindo a TG, diminuiu seu potencial de ligação a anticorpos IgE específicos. Os resultados obtidos por Van Esch e colaboradores (2013a) indicaram que, em camundongos já sensibilizados com caseinato, o caseinato polimerizado com TG não provocou respostas alérgicas mais pronunciadas que o caseinato. Além disso, os autores observaram sensibilização reduzida pelo caseinato polimerizado (VAN ESCH *et al.*, 2013a). Li e Damodaran (2017) usaram a TG para polimerizar sistemas contendo somente caseínas, somente IPS e ambos os grupos de proteínas do leite, caseínas e IPS. Os autores relataram que nos polímeros homólogos de IPS e caseínas ocorreu redução modesta da resposta de IgE às caseínas e β-Lg em comparação aos seus respectivos controles, enquanto, no polímero heterogêneo de IPS e caseínas, a reatividade da IgE contra às caseínas e β-Lg foi muito menor. A mistura de IPS e caseínas foi mais eficiente em impedir o acesso aos epítopos das caseínas e β-Lg (LI e DAMODARAN, 2017).

A razão de resíduos de glutamina para lisina bem como a distribuição desses aminoácidos ao longo da estrutura da molécula pode influenciar a reação de polimerização e, consequentemente, o mascaramento dos epítopos (LI e DAMODARAN, 2017). A β-Lg

tem 16 resíduos de glutamina e 15 resíduos de lisina, enquanto α-La possui oito resíduos de glutamina e 12 resíduos de lisina na sua cadeia proteica. Devido a sua estrutura globular estabilizada por pontes dissulfeto, essas proteínas do soro são substratos pobres para polimerização com TG no seu estado nativo e requerem modificações antes da reação de polimerização que aumentem a acessibilidade dos resíduos reativos de lisina e glutamina (GAUCHE *et al.*, 2008; GAUCHE, BARRETO e BORDIGNON-LUIZ, 2010).

Em estudos realizados em nosso laboratório observamos que a associação da desnaturação por cisteína (Cys), a qual promove o rompimento das ligações dissulfeto nas proteínas, e polimerização com TG diminuiu a resposta antigênica da β-Lg (VILLAS-BOAS *et al.*, 2010) e que os fragmentos liberados da β-Lg desnaturada e polimerizada, após digestão com pepsina e pancreatina, não foram reconhecidos por anticorpos IgE específicos (VILLAS-BOAS *et al.*, 2012). Olivier e colaboradores (OLIVIER *et al.* (2012a); 2012b) também relataram redução significativa na alergenicidade da β-Lg tratada com Cys e TG em comparação a β-Lg nativa por *skin prick test* em crianças e adultos alérgicos, respectivamente. O *skin prick test* é um teste cutâneo de alergia, no qual diferentes alérgenos são aplicados na pele do paciente e o surgimento de uma pápula indica uma reação contra o alérgeno testado.

Os resultados obtidos por Li e Damodaran (2017) indicaram, no entanto, que a polimerização com TG não eliminou alguns do epítopos lineares das proteínas do IPS e esses epítopos foram liberados intactos durante a digestão gastrointestinal, o que indica a necessidade de estratégias alternativas capazes de destruir esses epítopos lineares. A associação entre hidrólise com Alcalase e polimerização com TG foi estudada por Wróblewska e colaboradores (2008), que observaram maior redução ($p<0.05$) na alergenicidade das proteínas do soro de leite (α-La e β-Lg) hidrolisadas com Alcalase e polimerizadas com TG em comparação àquelas proteínas apenas hidrolisadas. Em trabalhos anteriores do nosso grupo de pesquisa (SABADIN *et al.*, 2012; VILLAS-BOAS *et al.*, 2015), a hidrólise com Alcalase, seguida ou não pela polimerização com TG, diminuiu significativamente a alergenicidade da β-Lg, avaliada por ensaios de imunoblot e ELISA usando soro de camundongos sensibilizados com β-Lg no primeiro estudo e por ELISA usando soro de crianças com ALV no segundo estudo. Em ambos os estudos, a polimerização usando TG após hidrólise com Alcalase não teve efeito significativo na resposta alergênica da proteína hidrolisada. No entanto, embora o mesmo epítopo tenha sido encontrado após a digestão gástrica, Villas-Boas e colaboradores (2015) observaram que a resposta da IgE específica à β-Lg hidrolisada e tratada com TG foi menor que à proteína apenas hidrolisada, indicando que o tratamento com TG pode ter atenuado a resposta alergênica.

2.4.3. Impacto da hidrólise enzimática e da polimerização nas características das proteínas

2.4.3.1. Características nutricionais

A qualidade nutricional de uma proteína depende da sua composição em aminoácidos essenciais na proporção requerida e da disponibilidade destes aminoácidos. A biodisponibilidade dos aminoácidos, por sua vez, pode ser influenciada pela digestibilidade da proteína, taxa de digestão, proporção relativa dos aminoácidos, efeito metabólico específico dos outros constituintes do alimento e estado fisiológico do indivíduo (POTIER e TOMÉ, 2008). As proteínas do soro de leite são altamente biodisponíveis e possuem perfil de aminoácidos que atende às necessidades fisiológicas do organismo humano (GAD e SAYED, 2009).

Apesar dos hidrolisados proteicos serem compostos por peptídeos de diferentes tamanhos e aminoácidos livres, seu perfil de aminoácidos é normalmente similar àquela da proteína antes do processo (BOZA, MARTÍNEZ-AUGUSTIN e GIL, 1995; CEZARD *et al.*, 1996; CALBET e HOLST, 2004; SINDAYIKENGERA e XIA, 2006; POTIER e TOMÉ, 2008) e a sua digestibilidade é similar ou melhor que a da proteína nativa (POTIER e TOMÉ, 2008). Cezard e colaboradores (1996) verificaram que dieta contendo proteínas de leite hidrolisadas apresentou taxa de absorção significativamente maior que a composta por proteínas intactas, enquanto, Calbet e Holst (2004) não encontraram diferenças na taxa de absorção intestinal de aminoácidos em humanos alimentados com caseínas e proteínas do soro intactas ou hidrolisadas.

As proteínas podem ser absorvidas tanto como peptídeos quanto como aminoácidos. Todavia, a absorção de peptídeos de cadeia curta, principalmente di- e tripeptídeos, é mais eficiente que absorção de quantidades equivalentes de aminoácidos livres (CLEMENTE, 2000). Isto pode ser explicado por diferenças entre os sistemas de transportes desses nutrientes: os di- e tripeptídeos são absorvidos nas porções proximal e distal, e os aminoácidos livres apenas na proximal; o mecanismo de transporte dos aminoácidos livres é competitivo e facilmente saturável, enquanto o transporte de peptídeos é menos afetado por alterações nutricionais e mais econômico energeticamente (FRENHANI e BURINI, 1999).

Como os aminoácidos livres são hipertônicos e menos absorvidos que peptídeos de baixa MM, sua presença em altas concentrações no hidrolisado pode limitar o uso em dietas clínicas e fórmulas hipoalergênicas (SVENNING *et al.*, 2000; EXL, 2001). A osmolalidade é um importante atributo físico-químico que caracteriza a qualidade de produtos alimentícios direcionados para crianças, dietas para idosos e dietas enterais. Produtos com alta

osmolalidade aumentam a quantidade de líquido no intestino, causando distúrbios no balanço eletrolítico e, consequentemente, diarreia, desidratação, náusea e vômito (HENRIQUES e ROSADO, 1999; NEKLYUDOV, IVANKIN e BERDUTINA, 2000).

A polimerização com TG tem sido cada vez mais aplicada na modificação das propriedades funcionais de proteínas pela indústria (AGYARE e DAMODARAN, 2010), o que faz surgir interesse a respeito dos aspectos nutricionais relacionados à digestibilidade da ligação isopeptídica ε-(γ-glutamil)lisina e à biodisponibilidade da lisina envolvida nessa ligação (JAROS *et al.*, 2006). O isopeptídeo ε-(γ-glutamil)lisina não é hidrolisado pelas enzimas proteolíticas do trato gastrointestinal dos mamíferos. Entretanto, esse dipeptídeo pode ser absorvido através da membrana intestinal (MOTOKI e SEGURO, 1998). A ligação isopeptídica é, então, clivada nos rins em L-lisina e 5-oxoprolina pela enzima γ-glutamil ciclotransferase, sendo a 5-oxoprolina metabolizada a ácido glutâmico por ação da 5-oxoprolinase (MOTOKI e KUMAZAWA, 2000). SEGURO *et al.* (1995) relataram que a ligação ε-(γ-glutamil)lisina também pode ser quebrada diretamente em lisina e ácido glutâmico por ação da γ-glutamil transpeptidase, enzima localizada principalmente nos rins, nas membranas em borda escova do intestino e no sangue. HULTSCH *et al.* (2005) investigaram o metabolismo da ligação isopeptídica ε-(γ-glutamil)lisina *in vivo* e mostraram a clivagem da ligação isopeptídica.

Além disso, a ligação isopeptídica ε-(γ-glutamil)lisina pode ser encontrada em alimentos crus e processados, uma vez que uma variedade de organismos vivos contém TG em seus tecidos e órgãos (SEGURO *et al.*, 1995) e que o aquecimento pode induzir a formação dessas ligações por meio da desidratação química. Isto significa que alimentos contendo isopeptídeo ε-(γ-glutamil)lisina são consumidos na dieta humana há muito tempo (MOTOKI e SEGURO, 1998). Portanto, parece que a formação de ligações cruzadas ε-(γ-glutamil)lisina em alimentos pela ação da TG não representa risco a saúde dos consumidores (HULTSCH *et al.*, 2005).

2.4.3.2. Características físico-químicas

A polimerização catalisada pela TG resulta na formação de polímeros proteicos de alto peso molecular e diminui a hidrofobicidade superficial das moléculas de proteína, com mudanças nas propriedades funcionais das proteínas podendo melhorar as características reológicas e sensoriais dos alimentos (GAUCHE *et al.*, 2008). Além disso, a formação de ligações cruzadas aumenta a repulsão eletrostática como resultado da desaminação parcial da glutamina e grupos ε-amino, o que pode alterar o ponto isoelétrico da proteína (BABIKER, 2000). Na ausência de resíduos de lisina ou aminas primárias, a água atua como

receptor de grupos acil e os grupos carboxiamida dos resíduos de glutamina são desaminados, formando resíduos de ácido glutâmico e amônia o que modifica, consequentemente, as cargas e a estabilidade da proteína (ROMEIH e WALKER, 2017).

Vários parâmetros moleculares, tais MM, conformação, estado de ionização e hidrofobicidade superficial, exercem um papel importante nas propriedades funcionais das proteínas (BABIKER, 2000). Por esse motivo, a TG tem sido amplamente utilizada para melhorar a textura e as propriedades funcionais de alimentos processados compostos por proteínas (GAUCHE *et al.*, 2008). A TG aumenta a firmeza do produto final, o que pode ser uma estratégia interessante para a produção carnes processadas com baixo teor de gordura e características sensoriais equivalentes aos produtos convencionais (NIELSEN, 1995).

As propriedades emulsificante e espumante e a habilidade de formação de gel das proteínas de soja melhoram após a polimerização com TG (BABIKER, 2000). A utilização da TG também é uma estratégia interessante para melhorar as propriedades funcionais de produtos derivados do leite. O tratamento com TG pode melhorar o rendimento da produção de queijo, a capacidade de retenção de água e a textura em queijos macios. A aplicação da TG em leites fermentados aumenta a viscosidade e a força do gel, e ainda melhora a capacidade de retenção de água, diminuindo significativamente a sinérese. Essas propriedades reológicas são de grande importância na produção de queijos e leites fermentados com baixo teor de gordura (ROMEIH e WALKER, 2017).

As propriedades emulsificante, espumante e de gelificação das proteínas podem ser diminuídas pela hidrólise excessiva (CHRISTIANSEN *et al.*, 2004). Babiker (2000) observou melhora significativa da atividade emulsificante e da estabilidade da emulsão das proteínas de soja hidrolisadas e tratadas com TG. Resultados semelhantes foram encontrados por Flanagan e FitzGerald (2003) e Tang e colaboradores (2005) para caseína polimerizada com TG.

2.4.3.3. Características sensoriais

Além do uso em fórmulas hipoalergênicas, as proteínas do soro de leite hidrolisadas são utilizadas na produção de barras nutricionais, bebidas esportivas e dietas enterais (PEDERSEN *et al.*, 2004; JOHNS *et al.*, 2011). A hidrólise enzimática extensiva da proteína com liberação de peptídeos menores que 1000 Da pode afetar negativamente o sabor dos hidrolisados proteicos devido à liberação de peptídeos amargos, o que dificulta sua incorporação em produtos alimentícios (NEWMAN *et al.*, 2015).

O peptídeo, para se ligar aos receptores de gosto amargo presentes nas papilas gustativas, deve conter em sua estrutura um grupo hidrofóbico, que age como a unidade

ligante ao receptor, e outro grupo hidrofóbico ou básico, que funciona como unidade estimulante (ISHIBASHI *et al.*, 1988). Além disso, é essencial que estes dois grupos, ligante e estimulante, sejam adjacentes na conformação estérica do peptídeo, possuindo uma distância ótima entre eles de 4,1 Å e com tamanho total de 15 Å. Isto permite o contato dos receptores de gosto amargo nas papilas com peptídeos contendo até oito aminoácidos (ISHIBASHI *et al.*, 1988; MAEHASHI e HUANG, 2009).

A presença e intensidade do gosto amargo nos peptídeos estão então associadas à presença de resíduos hidrofóbicos, à hidrofobicidade dos grupos, à MM dos peptídeos, e à orientação espacial das unidades responsáveis pela ligação aos receptores de gosto amargo (KIM *et al.*, 2008). A extensão (parcial ou extensiva) da hidrólise da proteína e a protease utilizada no processo são determinantes na liberação dos peptídeos amargos. Quanto mais extensa a hidrólise da proteína, maior tende a ser a exposição de resíduos hidrofóbicos e a quantidade de peptídeos com baixa MM (SPELLMAN, O'CUINN e FITZGERALD, 2009). Hidrolisados de proteínas de soja e do soro de leite com os maiores grau de hidrólise e peptídeos com MM menor que 600 Da ou entre 1000-4000 Da apresentaram maior intensidade de gosto amargo (CHO *et al.*, 2004; SPELLMAN, O'CUINN e FITZGERALD, 2005; CHEISON, WANG e XU, 2007; LEKSRISOMPONG, MIRACLE e DRAKE, 2010). Em um estudo conduzido por Pedrosa Delgado e colaboradores (2006), o elevado grau de hidrólise das proteínas hidrolisadas, que compõem as fórmulas hipoalergênicas, foi associado à menor aceitação em relação ao seu aroma, textura e, principalmente, seu sabor.

Assim como na redução da alergenicidade, a intensidade do gosto nos hidrolisados proteicos está associada à especificidade e seletividade da enzima, pois essas propriedades da protease determinam a composição e a MM dos peptídeos e a quantidade de aminoácidos livres liberadas pela hidrólise. Spellman, O'Cuin e Fitzgerald (2009) observaram que as proteínas do soro hidrolisadas com Alcalase apresentaram maior intensidade do gosto amargo que aquelas hidrolisadas com as enzimas Provile e Corolase. Seo, Lee e Baek (2008) também encontraram diferenças na intensidade do gosto amargo entre hidrolisados de proteínas de soja, dependendo da especificidade da enzima, GH e condições de reação utilizadas no processo.

Algumas estratégias têm sido indicadas para redução do gosto amargo em hidrolisados proteicos, incluindo cromatografia de interação hidrofóbica, tratamento com carvão ativado, hidrólise com exopeptidases, reação de plasteína e encapsulação (SAHA e HAYASHI, 2001; BARBOSA *et al.*, 2004). Entretanto, esses métodos possuem alguns inconvenientes tais como adsorção de aminoácidos essenciais (fenilalanina, triptofano),

produção excessiva de aminoácidos livres, reversibilidade da reação e baixo rendimento (SAHA e HAYASHI, 2001; BARBOSA et al., 2004).

O tratamento com TG pode ser uma alternativa na redução do gosto amargo normalmente presente nos hidrolisados proteicos sem prejuízos no valor nutricional. Babiker e colaboradores (1996) verificaram que a polimerização de hidrolisados de soja com TG diminuiu significativamente a intensidade do gosto amargo. Produtos da reação de Maillard feitos a partir de IPS hidrolisado e tratado com TG foram menos amargos que aqueles produzidos a partir de IPS apenas hidrolisado (SONG et al. 2013). A polimerização com TG pode diminuir o gosto amargo pelo aprisionamento dos resíduos de aminoácidos hidrofóbicos no interior das moléculas devido ao rearranjo da estrutura da proteína (BABIKER et al., 1996).

A intensidade do gosto amargo dos hidrolisados proteicos é normalmente determinada por análise sensorial utilizando-se escalas de intensidade e soluções padrões de cafeína e quinina como referência (BABIKER et al., 1996; SAHA e HAYASHI, 2001; SPELLMAN, O'CUINN e FITZGERALD, 2005; 2009; LEKSRISOMPONG et al., 2012; SONG et al., 2013). Kim e Li-Chan (2006), por meio da compilação de dados publicados na literatura, montaram um banco com a intensidade de gosto amargo de 224 peptídeos, determinada por avaliação sensorial.

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CAPÍTULO 3. ARTIGO 1. ***Physicochemical characteristics and antigenicity of whey protein hydrolysates obtained with and without pH control***

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Abstract

Protein hydrolysis on a laboratory scale is commonly carried out under controlled pH conditions, while, in industry, it is conducted without pH control. The impact of pH, with or without control, substrate concentration ($S_{\%}$) and enzyme-substrate ratios (E/S) on physicochemical and antigenic characteristics of whey protein hydrolysed with Alcalase were assessed. $S_{\%}$ and E/S affected the degree of hydrolysis; pH conditions resulted in hydrolysates with different physicochemical characteristics and concentrations of β-lactoglobulin and α-lactalbumin. Although pH plays a crucial role in the hydrolysates characteristics due to its influence on the enzyme cleavage pattern, the responses of anti-β-lactoglobulin, anti-α-lactalbumin IgE and IgG to hydrolysates were similar and independent of pH control. In the absent of control, pH increased response to anti-BSA antibodies. Overall, hydrolysis with Alcalase resulted in different peptide compositions, presenting possibly different bioactivities, but the cleavage of β-lactoglobulin and α-lactalbumin epitopes did not change.

3.1. Introduction

In the last few decades, there has been a significant increase in the prevalence of allergic diseases, including food allergies, related to changes in lifestyle, eating habits and epigenetic factors (Sicherer, 2011). Cow's milk allergy (CMA) is considered the most common food allergy in children under three years of age. It is characterized by an abnormal immune response mediated by IgE, triggered by one or more milk proteins, especially caseins, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) (Kumar, Verma, Das, & Dwivedi, 2012; Monaci, Tregoat, Van Hengel, & Anklam, 2006). When breastfeeding is not possible, formulas based on partially or extensively hydrolysed proteins are an alternative for feeding children with CMA or at risk of developing the condition (Van Esch et al., 2010).

Enzymatic hydrolysis is still the most common method used to reduce the antigenicity of milk proteins by destruction of epitopes (Agyare & Damodaran, 2010). However, several authors have reported residual antigenicity in hypoallergenic formulas due to the presence of unhydrolysed proteins and/or antigenic peptides released by hydrolysis (Brahim, Addou, Kheroua, & Saidi, 2012; Puerta, Diez-Masa, & De Frutos, 2006; Sabadin, Villas-Boas, de Lima Zollner, & Netto, 2012). The antigenicity reduction of milk proteins by hydrolysis depends on both the specificity of the enzyme and the reaction parameters, including pH, temperature, and enzyme to substrate ratio (Zheng *et al.*, 2008).

Alcalase is a low cost and broad specificity serine protease produced by selected strains of *Bacillus licheniformis* (Doucet, Otter, Gauthier, & Foegeding, 2003). Studies have shown that Alcalase is able to effectively reduce the antigenicity of bovine milk proteins (Izquierdo, Peñas, Baeza, & Gomez, 2008; Sabadin et al., 2012; Villas-Boas, Benedé, de Lima Zollner, Netto, & Molina, 2015; Wróblewska, Jędrychowski, Hajós, & Szabó, 2008). In most of these studies, hydrolysis was carried out under controlled pH conditions. The pH is an important parameter that changes as a consequence of peptide bond cleavage, which releases or captures H^+ ions during hydrolysis. The pH can be controlled by adding an acidic or alkaline solution to the reaction medium. The maintenance of constant pH has been used as a way to control and study hydrolysis reactions on the laboratory scale. However, from the industry point of view, hydrolysis without pH control is more feasible. It simplifies the process protocol, reduces the risk of contamination, and minimizes the addition of salts to the hydrolysate which may be inappropriate in the production of hypoallergenic formulas for children (Fernández & Kelly, 2016; Le Maux, Nongonierma, Barre, & FitzGerald, 2016).

In the industry, the initial pH is adjusted to the enzyme optimal pH and the hydrolysis is carried out without further addition of acid or alkali, allowing the pH to change during the course of proteolysis (Le Maux et al., 2016). The pH changes affect the charge and the

structure of the enzyme and its substrate, and hence enzyme selectivity, which is defined as the rate that the enzyme breaks each cleavage site within its specificity (Butré, Sforza, Gruppen, & Wierenga, 2014a; Butré, Sforza, Wierenga, & Gruppen, 2015; Vorob'ev, Dalgalarrondo, Chobert, & Haertlé, 2000). The enzyme selectivity determines the cleavage sequence that influences the profile of released peptides, thus influencing the antigenicity of protein hydrolysates (Butré et al., 2015), among other characteristics of the hydrolysates produced.

In a recent study, Le Maux et al. (2016) showed that whey protein hydrolysates obtained by hydrolysis under controlled and uncontrolled pH exhibited a similar degree of hydrolysis (DH), but with differences in peptide profiles, with different bioactivities such as dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant activities. Despite the industrial advantage of the process without pH control and the evidence of the impact of pH on the characteristics of hydrolysates, we have found no studies on the impact of hydrolysis without pH control on the potential antigenicity of milk protein hydrolysates.

The objective of this study was to evaluate the effect of pH control during hydrolysis reaction with Alcalase on the characteristics of the whey protein isolate (WPI) hydrolysates. Furthermore, we investigated the effect of pH control on the ability of Alcalase to reduce the antigenicity of the major WPI proteins (α-La, β-Lg, and BSA).

3.2. Materials and methods

3.2.1. Material

Whey protein isolate (WPI) PROVON® (Glanbia Nutritionals, Kilkenny, Ireland) was obtained from a local market. The total nitrogen content of WPI was determined by micro-Kjeldahl method (Horwitz, 2006) and the protein content, 90.4% ± 1.7, was calculated using 6.38 as conversion factor. The proteins α-lactalbumin (α-La, Type III, L6010), β-lactoglobulin (β-Lg, L3908) and bovine serum albumin (BSA, A2153) from bovine milk, and Alcalase (EC 3.4.21.62, protease from *Bacillus licheniformis*) were purchased from Sigma-Aldrich® (St.Louis, MO, USA).

The antibodies used were: mouse IgE Balb/c - isotype control (Abcam®, Cambridge, MA, USA), purified rat anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA, USA), HRP-conjugated goat anti-Rat IgG whole molecule (Sigma Chemical Co., St. Louis, MO, USA), normal mouse IgG (Merck Millipore, Darmstadt, Germany), alkaline phosphatase-conjugated anti-mouse IgG polyclonal antibody (Abcam®, Cambridge, UK).

The reagents tricine, sodium dodecylsulphate (SDS), and o-phthaldialdehyde (OPA) were purchased from Sigma-Aldrich®. Trifluoroacetic acid (TFA), β-mercaptoethanol, Coomassie Brilliant Blue G250, sodium hydroxide, and urea were from Merck (Hohenbrunn, Germany). Acrylamide and Tris [Tris(hydroxymethyl)aminomethane] were from Bio-Rad (Hercules, CA, USA). All other reagents were of analytical or chromatographic grade.

3.2.2 Hydrolysis of whey protein isolate

The activity of Alcalase was determined to standardize enzyme to substrate ratio (E/S) in terms of enzyme units per mg protein. Enzymatic activity of Alcalase was 8.96 U mg^{-1}, determined by the method described by Emi, Myers, &Iacobucci (1976). One unit (U) of enzyme activity was defined as the amount of enzyme that produces trichloroacetic acid (TCA) soluble peptide equivalent to 1 µg of tyrosine in 1 min (Emi et al., 1976).

A full factorial design was performed to evaluate the impact of independent variables: pH, substrate concentration ($S_{\%}$) and E/S on degree of hydrolysis (DH) of hydrolysates (dependent variable) as described in section 2.10. All hydrolysis experiments were carried out at 60 °C for 180 min under agitation using 3 or 7% of substrate and 50 or 100 U g^{-1} protein E/S. The hydrolysis reactions under controlled pH were performed using an automated titrator (Mettler dL-21, Schwerzenbach, Switzerland). The pH was initially adjusted by addition of 1 mol L^{-1} NaOH and maintained at pH 8.5. In the hydrolysis reactions carried out under uncontrolled pH, pH was initially adjusted to pH 8.5 using 1 mol L^{-1} Na_2CO_3. In both cases, pH was monitored during the reaction. After 180 min, the reaction was stopped by heating at 90 °C for 10 min. The hydrolysates obtained were freeze-dried and stored at -20 °C for further analysis. The total nitrogen content of hydrolysates was determined by micro-Kjeldahl procedure, and the protein content was calculated using 6.38 as conversion factor (Horwitz, 2006).

Aliquots were withdrawn every 15 min during the first hour and every 30 min during the remaining two hours to determine the DH. The DH was measured by the OPA method using serine as standard (Nielsen, Petersen, & Dambmann, 2001). The DH is defined as a ratio of the number of peptide bonds cleaved in relation to the total number of peptide bonds in the protein and is calculated according to equation 1:

$$\mathrm{DH}\ (\%) = \frac{number\ of\ peptide\ bonds\ cleaved}{number\ of\ total\ peptide\ bonds}\ x\ 100 = h/h_{tot}\ x\ 100 \qquad \text{(Equation 1)}$$

Where h_{tot} is the total number of peptide bonds in the protein substrate (8.8 mmol g^{-1} for WPI); h is the total number of peptide bonds cleaved and expressed as milliequivalents of serine-NH_2 per gram of protein and was calculated using the equation 2:

$$h = (SerineNH_2 - \beta)/\alpha \qquad \text{(Equation 2)}$$

Where α = 1.00 and β = 0.40 were values of the parameter of the model to whey proteins, experimentally obtained by Adler-Nissen (1979).

3.2.3. Electrophoresis

The electrophoretic profiles of WPI and hydrolysates were obtained by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970) using a Mini Protean II apparatus (Bio-Rad, Hercules, CA, EUA) and separation and stacking gels with 12% and 3% of acrylamide, respectively. The samples (1% w/v protein) were diluted in a reducing buffer (62.5 mmol L^{-1} Tris, 2% SDS, 20% glycerol and 5% β-mercaptoethanol, pH 6.8) and heated at 95°C for 5 min. Then, 10 µL aliquots of each sample were applied. The gels were stained with 0.1% Coomassie Blue R-250 and destained with acetic acid:methanol:distilled water (1:4:5). A 14.4–97.0 kDa MM marker kit (Bio-Rad) was used as standard.

The samples were also evaluated by SDS-PAGE/Tricine (Claeys, De Smet, Balcaen, Raes, & Demeyer, 2004) using a discontinuous system consisting of separation gel, spacer gel, and stacking gel with 16.5%, 10%, and 3% of acrylamide, respectively. The samples (1% protein) were diluted in the reducing buffer and heated at 95°C for 5 min. Aliquots of 10 µL of each sample were applied. The gels were fixed in methanol:acetic acid:distilled water (5:1:4) for 24 h and stained with 0.025% de Coomassie Blue G250 in 10% acetic acid for 48 h. De-staining was carried out in 10 % acetic acid. A 3.5-26.6 kDa MM marker kit (Bio-Rad) was used as standard. All electrophoretic analyses were performed in duplicate.

3.2.4. Reversed-phase high performance liquid chromatography

The peptide profiles of the hydrolysates were determined by reversed-phase high performance liquid chromatography (RP-HPLC) as described by Pessato et al. (2016). The chromatographic analyses were carried out using Agilent equipment equipped with diode array detector (DAD) and a Luna C_{18} column (250 mm x 4.6 mm, i.d. 5 µm - Phenomenex -

Woburn, MA, USA). To allow comparisons of the hydrolysates profiles, the chromatograms were divided into three regions: (I) high hydrophilicity – 16 min gradient from 10% to 30% B; (II) medium hydrophilicity –16 to 22 min gradient from 30 and 40% B; (III) low hydrophilicity – over 22 min – above 40% B. All chromatographic analyses were carried out in duplicate.

3.2.5. Surface hydrophobicity

The surface hydrophobicity (S_0) was determined using the anionic fluorescence probe 1-anililo-naphthalene-8-sulfonate (ANS), as described by Pessato et al. (2016). The relative fluorescence intensity (RFI) of each solution was measured against the blank (buffer with ANS) using a Synergy™ HT Multi-Mode Microplate Reader (BioTek®, Winooski, VT, USA). The S_0 was determined from the initial slope of linear regression analysis of the plot of RFI against protein concentration (%). Each sample was analyzed in triplicate.

3.2.6. Intrinsic fluorescence

The intrinsic fluorescence emission spectra of untreated WPI and hydrolysates were determined as described by Tang, Yang, Chen, Wu, &Peng (2005) using an *ISS PC1 Fluorimeter* (Champaign, IL, USA). The test samples were excited at 280 nm, and the emission intensities were measured from 290 to 500 nm.

3.2.7. *In silico* hydrolysis of β-Lg, α-La, and BSA

The *in silico* hydrolysis of β-Lg, α-La, and BSA was performed using the main enzymatic components of Alcalase: subtilisin (EC 3.4.21.62) available in BIOPEP (Dziuba, Minkiewicz, & Dabek, 2013) and glutamyl endopeptidase (3.4.21.19) available in ExPASy (Gasteiger et al., 2005). The primary protein structures were obtained from the UniProt (Universal Protein Resource) (Consortium, 2015).

3.2.8. Detection of antigenic proteins

The amount of residual α-La and β-Lg in WPI and hydrolysates obtained with and without pH control was determined using commercial ELISA kits, according to the manufacturer's protocol (Bethyl Laboratories Inc., Montgomery, AL, USA).

3.2.9. *Antigenicity evaluation*

Polyclonal antibodies (anti-α-La, anti-β-Lg, anti-BSA IgE and IgG) were obtained by sensitization of female BALB/c mice with α-La, β-Lg and BSA. BALB/c mice used in this study were obtained from the Multidisciplinary Centre for Biological Research, University of Campinas (Cemib-Unicamp). The experimental and control groups consisted of 20 mice fed *ad libitum* on a cow milk-free diet (Nuvilab®, Curitiba, Brazil) kept under specific pathogen-free (SPF) conditions, with controlled light, temperature and humidity in the Laboratory of Translational Immunology (LTI) facilities. The mice were divided into four groups (n = 5 mice per group): control group sensitized with adjuvant alum, first experimental group sensitized with α-La, second experimental group sensitized with β-Lg, and third experimental group immunized with BSA.

On day 1, BALB/c mice were sensitized by intraperitoneal injection with 50 µg of protein (α-La, β-Lg or BSA) adsorbed in a 10% of alum, in a final volume 0.2 mL per animal (Villas-Boas, Vieira, Trevizan, de Lima Zollner, & Netto, 2010). A booster subcutaneous injection was given on days 14 and 21 with 20 µg adsorbed in a 10% of alum and 50 µg of protein per mouse, respectively, in a final volume 0.2 mL/animal. One week after the last sensitization, the animals were sacrificed by intraperitoneal anesthesia comprising 150 mg kg^{-1} ketamine hydrochloride and 10 mg kg^{-1} xylazine hydrochloride (both from Vetbrands, Paulínia, Brazil) and peripheral blood was collected by cardiac puncture for serum separation. This experiment was approved by the Ethics Committee on Animal Experiments of University of Campinas protocol number 3295-1 (Campinas, SP, Brazil).

3.2.9.1. ELISA assays

The specific IgE and IgG binding capacity of hydrolysates were determined by ELISA as described previously (Villas-Boas et al., 2010), with adaptations. For the estimation IgE-binding, high-binding polystyrene microtitre plates (Corning, Cambridge, MA, USA) were used as solid support. Single wells were coated with 100 µL of an antigen (WPI, native BSA, α-La and β-Lg, or hydrolysates) at a concentration of 100 µg mL^{-1} in a carbonate-bicarbonate buffer (50 mmol L^{-1}, pH 9.6), and incubated overnight at 4°C. The plates were then washed with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS-T), using a microplate washer (BioTek ELx50, Thermo Scientific, Waltham, MA, USA). This washing system was used after each incubation step. Residual free binding sites were blocked using TBS-T for 2 h at 37°C. The plates were incubated with individual mouse serum diluted 1:500 (100 µL per well) in TBS-T and incubated overnight at 4°C. For the standard curve, purified mouse IgE with an initial concentration of 12,800 ng mL^{-1} was serially diluted up to a concentration of 25

ng mL^{-1} in carbonate-bicarbonate buffer (pH 9.6). After the washing step, the plates were incubated for 2 h at 37°C with 100 µL (per well) of purified rat anti-mouse IgE monoclonal antibody diluted 1:1500 in TBS-T. The plates were then incubated with 100 µL of HRP-conjugated goat anti-rat IgG whole molecule diluted 1:20,000 in TBS-T, for 2 h at 37°C. TMB (3,3´,5,5´-tetramethylbenzidine) was added to the plates (100 µL per well) and incubated for 30 min. The reaction was stopped using H_2SO_4 (0.18 mol L^{-1}). The color developed was read at 450 nm using an automated spectrophotometer reader (Spectra Max 190, Molecular Devices, Toronto, ON, Canada).

Specific IgG-binding was evaluated by ELISA as described above. For the standard curve, a serially diluted standard purified mouse IgG and anti-mouse IgG phosphatase alkaline conjugate and alkaline phosphatase yellow substrate were used. The reaction was stopped using NaOH (3.0 mol L^{-1}). The colour development was measured at 405 nm using an automated spectrophotometer reader.

3.2.9.2. Immunoblotting assays

The analyses were performed according to Villas-Boas et al. (2010), with adaptations. After electrophoresis carried out under the same conditions as described in section 3.2.3, the samples were transferred from the gel onto nitrocellulose membranes. The membranes were blocked and incubated overnight with the α-La or β-Lg-specific sera pool from five mice diluted 1:250 in TBS-T. The membranes were incubated with purified rat anti-mouse IgE monoclonal antibody and then with HRP-conjugated goat anti-rat IgG whole molecule. The antigen-antibody reaction was visualized by using Luminata Forte Western HRP Substrate (Merck Millipore, Taunton, MA, USA) and chemiluminescence signal was measured by Image Quant400 System (GE, Healthcare, USA).

3.2.10. Statistical analyses

Results were expressed as mean ± standard deviation. To evaluate the impact of pH, $S_{\%}$, and E/S on the DH of hydrolysates, a 2^3 full factorial design was carried out. The main factors were pH, substrate concentration and enzyme to substrate ratio at two levels as described in Table 3.1. All experiments were carried out in duplicate. For full factorial design, the analysis was performed using the Statistica 7.0 (Statsoft, Tulsa, OK, USA) software package. Unpaired t-test and ANOVA followed by the Tukey's were performed to verify differences in the DH of the hydrolysates. Surface hydrophobicity, antigenic proteins detection, specific IgG and IgE-binding results were analyzed by the non-parametric Mann-

Whitney and Kruskal-Wallis tests. For all analyses, p values ≤0.05 were considered significant. The statistical analyses were carried out using the software GraphPad PRISM 6 (GraphPad software, San Diego, CA, USA) and Action Stat (Software Action, São Carlos, Brazil).

Table 3.1
Experimental design, 2^3 full factorial design.

Trial	*pH control*	*$S_{\%}$*	*E/S*	*pH control*	*$S_{\%}$ (% protein)*	*E/S (U g^{-1} protein)*	*Nomeclature of hydrolysates*
1	−	−	−	With	3	50	HC3-50
2	+	−	−	Without	3	50	HUn3-50
3	−	+	−	With	7	50	HC7-50
4	+	+	−	Without	7	50	HUn7-50
5	−	−	+	With	3	100	HC3-100
6	+	−	+	Without	3	100	HUn3-100
7	−	+	+	With	7	100	HC7-100
8	+	+	+	Without	7	100	HUn7-100

3.3. Results and discussion

3.3.1. Impact of hydrolysis conditions on hydrolysates characteristics

To evaluate the impact of the pH condition (controlled and uncontrolled pH), $S_{\%}$, and E/S on DH of the hydrolysates, a 2^3 full factorial design was carried out. The analysis of variance (ANOVA) showed that the main effect of pH control was not significant ($p>0.05$), while the main effects of $S_{\%}$ and E/S were significant ($p<0.05$) (Table 3.2). The interactions between the main effects were not significant ($p>0.05$), thus the main effects were evaluated separately.

The hydrolysis parameters $S_{\%}$ and E/S are critical for hydrolytic processes and characteristics. Although higher DH ($p<0.05$) was obtained with 100 U g^{-1} of Alcalase than with 50 U g^{-1} of Alcalase and same $S_{\%}$ (Table 3.2), these differences were slight under both pH conditions. This possibly occurred due to the exhaustion of peptide bonds available for hydrolysis (Guadix, 2000; Ishibashi et al., 1988).

The increase in $S_{\%}$ from 3 to 7% led to a significant decrease by around 4% in the final DH of the hydrolysates obtained under controlled pH, and from 2.6 to 5.5% in DH of the hydrolysates produced without pH control (Table 3.3). According to Butré, Wierenga, &Gruppen (2014c), the decrease in DH with increasing $S_{\%}$ is associated with the higher amount of water required for protein hydration, which diminishes the free water of the system. Moreover, during hydrolysis, $–NH_3^+$ and $–COO^-$ groups are released at each cleaved peptide bond, increasing the amount of free water directed to the hydration of molecules containing these charged groups (Hardt, Janssen, Boom, & van der Goot, 2014). The reduced amount of free water in the system can hinder the diffusion of enzyme, substrate, and product, leading to the non-homogeneous distribution of substrate and enzyme and to a decrease both the hydrolysis rate and DH (Butré et al., 2014c; Hardt, van der Goot, & Boom, 2013).

No significant difference ($p<0.05$) was observed between the DH of the hydrolysates obtained with and without pH control and using the same $S_{\%}$ and E/S (Table 3.3). It is possible to observe in Fig. 3.1 that the hydrolysis curves at the same $S_{\%}$ and E/S, irrespective of the pH conditions, were similar. For all conditions, 50% of the total peptide bonds were cleaved after 15 min of reaction. The difference in the t=15min DH values fairly closely reflected the difference in the final DH values. Meanwhile, during the hydrolysis without pH control, under all $S_{\%}$ and E/S, the pH dropped from 8.5 to 7.0 within the first 15 min and leveled off until the end of the process. The pH of the reaction mixture remained within the optimum range of activity of Alcalase (6.5-8.5) (Yust, Pedroche, Millán-Linares, Alcaide-Hidalgo, & Millán, 2010). The decrease in pH during hydrolysis is due to the release of H^+ owing to cleavage of peptide bonds. At pH 7.0 the α-carboxyl groups (pKa 3.1) from peptides are fully deprotonated, while the α-amino groups (pKa 7.3) are partially protonated. After the first 15 min of hydrolysis, the α-amino groups capture the H^+ released, resulting in a buffering system in the reaction medium and maintaining the pH constant (Sousa Jr, Lopes, Pinto, Almeida, & Giordano, 2004).

Table 3.2
Analysis of variance (ANOVA) for factorial experimental.[a]

Effect	SS	Degree of fredoom	MS	F	p*
pH	0.002	1	0.002	0.001	0.970745
$S_{\%}$	58.523	1	58.523	33.513	0.000410
E/S	30.250	1	30.250	17.323	0.003156
pH*$S_{\%}$	0.010	1	0.010	0.006	0.941537
pH*E/S	0.903	1	0.903	0.517	0.492662
$S_{\%}$*E/S	2.103	1	2.103	1.204	0.304448
pH*$S_{\%}$*E/S	1.690	1	1.690	0.968	0.354045
Error	13.970	8	1.746		

[a] *p*-values < 0.05 indicate that the effect is significant.

Table 3.3
Final degree of hydrolysis (DH) of WPI hydrolysates obtained with Alcalase with and without pH control using different $S_{\%}$ and E/S.[a]

$S_{\%}$ (% protein)	E/S (U g^{-1} protein)	DH (%)	
		With pH control	Without pH control
3	50	23.2 ± 1.20 ^{A,b}	23.4 ± 1.28 ^{A,a,b}
7	50	19.1 ± 0.84 ^{A,d}	17.9 ± 1.04 ^{A,c}
3	100	25.3 ± 0.66 ^{A,a}	25.0 ± 1.32 ^{A,a}
7	100	21.6 ± 0.77 ^{A,c}	22.4 ± 0.72 ^{A,b}

[a] Values are the mean ± standard deviation; means in a row with different uppercase superscript letters are significantly different (t-test; $p < 0.05$); means in a column with different lowercase superscript letters are significantly different (ANOVA with Tukey's test; $p < 0.05$).

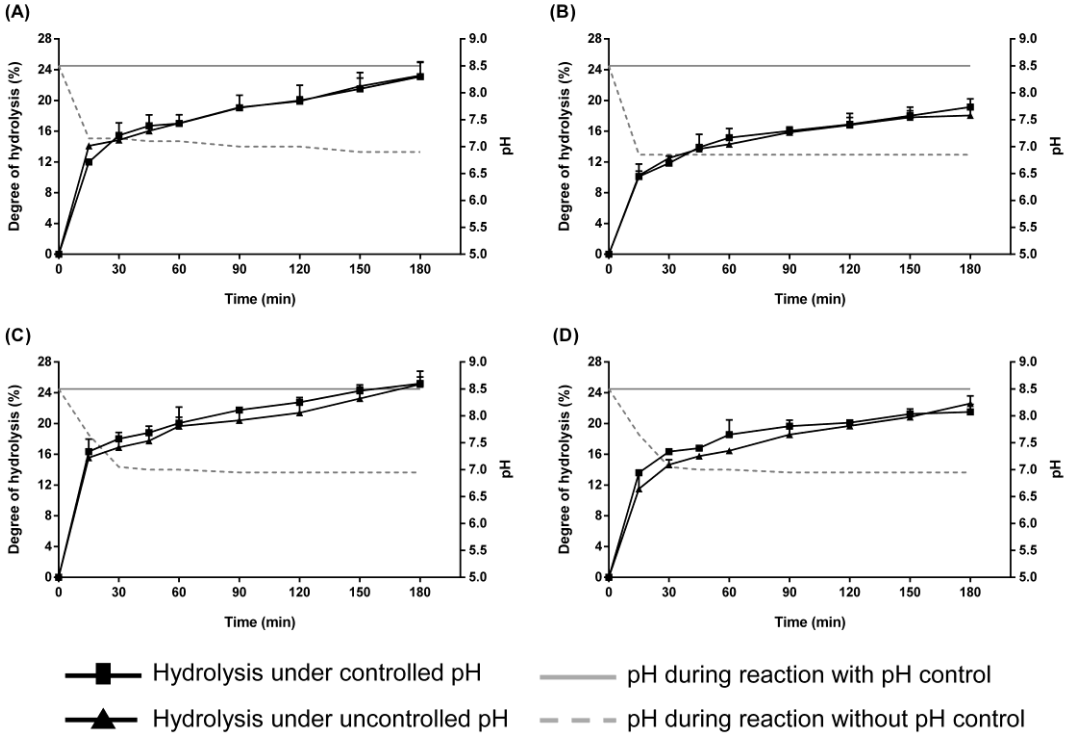

Fig. 3.1. DH curves of WPI hydrolysed by Alcalase with (■) and without (▲) pH control at different $S_{\%}$ and E/S: A, 3% and 50 U g^{-1}; B, 7% and 50 U g^{-1}; C, 3% and 100 U g^{-1}; D, 7% and 100 U g^{-1}. The change in pH with (solid grey line) and without (dashed grey line) during hydrolysis is also shown. Results are presented as the mean of two process of hydrolysis.

The electrophoretic profiles (SDS-PAGE and SDS-PAGE/Tricine) of the hydrolysates obtained at 15 and 180 min of reaction are shown in Fig. 3.2. The hydrolysates obtained at 15 min of reaction were also assessed, considering that the pH was stabilized at this point and over 50% of total peptide bonds cleavage had already occurred. Protein hydrolysis was evaluated by SDS-PAGE which revealed that the β-Lg (18.4 kDa) and α-La (14.4 kDa) proteins had been hydrolyzed after 15 min of hydrolysis in all hydrolysates, confirmed by the disappearance of the corresponding bands (Fig. 3.2A, lanes 3-10). Intact BSA (66.4 kDa) was observed in the profiles of all hydrolysates obtained at 15 min and, after 180 min, BSA was only observed in the profiles of HUn3-50, HUn7-50 and HUn3-100 (Fig. 3.2B, lanes 3, 5 and 7), but at a lower intensity than in WPI.

The molecular mass (MM) distribution profile of the hydrolysates obtained at 15 and 180 min was evaluated by SDS-PAGE/Tricine (Fig. 3.2C and D). At 15 min, the hydrolysates obtained under all conditions studied presented bands in the region 3.5 to 6.5 kDa (Fig. 3.2C). At 180 min, the hydrolysates produced without pH control (Fig. 3.2D, lanes 2, 4, 6 and 8) exhibited bands in the region 3.5 to 6.5 kDa. No band was observed in the profiles of the

hydrolysates obtained under controlled pH (Fig. 3.2D, lanes 3, 7, and 9), suggesting the presence of peptides smaller than 3.5 kDa, which may not have been stained with Coomassie Blue (Claeys et al., 2004). The exception was HC7-50 which exhibited bands in the region between 3.5 and 6.5 kDa (Fig. 3.2D, lane 5). The hydrolysates obtained without pH control, despite having a similar DH, contained peptides with a higher molecular mass than those obtained under controlled pH and with the same $S_{\%}$ and E/S. This finding indicates differences in patterns of hydrolysis, i.e., an order in which peptides are formed and released according to pH and $S_{\%}$.

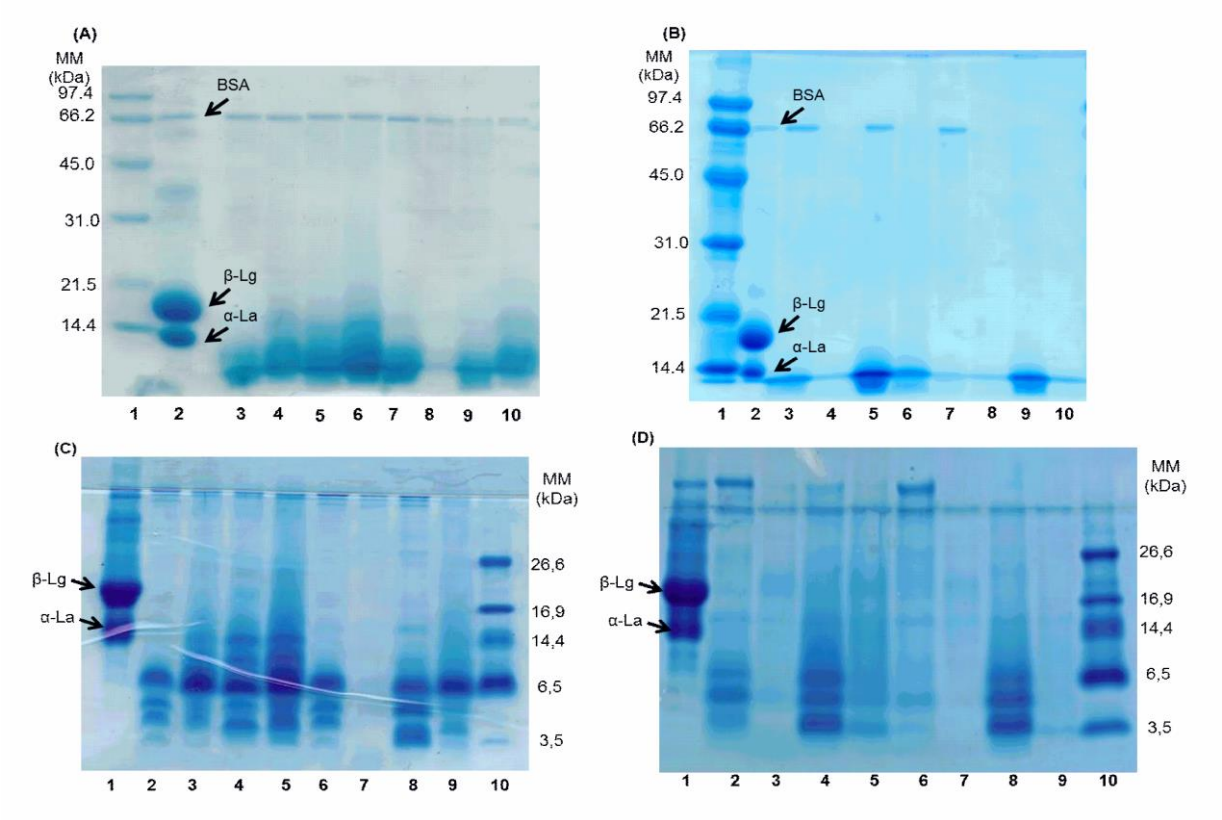

Fig. 3.2. Electrophoresis profiles of WPI and hydrolysates obtained with and without pH control using different S% and E/S. Panels A and B: SDS-PAGE profile (polyacrylamide gel 12%) of the hydrolysates at 15 and 180 min of hydrolysis, respectively. Lanes are: 1, molecular mass (MM) standard (14.4-97.4 kDa); 2, WPI; 3, HUn3-50; 4, HC3-50; 5, HUn7-50; 6, HC7-50; 7, HUn3-100; 8, HC3-100; 9, HUn7-100; 10, HC7-100. Panels C and D: SDS-PAGE/Tricine profile under reducing conditions of the hydrolysates at 15 and 180 min of hydrolysis, respectively. Lanes are: 1, WPI; 2, HUn3-50; 3, HC3-50; 4, HUn7-50; 5, HC7-50; 6, HUn3-100; 7, HC3-100; 8, HUn7-100; 9, HC7-100; 10, MM standard (3.5-26.6 kDa).

The chromatographic profiles (RP-HPLC) of the hydrolysates obtained at 15 and 180 min of reaction are shown in Fig. 3.3. The WPI chromatogram (Fig. 3.3A) showed that β-Lg eluted at 28.8 min and α-La and BSA fractions coeluted between 27.4 and 27.7 min (see Supplementary Fig. 3.S1). At 15 min of hydrolysis, the peak corresponding to β-Lg in chromatograms of the hydrolysates (Fig. 3.3B, C, D and E) was not observed, indicating that this protein had already been hydrolyzed. A small peak was observed between 27.1 and 27.3 min that is possibly a remaining of BSA, since the electrophoretic profiles displayed complete hydrolysis of α-La.

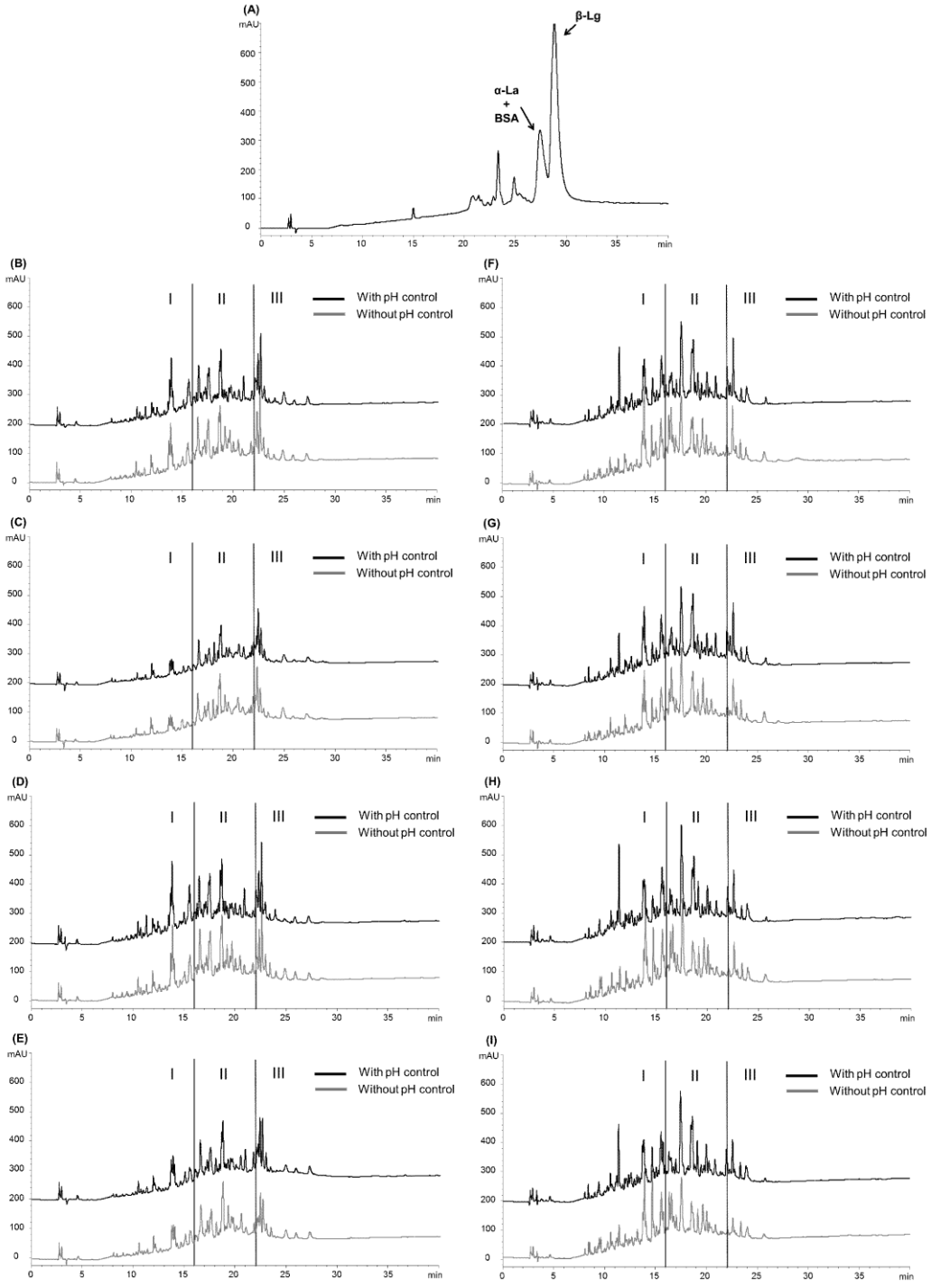

Fig. 3.3. Reverse phase-high performance liquid chromatography profiles of WPI hydrolysates obtained with (black upper trace) and without (grey lower trace) pH control using different $S_{\%}$ and E/S at 15 min (panels B-E) and 180 min (panels F-I) of hydrolysis. Panels are: A, untreated WPI; B and F, 3% and 50 U g^{-1}; C and G, 7% and 50 U g^{-1}; D and H, 3% and 100 U g^{-1}; E and I, 7% and 100 U g^{-1}. Regions labelled I, II and III are high hydrophilicity, medium hydrophilicity and low hydrophilicity, respectively.

The hydrolysates obtained with the same $S_{\%}$ and E/S, regardless of the pH control, produced chromatograms with similar peptide profiles at 15 min of hydrolysis. However, after 180 min of reaction, the hydrolysates obtained without pH control exhibited different peaks in the regions of high and medium hydrophilicity, as compared to those with the pH control. These findings suggest changes in the hydrolytic pattern of Alcalase after the first 15 min of hydrolysis when the pH stabilized at 7.0. These results can be explained by the pH change, which may have influenced the selectivity of the enzyme by modifying both substrate charge and structure (Vorob'ev et al., 2000), as well as by changing the ionization state of the amino acid residues of the enzyme active site (Butré et al., 2015). The catalytic site of Alcalase is composed of His, Asp, and Ser residues (Rehm, Kennedy, & Reed, 1987), and the proportion of positively charged His chains (9%) at pH 7.0 is 10 times greater than at pH 8.5 (0.9%). This increase in protonation of His at pH 7.0 may favour the breaking of cleavage sites with negatively charged amino acids (Glu, Tyr) in P1 position, i.e., the amino acid residues after which the peptide bond is cleaved by the protease (Schechter & Berger, 1967). Likewise, the pH can affect the charge state of the amino acid on the substrate and consequently influence the enzyme selectivity. For cleavage sites of the Alcalase with ionisable residues (Glu, Lys, and Tyr) in P1 position, the pH range (7.0-8.5) applied in the present study did not change the state charge of these amino acids. However, the interaction between the active and cleavage sites depends not only on the amino acid at P1 position, but also on the nature of another amino acid which forms the peptide bond (P1' position) and the amino acid residues located before P1 (P2, P3 , P4, etc.) and after P1' (P2', P3', etc.) in the polypeptide chain, called subsites (Schechter & Berger, 1967).

To evaluate how the charge state of these amino acids may influence the cleavage pattern, we assessed the hydrolysis of β-Lg, α-La, and BSA *in silico*. The *in silico* hydrolysis showed that some of the possible cleavage sites for Alcalase in the β-Lg, α-La, and BSA (see Supplementary Fig. 3.S2) contain Cys and His residues in nearby positions (P2, P3, P1', P2', P3'). The proportion of the positively charged His residues at pH 7.0 is 10 times greater than at pH 8.5, as already mentioned. Since the pK of the thiol group of the Cys residues is 8.18, the unprotonated Cys fraction (6%) at pH 7.0 is 10 times less than the deprotonated Cys side chains (67.6%) at pH 8.5. This increase in the proportion of positively charged His and the decrease in the deprotonated Cys fraction (not involved in disulfide bonds) at pH 7.0 may have affected the interaction between Alcalase and cleavage sites near these amino acid residues.

At 15 min of hydrolysis, hydrolysates obtained with the same $S_{\%}$ presented similar chromatographic profiles, irrespective of pH control or E/S. The hydrolysates obtained with 3% $S_{\%}$ presented similar profiles, though they were different from those obtained with 7% $S_{\%}$.

After 180 min of hydrolysis, all the hydrolysates obtained with the pH control, independent of $S_{\%}$ or E/S, exhibited similar peptide peaks, however, the intensities were different mainly in the regions of high and medium hydrophilicity. The same behavior was observed in the hydrolysates obtained without pH control. These findings, as well as the electrophoretic profiles, suggest that $S_{\%}$ may have influenced the pattern of Alcalase. Changes in enzyme selectivity at different substrate concentration may be related to differences in the accessibility of the enzyme to the substrate as a result of peptide aggregation (Butré, Sforza, Gruppen, & Wierenga, 2014b) and of the free water amount available in the system (Butré et al., 2014c).

The S_0 values are presented in Fig. 3.4A. The hydrolysates produced without pH control exhibited higher S_0 values ($p<0.05$) than those produced with the pH control. This result suggests that the formation of peptides aggregates in the hydrolysates obtained with pH control was predominantly stabilized by hydrophobic interactions. The increase of $S_{\%}$, regardless of pH control and E/S, led to higher S_0 values. In general, the hydrolysates obtained with 100 U g^{-1} E/S presented lower S_0 values than those obtained with 50 U g^{-1} and same $S_{\%}$. The different S_0 values of the hydrolysates may be related to differences in the peptides composition and aggregation as determined by the peptide profiling and molecular mass, and the pH (Creusot & Gruppen, 2007). For whey protein hydrolysates, extent of aggregation rose with increasing DH (Creusot, Gruppen, van Koningsveld, de Kruif, & Voragen, 2006; Pessato et al., 2016) which, in turn, resulted in lower S_0 values.

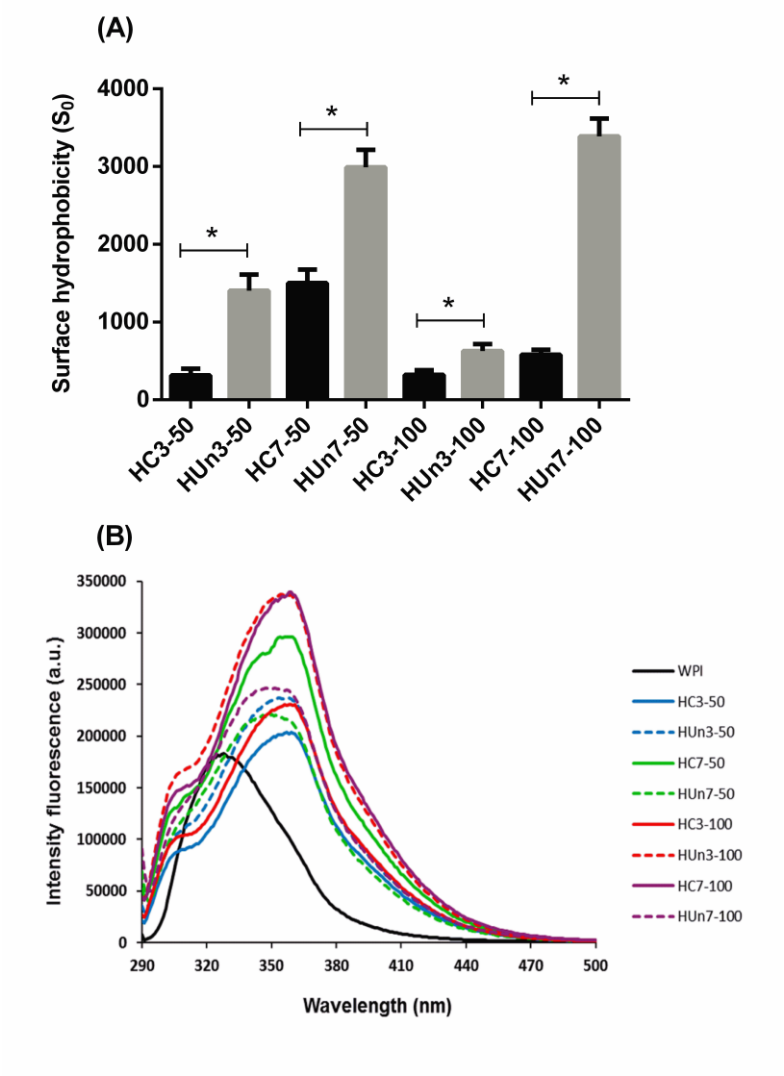

Fig. 3.4. Surface hydrophobicity (A) of the hydrolysates obtained with and without pH control at different $S_{\%}$ and E/S (results are expressed as mean ± standard deviations; an asterisk indicates $p < 0.05$ by Mann-Whitney) and fluorescence emission spectra (B; excitation wavelength 280 nm, emission wavelength 290-500 nm) of WPI and hydrolysates with Alcalase obtained with or without pH control.

Fig. 3.4B shows the fluorescence spectra of the hydrolysates and WPI. The λ_{max} of fluorescence emission of the hydrolysates was higher than that of WPI as a result of disruption of the structure or unfolding of proteins, which invariably led to a shift in the red emission (red-shifted) (Royer, 2006). The fluorescence intensity of the hydrolysates varied according to the reaction parameters. Among the hydrolysates produced with 3% $S_{\%}$, those obtained without pH control emitted higher fluorescence intensity than those obtained with the pH control. The hydrolysates produced with 7% $S_{\%}$, exhibited an opposite behavior: those obtained under controlled pH showed a high fluorescence. The fluorescence intensity is associated with both the microenvironment of Trp residues and the presence of quenching functional groups and molecules (Adams et al., 2002; Royer, 2006). The exposure of Trp

residues to solvent (more polar environment) decreases the fluorescence intensity (Damodaran & Agyare, 2013). The fluorescence emission of Trp residues may also be quenched by several amino acid side chains and the peptide bond through excited-state electron transfer (Adams et al., 2002). Differences in peptides profile and molecular mass may have resulted in different aggregation behaviour among the hydrolysates which may have affected the microenvironment of Trp and its proximity to quenching groups and consequently led to different fluorescence spectra.

3.3.2. Impact of hydrolysis conditions on the antigenic proteins

3.3.2.1. Detection of residual antigenic proteins

As measured by commercial ELISA kits, WPI contained 139.3 µg mg^{-1} of α-La and 677.1 µg mg^{-1} of β-Lg (Fig. 3.5A), which corresponds to 13.9 and 67.7% of the total protein, respectively. Hydrolysis of WPI with Alcalase under all conditions studied decreased concentrations of α-La and β-Lg in the hydrolysates by 99.9% compared to those in the WPI (Fig. 3.5B). All hydrolysates contained α-La in concentrations below <0.00078 µg mL^{-1}, which was the limit of detection by the ELISA kit. The exceptions were the hydrolysates HUn3-100 and HUn7-100, which had 0.023 and 0.021 µg mg^{-1} α-La, respectively. In general, β-Lg concentrations were significantly higher ($p<0.05$) in the hydrolysates obtained under uncontrolled pH than those produced with the pH control. The $S_{\%}$ and E/S had a significant impact on the amount of residual β-Lg in the hydrolysates. The hydrolysates obtained with 7% $S_{\%}$ exhibited the highest β-Lg concentration ($p<0.05$) under both pH conditions. In general, the increase of E/S led to decrement in β-Lg concentration ($p<0.05$) of the hydrolysates.

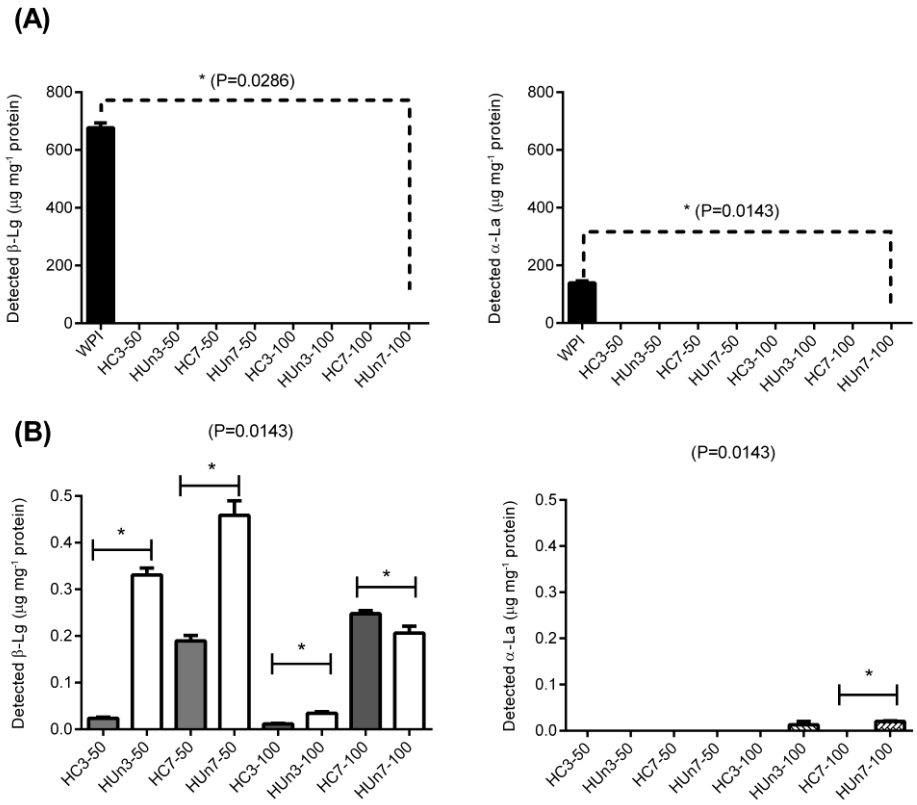

Fig. 3.5. Detection of residual β-Lg and α-La protein in hydrolysates obtained with and without pH control at different $S_{\%}$ and E/S: comparisons between (A) WPI and hydrolysates and between (B) hydrolysis performed with and without pH control. Results are presented as mean of 6 replications per sample (an asterisk indicates $p < 0.05$ by Mann-Whitney).

3.3.2.2. Evaluation of residual antigenicity

Considering the hydrolysis as a way to reduce protein antigenicity, we selected the two hydrolysates obtained without pH control which showed the lowest residual α-La and β-Lg concentrations (HUn3-100 with 0.023 µg mg^{-1} α-La, and 0.034 µg mg^{-1} β-Lg; and HUn7-100 with 0.021 µg mg^{-1} of α-La, and 0.206 µg mg^{-1} of β-Lg), and their respective counterparts obtained with pH control (HC3-100 and HC7-100) to evaluate the effect of hydrolysis on residual antigenicity. The ELISA method was used to quantify the immunoreactivity of WPI and hydrolysates to specific IgE and IgG (IgE and IgG anti-β-Lg, anti-α-La, and anti-BSA) and the results are shown in Fig. 3.6A. The specific IgE and IgG responses were significantly higher in groups sensitized with the proteins β-Lg, α-La, and BSA than those formed by non-sensitized mice (non-sens) (sensitized with alum without proteins). The sera from mice sensitized with α-La, β-Lg and BSA had a high reactivity to their respective antigenic proteins (Fig. 3.6A) and no cross-reactivity with other proteins was detected (data not shown), indicating that sensitization protocol was effective in obtaining specific IgE and IgG

antibodies. The level of cross-reactivity of HRP-conjugated anti-rat IgG antibody with mouse IgG was below 5%, highlighting the absence of false positive results and the data reliability.

The anti-β-Lg, anti-α-La IgE and IgG levels of the hydrolysates were below the ELISA detection limit (<0.025 μg mL^{-1} and <0.012 μg mL^{-1}, respectively), suggesting that the hydrolysis with Alcalase under the studied conditions decreased the β-Lg and α-La antigenicity. Despite the distinct characteristics of the hydrolysates, no difference in the anti-β-Lg and anti-α-La IgE and IgG responses to all hydrolysates was observed (Fig. 3.6A). These findings are important since suggest that Alcalase was able to cleave epitopes under different conditions, leading to more reliable processes, i.e, not dependent on pH control.

It should be considered that BSA-specific antibodies response to the hydrolysates obtained without pH control was greater than that to WPI. The hydrolysates HC3-100, HC7-100, and HUn7-100 exhibited lower anti-BSA IgE-binding capacity (42, 10, and 20 μg mL^{-1}, respectively) than WPI (255 μg mL^{-1}). While the hydrolysate HUn3-100 (532 μg mL^{-1}) showed high anti-BSA IgE-binding as compared with WPI. There was no significant difference between the anti-BSA IgG response to WPI (237 μg mL^{-1}) and to the hydrolysate HC3-100 (308 μg mL^{-1}). The hydrolysate HC7-100 exhibited a low anti-BSA IgG-binding (38 μg mL^{-1}) compared with WPI. The anti-BSA IgG response to the hydrolysates HUn3-100 (2793 μg mL^{-1}) and to HUn7-100 (579 μg mL^{-1}) was higher than that to WPI. The allergenicity of BSA is caused by linear and conformational epitopes (Restani et al., 2004). The exposure of hidden epitopes in the BSA structure during hydrolysis without pH control, along with the presence of intact BSA (Fig. 3.2B), may have increased the reactivity of the hydrolysates to anti-BSA antibodies as compared to WPI and native BSA. BSA is a minor allergen in milk but the major allergen in meats (Liccardi, Asero, D'Amato, & D'Amato, 2011).

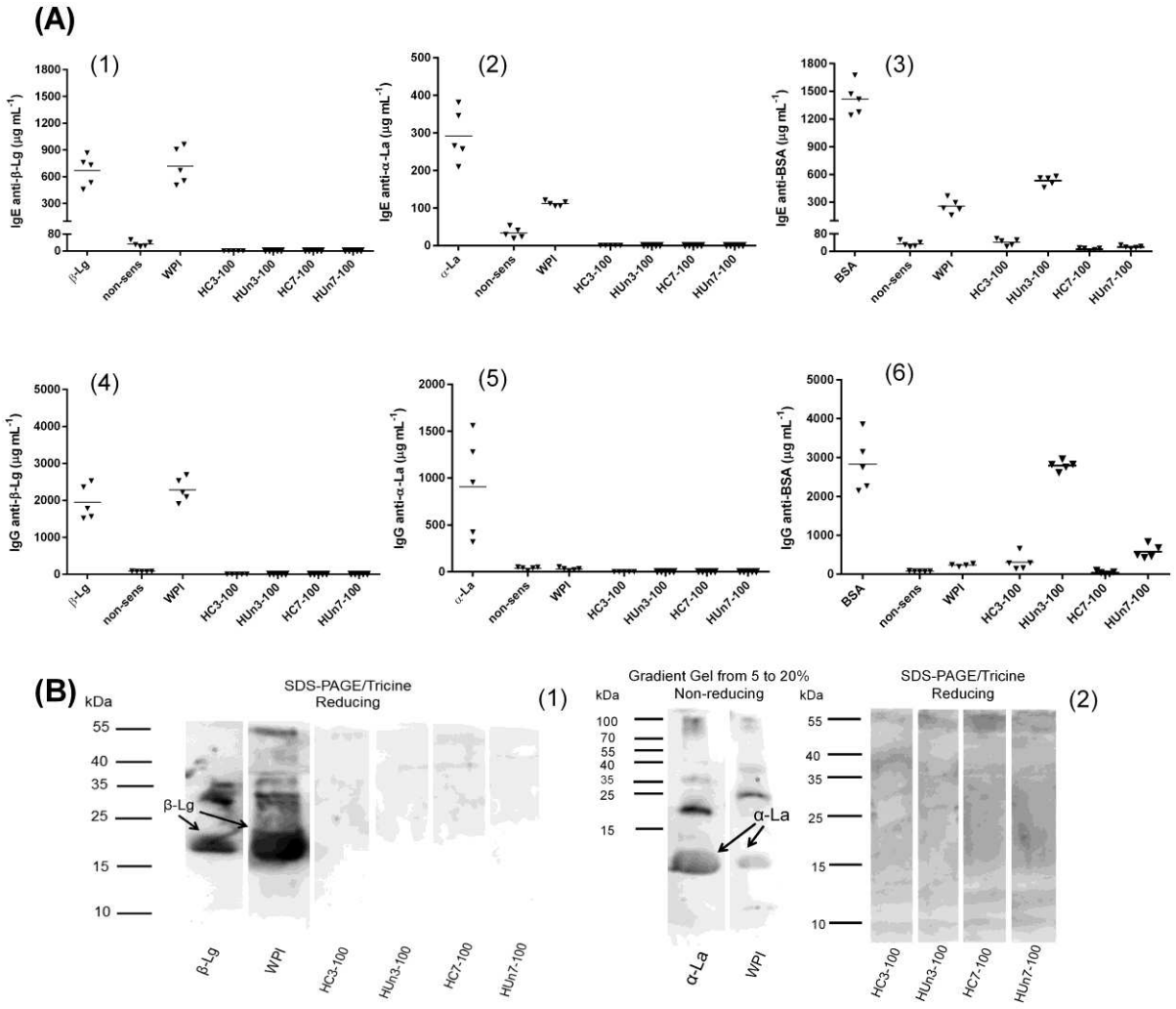

Fig. 3.6. Panel A: specific IgE and IgG response by ELISA assay; panels 1, 2 and 3 are the IgE-binding response in sera from mice sensitised to: β-Lg, α-La and BSA, respectively, and panels 4, 5 and 6 are the IgG-binding response in sera from mice sensitised to β-Lg, α-La, and BSA, respectively. Results are presented as individual values (n = 5 per group). Panel B: specific IgE response by immunoblotting assay; panels 1-2 are the IgE-binding response in sera pools from mice sensitised to: (1) β-Lg; and (2) α-La.

The binding capacity of hydrolysates to anti-β-Lg and anti-α-La IgE was performed by immunoblotting assay (Fig. 3.6B). After incubation of the membrane with anti-β-Lg sera, a band corresponding to β-Lg was detected in WPI (Fig. 3.6B, panel 1, lane 2), but not in the hydrolysates (Fig. 3.6B, panel 1, lanes 3-6), evidencing no anti-β-Lg IgE response to hydrolysates, which corroborates the results of the ELISA assays. The anti-α-La IgE response to α-La and WPI (Fig. 3.6B, panel 2, lanes 1 and 2) was only observed under non-reducing conditions and without heating, indicating that the anti-α-La sera responds mainly to conformational epitopes. Maynard, Jost, & Wal (1997) observed that 11 of the 19 sera from

patients with CMA responded exclusively to native α-La. In a recent study, Li et al. (2016) identified five IgE-binding and three IgG-binding conformational epitopes in bovine α-La. These findings corroborate with our results and highlighted that α-La epitopes are predominantly conformational. In agreement with ELISA assay results, no bands were recognized by the sera from mice sensitized with α-La, evidencing no anti-α-La IgE response to hydrolysates (Fig. 3.6B, panel 2, lanes 3-6).

Substantial decrease in the reactivity of β-Lg and α-La-specific IgE and IgG to hydrolysates is the first indication of the hydrolysates antigenicity reduction. However, for safety reasons, it is necessary to determine their residual sensitizing capacity. This evaluation should be performed using the oral sensitization route since similar process occurs in humans, and the digestion can either release or expose epitopes that were previously masked by the formation of aggregates in the whey hydrolysates (van Esch et al., 2011).

3.4. Conclusion

The differences in peptide profile, surface hydrophobicity, fluorescence spectra and antigen concentrations (α-La and β-Lg) of the hydrolysates, obtained under different pH conditions and the same $S_{\%}$ and E/S, show the pH crucial role on the hydrolysates characteristics. The pH drop during hydrolysis lead to a different charge state of enzyme and substrate, affecting Alcalase active site (His residue), and the subsites (Cys and His residues), as demonstrated by the *in silico* analysis, which influences in the interaction of the enzyme with the cleavage sites. Nevertheless, hydrolysis with Alcalase either with or without pH control was effective in reducing the antigenicity of the α-La and β-Lg protein since no differences were observed in anti-α-La and anti-β-Lg antibodies response to hydrolysates. However, additional studies are needed to develop other processes that may be associated with hydrolysis with Alcalase under uncontrolled pH condition to reduce the BSA antigenicity.

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Appendix A. Supplementary data

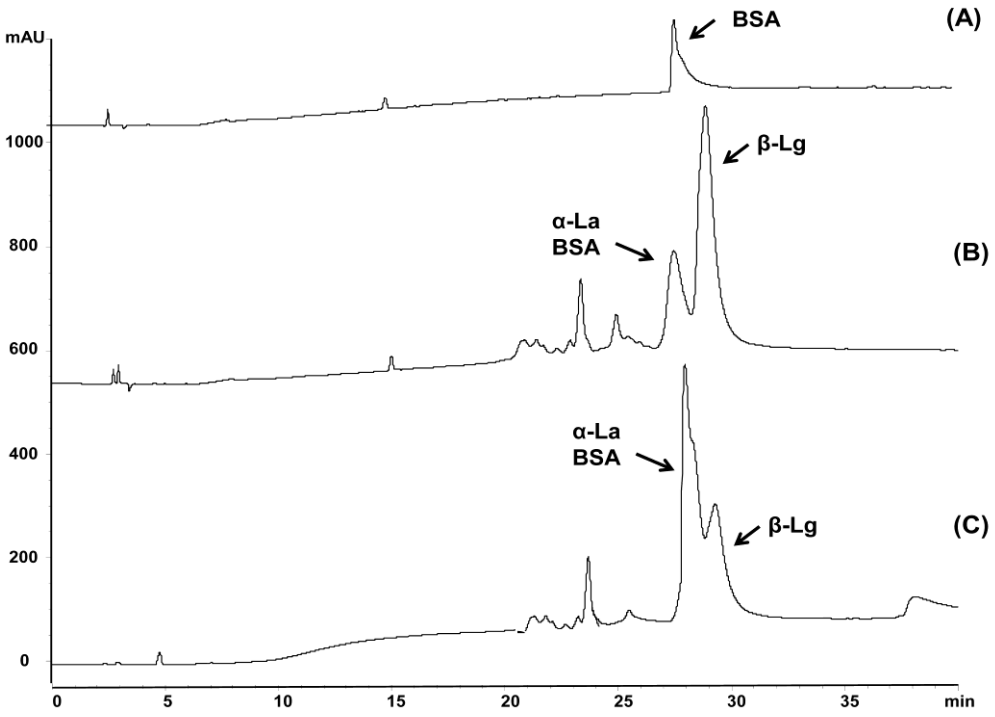

Fig. 3.S1. HLPC profile of (A) BSA protein; (B) WPI; and (C) WPI added BSA protein.

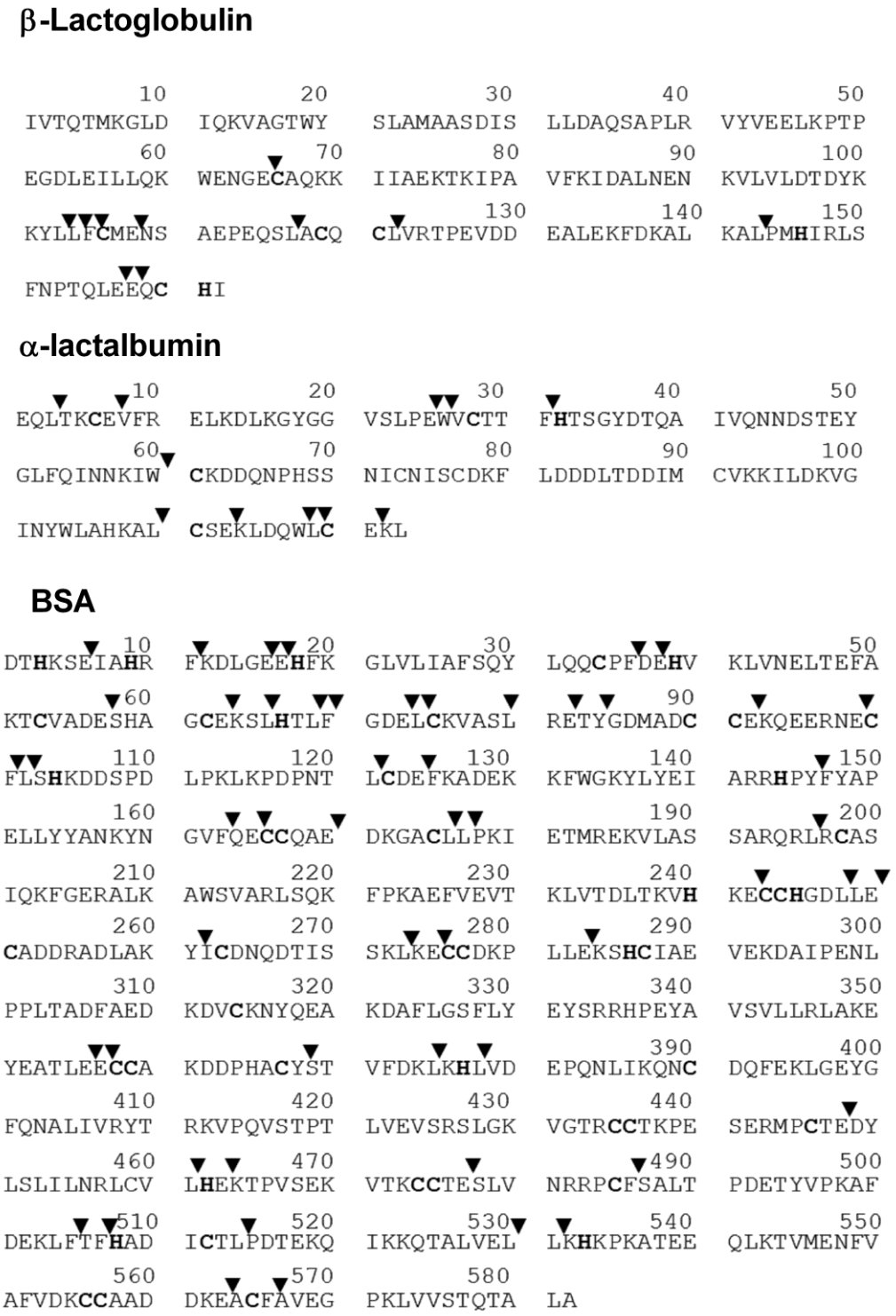

Fig. 3.S2. Amino acid sequence of β-lactoglobulin, α-lactalbumin, and BSA; the symbols ▼ refer to the possible cleavage sites for Alcalase which are close to Cys and His residues (positions P2, P3, P1', P2', P3').

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CAPÍTULO 4. ARTIGO 2. Physicochemical changes of transglutaminase treated whey protein hydrolysates and their effects on bitter taste

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Abstract

BACKGROUND: Extensive enzymatic hydrolysis of food proteins is widely used for production of hypoallergenic hydrolysates, however it results in the release of bitter-tasting peptides. The enzymatic hydrolysis followed by cross-linking catalyzed by transglutaminase (TG) has been considered a way to reduce the bitter taste of peptides. Therefore the effect of TG-catalyzed reactions on the physicochemical characteristics of whey protein hydrolysates (filtrate, molecular mass < 5 kDa) and their impact on the bitterness were investigated. RESULTS: TG treatment resulted in slight modifications in peptides structure and molecular mass profile. The peptide profile was altered after TG treatment, especially in the range of m/z 1600-3000. Despite changes on sample physicochemical characteristics as a result of TG treatment, no difference in the intensity or perception of bitter taste among samples was detected. CONCLUSION: Possibly there was a significant amount of short peptides with no lysine and glutamine residues, which were incapable to form cross-links and limited the action of TG. Under the studied condition, the polymerization with TG is not appropriated to reduce the bitterness of extensively hydrolyzed whey protein.

Key words: enzymatic hydrolysis, enzymatic cross-linking, MALDI-MS, bitter peptides

4.1. Introduction

Enzymatic hydrolysis of proteins is the most commonly used method to reduce antigenicity of milk proteins. [1] During hydrolysis, the cleavage of portions of the protein recognized by IgE antibodies (epitopes) occurs. [2] The conformational epitopes (specific three-dimensional shape) are easily disrupted by the process of hydrolysis, whereas the cleavage of linear epitopes (a sequence of amino acids) depends on enzyme specificity. [3] Van Beresteijn, et al. [4] found that whey peptides with minimum molecular mass (MM) of 3000 Da were required to elicit an immunological response. For antibody binding, an epitope must contain a minimum of 15 amino acid residues and an allergen must possess at least two IgE binding sites. [5] Commercially available hypoallergenic formulas based on extensively hydrolyzed milk protein predominantly contain peptides smaller than 1000 Da (63 to 95%). [6]

A high concentration of peptides lower than 1000 Da containing hydrophobic groups is associated with the bitterness of whey protein hydrolysates, [7, 8] which renders these formulas unpalatable and can limit their incorporation into foods. [9, 10] Bitterness is an important cause of food rejection, therefore the development of protein hydrolysates with low levels of bitterness is essential for their application into various foods. [11]

Enzymatic cross-linking using transglutaminase (TG) has been regarded as a promising strategy to reduce the bitter taste of protein hydrolysates. [12, 13] TG catalyzes acyl transfer reactions between γ-carboxyamide group of glutamine residue and a variety of primary amines, including ε-amine group of lysine residue. The ϵ-(γ-glutamyl)-lysyl isopeptides bonds form inter- and intramolecular cross-links in proteins/peptides. [1, 14] Since peptides with low MM and highly hydrophobic are generally regarded as being bitter [15] the changes catalyzed by TG can decrease the bitterness of protein hydrolysates. It has been reported that treatment with TG post-hydrolysis may result in enhanced bioactive and techno-functional properties such as solubility [12, 16] and emulsification capacity, [17, 18] which were related to MM and hydrophilicity increase. [16] Babiker, et al. [12] and Song, et al. [13] reported that TG-catalyzed polymerization decreased the bitter taste of soy protein hydrolysates as a result of the reduced exposition of the hydrophobic sites.

Furthermore, studies have shown that the combination of enzymatic hydrolysis of whey protein followed by TG treatment decreased or had no significant effect on the antigenic response of the hydrolysates. [19-22] Therefore this combination of enzymatic treatment could be used to produce hypoallergenic ingredients with improved sensory characteristics. The present study aimed to investigate the modifications caused by TG-catalyzed reactions on physicochemical characteristics of whey protein hydrolysates and their impact on the bitter taste of the hydrolysates.

4.2. Materials and methods

4.2.1. Materials

Whey protein isolate (WPI) PROVON® (Glanbia Nutritionals, Kilkenny, Ireland) was obtained from a local market. The protein content of the WPI, 90.4% ± 1.7, was determined by micro-Kjeldahl method [23] using 6.38 as conversion factor. Commercial preparation of TG (Activa®), a mixture containing 99% of maltodextrin and 1% TG, was donated by Ajinomoto Co. (São Paulo, SP, Brazil). Alcalase (EC 3.4.21.62, Protease from *Bacillus licheniformis*) were purchased from Sigma-Aldrich® (St.Louis, MO, USA).

The reagents tricine and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Trifluoroacetic Acid (TFA), β-mercaptoethanol, Coomassie Brilliant Blue G250, sodium hydroxide, and urea were purchased from Merck (Hohenbrunn, Germany). Acrylamide and Tris (Tris(hydroxymethyl)aminomethane) were purchased from Bio-Rad (Hercules, CA, USA). All other reagents used were of analytical or chromatographic grade.

4.2.2. Preparation of whey protein hydrolysates

Enzymatic activity of Alcalase was 8.96 U mg^{-1}, determined by the method described by Emi, et al. [24]. One unit (U) of enzyme activity was defined as the amount of enzyme that produces TCA (trichloroacetic acid) soluble peptides equivalent to 1 µg of tyrosine in 1 min. [24]

The hydrolysis of WPI with Alcalase was carried out according to the conditions defined for hydrolysis without pH control by Carvalho, et al. [25]. Briefly, WPI was diluted to obtain 3 or 7% of protein (w/v) and hydrolysis with Alcalase was performed using 100 U g^{-1} of protein, at pH 8.5 for 180 min at 60 °C. After 180 min, the reaction was stopped by heating at 90 °C for 10 min. Part of the hydrolysates obtained was freeze-dried and part was ultrafiltered. The hydrolysates were named H3 and H7, according to substrate concentration used.

The hydrolysates were ultrafiltered using a Pellicon® ultrafiltration system (Millipore, Bedford, MA, USA) and a membrane with cutoff of 5 kDa (Cartridge Prep/Scale: – TFF 6 ft2). The sample H7 was diluted to half of its concentration and the H3 was not diluted. Six diafiltration cycles were done, with addition of water at constant volume. The retentates (> 5 kDa fraction) were named as HR3 and HR7, and the filtrates (< 5 kDa fraction) were named as HF3 and HF7. The fractions were frozen, freeze-dried, and stored at −20 °C until their use.

4.2.3. Post-hydrolysis TG treatment

Enzymatic activity of TG was 43.6 U mg^{-1}, determined according to Folk and Cole [26]. One unit of the activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol min^{-1} of hydroxamic acid.

The TG treatment of the HF3 and HF7 samples was performed according to the conditions established by Villas-Boas, et al. [27]. Briefly, the samples were rehydrated to obtain 7% of protein (w/v) and the treatment with TG was carried out using 10 U g^{-1} of protein, at pH 8.0 for 180 min at 50 °C. After 180 min, the reaction mixture was cooled down in an ice bath, frozen and freeze-dried. The TG-treated samples were named HF3-TG and HF7-TG.

4.2.4. Protein determination

The total nitrogen content of hydrolysates was determined by micro-Kjeldahl method, and the protein content was calculated using 6.38 as conversion factor. [23]

4.2.5. Electrophoresis

The electrophoretic profiles of the samples were obtained by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under reducing conditions [28] using a Mini Protean II apparatus (Bio-Rad, Hercules, CA, EUA) and separation and stacking gels with 12% and 3% of acrylamide, respectively. The samples (1% w/v protein) were diluted in reducing buffer (62.5 mmol L-1 Tris, 2% SDS, 20% glycerol and 5% β-mercaptoethanol, pH 6.8) and heated at 95°C for 5 min. The gels were stained with 0.1% Coomassie Blue R-250 and de-stained with acetic acid:methanol:distilled water (1:4:5). A 14.4–97.0 kDa MM marker kit (Bio-Rad, Hercules, CA, USA) was used as standard.

The samples were also evaluated by SDS-PAGE/Tricine [29] using a discontinuous system consisting of separation gel, spacer gel, and stacking gel with 16.5%, 10%, and 3% of acrylamide, respectively. The samples (2% protein) were diluted in reducing buffer and heated at 95°C for 5 min. Aliquots of 10 µL of each sample were applied. The gels were fixed in methanol:acetic acid:distilled water (5:1:4) for 24 h and stained with 0.025% Coomassie Blue G250 in 10% acetic acid for 48 h. De-staining was carried out in 10% acetic acid. A 3.5-26.6 kDa MM marker kit (Bio-Rad, Hercules, CA, USA) was used as standard. All electrophoretic analyses were performed in duplicate.

4.2.6. Chromatography (HPLC)

All chromatographic analyses were performed using Agilent HPLC system (Waldbronn, Germany) equipped with a quaternary and semi preparative pump, a diode array detector (DAD). MM distribution of the peptides in the samples was estimated by size-exclusion high performance liquid chromatography (SE-HPLC). The analysis was performed using a TSK-GEL G2000 SWXL column (7.8 mm i.d., 300 mm - Tosoh BioSep - Montgomeryville, PA, USA) at a flow rate of 0.5 mL min^{-1}. The mobile phase was acetonitrile/water (25/75, v/v) with 0.1% trifluoroacetic acid. A volume of 5 µL of each samples (3 mg mL^{-1}) was injected and the absorbance was monitored at 214 nm. The following protein standards were used to estimate peptides MM: bovine serum albumin (BSA, 66.0 kDa), triosephosphate (26.6 kDa), β-lactoglobulin (β-Lg, 18.4 kDa), α-lactalbumin (α-La, 14.4 kDa), aprotinin (6.5 kDa), insulin (3.5 kDa) and bacitracin (1.4 kDa). The relative percentage of each range (> 66, 6.5–14.4, 3.5–6.5, 1.4–3.5, and < 1.4 kDa) was determined by the relationship between the peak area with the corresponding MM and the total area. The Star Chromatography Workstation software (Agilent) was used to record and process data.

The peptide profile of the hydrolysates was determined by reverse phase high performance liquid chromatography (RP-HPLC) as described by Pessato, et al. [30]. The mobile phase was a mixture of solvent A: water/trifluoracetic acid (0.04% v/v) and solvent B: acetonitrile/ trifluoracetic acid (0.03% v/v). The column was equilibrated with 100% of solvent B. A linear gradient of solvent B in A, from 0% to 70% in 40 min was performed, followed by 5 min at isocratic form and rebalanced to initial conditions of the column for 15 min. Absorbance was recorded at 214 nm using a diode-array detector (DAD). To allow comparisons of the hydrolysates profiles, the chromatograms were divided into three regions: (I) high hydrophilicity – 16 min gradient from 10% to 30% B; (II) medium hydrophilicity –16 to 22 min gradient from 30% to 40% B; (III) low hydrophilicity – above 22 min – above 40% B. All chromatographic analyses were carried out in duplicate.

4.2.8. Intrinsic fluorescence

The intrinsic fluorescence emission spectra of the filtrates before and after TG treatment were determined as described by Tang, et al. [17] using an *ISS PC1 Fluorimeter* (Champaign, IL, USA). The test samples (5 mg mL^{-1}) were excited at 280 nm, and the emission intensities were measured from 290 to 500 nm.

4.2.9. MALDI-MS peptide fingerprinting of filtrates before and after TG treatment

Sample preparation and data acquisition. The filtrates treated or not with TG were solubilized in 70% ethanol (HPLC grade - Sigma, Switzerland). A 1 µL drop of the samples was deposited on a spot of a polished steel target (MTP384 polished steel target; Bruker Daltonics, Bremen, Germany) and air-dried. Each spot was then covered with 1 µL of the matrix. The matrix was a daily prepared saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA - Sigma, Basel, Switzerland). The droplets were allowed to dry at room temperature prior to analysis. MALDI-TOF analysis was performed in a Bruker Autoflex III, equipped with Smartbeam TM laser technology (Bremen, Germany). MS data was acquired in positive reflector mode between *m/z* 700 to 3500.

Data processing. Raw data was pre-processed by Flex Analysis (3.4 software Bruker Daltonics) including baseline subtraction, spectra smoothing and alignment. In order to efficiently evaluate the data from a mass spectrum, it is necessary to properly generate a mass list. The SNAP (Sophisticated Numerical Annotation Procedure) algorithm was applied to data derived from samples. Since it generates a list of monoisotopic masses, which is preferable to a peak list containing averaged masses or a list of all the individual isotopic masses to improve mass accuracy and reduce the complexity and redundancy. Data was normalized by sum and auto-scaled. Pre-processed data was converted to .csv (Comma-Separated Values) files. After data pre-processing, samples were classified according to their peptide fingerprinting.

4.2.10. Sensory analysis

Sensory tests were carried out in individually air-conditioned booths in the Sensory Analysis Laboratory of the Faculty of Food Engineering (University of Campinas, Brazil). Sensory analyses were approved by the Research Ethics Committee of University of Campinas, protocol number CAAE 37308414.8.0000.5404 (Campinas, SP, Brazil). The sensory analyses were carried out in accordance with the Declaration of Helsinki. The time-intensity analysis was performed to evaluate the bitter taste of the filtrates before and after TG treatment.

Microbiological analysis. The samples were prepared in a food-grade laboratory and the hydrolysis process was adjusted to be safe for use in foods. The samples were tested for total and fecal coliforms, and *Salmonella* spp. before sensory analysis begins. The coliforms were determined at 30-35°C and at 45°C by the multiple-tubes, most probable number (MPN) technique, as described by ISO 4831:2006 and ISO7251:2005. The presence of *Salmonella* spp. was determined by Bax® System real-time PCR according to AOAC

2003.09 method. [31] All samples showed less than 0.3 MPN mL^{-1} of total and fecal coliforms. *Salmonella* spp. was absent in all samples. The results of microbiological analyses confirm that the samples were produced under adequate hygienic-sanitary conditions and pose no health risk to the panelists.

Pre-selection of panelists. Tetrad tests applied to Wald's sequential analysis [32] were used to select potential panelists with discriminatory ability for time-intensity analysis. Seventeen volunteers were recruited among students and staff at UNICAMP, and standard solution of caffeine ranging from 0.3 to 5.2 mg mL^{-1} were presented in order of increasing difficulty, i.e., from great to very small difference in concentration. Fifteen individuals were selected as potential panelists.

Training of sensory panel. The samples were compared against different caffeine solutions in order to determine the reference of strong bitterness. Caffeine concentration at 2.6 mg mL^{-1} was chosen as the reference of maximum intensity for bitter taste by a consensus of all judges. Training for the formation of sensory memory and equalization among the panelists was performed by direct contact of the individuals with the reference of maximum intensity for bitter taste in four training sessions.

Selection of panelists. The panelists were trained to use Time-Intensity Analysis of Flavors and Tastes (TIAFT) software. [33] The panelists evaluated the bitter taste of the samples using a computer mouse to record the perceived intensity of bitterness on a 10-continuum (0 = none, 4.5 = moderate and 9 = strong) over time. The test conditions were previously standardized using TIAFT software: (a) initial waiting time, 15 s, (b) time with sample in mouth, 10 s, and (c) time after ingestion, 60 s. The software analyzed the data collected during each sensory evaluation session and provided the following parameters: I_{max} (maximum intensity recorded by the judge); Ti_{max} (time in which the maximum intensity was recorded); Area (area of the time curve × intensity) and T_{tot} (total duration time of the stimulus). [30] The samples were presented monadically with four repetitions. Twelve panelists were selected based on their discrimination power, repeatability and agreement with the panel [31] as verified by two-factor analysis of variance (sample and repetition), using significant F_{sample} as $p < 0.50$ and non-significant repetition as $p > 0.05$.

Evaluation of bitter taste. The bitter taste of the samples was assessed by time-intensity analysis. The samples were rehydrated at 2% of protein (w/v) with sterile deionized water. This protein concentration was within the range of commercially available hypoallergenic formulas (1.6 to 2.1 g/100 mL), [6] and of the recommended protein content by European Society for Pediatric Gastroenterology, Hepatology and Nutrition (1.8 to 3.0 g/100 kcal). [34] All samples were presented monadically to panelists at room temperature in 7 mL

sample cups coded with random three-digit number. In each session, water and unsalted crackers were provided for palate cleansing between samples. The selected panelists (n = 12) evaluated all samples with four repetitions. At the end of the test, the time–intensity curves were obtained and the data was collected ($I_{max,}Ti_{max}$, Area, and T_{tot}).

Statistical analysis. The statistical analysis of time-intensity test was performed considering each panelist and their repetition. The parameters obtained by time-intensity curves were evaluated by analysis of variance and Tukey's test ($p< 0.05$) using the Statistical Analysis System (SAS) 9.4 software.

4.3. Results and discussion

4.3.1. Electrophoretic characterization of WPI hydrolysates and their ultrafiltered fractions

In a previous study, we found that the residual antigenicity of WPI hydrolysates obtained with Alcalase under uncontrolled pH condition was mainly related to the presence of intact BSA and/or the exposure of hidden epitopes by hydrolysis process. [25] Thus, in the present study, the hydrolysates were ultrafiltered using a 5 kDa cutoff membrane to separate low MM peptides from higher MM peptides and unhydrolysed and partially hydrolyzed BSA

The electrophoretic profile (SDS-PAGE) of the hydrolysates and their fractions are shown in Figure 1A. Electrophoresis revealed that the α-La (14.4 kDa) and β-Lg (18.4 kDa) were cleaved in both hydrolysates, H3 and H7, confirmed by the complete disappearance of their correspondent bands while intact BSA (66.4 kDa) remained in both hydrolysates, confirming our previous results. [25] After ultrafiltration, BSA was only observed in the profiles of the retentates (> 5 kDa), showing the process effectiveness in separating this protein. In the SDS-PAGE/Tricine analysis (Figure 1B) no band was observed in the profiles of the HF3 and HF7 (Figure 1B, lanes 3 and 6), suggesting that these fractions contain mostly peptides smaller than 3.5 kDa, which are not stained by Coomassie Blue. [29]

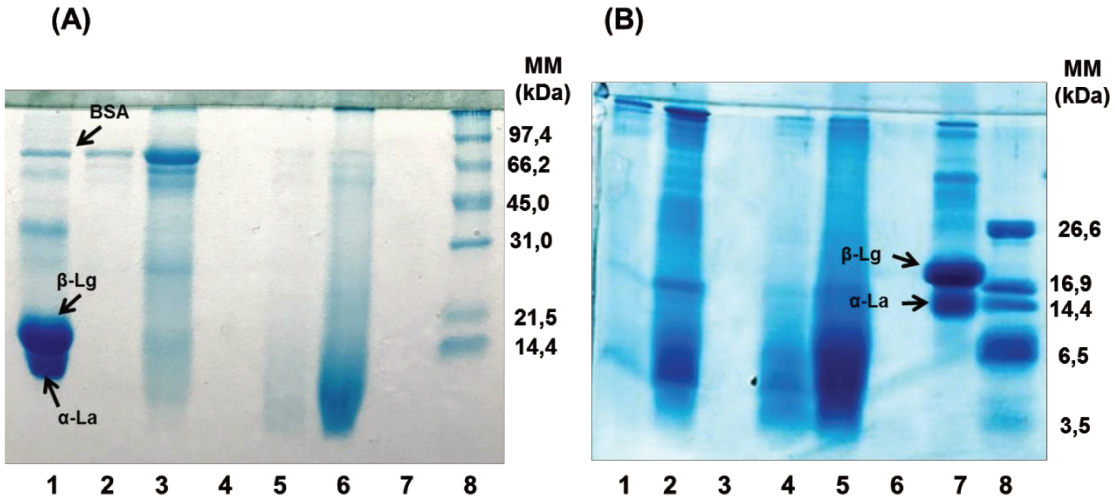

Figure 4.1: Electrophoresis profile of WPI, hydrolysates and their fractions. (A) SDS-PAGE profile (polyacrylamide gel 12%): (1) WPI; (2) H3; (3) HR3; (4) HF3; (5) H7; (6) HR7; (7) HF7; (8) Molecular mass (MM) standard (14.4 – 97.4 kDa). (A) SDS-PAGE/Tricine profile under reducing conditions of the: (1) H3; (2) HR3; (3) HF3; (4) H7; (5) HR7; (6) HF7; (7) WPI; (8) MM standard (3.5 – 26.6 kDa).

4.3.2. TG treatment: effect on low molecular mass hydrolysates fractions characteristics

Considering that low MM peptides are usually used in hypoallergenic formulas because they are less antigenic and are better absorbed by the gastrointestinal tract, [5, 35, 36] the filtrates (MM < 5 kDa) were used to be treated with TG to continue this study.

The MM distribution obtained by SE-HPLC is shown in Table 4.1. HF3 and HF7 presented approximately 16% of peptides between 1.4 and 3.5 kDa and 83% of peptides smaller than 1.4 kDa. After the TG treatment, the samples showed a slight increase in peptides with MM from 1.4 to 3.5 kDa (Table 4.1).

Table 4.1: Peptide molecular mass (MM) relative composition (%) of whey protein hydrolysates and their filtrates, before and after treatment with transglutaminase (TG).

Samples	***Molecular mass***			
	> 66 kDa	**6.5-14.4 kDa**	**1.4-3.5 kDa**	**< 1.4 kDa**
H3	n.d.	1.51	16.32	82.17
HF3	n.d.	n.d.	16.11	83.90
HF3-TG	n.d.	n.d.	17.60	82.90
H7	0.23	2.36	18.26	79.15
HF7	n.d.	n.d.	16.95	83.06
HF7-TG	n.d.	n.d.	19.84	80.16

H3: hydrolysate obtained using 3% of protein; H7: hydrolysate obtained using 7% of protein; HF3: H3 hydrolysate fraction containing peptides < 5 kDa; HF7: H7 hydrolysate fraction containing peptides < 5 kDa; HF3-TG: HF3 treated with transglutaminase; HF7-TG: HF7 treated with transglutaminase.
n.d. = not detect.

The chromatographic profiles obtained by RP-HPLC are shown in Figure 4.2. HF3 and HF7 presented some differences in their profiles, especially the intensity of the peaks in the regions of high and medium hydrophilicity. This result is in accordance with a previous study, in which we found that different WPI concentrations altered the cleavage pattern of Alcalase and, consequently, the profile of released peptides. [25] The chromatograms of the HF3-TG and HF7-TG showed different peak intensities in the regions of high and medium hydrophilicity and disappearance of a peak eluted at 26.0 min (in the region of low hydrophilicity) which was observed in the chromatograms of their non-TG treated counterparts (Figure 4.2).

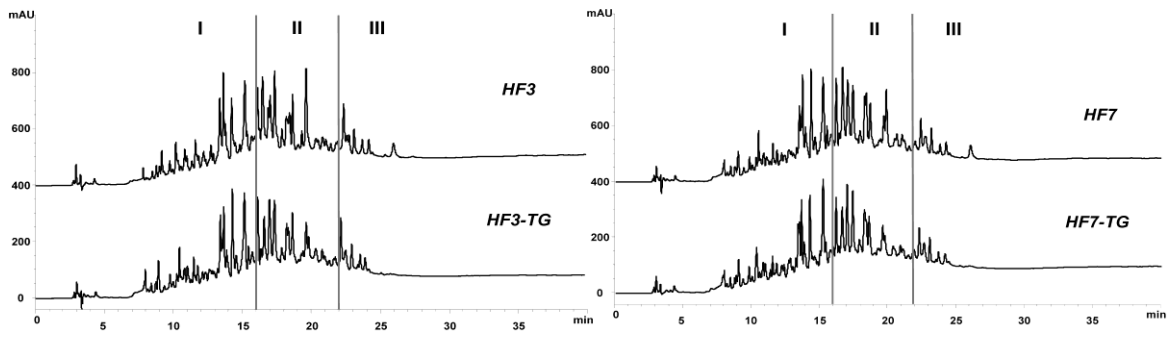

Figure 4.2: Reverse phase-high performance liquid chromatography profiles. Comparison between peptide profiles of the filtrates before (HF3 and HF7) and after treatment with transglutaminase (HF3-TG and HF7-TG). Regions labeled I, II and III are high hydrophilicity, medium hydrophilicity and low hydrophilicity, respectively.

The fluorescence spectra of the filtrates before and after TG treatment are shown in Figure 4.3. No λ_{max} shift was observed as a result of TG-catalyzed reaction (Figure 4.3), which indicates that the dielectric environment of Trp residues was not altered in any significant way. [1] However, a slight decrease in fluorescence intensity was observed in the both TG-treated samples as compared to their respective non treated counterpart, which was less intense for HF7. This decrease in fluorescence intensity may be provoked by the exposure of the Trp residues to a more polar environment [37] and/or their relocation next to amino acids residues and the peptide bond. [38] The TG treatment may have altered the spatial conformation of peptides, impacting the microenvironment of Trp residues and its proximity to quenching groups and leading to lower intensities of fluorescence emission.

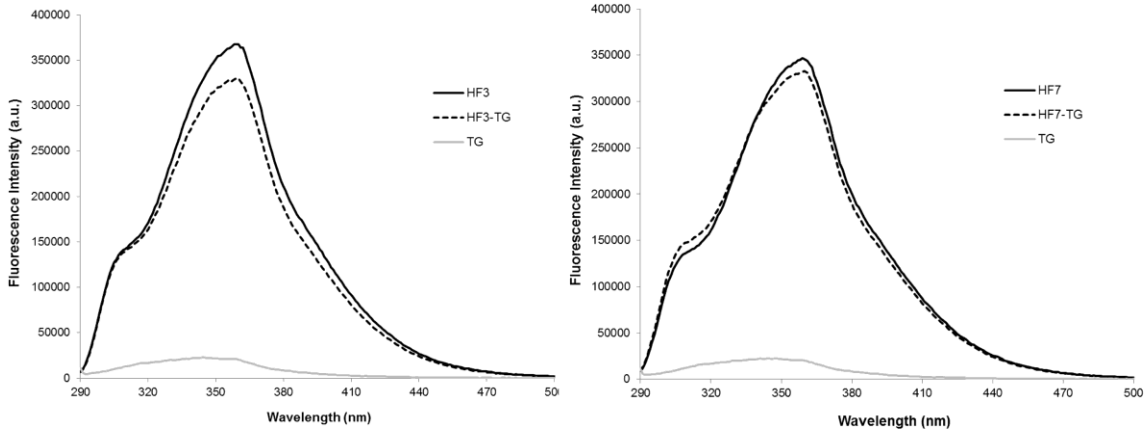

Figure 4.3: Fluorescence emission spectra (excitation wavelength 280 nm, emission wavelength 290-500 nm) of the filtrates before (HF3 and HF7) and after treated with transglutaminase (HF3-TG and HF7-TG). TG corresponds to the fluorescence spectrum of transglutaminase enzyme.

Samples were also analyzed by MALDI-MS (Figure 4.4). Both HF3 and HF7 displayed peaks in the range of *m/z* 700-1600, however, with different relative intensities. The algorithm SNAP detected a greater number of peaks for HF3, whereas HF7 exhibited only two peaks at *m/z* 2109 and *m/z* 2534, which were also observed in HF3 spectrum.

After TG treatment, changes in peptides profile were observed (Figures 4.4). In contrast to HF3, peptides at *m/z* lower than 1600 were not present in HF3-TG and the peptides at *m/z* between 700 and 1600 exhibited different relative intensities. The majority of peptide signals between *m/z* 700-1600 were not detected in HF7-TG, and the signals at *m/z* 2109 and 2534 were no longer detected. The reaction catalyzed by TG between the residues of Glu and Lys may have suppressed peptide ionization, leading to the non-detection of these peptide signals. Figure 4.5 shows the results graphically as heatmaps that provide

intuitive visualization of the ions distribution over the samples. Ions at *m/z* 904 and *m/z* 1058 have highest intensity in HF3, whereas ions at *m/z* 852 and 1014 showed highest intensity in HF3-TG. On the other hand, for HF7 and HF7-TG, the ions with highest intensity were those at *m/z* 942 and at *m/z* 948 for HF7, and at *m/z* 1124 and *m/z* 1146 for HF7-TG. These peptide profile changes suggest that ions ranging from *m/z* 1600 to 3000 for both samples and ions ranging from *m/z* 700 to 1600 for HF7 were involved in the TG-catalyzed reaction.

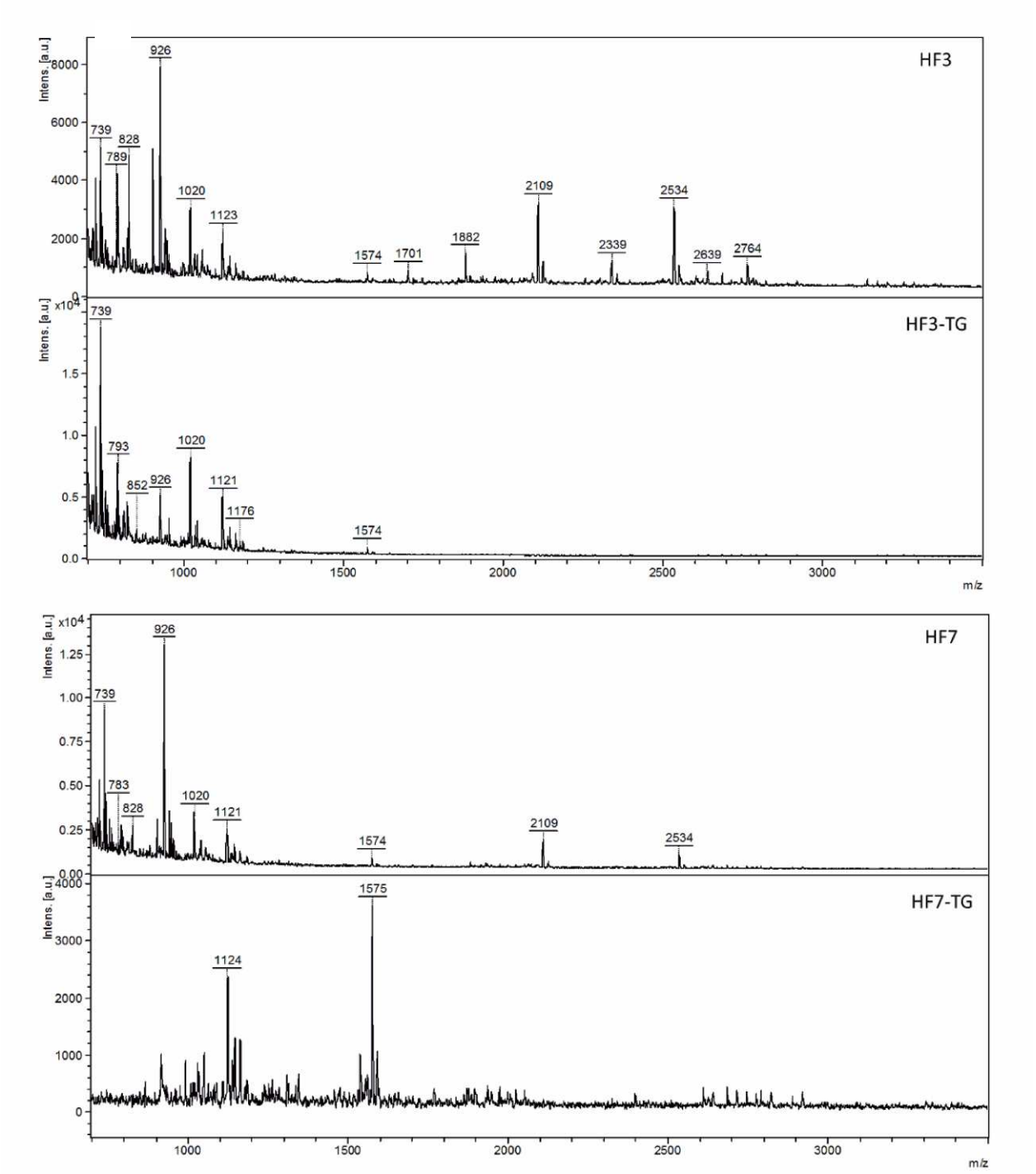

Figure 4.4: MALDI MS fingerprinting of the samples: HF3, filtrate from whey protein hydrolysate obtained with 3% protein and HF3-TG, HF3 treated with transglutaminase (TG); and HF7, filtrate from whey protein hydrolysates obtained with 7% protein and HF7-TG, HF7 treated with TG.

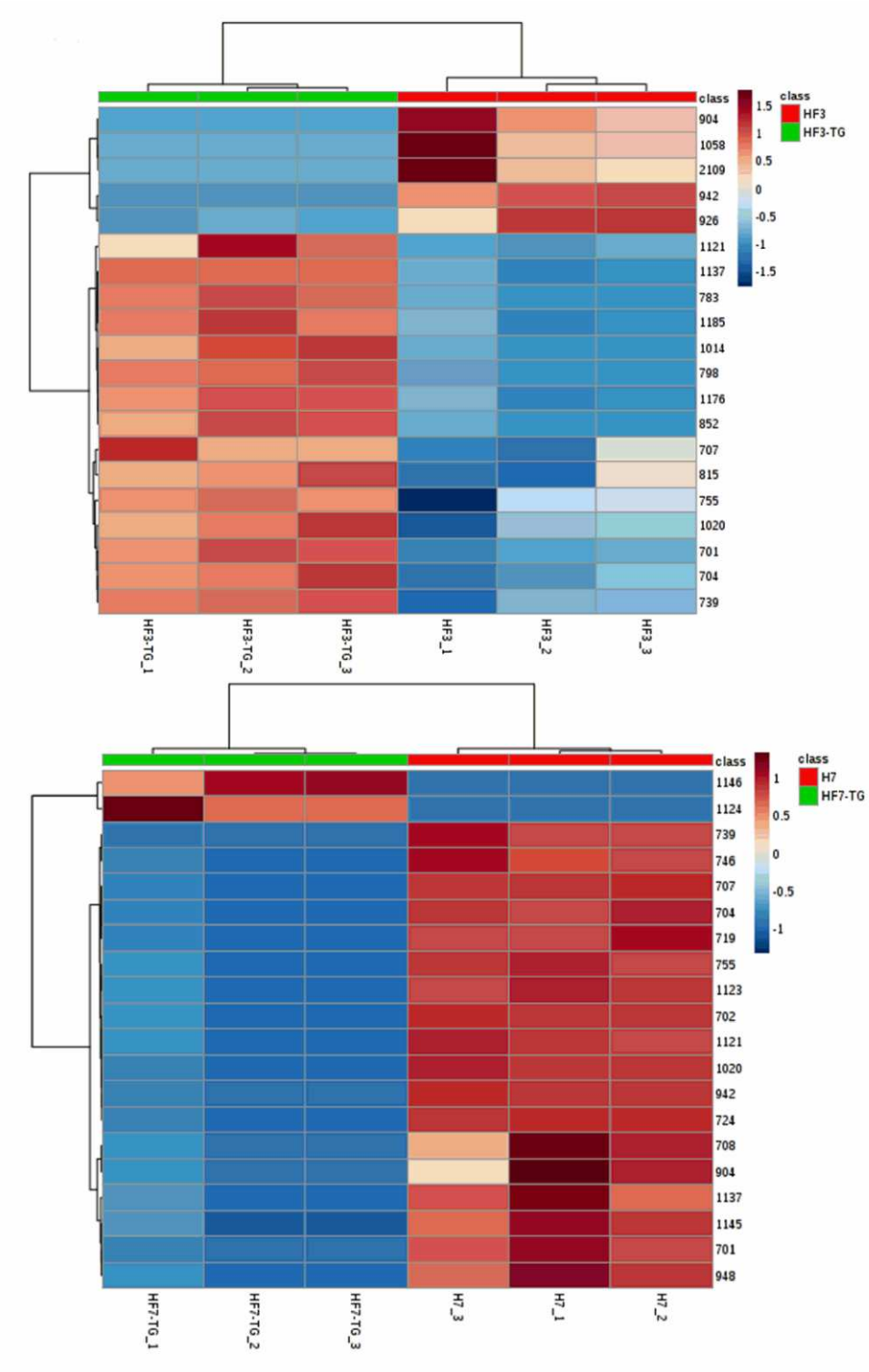

Figure 4.5: Heatmaps comparing for samples: HF3, filtrate from whey protein hydrolysate obtained with 3% protein and HF3-TG, HF3 treated with transglutaminase (TG); and HF7, filtrate from whey protein hydrolysates obtained with 7% protein and HF7-TG, HF7 treated with TG. Each colored cell on the map corresponds to a relative intensity value in their data table; samples are organized in rows and features/compounds in columns.

After TG treatment, the majority of the peptides (≥ 80%) remained smaller than 1.4 kDa. This occurred possibly because a significant amount of short peptides with no lysine and glutamine residues were generated, which are incapable to form cross-links, limiting the action of TG and, consequently, the MM increase. [17] Slight modifications in peptides structure and peptide profile, indicated by intrinsic fluorescence and RP-HPLC, were observed as a result of TG treatment. MALDI-MS results also showed changes in peptide profile after TG treatment, especially concerning ions at *m/z* 1600 to 3000. Figure 4.6 illustrates the TG action in peptides < 5 kDa of the WPI hydrolysates.

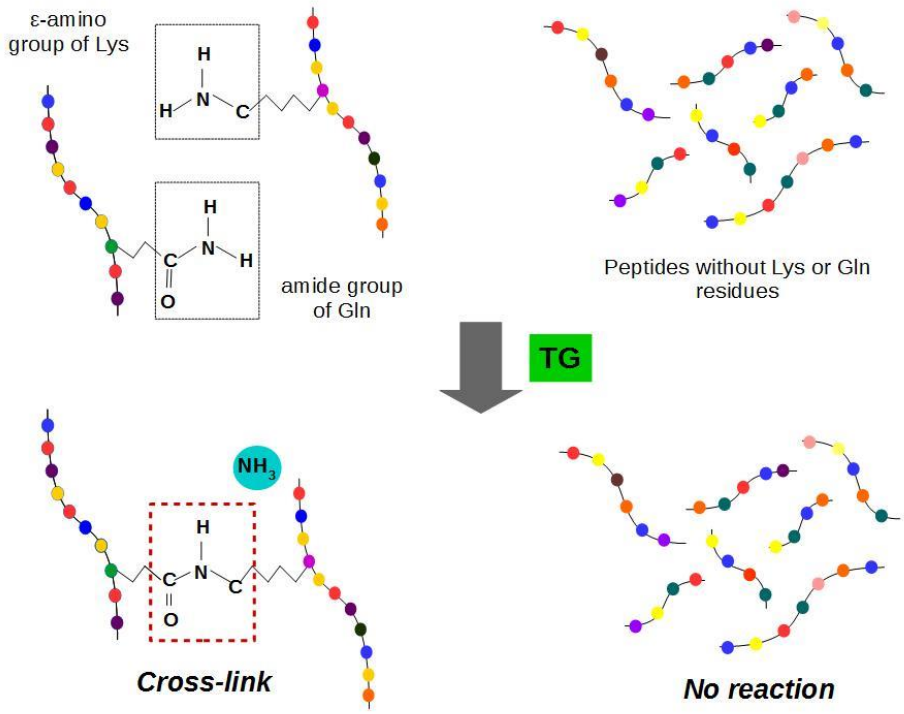

Figure 4.6: Proposed model for whey protein (WPI) peptides (< 5 kDa) modifications by the transglutaminase (TG).

4.3.3. Impact of the TG treatment on the bitter taste

The bitterness of the filtrate fractions (< 5 kDa) untreated and TG treated was evaluated by time-intensity analysis with twelve selected panelists and the average of parameters of the time-intensity curves are shown in Table 4.2. The characteristics of the time-intensity curves recorded for the bitter stimulus are represented graphically in Figure 4.7. The HF3-TG and HF7-TG samples did not differ ($p > 0.05$) from non-TG treated samples in any parameters of time-intensity curve (Table 4.2). All samples showed high intensity stimulus and prolonged time for bitterness sensation, resulting in large area of the time-

intensity curves. For all samples the stimulus to bitterness demanded a long time to be perceived by the panelists and the bitter taste remained in the mouth for a long time.

Despite some structural changes observed as a result of TG treatment, no difference in the intensity or perception of bitter taste among samples was detected, suggesting that they were not sufficient to modify the interaction between peptide and taste receptor. In order to bind to bitter taste receptors, peptides must possess two determinant sites, a bulky hydrophobic group as the binding unit and a bulky basic (including α-amino groups) or hydrophobic group as stimulating unit. [37] The vicinity of these two sites in the steric conformation of peptides play an essential role, the optimal steric distance between the two sites is estimated as 4.1 Å, with a pocket size as 15 Å, allowing the contact of the taste buds with the peptide of up to eight amino acids. [15, 39] The cross-linking reaction slightly increased the peptide MM but most peptides remained with less than 1.4 kDa, which are still capable to bind to the taste buds. Furthermore, the large amount of peptides with less than 1.4 kDa may have hindered the formation of hydrophobic interactions, since peptides with MM range of 3.0 to 6.0 kDa are required to form hydrophobic interactions among themselves. [7]

Table 4.2: Time-intensity curve parameters of the filtrates before and after treatment with transglutaminase (TG).

Samples	TIMAX	IMAX	AREA	TTOT
HF3	10.36 [a]	6.55 [a]	156.88 [a]	37.99 [a]
HF3-TG	10.88 [a]	6.61 [a]	143.17 [a]	38.45 [a]
HF7	10.73 [a]	6.28 [a]	137.91 [a]	37.46 [a]
HF7-TG	10.43 [a]	6.51 [a]	147.46 [a]	37.70 [a]

HF3 and HF7: filtrates; HF3-TG and HF7-TG: HF3 and HF7 (respectively) treated with TG.. TIMAX, time in which the maximum intensity was recorded; IMAX, maximum intensity recorded by the judge; AREA, area of the time curve × intensity; and TTOT (total duration time of the stimulus)
* Means followed by different lowercase letters in the same column are significantly different (Tukey's test, $p < 0.05$).

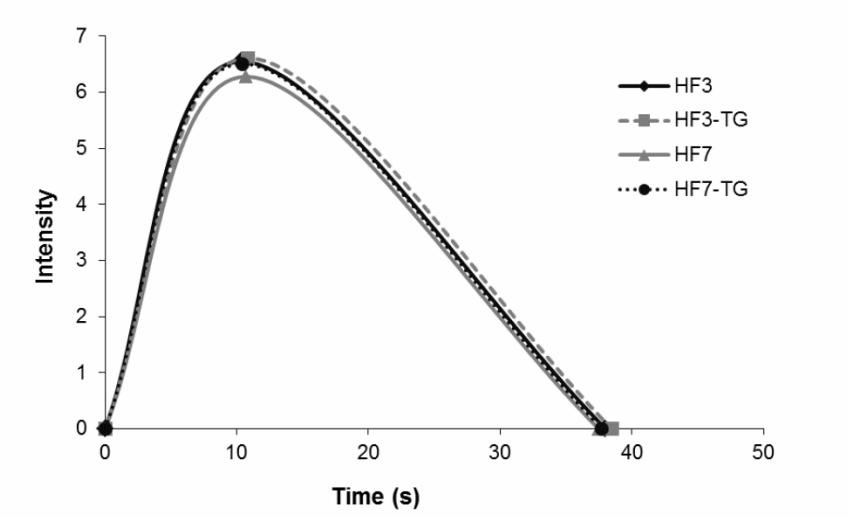

Figure 4.7: Time-intensity curve for bitter taste stimulus of filtrates before and after treatment with transglutaminase.

4.4. Conclusion

As a consequence of TG treatment of the filtrates, cross-linking catalyzed by TG occurred, leading to a slight increase of MM and changes in spatial conformation of the peptides. The differences in peptides profiles between the hydrolysates influenced TG action. No reduction in bitterness of the hydrolysates was observed as a consequence of TG treatment, possibly because the bitterness of the protein hydrolysates is associated with peptides smaller than 1000 Da and the significant amount of short peptides with no lysine or glutamine residues released by WPI hydrolysis limited the TG action.

Acknowledgements

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CAPÍTULO 5. ARTIGO 3. Sensitizing capacity of whey protein hydrolysate submitted to transglutaminase treatment in a mouse model for cow's milk allergy

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Background

Enzymatic hydrolysis is the widely used for reducing the antigenicity of milk proteins. However, linear epitopes may survive the hydrolysis process. The combination of enzymatic hydrolysis followed by treatment with transglutaminase (TG) of whey protein isolate (WPI) has been studied as way to reduce immunoreactivity of protein hydrolysates. Apart from in vitro assays, to demonstrate that modified proteins may be considered hypoallergenic, it is required that the sensitizing capacity should be tested in an animal model of allergenicity.

Objective

Our objective was to assess the impact of TG treatment of filtrates (molecular mass < 5.0 kDa) obtained from whey protein hydrolysate on the IgE-binding capacity *in vitro* and on the ability to induce sensitization in a mouse model.

Methods

The ELISA method was used to evaluate the specific IgE-binding response of the samples using serum antibodies from sensitized animals. Mice were orally sensitized with WPI or with filtrate treated or not with TG using cholera toxin as adjuvant. Whey protein specific IgE, IgG1 and IgG2a concentrations were determined after challenge with whey protein isolate.

Results

The filtrates before and after TG treatment showed no specific IgE-binding response. In contrast to whey protein isolate sensitization, no detectable level of WPI-specific IgE, IgG1, and IgG2a antibodies were observed in sensitized mice with filtrates treated or not with TG.

Conclusion

The TG treatment had no further effect on reducing sensitizing capacity of filtrates. Both filtrates did not react to specific IgE and the filtrate obtained from hydrolysate produced with 7% of protein did not promote WPI-specific IgE or IgG1 antibodies.

5.1. Introduction

The cow's milk is the leading cause of food allergy in infants and children under the age of three [1]. Cow's milk allergy corresponds to an inappropriate immune response characterized by a disruption of the Th1/Th2 balance toward a predominantly Th2-profile. The shift toward Th2 determines allergic responses, while Th1 cytokines are supposed to suppress these reactions [2]. The Th1-derived cytokine IFN-γ inhibits the proliferation of Th2 lymphocytes [3]. The Th2 effector-cells secrete cytokines IL-4 and IL-13 which induce B cell activation and class switching to IgE. Binding of IgE to the high affinity receptor FcεRI on mast cells or basophils followed by cross-linking of the IgE antibodies to food allergen elicits degranulation and the release of inflammatory mediators [4].

Cow's milk proteins hydrolysates produced by enzymatic hydrolysis followed or not by ultrafiltration are widely used in hypoallergenic formulas. These formulas are generally categorized as partial or extensively hydrolyzed based on the degree of hydrolysis and the length of the peptides [5]. However, residual antigenicity of protein hydrolysates cannot be predicted only by the MM distribution of the peptides. The American Academy of Pediatrics and the European Commission establish that the hypoallergenicity of new infant formulas needs to be assessed by preclinical testing before to be tested in trials using humans [6, 7].

Residual antigenicity of new infant formulas can be determined by measuring the binding response to serum antibodies from sensitized animals or humans using *in vitro* methods. The decrease in the ability of IgE to bind to the modified protein gives a first indication of reduced antigenicity of the formula [8, 9]. According to the America American Pediatrics besides the *in vitro* data, for safety reasons, the hypoallergenic formulas should not be able to sensitize animals [6]. During the sensitization phase, the production of allergen-specific IgE antibodies may occur upon the exposure to a food protein, once this phase is completed, the individual is primed to react to the allergen [4].

Enzymatic hydrolysis persists as the most commonly used method for destroying epitopes and producing hypoallergenic formulas [10]. However, the effectiveness of hydrolysis in reducing protein antigenicity depends on the enzyme specificity and reaction conditions [11]. Studies have been reported residual antigenicity in commercial hydrolyzed proteins-based formulas due to the presence of intact epitopes which are not disrupted by hydrolysis process [12-15]. Transglutaminase (TG) has been considering a promising strategy to modify protein antigenicity [16-18]. TG catalyzes acyl transfer reactions between glutamine residues and an primary amine, including ε-amine group of lysine residue in proteins/peptides [10, 19], which can change both conformational and linear epitopes and hence may modify antigenic response of proteins [20].

Studies have showed that the post-hydrolysis treatment with TG significantly decreased or did not have significant effect on antigenic response of the hydrolysates [20-24]. In the present study, whey protein isolate (WPI) was hydrolyzed using Alcalase, ultrafiltered, and filtrates (< 5.0 kDa fractions) were treated with TG. The impact of the combinations of these treatments on IgE-binding capacity and ability to induce sensitization were evaluated. The sensitizing capacity was assessed by an *in vivo* mouse model using oral sensitization in order to consider digestion process and gastrointestinal barriers, which mimics the human route [5].

5.2. Material and methods

5.2.1. Materials

WPI PROVON® (Glanbia Nutritionals, Kilkenny, Ireland) was obtained from a local market. The total nitrogen content of WPI was determined by micro-Kjeldahl method (Horwitz, 2006) and the protein content, 90.4% ± 1.7, was calculated using 6.38 as a conversion factor. The proteins α-lactalbumin (α-La; Type III, L6010), β-lactoglobulin (β-Lg; L3908) and bovine serum albumin (BSA; A2153) from bovine milk, and Alcalase (EC 3.4.21.62; protease from *B. licheniformis*) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). A commercial preparation of TG (Activa®), consisting of a mixture containing 99% maltodextrin and 1% TG, was donated by Ajinomoto Co. (São Paulo, SP, Brazil).

The antibodies used were: mouse IgE Balb/c isotype control (Abcam®, Cambridge, MA, USA), purified rat anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA, USA), HRP-conjugated goat anti-Rat IgG whole molecule (Sigma Chemical Co., St. Louis, MO, USA), purified mouse IgG1 κ isotype control and purified mouse IgG2a κ isotype control (BD Biosciences, San Diego, CA, USA), goat anti-mouse IgG1 heavy chain (HRP) and goat anti-mouse IgG2a heavy chain (HRP) (Abcam®, Cambridge, MA, USA).

5.2.2. Preparation of whey protein isolate hydrolysates

The WPI hydrolysates with Alcalase were obtained under the conditions established by Carvalho, Pessato, Fernandes, Zollner and Netto [25]. Briefly, WPI was diluted to obtain 3 or 7% (w/v) of protein and hydrolysis with Alcalase was performed using 100 U g^{-1} of protein. The enzymatic activity of Alcalase was 8.96 U mg^{-1}, determined according to Emi, Myers and Iacobucci [26]. One unit (U) of enzyme activity corresponds to the amount of enzyme that produces 10% TCA (trichloroacetic acid) soluble peptide equivalent to 1 µg of tyrosine in 1 min. The hydrolysis reactions were carried out for 180 min at 60 °C and pH was initially

adjusted to 8.5 using 1 mol L^{-1} Na_2CO_3. After 180 min, the reaction was stopped by heating at 90 °C for 10 min. The hydrolysates were ultrafiltered according to using an ultrafiltration system Pellicon® (Millipore, Bedford, MA, USA) and a membrane with cutoff of 5 kDa (Cartridge Prep/Scale: – TFF 6 ft2) to obtain filtrate (MM < 5 kDa). For ultrafiltration, the hydrolysate obtained with 7% of protein was diluted to half of its concentration and the hydrolysate obtained with 3% of protein was not diluted. Six diafiltration cycles were done, with the addition of water at constant volume. The filtrates were named as HF3 and HF7 according to the substrate concentration of the hydrolysate. The filtrates were frozen and freeze-dried.

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5.2.3. Post-hydrolysis TG treatment

The filtrates were treated with TG according to the conditions established by Villas-Boas, Vieira, Trevizan, de Lima Zollner and Netto [16]. Briefly, the hydrolysates were rehydrated to obtain 7% of protein and 10 U $g^{-1}$of protein TG were added. The enzymatic activity of TG was 43.6 U mg^{-1} measured according to Folk and Cole [27]. One unit of TG activity corresponds to the amount of enzyme that catalyzes the formation of 1 µmol min^{-1} of hydroxamic acid. The reactions were carried out at pH 8.0 for 180 min at 50 °C. After 180 min, the reaction mixture was cooled down in an ice bath, frozen and freeze-dried. The TG-treated samples were indicated by HF3-TG and HF7-TG.

5.2.4. Protein determination

The total nitrogen content of the samples was determined by micro-Kjeldahl method, and the protein content was calculated using 6.38 as conversion factor [28].

5.2.5. Mice

Four-week-old specific pathogen-free female BALB/c and C3H/HePas mice were obtained from the Multidisciplinary Centre for Biological Research, University of Campinas (Cemib-Unicamp), and maintained on a cow milk-free diet *ad libitum* (Nuvilab®, Curitiba, Brazil) and kept under specific conditions with controlled light, temperature and humidity in the Laboratory of Translational Immunology (LTI) facilities. The experiment was approved by the Ethics Committee on Animal Experiments of University of Campinas protocols numbers 3295-1 and 4285-1 (Campinas, SP, Brazil).

5.2.6. IgE-binding capacity by ELISA

The IgE-binding capacity of the filtrates before and after TG treatment was evaluated by indirect ELISA using sera from sensitized mice with α-La, β-Lg and BSA. On day 1, BALB/c mice were sensitized by intraperitoneal injection with 50 mg of protein (α-La, β-Lg or BSA) adsorbed in a 10% of alum, in a final volume 0.2 mL per animal [16]. A booster subcutaneous injection was given on days 14 and 21 with 20 mg adsorbed in a 10% of alum and 50 mg of protein per mouse, respectively, in a final volume 0.2 mL per animal. One week after the last sensitization, the animals were sacrificed by intraperitoneal anesthesia comprising 150 mg kg^{-1} ketamine hydrochloride and 10 mg kg^{-1} xylazine hydrochloride (both from Vetbrands, Paulínia, Brazil) and peripheral blood was collected by cardiac puncture for serum separation.

Concentration of specific IgE was determined in sera by ELISA as described previously [25]. In short, high-binding polystyrene microtiter plates (Corning, Cambridge, MA, USA) were coated with WPI, native BSA, α-La and β-Lg, or hydrolysates overnight at 4 °C. Plates were washed with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS-T) after each incubation step, using a microplate washer (BioTek ELx50, Thermo Scientific, Waltham, MA, USA). Residual free binding sites were blocked for 2 h at 37 °C. Serum samples (1:500) were applied and incubated overnight at 4 °C. The plates were incubated with purified rat anti-mouse IgE monoclonal antibody (1:1500) for 2 h at 37 °C, followed by incubation with HRP-conjugated goat anti-rat IgG whole molecule (1:20,000) for 2 h at 37 °C. TMB (3,30,5,50 -tetramethylbenzidine) was added to the plates (100 mL per well) and incubated for 30 min. The reaction was stopped using H_2SO_4 (0.18 mol L^{-1}). Absorbance was measured at 450 nm using an automated spectrophotometer reader (Spectra Max 190, Molecular Devices, Toronto, ON, Canada).

5.2.7. Residual sensitizing capacity of C3H/HePas mice

To investigate the residual sensitizing capacity of WPI, HF7 and HF7-TG samples, C3H/HePas mice were orally sensitized with a blunt needle on day 0 with 1.0 mg/g, on day 7 with 0.5 mg /g and on day 14 and 21 with 0.25 mg/g of sample dispersed in PBS (phosphate buffered saline) mixed with 0.3 µg/g cholera toxin (Sigma-Aldrich®, St. Louis, MO, USA) as an adjuvant. Non-sensitized mice received cholera toxin in PBS only. One week after the last oral sensitization, mice were challenged orally with 35 mg of WPI and 30 min later blood samples were collected. Blood samples were centrifuged and sera were stored at -20 °C.

5.2.8. Measurement of WPI-specific serum IgE, IgG1 and IgG2a

WPI-specific serum IgE was measured by indirect ELISA as described above. WPI-specific serum IgG1 and IgG2a were measured by direct ELISA. In brief, high-binding polystyrene microtitre plates (Corning, Cambridge, MA, USA) were coated with WPI overnight at 4 °C. Plates were washed with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS-T) after each incubation step, using a microplate washer (BioTek ELx50, Thermo Scientific, Waltham, MA, USA). Residual free binding sites were blocked for 2 h at 37 °C. For IgG1 ELISA, serum samples (1:1000) were applied and incubated for 2 h at 37 °C. The plates were incubated with goat anti-mouse IgG1 heavy chain (HRP) (1:400,000) for 1 h at 37 °C, followed by incubation with TMB for 15 min. For IgG2a ELISA, serum samples (1:500) were applied and incubated for 2 h at 37 °C. The plates were incubated with goat anti-mouse IgG2a heavy chain (HRP) (1:500,000) for 1 h at 37 °C, followed by incubation with TMB for 15 min. The reaction was stopped using H_2SO_4 (0.18 mol L^{-1}). Absorbance was measured at 450 nm using an automated spectrophotometer reader.

5.3. Results and discussion

5.3.1. IgE-binding capacity of the filtrates before and after TG treatment

In a previous study, we found residual antigenic activity in WPI hydrolysates obtained with Alcalase under uncontrolled pH condition which was related to intact BSA and/or antigenic peptides [25]. In the present study, the hydrolysates were ultrafiltered using a 5.0 kDa cutoff membrane, in order to separate unhydrolysed and partially hydrolyzed BSA. Although low MM peptides are less antigenic and usually used in hypoallergenic formulas, whey peptides of 3000 Da are still capable to elicit an immunological response [29]. Thus, the filtrates were treated with TG.

The ELISA method was used to evaluate the impact of the changes induced by TG on specific IgE-binding response of the filtrates (Figure 5.1). The specific IgE response was significantly higher in groups sensitized with intact whey proteins (α-La, β-Lg, or BSA) than in control group (sensitized with alum without proteins, non-sens). The sera from α-La-, β-Lg- and BSA-sensitized mice had a high reactivity to their respective antigenic proteins and no cross-reactivity with other proteins was detected (data not shown), indicating that sensitization protocol was effective to elicit specific IgE antibodies.

The samples HF3 and HF7 - before and after TG treatment - showed no specific IgE-binding response, with values of anti-α-La, anti-β-Lg and anti-BSA IgE below the ELISA detection limit (< 0.025 μg mL^{-1}). The decrease in IgE-binding capacity suggests that epitopes of all proteins tested were cleaved during hydrolysis with Alcalase and/or were

excluded by the ultrafiltration, TG-catalyzed reactions had no further impact on specific IgE-binding of the samples. Similar result was obtained for β-Lg hydrolysates obtained with Alcalase [21, 22]. However, β-Lg hydrolyzed with Alcalase showed an increment in the response to specific IgE after *in vitro* gastric digestion whereas the immunoreactivity of TG-treated β-Lg hydrolysate remained almost null [22], suggesting that the combination of hydrolysis with Alcalase followed by TG treatment might have implications in sensitizing capacity and in eliciting allergy response. Conformational epitopes collapse during hydrolysis, while linear epitopes may survive the process and be masked by formation of peptides aggregates, which can be exposed by digestion process.

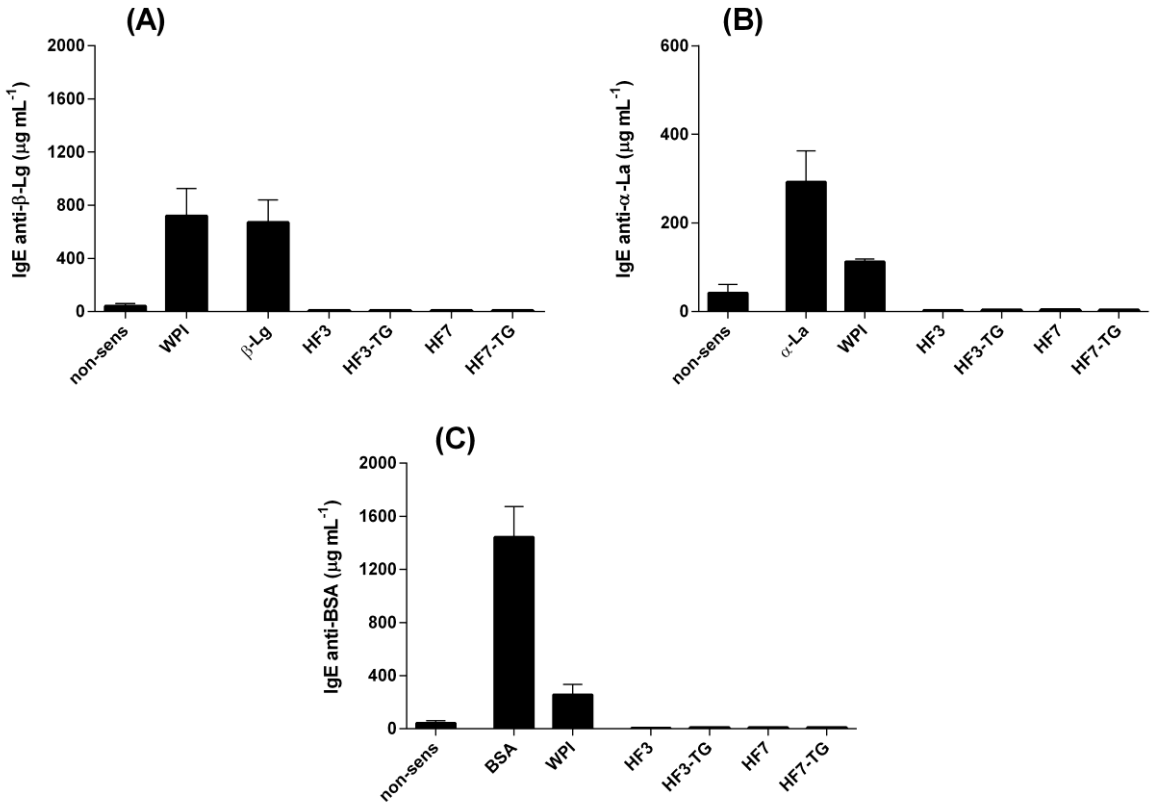

Figure 5.1: IgE-binding capacity of the filtrates before (HF3 and HF7) and after treatment with transglutaminase (TG) (HF3-TG and HF7-TG) determined by ELISA assays using sera from mice sensitized to: (A) β-Lg; (B) α-La; and (C) BSA. The mice sensitized with alum without protein were named as non-sens (A, B, and C). Data were expressed as mean ± SD of five mice per group.

5.3.2. Impact of TG treatment on residual sensitizing capacity

Since there was no difference in IgE-binding capacity among the filtrates treated or not with TG, the HF7 and HF7-TG samples were selected because the HF7 sample seems to have undergone more changes after TG treatment in previous study [30]. Therefore the

sensitizing capacity of HF7 before and after TG treatment was assessed by an oral sensitization in order to evaluate the influence of digestion process and gastrointestinal barriers involved in sensitizing phase of allergy.

Mouse model of orally induced CMA has been used successfully to study the sensitizing capacity of whey protein hydrolysates [5, 9]. In mice, the Th2 response induces the IgE and IgG1 production, while Th1 response leads to the IgG2a production [31]. Thus, IgG2a and IgG1 immunoglobulin isotypes as markers for Th1 and Th2 lymphocytes, respectively, were investigated in mice exposed to WPI, HF7 or HF7-TG samples. The WPI-specific IgE and IgG1 levels were strongly enhanced in mice sensitized with WPI compared to mice sensitized only with cholera toxin (non-sens), whereas sensitization with WPI did not induce WPI-specific IgG2a response in three mice and resulted in low IgG2a response in one mouse (Figure 5.2). These results show a response essentially Th2-profile, confirming that the mouse model was adequate to assess the sensitizing capacity of whey proteins.

In contrast to sensitization with WPI, sensitization with HF7 or HF7-TG did not induce any detectable level of WPI-specific IgE, IgG1, and IgG2a antibodies, which indicate that the digestion did not release any antigenic peptides capable to stimulate the production of WPI-specific antibodies. Administration of HF7 and HF7-TG plus cholera toxin did not lead to production of WPI-specific antibodies. Therefore we can consider that these samples are complying with the recommendations of American Academy Pediatrics and European Guidelines which establish that hypoallergenic formulas must prevent potential sensitization in animals. No significant difference was observed between HF7 and HF7-TG sensitized mice (Figura 5.2), indicating that the decrease in sensitizing capacity of WPI peptides lower 5.0 kDa seems to be independent of the TG treatment.

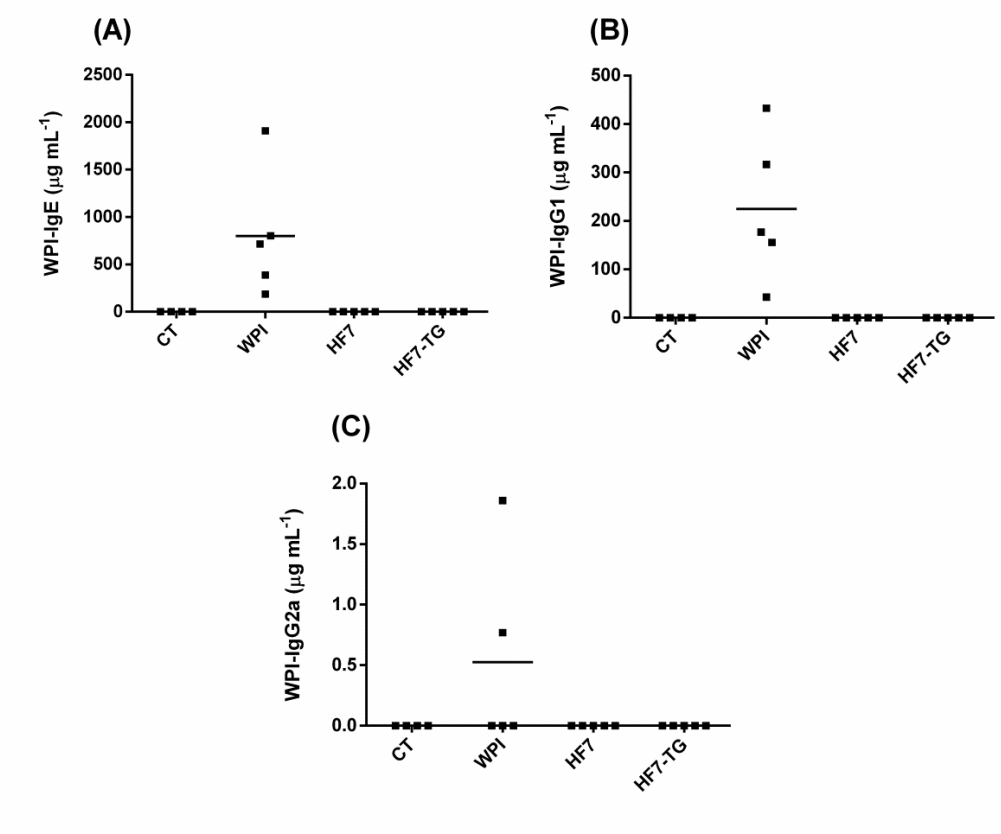

Figure 5.2: Levels of WPI-specific IgE (A), IgG1 (B), and IgG2a (C) in sera from mice sensitized with cholera toxin (CT), WPI, filtrate before (HF7) or after TG treatment (HF7-TG). Data are expressed as individual values.

5.4. Conclusion

The TG treatment had no effect on reducing the antigenicity of the filtrates (< 5. kDa) from WPI hydrolysates with Alcalase. The results from orally sensitizing mouse model showed that the digestion of the samples - TG-treated or not - did not release any additional epitope. Both filtrates studied showed no reaction toward specific IgE and the HF7 filtrate did not generate WPI-specific IgE or IgG1 antibodies in mice, therefore showing reduced possibility to elicit allergenic response. Therefore the WPI hydrolysis with Alcalase followed by ultrafiltration is a potential alternative for obtaining of hypoallergenic food products.

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CAPÍTULO 6. DISCUSSÃO GERAL

6.1. Discussão Geral

A hidrólise enzimática ainda é o método mais utilizado para a destruição de epítopos nas proteínas e obtenção de fórmulas hipoalergênicas, especialmente fórmulas infantis direcionadas para crianças alérgicas às proteínas do leite de vaca (AGYARE e DAMODARAN, 2010). No entanto, essas fórmulas podem ainda apresentar antigenicidade residual (PUERTA, DIEZ-MASA e DE FRUTOS, 2006; BRAHIM *et al.*, 2012) e possuem forte gosto amargo (NEWMAN *et al.*, 2015). A combinação da hidrólise enzimática seguida pelo tratamento com TG mostrou-se como uma estratégia promissora para hipoalergênicos redução da antigenicidade das proteínas do soro de leite (WRÓBLEWSKA *et al.*, 2008) e diminuir o gosto amargo de hidrolisados proteicos (BABIKER *et al.*, 1996).

Dessa forma, este trabalho teve como objetivos estudar as alterações no IPS provocadas pela combinação da hidrólise com Alcalase e tratamento com TG e o impacto no gosto amargo dos hidrolisados e na antigenicidade das proteínas. Seguindo os critérios definidos pela *American Academy of Pediatrics* (AAP, 2000), para a avaliação da hipoalergenicidade de fórmulas, a antigenicidade das amostras foi avaliada pela medida da sua capacidade de ligação a anticorpos IgE específicos obtidos do soro de animais imunizados e capacidade de induzir a sensibilização em modelos animais de alergia.

Em estudos anteriores, verificamos que a hidrólise com Alcalase foi capaz de reduzir significativamente a antigenicidade da β-Lg *in vitro*. Todavia, esse resultado foi obtido pela hidrólise desta proteína sob condições de pH controlado (SABADIN *et al.*, 2012; VILLAS-BOAS *et al.*, 2015). Do ponto de vista industrial, hidrólise sem controle de pH é mais viável, uma vez que simplifica o processo, diminui os riscos de contaminação e minimiza a adição de sais ao produto (LE MAUX *et al.*, 2016), aspectos relevantes especialmente na produção de alimentos voltados para o público infantil. Dessa forma, neste trabalho optamos por realizar as reações de hidrólise sem o controle de pH a fim de simular a condição empregada na indústria. Antes disso, no entanto, o impacto dos processos de hidrólise enzimática conduzidos com e sem controle do pH na antigenicidade dos hidrolisados foi investigado.

Os hidrolisados foram obtidos a partir da hidrólise do IPS com Alcalase sob condições de pH controlado e não-controlado, utilizando duas concentrações de substrato ($S_{\%}$), 3 e 7% de proteína, e duas relações de enzima:substrato (E/S), 50 e 100 U g^{-1}. Nas hidrólises sem controle, o pH diminuiu de 8,5 para 7,0 nos primeiros 15 minutos da reação e permaneceu constante até o final do processo, devido à formação de um sistema tampão no

meio reacional (SOUSA JR *et al.*, 2004). Apesar da diferença de pH nas hidrólises sem controle de pH, não houve diferença significativa no grau de hidrólise (GH) entre os hidrolisados obtidos com as mesmas $S_{\%}$ e E/S (Tabela 3.2), possivelmente porque o pH manteve-se na faixa de atividade da Alcalase (YUST *et al.*, 2010).

O GH dos hidrolisados, no entanto, foi significativamente afetado pela $S_{\%}$ e E/S (Tabela 3.2). O GH aumentou com o aumento da E/S, entretanto esse aumento não foi proporcional à quantidade de enzima adicionada a mais (Tabela 3.3) devido à exaustão dos sítios de clivagem no substrato (ISHIBASHI *et al.*, 1988b). O aumento da $S_{\%}$, por sua vez, resultou na diminuição no GH dos hidrolisados (Tabela 3.3). O aumento da $S_{\%}$ levou a um deslocamento de maior quantidade de água para hidratação das proteínas e peptídeos o que, consequentemente, diminuiu a quantidade de água livre disponível no sistema para o desenvolvimento da reação enzimática (HARDT, VAN DER GOOT e BOOM, 2013; BUTRÉ *et al.*, 2014; HARDT *et al.*, 2014).

Aos 15 minutos de hidrólise, os hidrolisados obtidos com e sem controle de pH e iguais $S_{\%}$ e E/S apresentaram perfil cromatográfico (Figura 3.3 B-E) e de distribuição de massa molecular (MM) dos peptídeos (Figura 3.2 C) muito similares. Entretanto, aos 180 minutos de hidrólise, foram observadas diferenças entre esses hidrolisados. Os hidrolisados obtidos sem controle de pH apresentaram peptídeos de maior MM que aqueles obtidos com controle de pH e iguais $S_{\%}$ e E/S (Figura 3.2 D). Houve também diferença no perfil cromatográfico entre os hidrolisados obtidos com e sem controle de pH, especialmente nas regiões de média e alta hidrofilicidade (Figura 3.3 F-I). Esses resultados sugerem a ocorrência de mudanças no padrão de hidrólise da Alcalase após os 15 minutos de hidrólise quando o pH estabilizou em 7,0.

A alteração no padrão de hidrólise pode ser explicada pela queda no pH de 8,5 para 7,0, a qual pode ter influenciado a seletividade da Alcalase devido às mudanças no estado de ionização dos resíduos de His que compõe o sítio de clivagem da enzima e dos resíduos de His e Cys localizados nos subsítios do substrato (VOROB'EV *et al.*, 2000; BUTRÉ *et al.*, 2015). O aumento na proporção de resíduos de His carregados positivamente e a redução na proporção de resíduos de Cys desprotonados podem ter afetado a interação entre a Alcalase e os sítios de clivagem próximos a esses resíduos de aminoácidos.

Também foram observadas diferenças nos perfis cromatográficos (Figura 3.3) e de distribuição de MM dos peptídeos (Figura 3.2) entre os hidrolisados obtidos com diferentes $S_{\%}$, indicando que a concentração de substrato pode ter influenciado no padrão de hidrólise da Alcalase. Alterações na seletividade da enzima em função da $S_{\%}$ podem estar associadas a diferenças na acessibilidade da enzima ao substrato devido à agregação dos

peptídeos (BUTRÉ *et al.*, 2014) e à quantidade de água livre disponível no sistema (BUTRÉ, WIERENGA e GRUPPEN, 2014).

As diferenças no perfil de peptídeos e na distribuição de MM entre os hidrolisados refletiram no comportamento de agregação e conformação espacial dos peptídeos (Figura 3.4), e nas concentrações de β-Lg e α-La detectadas pelos kits comerciais de ELISA (Figura 3.5). De maneira geral, os hidrolisados obtidos sem controle de pH apresentaram maiores concentrações de β-Lg ($p<0,05$) que aqueles obtidos com controle de pH. Os hidrolisados obtidos com 7% de proteína exibiram as maiores concentrações de β-Lg ($p<0,05$) em ambas as condições de pH. Nossos resultados mostraram que a concentração de alérgenos determinada por kits comerciais necessariamente não prediz a resposta de IgE e IgG, uma vez que a resposta de ligação de IgE e IgG anti- α-La e anti- β-Lg aos hidrolisados não diferiu entre as condições de pH e $S_{\%}$ (Figura 3.6).

Os resultados de ELISA e imunoblot (Figura 3.6) indicaram que, nas condições estudadas, a hidrólise com Alcalase é capaz de reduzir significativamente a capacidade da α-La e β-Lg de se ligarem às IgE e IgG específicas, independente da condição de pH e $S_{\%}$. No entanto, a hidrólise conduzida sem controle de pH aumentou a resposta dos anticorpos anti-BSA frente aos hidrolisados em comparação ao IPS, evidenciando que a hidrólise sem controle, além de não ter hidrolisado totalmente a BSA (Figura 3.2 B), possivelmente expôs epítopos ocultos na estrutura desta proteína.

Uma vez que nos hidrolisados obtidos sem controle de pH ainda foi detectada BSA intacta, os hidrolisados foram ultrafiltrados usando membrana de corte de 5,0 kDa para a separar a BSA não hidrolisada e obter IPS extensivamente hidrolisado com peptídeos < 5,0 kDa. Os filtrados foram então tratados com TG.

Os filtrados obtidos com 3 e 7% de proteína ($S_{\%}$) foram compostos por cerca de 83 a 84% de peptídeos menores que 1,4 kDa e de 16 a 17% peptídeos com MM entre 1,4 e 3,5 kDa (Tabela 4.1). Após o tratamento com TG, observamos aumento na porcentagem de peptídeos com MM entre 1,4 e 3,5 kDa de 1,5% no filtrado obtido com 3% $S_{\%}$ e de 3,0% no filtrado obtido com 7%. Também foram observadas pequenas alterações na estrutura dos peptídeos, como indicado pelo espectro de fluorescência (Figura 4.3), e no perfil de peptídeos determinado por CLAE-FR (Figura 4.2), sugerindo que os peptídeos sofreram algumas alterações devido ao tratamento com TG. Os resultados do MALDI-MS exibiram mudanças no espectro de massas após o tratamento com TG, especialmente nos peptídeos com *m/z* entre 1600 e 3000. Possivelmente, a presença de significativas quantidades de peptídeos pequenos sem resíduos de glutamina e lisina, os quais são incapazes de formar

ligações cruzadas, limitou a ação da TG e, consequentemente, as alterações provocadas nos filtrados.

Embora o tratamento com TG tenha provocado alterações na estrutura e tamanho dos peptídeos, essas mudanças não foram suficiente para reduzir a intensidade do gosto amargo ou o residual amargo dos filtrados (Tabela 4.3 e Figura 4.5). Apesar do aumento da MM dos peptídeos observado após tratamento com TG, a maioria dos peptídeos permaneceram menores que 1,4 kDa, tamanho suficiente para permitir a ligação aos receptores de gosto amargo (ISHIBASHI *et al*., 1988a). Além disso, possivelmente, a grande quantidade de peptídeos menores que 1,4 kDa impediu a formação de interações capazes de bloquear grupos hidrofóbicos, uma vez que são necessários peptídeos com MM a partir de 3,0 kDa para formar interações hidrofóbicas entre eles (CHEISON, WANG e XU, 2007).

Os filtrados antes e após o tratamento com TG foram avaliadas por ELISA quanto à resposta de IgE específicas (anti-α-La, anti-β-Lg e anti-BSA). As respostas de IgE anti-α-La, anti-β-Lg e anti-BSA foram praticamente nulas frente aos filtrados tratados ou não com TG, apresentando valores de IgE específicas abaixo do limite de detecção do ELISA (< 0,025 ug mL^{-1}) (Figura 5.1). A redução na capacidade de ligação à IgE parece ter sido independente do tratamento com TG. Esse resultado corrobora com os estudos anteriores nos quais o tratamento de hidrolisados de β-Lg com TG não teve efeito adicional na redução da capacidade desses hidrolisados de se ligarem às IgE anti-β-Lg (SABADIN *et al*., 2012; VILLAS-BOAS *et al*., 2015). No entanto, após a digestão gástrica *in vitro*, a resposta antigênica da β-Lg hidrolisada foi maior que a da hidrolisada tratada com TG, sugerindo que a TG atenuou a capacidade de ligação às IgE específicas (VILLAS-BOAS *et al*., 2015). Esse resultado nos sugeriu que a combinação de hidrólise e tratamento com TG poderia ter implicações na capacidade de sensibilização e de indução de resposta alérgica *in vivo*.

Dessa forma, a capacidade de sensibilização de HF7 e HF7-TG foi avaliada em modelo animal utilizando um protocolo de sensibilização por via oral, a fim de avaliar o impacto do processo de digestão e das barreiras naturais do trato gastrointestinal envolvidas na fase de sensibilização da alergia, aproximando-se assim da realidade em humanos (VAN ESCH *et al*., 2011).

Os níveis de IgE e IgG1 específicos (anti-IPS) foram elevados nos camundongos sensibilizados com IPS comparado ao grupo não-sensibilizado (tratado apenas com adjuvante), enquanto a sensibilização com IPS resultou em baixa ou nenhuma indução da produção de IgG2a anti-IPS (Figura 5.2). Esse resultado mostra uma resposta essencialmente de perfil Th2 (T *helper* 2), indicando que esse modelo foi adequado para o estudo da resposta alérgica às proteínas do soro de leite. Em contraste com animais

sensibilizados com IPS, a sensibilização com HF7 ou HF7-TG não induziu níveis detectáveis de IgE, IgG1 e IgG2a específicos (Figura 5.2), mostrando que a digestão não liberou nenhum epítopo capaz de induzir a produção de anticorpos anti-IPS em ambas as amostras. Portanto, tratamento com TG não teve efeito adicional na redução da capacidade de sensibilização do filtrado.

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CAPÍTULO 7. CONCLUSÃO GERAL

A concentração de substrato e a relação de enzima tiveram um impacto significativo no GH do IPS, portanto, esses parâmetros são fatores importantes a serem considerados na obtenção de hidrolisados com finalidades específicas, como redução a antigenicidade de proteínas. A condição de pH também é um parâmetro importante nas reações de hidrólise, uma vez que alterou o padrão de hidrólise da Alcalase, resultando em hidrolisados com distintas características físico-químicas. A queda do pH durante a hidrólise realizada sem controle provocou mudanças no estado de ionização da enzima e substrato, afetando a interação entre eles. Apesar da diferença no padrão de hidrólise, ambas as condições de pH, constante e sem controle, foram efetivas na redução da antigenicidade da α-La e β-Lg. Entretanto, a hidrólise sem controle de pH aumentou a antigenicidade da BSA, pois, nesta condição de pH, a reação resultou na exposição e/ou liberação de epítopos desta proteína.

O tratamento dos filtrados com TG levou à formação de ligações cruzadas que resultou em discreto aumento na MM dos peptídeos bem como em alterações na conformação espacial e perfil de hidrofilicidade. Apesar das alterações nas características dos filtrados, o tratamento com TG não diminuiu a intensidade do gosto amargo. A ação da TG foi limitada pela presença de grande quantidade de peptídeos de baixa MM (< 1,4 kDa) sem resíduos de Glu e Lys.

O tratamento com TG não teve benefício adicional na redução da antigenicidade dos filtrados. A resposta das IgE específicas foi praticamente nula frente ambos os filtrados, tratados ou não com TG. O filtrado obtido com 7% $S_{\%}$, antes ou após o tratamento com TG, não induziu a produção de IgE e IgG1 especificas em camundongos, demonstrando sua incapacidade de sensibilizar o sistema imune desses animais. A hidrólise do IPS com Alcalase seguida por ultrafiltração constitui em uma combinação de tratamentos em potencial para obtenção de alimentos hipoalergênicos, de acordo com os critérios de avaliação definidos pela *American Academy of Pediactrics*.

Embora as hipóteses desse trabalho, de que a hidrólise do IPS seguida pelo tratamento com TG diminui o gosto amargo e é mais eficaz na redução da antigenicidade do IPS, não tenham sido confirmadas, a combinação desses tratamentos enzimáticos não devem ser descartados. A combinação de hidrólise enzimática e polimerização com TG tem potencial para melhorar as propriedades tecnofuncionais (capacidade emulsificante e espumante, gelificação) de proteínas. Além disso, a aplicação da TG na redução do gosto amargo de hidrolisados pode ser uma alternativa viável quando associada a proteínas parcialmente hidrolisadas.

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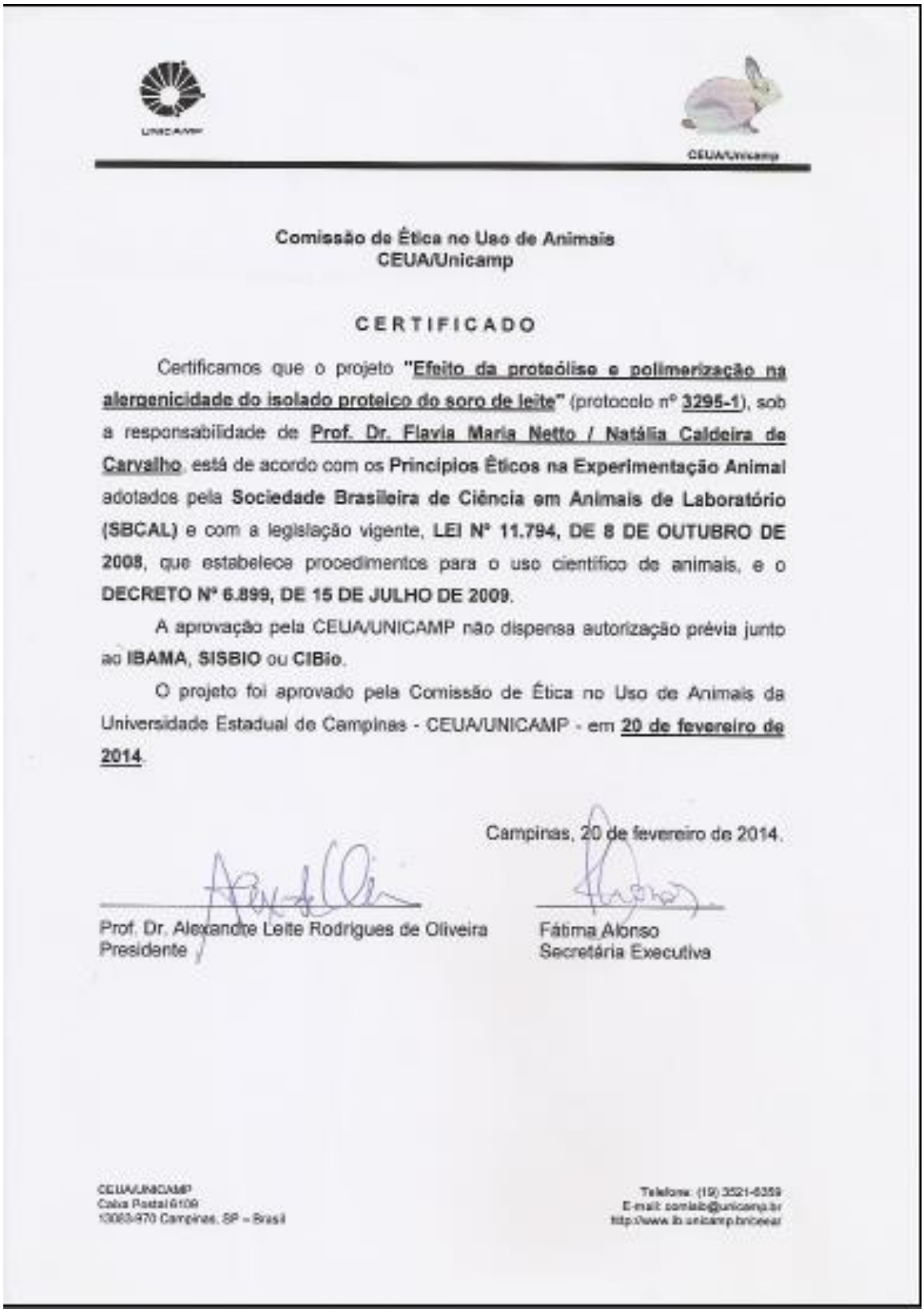
ANEXO 1. Posição da editora sobre a inclusão do Artigo 1 na tese

ANEXO 2. Certificado de aprovação CEUA (Comissão de Ética para Uso de Animais)



UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto **"Efeito da proteólise e polimerização na alergenicidade do isolado proteico do soro de leite"** (protocolo nº **3295-1**), sob a responsabilidade de **Prof. Dr. Flavia Maria Netto / Natália Caldeira de Carvalho**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **20 de fevereiro de 2014**.

Campinas, 20 de fevereiro de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comiab@unicamp.br
http://www.ib.unicamp.br/ceua

ANEXO 3. Certificado de aprovação CEUA 2 (Comissão de Ética para Uso de Animais)

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **Efeito da proteólise e polimerização na alergenicidade de isolado proteico do soro de leite**, registrada com o nº **4285-1**, sob a responsabilidade de **Profa. Dra. Flavia Maria Netto e Natália Caldeira De Carvalho**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, tendo sido aprovada pela **Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **04 de julho de 2016**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**01/06/2016-01/06/2017**
Vigência da autorização para manipulação animal:	**01/06/2016-01/06/2017**
Espécie / linhagem/ raça:	**Camundongo isogênico / C3H/He**
No. de animais:	**40**
Peso / Idade:	**06 semanas / 15g**
Sexo:	**fêmeas**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA, SISBIO** ou **CIBio**.

Campinas, 04 de julho de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação do relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

ANEXO 4. Certificado de aprovação CEP (Comissão de Ética em Pesquisa)

FACULDADE DE CIENCIAS MEDICAS - UNICAMP (CAMPUS CAMPINAS)

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da associação de hidrólise enzimática e polimerização com transglutaminase no desenvolvimento de gosto amargo em isolado proteico do soro de leite.

Pesquisador: Natália Caldeira de Carvalho
Área Temática:
Versão: 1
CAAE: 37308414.8.0000.5404
Instituição Proponente: Faculdade de Engenharia de Alimentos
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 841.224
Data da Relatoria: 20/10/2014

Apresentação do Projeto:

A alergia alimentar é uma resposta imunológica adversa provocada por alimentos em indivíduos sensíveis, que atinge mais de 6% das crianças e 3-4% dos adultos. A alergia ao leite de vaca (ALV) é, dentre as alergias alimentares, a de maior incidência em crianças, sendo o leite responsável por 80% das alergias alimentares em crianças menores de um ano de vida. Os principais alérgenos do leite são as suas mais abundantes proteínas, as caseínas, a -lactoglobulina e -lactalbumina. O tratamento da ALV consiste na exclusão do leite e derivados da dieta. Crianças diagnosticadas com ALV podem ser alimentadas com leite materno, mas no caso de crianças alérgicas que não estão em aleitamento materno, a alternativa indicada é o uso de fórmulas hipoalergênicas na alimentação infantil. Essas fórmulas são compostas por proteínas extensamente hidrolisadas. A hidrólise enzimática das proteínas do leite é o método mais utilizados para destruição de epítopos alérgicos e redução da sua antigenicidade. Fórmulas hipoalergênicas compostas de hidrolisados proteicos, especialmente de caseína e proteínas do soro de leite, são comercializadas há mais de 50 anos. No entanto, essas fórmulas são pouco palatáveis, pois a hidrólise das proteínas pode produzir produtos com gosto amargo acentuado e hipertônico. Algumas técnicas têm sido utilizadas para redução do gosto amargo em hidrolisados proteicos; entretanto, apresentam alguns inconvenientes tais como adsorção de aminoácidos essenciais, produção excessiva de

aminoácidos livres, reversibilidade da reação e baixo rendimento. A transglutaminase (TG) é uma enzima muito utilizada na indústria para melhorar características tecno-funcionais e sensoriais de proteínas utilizadas em alimentos. Sua capacidade de introduzir ligações cruzadas em proteínas tem sido estudada para redução da antigenicidade e gosto amargo de hidrolisados proteicos. Estudos demonstraram que a hidrólise com alcalase, associada à polimerização com TG, diminuiu significativamente a antigenicidade de proteínas do soro de leite. No entanto, o efeito dessa combinação de tratamentos enzimáticos no desenvolvimento de gosto amargo ainda tem sido pouco estudado.

Objetivo da Pesquisa:

Objetivo Primário:

O projeto visa avaliar o efeito da hidrólise de isolado proteico do soro de leite (IPS) bovino com a enzima alcalase associada à polimerização com a enzima transglutaminase (TG) no desenvolvimento de gosto amargo.

Objetivos Específicos

1) Produzir hidrolisados de IPS com alcalase utilizando processo com baixo teor de sais (sem alteração de pH) e em condições sanitárias adequadas para os experimentos sensoriais.

2) Produzir polimerizados a partir desses hidrolisados utilizando TG.

3) Avaliar as condições higiênico-sanitárias dos hidrolisados e polimerizados por meio de testes microbiológicos.

4) Medir a intensidade e persistência do gosto amargo do hidrolisado e polimerizados.

Avaliação dos Riscos e Benefícios:

Riscos:

Não há riscos previsíveis.

Benefícios:

O desenvolvimento de metodologia para produção de fontes proteicas com baixo potencial alergênico e mais palatáveis que as disponíveis atualmente para formulação de produtos hipoalergênicos poderia contribuir para diminuir a rejeição das fórmulas hipoalergênicas pelos indivíduos e, assim, contribuir para a nutrição adequada das pessoas alérgicas especialmente lactentes e crianças.

FACULDADE DE CIENCIAS MEDICAS - UNICAMP (CAMPUS CAMPINAS)

Continuação do Parecer: 841.224

Comentários e Considerações sobre a Pesquisa:

Trata-se de um projeto de pesquisa relativo à Tese (Doutorado) de uma aluna matriculada ano programa de Pós Graduação, a ser desenvolvida no Departamento de Alimentos e Nutrição da Faculdade de Engenharia de Alimentos (FEA)/Unicamp, orientada por uma docente dessa Unidade. As enzimas utilizadas nos processos são GRAS (Generally Recognized As Safe). Os hidrolisados serão produzidos a partir da hidrólise do IPS com a enzima alcalase, em concentrações adequadas. A reação será conduzida em reator apropriado, sob condições sanitárias adequadas, a 60° e pH 8,5. A reação será interrompida após 180 min por aquecimento a 90°C durante 10 min para inativar a enzima. Os hidrolisados serão imediatamente congelados e, posteriormente, liofilizados. Em seguida, suspensões das amostras de hidrolisados contendo 7% (m/v) de proteína serão preparadas e polimerizadas com transglutaminase nas concentrações de 10 ou 25 U/g de proteína em pH 8,0. A reação será realizada a 50 °C por 180 min em reator, em condições higiênico-sanitárias, e será interrompida por resfriamento das amostras a 4°C. As amostras serão liofilizadas e, após, serão submetidas a análises microbiológicas para avaliar a qualidade higiênico-sanitária. O gosto amargo das amostras será avaliado por análise de tempo-intensidade no Laboratório de Análise Sensorial/FEA. As amostras serão servidas em cabines individuais, sob luz branca e temperatura ambiente, e serão apresentadas em copos plásticos descartáveis, assegurando conforto e privacidade aos provadores. Serão recrutados pelo menos 25 voluntários adultos independente de sexo, cor, classes ou grupos sociais que não sejam alérgicos à cafeína e/ou leite e não possuam aversão a gosto amargo. Os voluntários serão escolhidos para compor a equipe sensorial pelo seu poder de discriminar diferentes intensidades de gosto amargo, o qual será avaliado por meio de teste triangular e análise sequencial de Wald. Os provadores serão, então, treinados para formação de memória sensorial e padronização entre as respostas pelo contato direto dos indivíduos com as referências de intensidade moderada e máxima do estímulo gosto amargo. Soluções de cafeína serão utilizadas como referência de intensidade de gosto amargo e água como referência de nenhum gosto amargo. Os provadores serão selecionados segundo o seu poder de discriminação, repetibilidade e concordância dos provadores, verificados através dos valores de F amostra significativo (p0,30) e de F repetição não significativo (p>0,05) em relação ao parâmetro gosto amargo. As amostras de hidrolisados e polimerizados para o teste de tempo-intensidade serão diluídos em água deionizada a 2,0% (m/v) de proteína. A análise será realizada com 5 mL da amostra servidos à temperatura ambiente em copo descartável codificado com número de três dígitos e utilizando uma escala linear estruturada de 0 a 9 (0 = nenhum; 4,5 = gosto amargo moderado; 9 = gosto amargo forte). A coleta de dados para a análise de tempo-

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intensidade será realizada em computador utilizando o programa Time-Intensity Data Collection System (SCDTI desenvolvido no Laboratório de Análise Sensorial da FEA. Este programa analisará os dados coletados durante cada sessão de teste sensorial e fornecerá os parâmetros: Timax (tempo no qual a intensidade máxima foi percebida), Imax (intensidade máxima reconhecida pelo provador), área (área da curva) e Ttot (tempo total de duração do estímulo).

Critério de Inclusão:
Disponibilidade e interesse em participar de uma equipe treinada de provadores e poder de discriminação para diferentes intensidades de gosto amargo.

Critério de Exclusão:
Não poderão participar dos testes sensoriais indivíduos alérgicos à cafeína e/ou leite e que tenham aversão a alimentos com gosto amargo.

Considerações sobre os Termos de apresentação obrigatória:
Foram apresentadas a Folha de Rosto, assinada apelo Diretor da FEA, o documento com "Informações Básicas do Projeto", o Projeto e o TCLE. O questionário a ser aplicado aos participantes da pesquisa está anexado ao Projeto.

Recomendações:
Nenhuma. Porém, é preciso mencionar que não se pode medir a intensidade e persistência do gosto amargo do hidrolisado e polimerizados, como consta dos Objetivos Específicos, mas sim avaliá-las.

Conclusões ou Pendências e Lista de Inadequações:
Nenhuma.

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:
- O sujeito de pesquisa deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado.

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- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado.

- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado. Se o pesquisador considerar a descontinuação do estudo, esta deve ser justificada e somente ser realizada após análise das razões da descontinuidade pelo CEP que o aprovou. O pesquisador deve aguardar o parecer do CEP quanto à descontinuação, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de uma estratégia diagnóstica ou terapêutica oferecida a um dos grupos da pesquisa, isto é, somente em caso de necessidade de ação imediata com intuito de proteger os participantes.

- O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo. É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

- Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projetos do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma, junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial.

- Relatórios parciais e final devem ser apresentados ao CEP, inicialmente seis meses após a data deste parecer de aprovação e ao término do estudo.

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FACULDADE DE CIENCIAS MEDICAS - UNICAMP (CAMPUS CAMPINAS)

Continuação do Parecer: 841.224

CAMPINAS, 22 de Outubro de 2014

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador)

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