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JOÃO PEDRO BRASIL DE OLIVEIRA

USO DE PEPTIDASES PARA PRODUÇÃO DE HIDROLISADO HIPOALERGÊNICO DO LEITE BOVINO

FORTALEZA

2018

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Dissertação apresentada ao programa de Pós-graduação em Bioquímica da Universidade Federal do Ceará, como parte dos requisitos para obtenção de título de Mestre em Bioquímica

Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas

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À Deus

À minha família

E a todos que contribuíram com esse trabalho

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RESUMO

A alergia ao leite bovino é um problema de saúde global que afeta principalmente os lactentes. Embora a maioria desses casos sejam transitórios, estes devem ser tratados para evitar problemas no desenvolvimento da criança. Assim, as fórmulas hidrolisadas se destacam por ser a alternativa mais viável para substituir o consumo do leite bovino. Nesse contexto, este estudo teve como objetivo estudar peptidases de origem vegetal (plantas laticíferas) e animal (intestino de insetos) com habilidade de hidrolisar as proteínas do leite bovino visando a produção de hidrolisados hipoalergênicos. A hidrólise das proteínas do leite foi realizada variando os seguintes parâmetros: proporção enzima:substrato, pH e tempo de incubação. Em seguida, os hidrolisados foram caracterizados de acordo com o grau de hidrólise e a antigenicidade residual foi determinada. As peptidases serínicas purificadas do intestino médio de *Danaus plexippus* (DpGp) foram capazes de hidrolisar as caseínas na proporção 1:25 (enzima:substrato), após 4 horas de incubação a pH 9,0. Utilizando os mesmos parâmetros, a hidrólise das proteínas do lactosoro só foi observada após 24 horas de incubação. Contudo, a hidrólise do lactosoro pré-aquecido (85 °C por 30 min) resultou em uma diminuição no tempo de reação e redução da antigenicidade. Os ensaios imunoenzimáticos mostraram que o leite hidrolisado por DpGp apresentou antigenicidade residual semelhante às formulas parcialmente hidrolisadas vendidas comercialmente. Em relação as plantas laticíferas, somente as peptidases do látex de *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP) e *Carica papaya* (CapLP) foram capazes de hidrolisar as caseínas na proporção 1:225 (enzima:substrato) após 30 min de incubação a pH 6,5. Apenas CapLP hidrolisou de forma significativa as proteínas do lactosoro após 24 horas de incubação. O pré-aquecimento do lactosoro também resultou em um aumento no grau de hidrólise e consequentemente na redução da antigenicidade dos hidrolisados. O leite bovino hidrolisado com CpLP desencadeou respostas imunológicas em um modelo animal de alergia ao leite. Contudo, os leites hidrolisados com CgLP e CapLP não elicitaram sintomas clínicos aparentes após administração oral em animais sensibilizados. As peptidases de DpGp, CgLP e CapLP foram promissoras para o desenvolvimento de novas formulas hipoalergênicas para crianças com alergia ao leite.

Palavras-chaves: Monarca. Látex. Alergia. Leite.

ABSTRACT

Cow's milk allergy is a global health problem that generally affect infants and children. The disorder in most cases is transitory, however it must be managed to avoid development issues. Hydrolyzed formulas are the most suitable alternative to cow's milk. Thus, the present study aimed to study the peptidases from laticifer plants and insect midgut able to perform cow's milk proteins hydrolysis. The hydrolysis assays were performed varying the following parameters: enzyme:substrate ratio, pH and time of reaction. In addition, it was measured the degree of hydrolysis and the residual antigenicity of the hydrolysates. The serine peptidases purified from *Danaus plexippus* midgut (DpGp) were able to perform caseins hydrolysis at 1:25 (enzyme:substrate ratio), after 4 hours of reaction at pH 9.0. Using the same parameters, whey proteins hydrolysis were performed after 24 hours; however the hydrolysis of pre-heated whey proteins (85 °C for 30 min) caused a reduction in the time of reaction as well as in its antigenicity. The immunoenzymatic assays showed that milk hydrolyzed by DpGp exhibited residual antigenicity similar to commercial partially hydrolyzed formulae. The latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP) e *Carica papaya* (CapLP) were able to perform caseins hydrolysis at 1:225 (enzyme:substrate ratio), after 30 min of reaction at pH 6.5. Only CapLP hydrolyzed the whey proteins after 24 hours of reaction. The heat denaturation of the whey proteins also caused an enhance in the degree of hydrolysis and reduced the residual antigenicity of the hydrolysates. The cow's milk hydrolyzed by CpLP trigged immune reactions in cow's milk allergy mouse model. However, the milk hydrolyzed by CgLP and CapLP did not elicited clinical symptoms after oral administration in sensitized mice. The peptidases of DpGp, CgLP and CapLP are promising enzymes to develop novel hypoallergenic formulas for children with milk allergy

Key words: Monarch. Latex. Peptidases. Allergy.

SUMÁRIO

1. INTRODUÇÃO

1.1 Plantas laticíferas

As plantas laticíferas somam mais de 20.000 espécies e estão agrupadas em 40 famílias distribuídas por diversos ambientes (Lewinsohn, 1991). Dentre tais famílias, pode-se destacar Apocynaceae, Asclepiadaceae, Caricaceae, Euphorbiaceae, Moraceae, Convolvulaceae e Sapotaceae, como as mais representativas (Badgujar et al., 2009). Uma característica marcante das plantas laticíferas é a presença de um conjunto de células altamente especializadas formando tubos contínuos ou descontínuos capazes de produzir um fluido de aspecto leitoso conhecido como látex. De acordo com Agrawal e Kono (2009), o látex é uma emulsão viscosa exsudada após a planta sofrer uma lesão, seja por herbivoria ou injuria mecânica. Embora esteja mais associado à defesa contra patógenos, o látex vegetal tem recebido destaque devido a sua composição, em que pode ser encontrado uma grande quantidade de peptidases, além de outras moléculas cujo potencial precisa ser melhor investigado (Agrawal; Kono 2009).

1.1.1 *Cryptostegia grandiflora* R. Br.

Cryptostegia grandiflora é uma espécie de porte arbustivo originária do sudoeste de Madagascar, que atualmente encontra-se distribuída pelas regiões de clima tropical e subtropical do planeta (Figura 1D). No Brasil, é encontrada principalmente na região nordeste. Pertencente à família Apocynaceae, *C. grandiflora* é popularmente conhecida como unha de onça, alamanda-roxa, boca-de-leão, leite-de-bom-jesus, margarida e viúva-alegre. Essa espécie é produtora de látex, sendo este coletado principalmente de seus frutos.

A fração proteica do látex de *C. grandiflora* apresentou forte atividade proteolítica, com predomínio de peptidases cisteínicas, além de atividade quitinolítica e antioxidante (Freitas et al., 2010). Também pode ser destacado as propriedades farmacológicas do látex de *C. grandiflora*, que apresentou atividade fibrinogenolítica, pró-coagulante (Viana et al., 2013) e pró-inflamatória (Albuquerque et al., 2009). Trabalhos anteriores destacam a atividade inseticida contra o mosquito *Aedes aegypti* (Ramos et al., 2009) e a potencial aplicação das peptidases laticíferas como coalho vegetal na produção de queijo vegetariano (Freitas et al., 2016). Além disso, uma peptidase cisteínica do látex de *C. grandiflora* (Cg24-I) foi purificada e apresentou atividade contra o fungo fitopatogênico *Fusarium solani* (Ramos et al., 2014).

1.1.2 Carica papaya L.

Carica papaya é uma espécie de porte herbáceo originária da América Central, que atualmente encontra-se distribuída pelas regiões de clima tropical do planeta (Figura 1C). Pertencente à família Caricaceae, *C. papaya* é popularmente conhecida como mamoeiro, papaeira, ababaia ou pinoguaçu. No Brasil, o mamoeiro é explorado comercialmente, sendo amplamente produzido na região norte do Espírito Santo, sul da Bahia e Pará.

Além dos frutos serem bastante valorizados, o látex dessa espécie possui diversas aplicações. O látex dos frutos verdes de *C. papaya* possui propriedades cicatrizantes (Gurung; Skalko-Basnet, 2009), anti-helmíntica (Satrija et al., 1994), atividade antifúngica (Giordani et al., 1997), atividade contra insetos herbívoros (Konno et al., 2004) e além disso, uma de suas peptidases (quimopapaína) apresenta propriedades imunológicas (Buttle; Barrett, 1984).

O látex de *C. papaya* é rico em peptidases cisteínicas e dele pode ser obtido papaína, quimopapaína, glicil endopeptidase e caricaína (Azarkan et al., 2003). Dentre estas, a papaína é mais facilmente purificada e, consequentemente, a mais estudada, possuindo diversas aplicações biotecnológicas. Dentre as aplicações da papaína, pode-se destacar o amaciamento de carnes (Iizuka; Aishima, 2000), participação na composição de cosméticos (Sim et al., 2000), capacidade de coagulação do leite bovino, fabricação de cervejas, além da utilização na indústria têxtil e na fabricação de borracha (Boshra; Tajul, 2013).

1.1.3 Plumeria rubra Linn.

Plumeria rubra é uma espécie de porte arbóreo originária da América Central, que atualmente encontra-se distribuída pelas regiões de clima tropical do planeta (Figura 1B). Pertencente à família Apocynaceae, *P. rubra* é popularmente conhecida como jasmim, jasmim-manga, frangipani ou árvore-pagode.

Na Índia, as plantas do gênero Plumeria são bastante valorizadas na medicina popular, podendo ser usadas no tratamento de infecções, doenças digestivas e artrites, possuem ação anti-inflamatória, antipirética e são potencialmente antitumorais (Choudhary; Kumar; Singh, 2014). Além disso, estudos apontam a presença de enzimas relacionadas ao metabolismo anti-oxidativo no látex dessa espécie (Freitas et al., 2010). A fração proteica de látex de *P. rubra*

também apresentou efeito repelente contra insetos (Ramos et al., 2011), mas não foi observado atividade de inibição de crescimento contra fungos fitopatogênicos (Sousa et al., 2011).

No látex de *P. rubra* foram detectadas quatro peptidases, mas este apresentou atividade proteolítica moderada, com predomínio de peptidases cisteínicas e serínicas (Freitas et al., 2010). Uma peptidase foi purificada do látex de *P. rubra* (Plumerin-R) com atividade ótima a pH 7.0 e 55 °C, apresentando propriedades anti-inflamatórias e cicatrizantes (Chanda et al., 2011a, 2011b).

1.1.4 *Calotropis procera* (Ait.) R. Br.

Calotropis procera é uma espécie de porte arbustivo, originária da África e sudoeste Asiático, que atualmente encontra-se distribuída pelas regiões de clima tropical e subtropical do planeta (Figura 1A). Pertencente à família Apocynaceae, uma característica marcante desta espécie é sua grande capacidade de produzir látex, justificando assim o seu nome popular, leiteiro. Contudo, *C. procera* também é conhecida por outros nomes populares, como algodão-de-seda, flor-de-seda, hortência e ciúme, dependendo da região encontrada. O látex de *C. procera* é abundante em todas as partes verdes da planta, porém não há relato de sua presença nas sementes e raízes (Ramos et al., 2007).

Diferentes partes da planta são utilizadas na medicina popular, porém o látex se destaca possuindo um grande número de atividades biológicas. As proteínas do látex de *C. procera* apresentam propriedades anti-inflamatória (Alencar et al., 2004), coagulante (Ramos et al., 2012), antitumoral (Oliveira et al., 2007), além de serem usadas no tratamento de lepra, úlceras, hemorroidas, doenças do baço, fígado e abdómen (Kumar; Arya, 2006). Trabalhos anteriores também destacam a atividade inseticida contra o mosquito *Aedes aegypti* e diferentes pragas agrícolas (Ramos et al., 2006, 2007), assim como atividade contra diferentes fungos fitopatogênicos (Sousa et al., 2011).

Várias peptidases cisteínicas do látex de *C. procera* já foram purificadas e caracterizadas. Dubey e Jagannadham (2003) reportaram uma peptidase nomeada Proceraína com atividade em uma ampla faixa de pH e temperatura. Outra peptidase purificada do látex de *C. procera*, Proceraína B, também apresentou boa atividade em ampla faixa de pH e temperatura, distinguindo-se da Proceraína em termos de peso molecular, ponto isoelétrico, local de reconhecimento de clivagem, etc. Posteriormente, Ramos e colaboradores (2013)

purificaram as peptidases CpCP-1, CpCP-2 e CpCP-3, capazes de mediar a coagulação plasmática em um mecanismo semelhante ao da trombina.

1.2 Plantas laticíferas e insetos

Apesar da ação defensiva do látex, alguns organismos são capazes de sobrepor essa barreira natural através da ação de suas enzimas digestivas. Um exemplo comumente observado é o fato de algumas lagartas serem capazes de se alimentar das folhas/látex da espécie *C. procera* durante os diferentes estágios do desenvolvimento larval. Neste contexto, as enzimas presentes no intestino de muitas espécies de insetos também podem ser estudadas como novas biomoléculas visando diferentes aplicações biotecnológicas.

Figura 1. Plantas laticíferas

Fontes: (A) Brito, 2015; (B) https://www.indiamart.com; (C) https://www.pinterest.pt; (D) https://www.davesgarden.com.

1.2.1 Intestino de insetos

A classe Insecta é a maior e mais distribuída entre os grupos de animais, possuindo representantes em quase todos os ecossistemas do planeta (Terra et al., 1996). Como consequência, é observado uma grande variedade de hábitos alimentares no grupo, como por exemplo tecidos vegetais, lã, cera, sangue ou alimentos que seriam tóxicos para outros animais (Terra et al., 1996). O trato digestório dos insetos é dividido em intestino anterior, intestino médio e intestino posterior. No intestino anterior, estão presentes as glândulas salivares e as primeiras câmaras do trato digestivo. No intestino médio é encontrado a membrana peritrófica e as principais enzimas digestivas. No intestino posterior estão a maioria dos microrganismos simbiontes (Willis; Mazarei; Stewart, 2016). No trato digestório de larvas da ordem Lepdoptera já foram identificadas peptidases similares a tripsina, quimitripsina, elastase, aminopeptidases e carboxipeptidases A e B. Estima-se que as peptidases serínicas sejam responsáveis por 95% do processo digestivo destes insetos (Srinivasan; Giri; Gupta, 2006), contudo peptidases aspárticas e cisteínicas já foram identificadas (Seddigh; Darabi, 2016; Wu et al., 2016). Em contrapartida, metalopeptidases parecem não exercerem influência significativa na digestão enzimática no intestino médio (Christeller et al., 1989).

1.2.2 *Danaus plexippus* L.

Danaus plexippus, também conhecida pelo nome popular Monarca (figura 2), é um inseto da ordem Lepdoptera originário das Américas do Sul e do Norte. Essa espécie é conhecida por realizar grandes migrações, podendo percorrer mais de 2.200 km devido às mudanças de estações (Oberhauser; Solensky, 2011). Atualmente, existe um banco de dados genômico de *D. plexippus*, Monarch Base (Zhan et al., 2011), criado com o objetivo de proporcionar um melhor entendimento em relação à capacidade migratória dessa espécie.

A ovoposição da borboleta-monarca ocorre frequentemente em plantas da espécie *C. procera* e o ciclo de vida é dividido em cinco estágios larvais e uma fase reprodutiva na forma de borboleta (Pereira et al., 2010). Em estudos anteriores, Ramos e colaboradores (2015) mostraram os efeitos das peptidases laticíferas na alimentação de larvas de *D. plexippus*. Foi observado, em ensaios *in vitro*, que a membrana peritrófica do animal é danificada pelas peptidases laticíferas, contudo o mesmo não foi observado nos ensaios *in vivo*, corroborando com o observado por Pereira e colaboradores (2010) em que larvas de monarca alimentadas

com o látex de *C. procera* apresentaram maior aumento na massa corporal comparado ao grupo controle. No mesmo trabalho, também foi observado que as peptidases intestinais de *D. plexippus* foram capazes de hidrolisar toda a fração proteica do látex de *C. procera*, mostrando assim uma estratégia evolutiva em que as larvas de *D. plexippus* sobrepõe uma das defesas da planta.

O extrato intestinal de D. plexippus foi caracterizado por Pereira e colaboradores (2010). Foi observado uma predominância de peptidases serínicas com massa molecular aparente superior a 45 kDa. Além disso, a atividade enzimática foi maior em meio alcalino, sendo máxima em pH 10, e a temperatura ótima 37 °C, ocorrendo inativação das enzimas em temperaturas superiores a 60 °C.

Figura 2. *Danaus Plexippus* se alimentando de folhas de *Calotropis procera*

Fonte: autor, a barra equivale a 1 cm.

1.3 Peptidases e suas aplicações biotecnológicas

O látex de diferentes espécies vegetais e o intestino médio de *D. plexippus* mostraram-se uma rica fonte de biomoléculas com elevado potencial para aplicações biotecnológicas. Dentre essas moléculas, as peptidases se destacam pela sua abundância nesses extratos, além do fato de serem bastante valorizadas em diversos segmentos na indústria.

As peptidases, também conhecidas como proteases, proteinase ou enzimas proteolíticas, são catalizadores biológicos responsáveis pela clivagem de ligações peptídicas em uma proteína. De acordo com o Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (NC-IUBMB), as peptidases pertencem a classe das hidrolases (EC 3) e são agrupadas entre as que hidrolisam ligações peptídicas (EC 3.4). Com isso, as peptidases podem ser classificadas de acordo com a posição da ligação peptídica clivada. As exopeptidases clivam ligações no exterior do polipeptídeo, subdividindo-se em aminopeptidases (ligações do lado N-terminal) e carboxipeptidases (ligações do lado C-terminal); e as endopeptidases, que clivam ligações no interior do polipeptídeo (Tavano et al., 2018). Baseado no mecanismo de ação, as peptidases podem ser agrupadas como do tipo serina, cisteína, aspártica, glutâmica, asparagina ou treonina, de acordo com o aminoácido presente no seu sítio ativo, ou metalopeptidase, quando é necessário um metal para a sua ativação (Rawlings et al., 2017). Além disso, as peptidases podem ser classificadas em famílias com base na similaridade das sequências aminoacídicas e famílias homologas são agrupadas em clãs (Rawlings et al., 2017).

As peptidases estão presentes em todos os organismos vivos e são fundamentais para o controle preciso dos processos biológicos. Essas moléculas também apresentam grande importância biotecnológica, estando envolvidas em diversos setores industriais (Tavano et al., 2018). Com base nisso, um novo termo foi criado para designar o estudo das peptidases. De acordo com López-Ótin e Overall (2002), a degradômica foca em elucidar o conjunto de peptidases em uma célula, tecido ou organismo, assim como a relação com seus substratos e inibidores. Atualmente, é crescente o interesse na identificação e caracterização funcional de enzimas proteolíticas de diferentes organismos, possibilitando assim o crescimento da degradômica junto com as outras ‘ômicas’.

Com os avanços nas técnicas de purificação e produção em larga escala, somado ao desenvolvimento da tecnologia do DNA recombinante e engenharia de proteínas, foi possível estender o número de peptidases utilizadas. O principal fator que impulsiona o crescente mercado das peptidases é a sua utilização em diversos segmento da indústria, onde são utilizadas como insumos ou, em alguns casos, podem ser o produto final. Essas moléculas tornaram-se parte indispensável em muitos processos visando a produção em larga escala de diversos produtos, como detergentes, ração animal, couro, papel, na indústria farmacêutica e indústria alimentar (Tavano et al., 2018). Atualmente, a maioria das peptidases utilizadas na

indústria são de origem microbiana (Singh et al., 2016) contudo a busca por enzimas proteolíticas de origem vegetal ou animal contribui para enriquecimento do mercado mundial dessas moléculas.

1.3.1 Peptidases na indústria alimentar

Na indústria alimentar, a escolha da enzima é uma etapa fundamental. Papaína e bromelaína, por exemplo, são usadas como amaciadores de carnes, clarificadores de bebidas, na produção de queijo e hidrolisados proteicos (Gupta et al., 2016). Em relação as peptidases de origem animal, pode-se destacar a quimosina que desde 5.000 a.C. é utilizada na coagulação do leite bovino por realizar a clivagem específica da proteína *k*-caseína (Rosell et al., 2017). Além disso, peptidases de peixes e invertebrados aquáticos também são bastante valorizadas na indústria (Shahidi; Kamil, 2001). De forma geral, as peptidases são utilizadas na produção de hidrolisados proteicos, sendo responsáveis por melhorar o valor nutricional dos alimentos ao facilitar a digestão das proteínas, eliminar proteínas tóxicas, além de promover mudanças na textura, sabor e odor de alguns alimentos (Tavano, 2013). Além disso, a hidrolise enzimática das proteínas alimentares pode ser usada para a redução da alergenicidade de muitos alimentos, como é o caso do leite bovino.

1.4 Alergia – visão geral

De acordo com a Organização Mundial de alergia (WAO), o termo alergia descreve uma reação de hipersensibilidade iniciada por mecanismos imunológicos específicos. Essa é uma condição crônica que envolve reações anormais do corpo a substâncias (alérgenos) do meio que são inofensivas à maioria das pessoas. Em geral, a exposição aos alérgenos ocorre pelo contado com a pele, inalação, ingestão ou injeção. Uma vez no organismo, os alérgenos são reconhecidos por células do sistema imunológico que são responsáveis por desencadear as respostas inflamatórias, através das reações de hipersensibilidade (Docena et al., 2016). Geralmente, as respostas alérgicas são classificadas como leves ou moderadas, contudo em alguns casos, pode haver uma reação mais grave e generalizada, conhecida como anafilaxia. As reações anafiláticas podem ocorrer minutos após a exposição ao alérgeno e, se não tratadas com injeções de epinefrina, podem ser fatais.

Dentre os principais alergenos, pode-se destacar aqueles presentes nos alimentos. A alergia alimentar é muito comum em crianças e, a princípio, pode ser desencadeada por qualquer alimento. Contudo, em 90% dos casos, esta é causada por um grupo de alimentos conhecido como “big-8”, em que estão presentes o leite, ovo, crustáceos, peixe, nozes, trigo, amendoim e soja. Destes, o leite é responsável por 77% dos casos de alergia alimentar na infância.

1.4.1 Alergia ao leite bovino

Estima-se que o hábito de consumir leite proveniente de animais domesticados teve início há aproximadamente 9.000 anos (Hochwallner et al., 2014). Assim, os primeiros casos de alergia ao leite bovino devem datar do mesmo período, contudo foi só em torno de 370 a.C. que as primeiras reações adversas, provenientes do consumo desse alimento, foram descritas por Hipócrates (Hochwallner et al., 2014). Os principais alérgenos do leite são as proteínas e embora possa ser encontrado cerca de 25 tipos diferentes dessas moléculas, apenas uma parte é responsável por desencadear as reações alérgicas (Martorell-Aragonés et al., 2015). Em geral, a sensibilização ocorrer devido à exposição de recém-nascidos às proteínas do leite bovino, pois nesta fase existe uma baixa atividade das enzimas digestivas. Estima-se que 6 a 8% das crianças sejam alérgicas ao leite bovino, contudo geralmente ocorre o desenvolvimento de uma tolerância oral, durante o crescimento, resultando em uma estimativa de 1 a 3% de adultos alérgicos. Durante as reações alérgicas, podem ser observados sintomas dermatológicos (em até 90% dos casos), gastrointestinais (até 60%) e anafilaxia (até 9%).

Muitas vezes a alergia ao leite é confundida com intolerância ao leite uma vez que ambas podem apresentar sintomas semelhantes. Contudo, a intolerância ao leite não é mediada por anticorpos, mas sim atribuída a deficiência de uma enzima específica, a lactase.

1.4.2 Principais antígenos do leite bovino

O leite bovino pode ser dividido em duas frações de acordo com a solubilidade em pH 4,6. A fração caseína é constituída por quatro proteínas diferentes (α_{s1}, α_{s2}, β e *k*-caseína), que juntas correspondem a aproximadamente 80% do conteúdo proteico total do leite. As caseínas possuem baixa solubilidade em pH 4,6 devido ao seu ponto isoelétrico, o que causa a

sua precipitação neste pH. No leite, as caseínas estão organizadas em partículas coloidais denominadas micelas, que possuem o interior hidrofóbico formado por α_{s1}, α_{s2} e β-caseína e uma superfície hidrofílica formada principalmente por *k*-caseína. As micelas de caseínas estão em suspensão no lactosoro e de acordo com Holt et al. (2013) são responsáveis pelo transporte e secreção de cálcio e fosfato para os lactentes. As caseínas possuem uma estrutura aberta e flexível devido ao alto conteúdo de resíduos de prolina que dificulta a formação de estruturas secundárias. Além disso, essas proteínas são os principais antígenos no leite bovino, sendo α_{s1}, α_{s2} e *k*-caseína as mais relevantes no desencadeamento das reações alérgicas (Docena et al., 1996).

Na fração lactosoro estão presentes as proteínas α-lactalbumina, β-lactoglobulina, albumina sérica bovina, lactoferrina e imunoglobulinas, que juntas correspondem a 20% do conteúdo proteico total do leite. A proteína α-lactalbumina é sintetizada nas glândulas mamárias e participa da regulação da síntese de lactose, sendo encontrada em todos os leites já estudados (Fiocchi et al., 2010). A α-lactalbumina é uma proteína monomérica, globular, que possui sítios de ligação a Ca^{2+} e é estabilizada por quatro ligações dissulfeto. Assim, essa proteína possui alta estabilidade térmica e capacidade de reenovelamento. A proteína β-lactoglobulina é sintetizada nas glândulas mamárias e está envolvida no transporte de retinol. Embora não seja sintetizada em humanos, essa proteína é constantemente detectada no leite materno, pois é absorvida pela mucosa do intestino e depositada nas glândulas mamárias (Wal, 2004). Com isso, β-lactoglobulina é um dos principais antígenos do lactosoro. A β-lactoglobulina é uma proteína globular estabilizada por duas pontes dissulfeto, além de possuir um tiol livre. Em condições fisiológicas, ela pode estar presente na forma de monômeros ou dímeros, porém em seu ponto isoelétrico pode se organizar em octâmeros. Embora os principais antígenos do lactosoro sejam as proteínas α-lactalbumina e β-lactoglobulina, também é relatado a antigenicidade das proteínas albumina sérica bovina, imunoglobulinas e lactoferrina (Hochwallner et al., 2014).

1.4.3 Fórmulas hipoalergênicas

Uma vez diagnosticado a alergia ao leite, a forma mais efetiva de evitar os sintomas é cessar o consumo deste alimento. Contudo, devido ao seu alto valor nutricional, essa pratica pode provocar problemas no desenvolvimento da criança, em consequência da redução da

ingestão de calorias e alguns aminoácidos essenciais. Substituir o leite bovino pelo leite de outros mamíferos (cabra, ovelha, búfalo e camelo) é uma prática comum, porém não recomendável pelo ao alto risco de reações-cruzadas, pois muitas das proteínas desses leites são similares às do leite bovino (Monaci et al., 2006). Reações-cruzadas também foram detectadas entre várias proteínas da soja e as caseínas, dificultado o uso de leite de soja por crianças alérgicas (Docena et al., 2015; 2014). Com isso, a alternativa mais efetiva é o processamento das proteínas do leite com o objetivo de destruir ou modificar a estrutura dos antígenos, que pode ser alcançado através de altas pressões, desnaturação térmica, reações de glicação, fermentação ou hidrólise enzimática (Bu et al., 2013). Dentre esses, a hidrólise enzimática se destaca pois é a forma mais eficiente de interromper os epítopos das proteínas e prevenir as interações com as imunoglobulinas, principalmente IgE.

As fórmulas hipoalergênicas sugiram na década de 1940 e atualmente existem diversos produtos no mercado capazes de substituir o leite bovino por crianças alérgicas. Essas fórmulas podem ser compostas exclusivamente por aminoácidos livres, sendo indicadas nos casos mais extremos de alergia. Porém, na maioria dos casos, são utilizadas fórmulas de leite hidrolisado. As fórmulas hidrolisadas podem ser divididas, de acordo com o grau de hidrólise, em parcialmente ou extensivamente hidrolisadas, porém apesar do constante uso desses términos na literatura, não existe um consenso entre a comunidade científica de um critério para a classificação em uma dessas categorias.

Apesar dos benefícios à saúde, alguns fatores são limitantes à utilização dessas fórmulas hipoalergênicas. Fórmulas extensivamente hidrolisadas possuem o gosto amargo decorrente da alta concentração de peptídeos hidrofóbicos em sua composição (Rosendal; Barkholt, 2000). A hidrólise extensiva também gera uma concentração elevada de aminoácido livres o que torna as fórmulas hiperosmóticas, podendo causar diarreia em alguns lactentes. Além disso, fórmulas extensivamente hidrolisadas possuem preços elevados, sendo muitas vezes inacessível. Em contrapartida, algumas fórmulas parcialmente hidrolisadas podem ainda reter uma elevada antigenicidade residual, provocando reações imunológicas em crianças alérgicas (Halken; Høst, 1997). Ainda assim, o uso das fórmulas hidrolisadas, seja parcialmente ou extensivamente hidrolisadas, é considerado como a opção mais viável para a alimentação de crianças alérgicas (salvo os casos mais graves), uma vez que o leite possui elevado valor energético, excelente composição aminoacídica, além imunoglobulinas que podem ajudar na defesa do organismo.

2. OBJETIVOS

2.1 Objetivo Geral

Prospectar novas peptidases capazes de hidrolisar as proteínas do leite bovino para a produção de fórmulas de leite hipoalergênico

2.2 Objetivos Específicos

- Purificar e caracterizar peptidases do intestino de lagartas de *Danaus plexippus*;

- Avaliar a ação das peptidases intestinais sobre as proteínas purificadas do leite bovino através de zimogramas, ensaios enzimáticos, eletroforeses e ELISA;

- Testar a capacidade de diferentes peptidases laticíferas em hidrolisar as proteínas do leite bovino através de ensaios colorimétricos, eletroforese e ELISA;

- Avaliar o potencial hipoalergênico dos hidrolisados do leite bovino em modelo de camundongos alérgicos a proteínas do leite bovino.

Gut peptidases from a specialist herbivore of latex plants are capable of milk protein hydrolysis: inputs for hypoallergenic milk formulas

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Abstract

Transitory allergies to cow milk proteins in infants or adults have become a public health problem. Although extensively or partially hydrolyzed cow milk protein formulas are available, these products are costly. Therefore, studies into innovative enzymes to digest cow milk proteins are needed. *Danaus plexippus* gut peptidases were purified and examined with regard to cow milk protein hydrolysis. The peptidases hydrolyzed caseins and whey proteins. However, after heat treatment, there was a significant improvement in β-lactoglobulin hydrolysis. The hydrolyzed cow milk proteins were not recognized by anti-casein antibodies and only reacted slightly with antibodies against whey proteins. This performance was better than that of partially hydrolyzed formulas and similar to that of an extensively hydrolyzed formula. These results suggest that *D. plexippus* gut peptidases are suitable and innovative enzymes to produce hypoallergenic cow milk protein formulas.

Keywords: Casein. Insect. Milk. Monarch. Proteases.

1. Introduction

Milk allergy is an immunological reaction induced by undigested proteins present in milk. It is the most common food allergy during infancy and childhood (Sicherer & Sampson, 2014). Although, in more than 80% of cases the allergy is transitory, this immunological reaction can result in intensified gastrointestinal, respiratory and dermatological symptoms (Fiocchi et al., 2010). Consequently, the supply of energy and amino acids for the growth and development of children is reduced to some extent (Luyt et al., 2014). Although cow milk contains more than 25 different proteins (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014), caseins, β-lactoglobulin and α-lactalbumin (whey proteins) are reported as the major milk allergens (Monaci, Tregoat, van Hengel, & Anklam, 2006). Therefore, the partial or extensive cleavage of cow milk proteins (CMP) achieved by peptidases is one of the most effective methods to reduce allergic reactions to these proteins (Bu, Luo, Chen, Liu, & Zhu, 2013). Even though very effective, these formulas are expensive, making them unaffordable to many people. Children suffering from milk allergy have few alternative protein sources to supply their daily intake to minimally support their growth and development. Soy protein formulas, which are the immediate substitute for CMP, are not adequate for the nutritional needs of infants and in some cases cross-reactions between antibodies against CMP with soy proteins have been documented (Candreva, Smaldini, Curciarello, Fossati, Docena, & Petruccelli, 2016). To confront this complex scenario, the identification of novel enzymatic sources suitable for efficiently digesting CMP and producing hypoallergenic formulas is needed. Therefore, the study of new peptidases able to hydrolyze milk proteins should be encouraged.

The caterpillar of the Monarch butterfly is extremely well adapted to feed on the leaves of latex-producing plants, such as *Calotropis procera*. A previous study by Pereira and collaborators (2010) showed that *D. plexippus* gut peptidases are able to hydrolyze all proteins present in the latex of *C. procera* within a few minutes. Interestingly, this same protein fraction

from *C. procera* latex is toxic to other insects (Ramos et al., 2007). Moreover, *D. plexippus* gut peptidases are resistant to hydrolysis by latex peptidases even after 24 h of incubation (Pereira et al., 2010). Therefore, our hypothesis was that *D. plexippus* gut peptidases can be used as powerful biomolecules to hydrolyze other proteins, including food proteins. Therefore, the aim of the present work was to purify *D. plexippus* gut peptidases by affinity chromatography and evaluate their hydrolytic action on CMP (caseins, β-lactoglobulin and α-lactalbumin) and to compare the resulting hydrolysate with commercial milk formulas.

2. Materials and methods

2.1. Materials

Acrylamide (17-1302-02), bis-acrylamide (17-1304-02), sodium dodecyl sulfate (SDS) (17-1313-01), HiTrap™ Benzamidine Sepharose 4 Fast Flow column (17-5143-01), HiTrap DEAE FF Sepharose Fast Flow (17-5055-01), HiTrap CM FF Sepharose Fast Flow (17-5056-01), HiTrap Protein A Sepharose high performance column (17-0402-01) and low molecular mass markers (17-0446-01) were acquired from GE Healthcare Life Science (São Paulo, SP, Brazil). Sequencing-grade modified trypsin was obtained from Promega (V511B) (São Paulo, SP, Brazil). Azocasein (A2765), porcine pepsin (P7012), Freund's complete (F5881) and incomplete adjuvant (F5506), goat anti-rabbit IgG conjugated with alkaline phosphatase (A6066) and *p*-nitrophenylphosphate disodium (N2765) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). All other chemicals were of analytical grade.

2.2. Gut protein extract

Gut protein extracts of *Danaus plexippus* larvae (5^{th} instar) were obtained according to Pereira et al. (2010), dialyzed against distilled water, lyophilized and stored at -20 ºC until

further assays were performed. Soluble proteins were quantified by Bradford's method (Bradford, 1976).

2.3. Gut peptidase purification

D. plexippus gut peptidases (DpGp) were purified by affinity chromatography using a HiTrap™ Benzamidine Sepharose 4 Fast Flow column coupled to an AKTA chromatography system (GE HealthCare). The unbound proteins (PI) were washed with equilibrium buffer (50 mM Tris-HCl pH 7.4, containing 0.5 M NaCl) and the bound proteins (PII, DpGp) were eluted with 50 mM glycine-HCl (pH 3.0) at a flow rate of 1 mL/min. Proteins were monitored at 280 nm. Both peaks (PI and PII) were dialyzed against distilled water and lyophilized.

In order to purify the peptidases, the PII fraction from the HiTrap™ Benzamidine Sepharose 4 Fast Flow column was submitted to ion exchange chromatography in DEAE-Sepharose fast flow and CM-Sepharose fast flow columns, which were previously equilibrated with 25 mM Tris-HCl buffer (pH 7.0). The proteins were eluted from the column by using a linear gradient from 0 up to 1 M NaCl in 25 mM Tris-HCl buffer (pH 7.0). Additionally, the PII fraction was also submitted to reversed-phase high-performance liquid chromatography (RP-HPLC). Samples of 50 μL (1 mg/mL) were loaded in the C2/C18 column (μRPC C2/C18 ST, bed length 100 mm, i.d. 4.6 mm, 3 μm particle size, 12 nm porosity, Amersham Bioscience) coupled to a Jasco CO-2060 Plus HPLC device. The proteins were eluted using a linear gradient from 5% to 80% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate. Proteins were detected at 280 nm.

2.4. Proteomic analysis

2.4.1. One-dimensional gel electrophoresis

Polyacrylamide gel electrophoresis (15%) in the presence of SDS (1D-SDS-PAGE) was performed according to Laemmli (1970). The samples were dissolved in sample buffer [0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS] and the runs were performed at 25 mA (25 ºC for 2 h) using 10 x 10 cm gels. Proteins were stained using Coomassie brilliant blue R-350.

2.4.2. Protein identification by mass spectrometry (LC–MS/MS)

The protein bands from the 1D-SDS-PAGE gels were removed, destained, and digested with sequencing-grade modified trypsin according to Hellman, Wernstedt, Góñez & Heldin (1995). The tryptic peptides were analyzed using a Synapt G1 HDMS mass spectrometer (Waters, Manchester, UK) coupled to a Nano UPLC-ESI system, as described by Freitas et al. (2016). Proteins were identified using the NCBI database and MASCOT search engine (Matrix Science Ltd., London, UK; website: http://www.matrixscience.com).

2.5. Proteolytic activity

Total proteolytic activity was evaluated using azocasein as non-specific substrate (Freitas et al., 2007). Briefly, 50 µL of total gut protein extract or peaks from affinity chromatography (1 mg/mL) were mixed with 1% azocasein (200 µL) and the final volume of the reaction was adjusted to 300 µL with 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5). The reactions were performed at 37 °C for 1 h, and then stopped with 10% TCA solution. The reaction products were measured at 420 nm.

2.6. Autolysis assays

D. plexippus gut peptidases (DpGp) [1 mg/mL in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5)] were incubated at different temperatures (4, 25 and 37 °C) and for different time intervals (1, 2, 4 and 24 h). After incubation, aliquots (50 µL) were used to determine the residual proteolytic activity, as described in section 2.5. Protein autolysis was verified by 15% polyacrylamide gel electrophoreses in the presence of SDS (1D-SDS-PAGE), as described previously in section 2.4.1.

2.7. Caseins and whey protein purification

Caseins and whey proteins were purified according to Egito et al. (2007). Briefly, raw bovine milk (obtained from a local market) was centrifuged (2,100 x g at 32 °C for 30 min) and the supernatant (skimmed milk) was separated and acidified with 0.1 M HCl to pH 4.6. Then, caseins and whey proteins were separated by centrifugation at 1,500 x g at 20 °C for 20 min. The supernatant (whey proteins) was collected and the precipitate (sodium caseinate) was washed three times with distilled water and centrifuged again. Both fractions were dialyzed against distilled water at 4 °C for two days using membranes with an 8,000 Da cut-off, and then lyophilized for further use.

2.8. β-Lactoglobulin purification

β-lactoglobulin was purified as described by Kitabatake & Kinekawa (1998). The whey protein fraction (non-lyophilized) was adjusted to pH 2.0 with 1 M HCl and porcine pepsin was added to a ratio of 1:200, enzyme:substrate (mass:mass). After 1 h at 37 °C, the proteins were precipitated overnight with 75% ammonium sulfate and then centrifuged at 20,800 x g for 30 min at 25 ºC. The supernatant (β-lactoglobulin) was dialyzed against distilled water at 4 °C for two days using membranes with an 8,000 Da cut-off, and then lyophilized.

The homogeneity of β-lactoglobulin was checked by 15% 1D-SDS-PAGE as described in section 2.4.1.

2.9. Hydrolysis of milk proteins

The hydrolytic potential of DpGp on the milk proteins (caseins, whey proteins and purified β-lactoglobulin) was assessed by zymograms and *in vitro* assays. For the zymograms, the gels were supplemented with the four protein substrates at 0.2% (m/m): gelatin (control), caseins, whey proteins and purified β-lactoglobulin. After the electrophoretic runs of DpGp, the gels were washed twice with 2.5% Triton X-100 for 30 min to remove all SDS. Afterward, the gels were incubated in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5) for 1 h at 37 °C. Protein hydrolysis was detected as transparent bands in blue gels after staining with 0.1% Coomassie brilliant blue R-350.

The *in vitro* assays were performed by incubating DpGp with the different substrates: caseins, whey proteins, native β-lactoglobulin and heated β-lactoglobulin (85 °C for 30 min) in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5). For the reactions, each substrate (10 mg/mL) was mixed with different aliquots of DpGp (4 mg/mL) to obtain the ratios of 1:100, 1:50 and 1:25 (enzyme:substrate, m/m). After 1, 2, 4 and 24 h of incubation at 37 ºC, aliquots were taken to measure the extensiveness of protein hydrolysis. To estimate the degree of hydrolysis by absorbance, samples obtained in each time interval were mixed with 20% trichloroacetic acid (TCA) solution in a 1:1 ratio (hydrolysate:TCA solution) and centrifuged at 10,000 x g, 10 °C for 10 min. Afterward, the absorbance of the supernatants were measured at 280 nm. The degree of hydrolysis was also quantified from 1D-SDS-PAGE by using the IMAGEJ software (https://imagej.nih.gov/ij/).

2.10. Polyclonal antibody production

Rabbit polyclonal antibodies against bovine casein and whey protein fractions were produced as described by Freitas, Silva, Bruno-Moreno, Monteiro-Moreira, Moreira & Ramos (2015). The protocol was approved by the institutional ethics committee for animal experimentation of Federal University of Ceará. Briefly, two adult New Zealand male rabbits were sensitized intramuscularly, one with caseins and the another with whey proteins (1 mg, dissolved in 0.5 mL saline and 0.5 mL of Freund's complete adjuvant) and booster injections were applied subcutaneously at 21, 35 and 42 days after the first sensitization using Freund's incomplete adjuvant containing the protein fractions. Blood samples were collected and allowed to coagulate at 37 °C for 5 h. The sera were centrifuged (2,000 x g at 25 °C for 5 min) and IgGs were purified by immunoaffinity chromatography using Protein A immobilized in a Sepharose 4B column (Freitas et al., 2017).

2.11. Enzyme-linked immunosorbent assay (ELISA)

The hydrolysis of milk proteins (caseins, whey proteins and purified β-lactoglobulin) by DpGp was also estimated by ELISA using anti-casein and anti-whey protein IgGs as the primary antibodies (1:20,000 dilution) and anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000 dilution) as the secondary antibody. The same samples used in the *in vitro* assays (section 2.9, 150 µL) were diluted in 96-well microplates. After the removal of unbound proteins, the residual binding sites were blocked with 200 µL of gelatin (10 mg/mL) in 50 mM sodium phosphate buffer (pH 7.0). Subsequently, the primary and secondary antibodies (150 µL) were added and the reactions were detected after adding the substrate *p*-nitrophenyl phosphate disodium and measuring absorbance at 405 nm (Freitas et al., 2015). Commercial non-hydrolyzed and hydrolyzed milk formulas were used as references: Itambé® and Nestogeno® (Nestlé): non-hydrolyzed cow milk; Nan Supreme1® (Nestlé) and Enfamil®

Gentlease® (Mead Johnson Nutrition): partially hydrolyzed cow milk; and Aptamil Pepti® (Danone): extensively hydrolyzed cow milk.

3. Results

3.1. Purification, identification and characterization of gut peptidases

Two peaks were obtained when the gut extract of *D. plexippus* was chromatographed in an affinity column with immobilized benzamidine (Supplementary Fig. 1a). The retained proteins (PII fraction) were eluted with 50 mM glycine-HCl buffer (pH 3.0). The 1D-SDS-PAGE showed that the PII fraction was rich in proteins with high molecular masses (45-70 kDa) (Supplementary Fig. 1b). All protein bands detected in the PII fraction were excised from the gels, digested with trypsin and analyzed by mass spectrometry (LC-MS/MS). All analyzed proteins matched with peptidases (Supplementary Table 1). To purify the gut peptidases, different chromatographic steps were performed, including the use of DEAE- and CM-Sepharose columns (pH 7.0) and reversed-phase high-performance liquid chromatography (RP-HPLC, C2/C18 column). However, these chromatographic steps were not effective, because only one peak was obtained in the CM-Sepharose column, and the protein peaks from DEAE-Sepharose and RP-HPLC columns were not separated accordingly (Supplementary Fig. 2). The 1D-SDS-PAGE (15%) of different fractions from DEAE-Sepharose fast flow showed that the peptidases were not isolated (data not shown). Therefore, the PII fraction from the HiTrap™ Benzamidine Sepharose column was used in all further experiments, and termed *D. plexippus* gut peptidases (DpGp).

To better characterize the hydrolytic potential of DpGp, an assay was performed to determine its proteolytic activity at two pH levels: pH 6.5 (milk pH) and pH 9.0 (optimal pH of *D. plexippus* gut peptidases)(Pereira et al., 2010). The proteolytic activity at pH 9.0 was 2.6-fold higher than at pH 6.5 (Supplementary Fig. 1c).Thus, further assays were performed at pH

9.0. The proteolytic activity of DpGp was monitored after different storage times and temperatures, both important parameters to evaluate protein autolysis and stability. The proteolytic activity of DpGp remained unaltered after 1 h at 37 ºC (100% activity), with a slight decrease in activity (10%) when the sample was incubated for 2 h. Only after 24 h was there a considerable loss (55%) of activity. On the other hand, DpGp were stable at 4 ºC even after 24 h, retaining 80% of the initial activity (Supplementary Fig. 2a). The evaluation of protein autolysis was observed by changes in the protein profiles and the results were in accordance with those of proteolytic activity *in vitro*. Autolysis began after 2 h and reached more than 50% after 24 h at 37 ºC. In contrast, DpGp were very stable after 24 h of incubation at 4 °C (Supplementary Fig. 2b). All these results concerning the purification and enzymatic characterization of DpGp are detailed in the supplementary materials.

3.2.Hydrolysis of milk proteins

Once characterized, DpGp were screened for the ability to hydrolyze CMP by zymography in gels containing caseins, whey proteins and purified β-lactoglobulin as substrates. After protein migration by electrophoresis, the gels were incubated at pH 6.5 or pH 9.0 for 1 h (37 °C) and proteolytic activities were detected as transparent bands in dark gels (Fig. 1). Although all gels showed the same total proteolytic activity (determined using gelatin substrate as control), significant differences in the hydrolysis patterns were observed at pH 6.5 and pH 9.0 and among the different protein substrates (Fig. 1). In all assays, protein hydrolysis was better at pH 9.0, corroborating data from the *in vitro* assays (Supplementary Fig. 1c), and using the caseins as the substrate. Interestingly, the zymograms showed several proteolytic bands able to degrade caseins and whey proteins, including purified β-lactoglobulin (Fig. 1). These results indicate that the different gut peptidase isoforms of *D. plexippus* were able to hydrolyze the major CMP at pH 9.0.

The digestibility of milk proteins by DpGp was further assessed by *in vitro* assays followed by electrophoresis analysis. DpGp digested caseins in a dose-dependent manner. The hydrolysis was remarkable at a 1:100 ratio (enzyme:substrate, m:m) and after 1 h of incubation at 37 ºC (pH 9.0). Complete hydrolysis (100%) was obtained after 4 h using a 1:25 ratio or after 24 h using a 1:100 ratio (Fig. 2). In contrast, whey proteins were more resistant than caseins to hydrolytic action by DpGp (Fig. 3). Only at the highest ratio (1:25, enzyme:substrate) and after 24 h of incubation at 37 ºC (pH 9.0) was effective hydrolysis (100%) of whey proteins (β-lactoglobulin and α-lactoalbumin) observed (Fig. 3). On the other hand, when hydrolysis assays were performed at pH 6.5 (milk pH), DpGp were able to digest only caseins at highest dose tested (1:25 ratio) (Supplementary Fig. 4).

To improve hydrolysis performance, purified β-lactoglobulin was heated at 85 °C for 30 min and then incubated with DpGp at different time points and ratios at pH 9.0. Heat treatment led to an enhancement of β-lactoglobulin processing by gut peptidases of the caterpillar (Fig.4). A comparison between native and heat denatured β-lactoglobulin hydrolysis revealed that heat denaturation supported more efficient hydrolysis even in the first hour (30%) at a 1:25 (enzyme:substrate) ratio. After 24 h, hydrolysis at the 1:100, 1:50 and 1:25 ratios were 50%, 75% and 100%, respectively (Fig. 4).

3.3. Residual antigenicity of peptides from hydrolyzed milk proteins

To confirm the hydrolytic potential of DpGp, whole skimmed milk (Itambé®) was used as the substrate (Fig. 5) and the resulting hydrolysis was compared with commercial non-hydrolyzed (Itambé® and Nestogeno®), partially hydrolyzed (Nan Supreme® and Enfamil®) and extensively hydrolyzed (AptamilPepti®) milk formulas. After 24 h of incubation at 37 ºC (pH 9.0), caseins and whey proteins were completely hydrolyzed by DpGp, using the1:25

(enzyme:milk proteins) ratio. This pattern was very similar to those observed for all hydrolyzed commercial formulas (Fig. 5a) and corroborated the previous results.

The possible residual antigenicity of peptides from hydrolyzed milk by DpGp was measured by ELISA, using anti-casein and anti-whey protein antibodies (Fig. 5b). The detection of casein peptides was minimal for milk hydrolyzed by DpGp when compared to non-hydrolyzed milk (Itambé® and Nestogeno®), and very similar to partially and extensively commercial milk (Nan Supreme®, Enfamil® and AptamilPepti®). On the other hand, antigenic peptides from whey proteins were detected in milk hydrolyzed by DpGp, although at a much lower level than in non-hydrolyzed milk (Itambé® and Nestogeno®) and the partially hydrolyzed milk Enfamil®(Fig. 5b).The extensively hydrolyzed milk formula (Aptamil Pepti®) did not show any residual antigenicity for caseins and whey proteins.

4. Discussion

An important industrial application of peptidases is in the hydrolysis of food proteins, particularly to reduce their allergenicity. Food allergies are important medical and social problems. Around 8% of infants and 2% of adults in the world have some type of food allergy (Gendel, 2013). Therefore, it is estimated 700 million people (considering a current population of 7 billion) have some kind of food allergy, representing a major global health problem. In particular, cow milk allergy constitutes the main food allergy (Fiocchi et al., 2010). Therefore, peptidases from microbial, animal and plant sources have been studied for the hydrolysis of CMP (Tavano, 2013; Patel, Singhania, & Pandey, 2016). However, gut peptidases from insects have not received much attention. Here, the gut peptidases of *D. plexippus* larvae were purified, identified, partially characterized and evaluated to digest CMP.

Because previous studies have reported predominance of serine peptidases in the *D. plexippus* larval gut extract (Pereira et al., 2010), these enzymes were cleaned from the extract using an affinity column containing immobilized benzamidine, which is a reversible competitive inhibitor for serine peptidases. The efficiency of the affinity chromatography was confirmed by proteomic analysis, since only peptidases were identified (Supplementary Table 1). Accordingly, the same method was successfully used in the purification of peptidases from other insects (Rascón, Gearin, Isoe, & Miesfeld, 2011). Because additional chromatographic steps failed to purify the gut peptidases to homogeneity (Supplementary Fig. 2), the mixture of peptidases (PII fraction) from the HiTrap™ Benzamidine Sepharose column was used in all further experiments.

Beyond high activity, industry demands peptidases that do not easily undergo inactivation or autolysis during the proteolytic process. Thus, two biochemical parameters are prerequisites for new alternative enzymes: the effect of reaction time and temperature on peptidase stability. *D. plexippus* gut peptidases remained stable even after two hours at 37 ºC. Only after 24 h at 37 ºC was there a considerable loss of activity and autolysis (Supplementary Fig. 2). Similar results were obtained for a thermo active alkaline serine peptidase from alkaliphilic *Bacillus sp*. (Ibrahim, Al-Salamah, El-Badawi, El-Tayeb, & Antranikian, 2015). Although the peptidases still exhibited the problems of inactivation and autolysis, with additional understanding of their three-dimensional structures and the specific amino acids involved in the mechanisms of action, it may be possible to manipulate and modify them to optimize their use in hydrolytic processes (Olivera-Nappa, Reyes, Andrews, & Asenjo, 2013).

The hydrolysis of caseins by DpGp was very effective. In a similar work, Mika, Gorshkov, Spengler, Zorn & Rühl (2015) tested gut extracts from six insects for the hydrolysis of different food proteins (casein, gluten and rice proteins). The gut peptidases of the beetles *Tribolium castaneum* and *Sitophilus granarius* exhibited no activity, while the enzymes from

Rhyzopertha dominica, Oryzaephilus surinamensis, Tenebrio molitor and *Alphitobius diaperinus* were able to digest casein (Mika et al., 2015). Because caseins constitute the most abundant CMP, corresponding to almost 80% of all proteins, they have been identified as major milk allergens (Docena, Fernandez, Chirdo, & Fossati, 1996). By analyzing 80 milk-atopic patients on the basis of the presence of cow milk-specific IgE antibodies in serum and compatible clinical history, Docena and collaborators (1996) showed that casein-specific IgE antibodies were present in all sera examined (100%), whereas 12% were reactive to β-lactoglobulin and 6% to α-lactalbumin. Consequently, peptidases able to extensively hydrolyze caseins are potentially good biotechnological tools for producing hypoallergenic milk formulas.

Although to a lesser extent when compared to caseins, whey proteins (α-lactoalbumin and β-lactoglobulin) can also be considered cow milk allergens (Fiocchi et al., 2010). In particular, β-lactoglobulin has been studied because this protein is not present in human milk. Moreover, it is very resistant to enzymatic action (Fiocchi et al., 2010). In the present study, only after 24 h at 37 ºC and using the highest concentration (1:25, peptidase:substrate ratio) were DpGp able to totally digest whey proteins (α-lactoalbumin and β-lactoglobulin) (Fig. 3). Similar results were observed with other enzymatic sources, such as trypsin, chymotrypsin, pepsin, chymosin, rat gastric juice and acid peptidases from the fungus *Monascus pilosus* (Kitabatake & Kinekawa, 1998; Salami et al., 2008; Lakshmana, Toyokawa, Tachibana, Toyama, Taira, & Yasuda, 2011). The high resistance of whey proteins to enzymatic digestion can be explained by the stability of the tertiary structure (Reddy, Kella, & Kinsella, 1988). Therefore, some researchers have added physical treatments, such as pressure and heating, in order to increase the hydrolysis of whey proteins (Patel et al., 2016). Here, we showed that the hydrolytic action of DpGp on β-lactoglobulin was significantly increased after heat treatment (Fig. 4). Accordingly, the hydrolysis of whey proteins by trypsin and pepsin were slightly increased after the same treatment (Adjonu, Doran, Torley, & Agboola, 2013). It

is worth noting that heat treatment can also promote the formation of aggregates of β-lactoglobulin, thereby decreasing the hydrolysis performance by hindering the cleavage sites. Therefore, increasing/decreasing β-lactoglobulin hydrolysis during thermal treatment is affected by the temperature and the duration of heating (Leeb, Götz, Letzel, Cheison, & Kulozik, 2015).

The pH is another important parameter to be considered for effective protein hydrolysis by peptidases. DpGp showed better hydrolytic action on milk proteins at pH 9.0 (optimal pH) than at pH 6.5 (milk pH), corroborating other studies that showed better hydrolytic action when the assays were performed at optimal pH of enzymes. Lakshman and collaborators (2011) tested an acid peptidase from *Monascus pilosus* to reduce the antigenicity of milk whey protein and the pH of the reaction mixtures was maintained at 3.0. In another work, whey proteins were hydrolyzed by pepsin, chymotrypsin and trypsin always using enzyme working pH (Adjonu et al., 2013). Therefore, there is no discrepancy if the optimal pH of enzyme is different from milk pH, because after the hydrolysis, the milk pH can be adjusted to neutrality with NaOH or HCl.

Different commercial hypoallergenic milk formulas were used as standards to compare with the milk protein hydrolysate obtained by DpGp (Fig. 5). Residual peptides from the casein fraction were not detected by the ELISA at all, similar to commercial hydrolyzed milk formulas. However, peptides from whey proteins were detected, although in a much lower proportion when compared to commercial non-hydrolyzed milk and the partially hydrolyzed milk formula Enfamil® (Fig. 5). Rosendal & Barkholt (2000) studied potentially allergenic peptides in 12 commercially available hydrolyzed milk formulas, all of which are listed by the European Society of Pediatric Allergy and Clinical Immunology. They concluded that proteins with molecular masses above 20 kDa were detected in some products, including extensively hydrolyzed formulas. Additionally, they found residual antigenic β-lactoglobulin in all products

by ELISA. Therefore, after performing hydrolysis with peptidases, it is very important to use complementary techniques such as ultrafiltration to eliminate any potential remaining allergenic peptides (Quintieri, Monaci, Baruzzi, Giuffrida, Candia, & Caputo, 2017).

In this study, the gut peptidases of the Monarch caterpillar were found to be a suitable source of proteolytic activity for CMP processing. The genomic data of the Monarch is available; therefore, given the availability of genomic information on their peptidases, heterologous expression in desired cellular systems would be feasible.

5. Conclusion

Gut peptidases of the Monarch caterpillar efficiently digested cow milk proteins and would be suitable for producing partially hydrolyzed milk formulas. Further studies could extend their usefulness to produce fully hydrolyzed milk formulas and other related applications.

Conflict of interest

The authors confirm that the contents of this article impose no conflicts of interest.

Contributions

JPBO and CDTF performed the main research work in this study, including gut peptidase purification, gel electrophoresis, proteolytic activity assays and hydrolysis assays. FESL, ICS and JSO produced polyclonal antibodies and performed ELISA. MDPL and ACOMM performed the proteomic analysis. CDTF and MVR contributed to data analysis, discussion and wrote the manuscript.

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Figure Captions

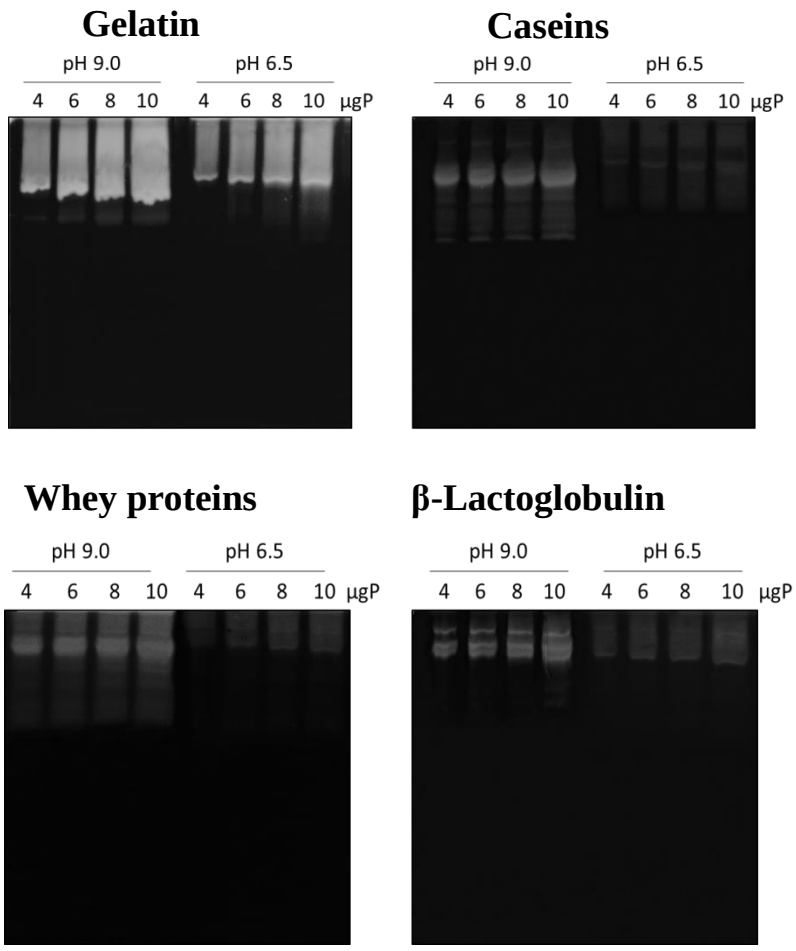
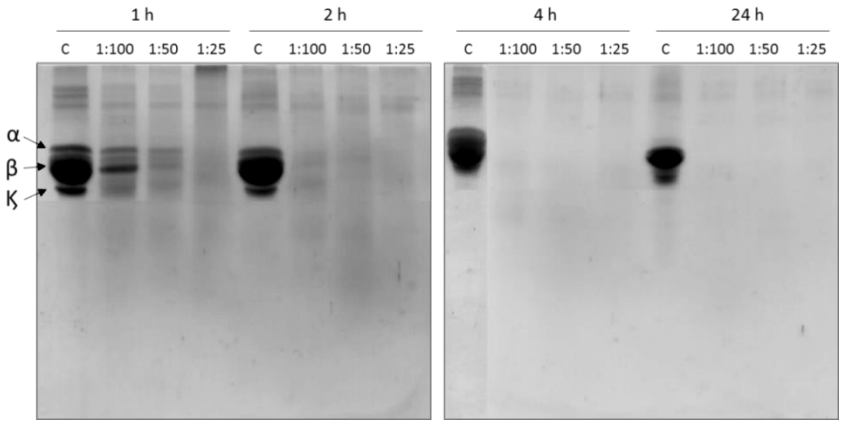


Fig. 1. Zymograms for proteolytic activity detection in gels containing gelatin, mixed caseins (α-, β-, and κ-caseins), whey proteins (α-lactoalbumin and β-lactoglobulin) or purified β-lactoglobulin as substrates at 0.2%. After 1D-SDS-PAGE, the gels were washed with Triton X-100 and incubated for 1 h at 37 °C at pH 9.0 or 6.5. Transparent bands indicate protein hydrolysis by DpGp.

(a)



(b)

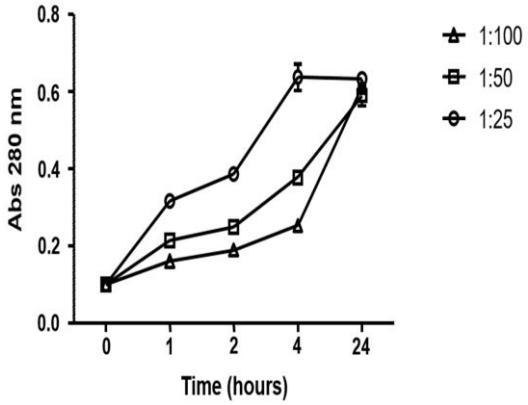


Fig. 2. Casein hydrolysis by DpGp performed at 37 °C and pH 9.0. **(a)** 1D-SDS-PAGE (15%) of the casein hydrolyzed at different time points and enzyme:substrate ratios (1:100, 1:50 and 1:25); 50 µg of protein was added to each well. C: non-digested casein. (**b**) The same samples in **(a)** were used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

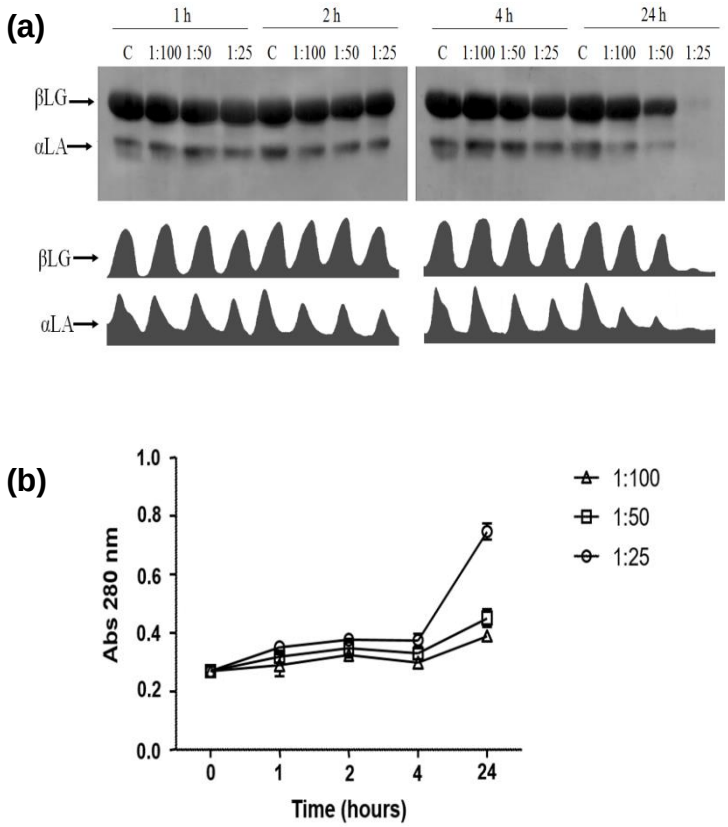


Fig. 3. Whey protein hydrolysis by DpGp performed at 37 °C and pH 9.0. **(a)** 1D-SDS-PAGE (15%) of the whey protein hydrolyzed at different time points and enzyme:substrate ratios (1:100, 1:50 and 1:25); 50 µg of protein was added to each well. C: non-digested whey proteins. βLG: β-lactoglobulin. αLA: α-lactoalbumin. The degree of hydrolysis was quantified by densitometry using the IMAGEJ software (lower panel). (**b**) The same samples in (**a**) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

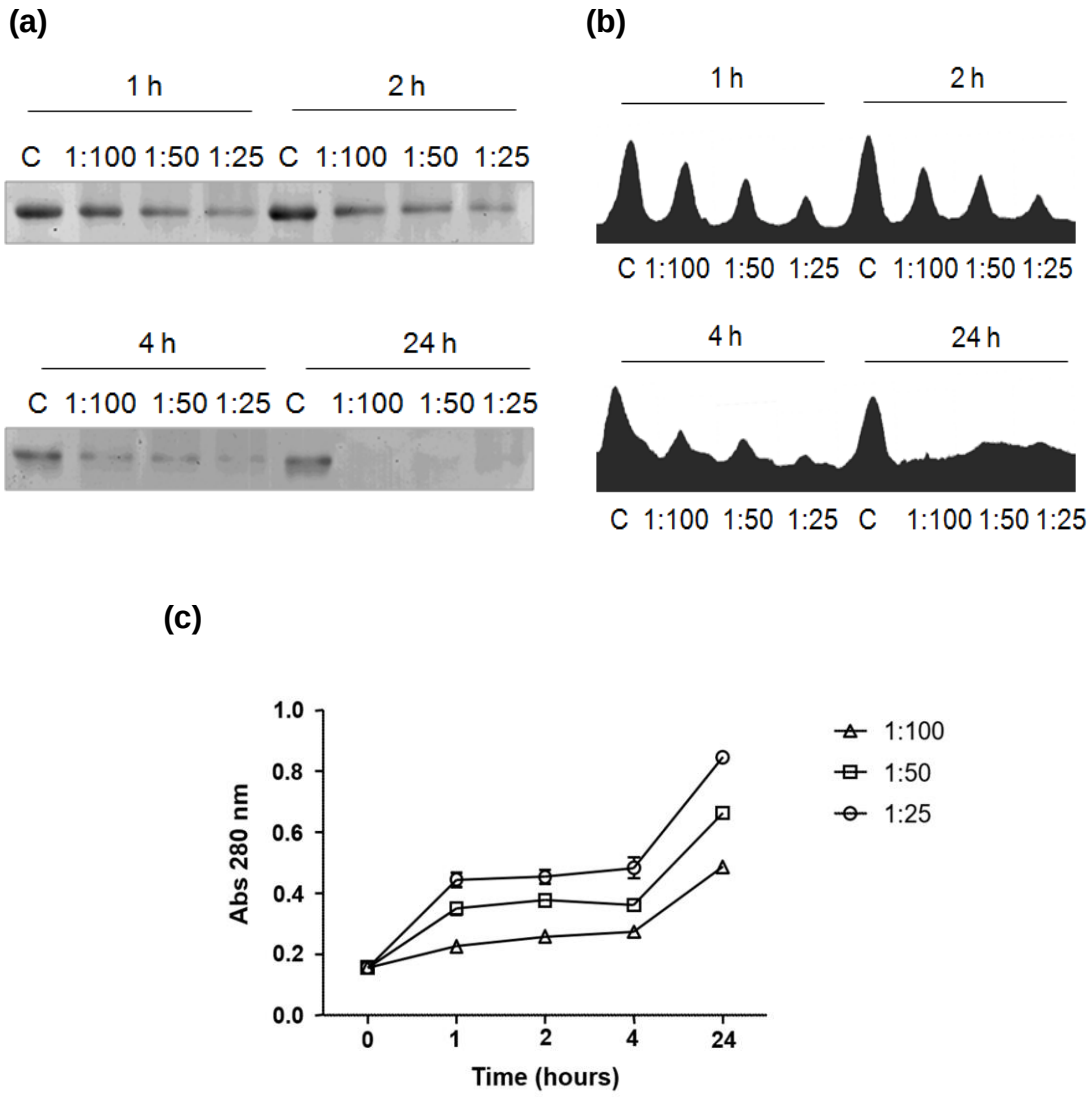


Fig. 4. Heated β-lactoglobulin hydrolysis by DpGp performed at 37 °C and pH 9.0. **(a)** 1D-SDS-PAGE (15%) of the heated β-lactoglobulin hydrolyzed at different time points and enzyme:substrate ratios; 50 µg of protein was added to each well. C: non-digested heated β-lactoglobulin. **(b)** Densitometry of the protein bands in (**a**) using the IMAGEJ software. **(c)** The same samples in (**a**) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

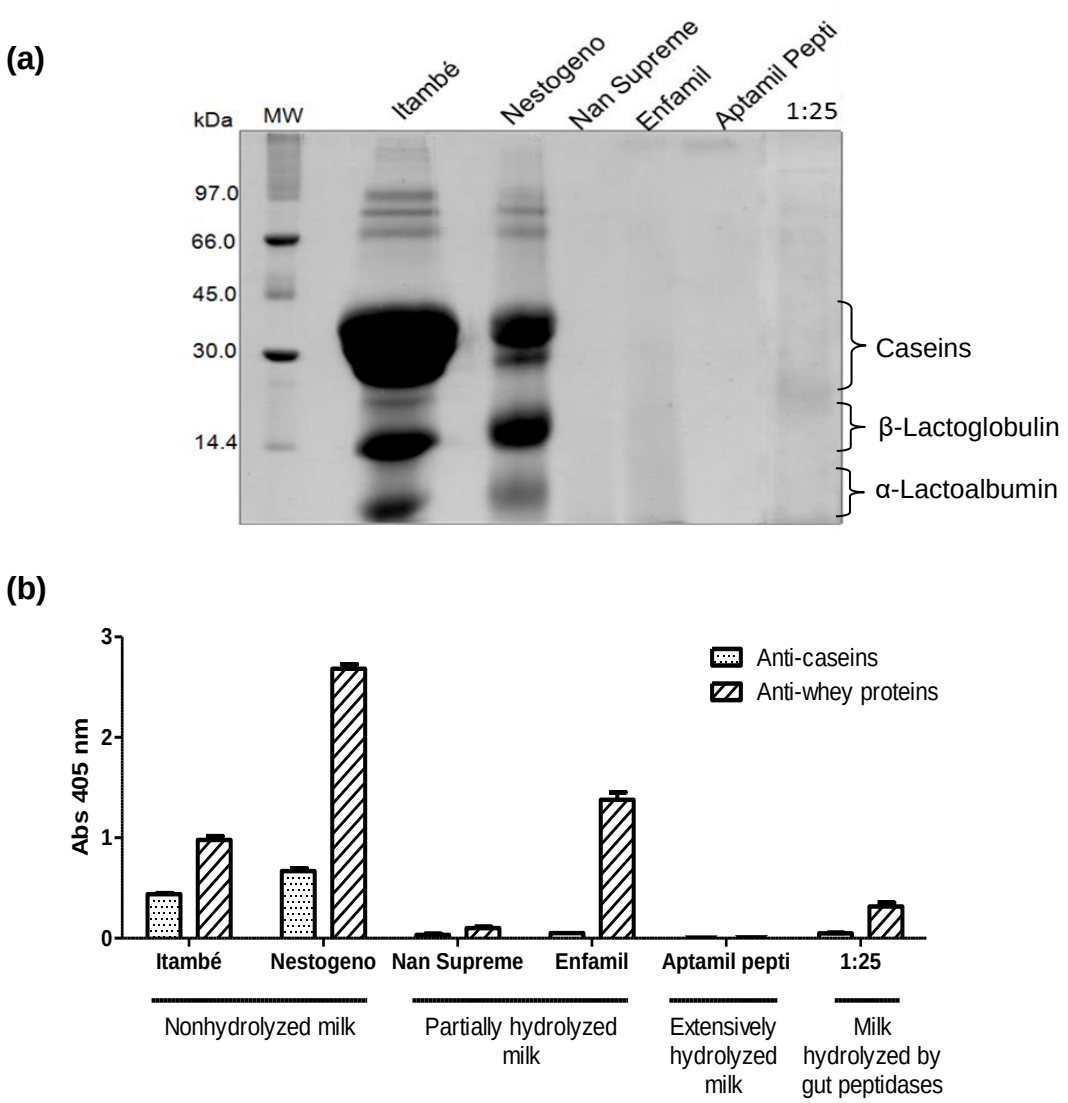
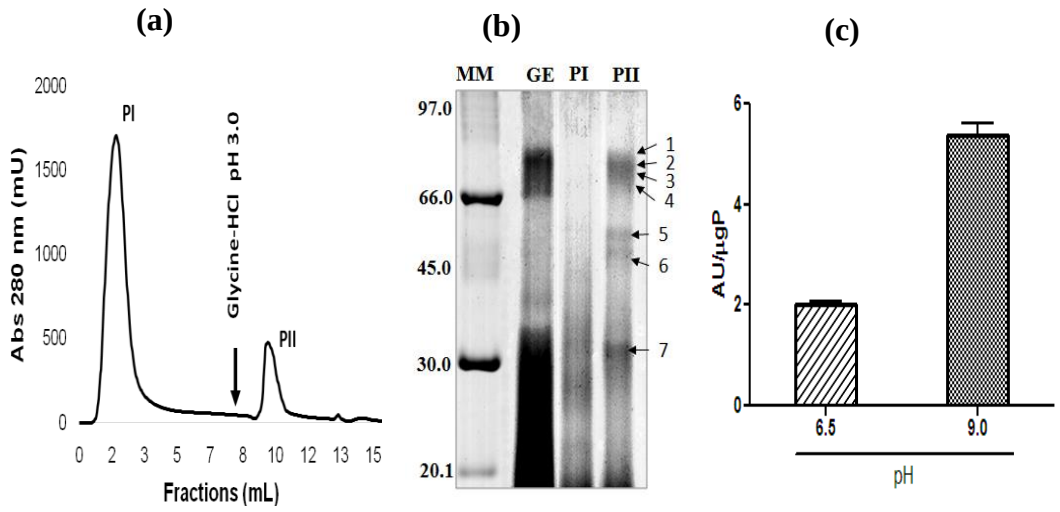
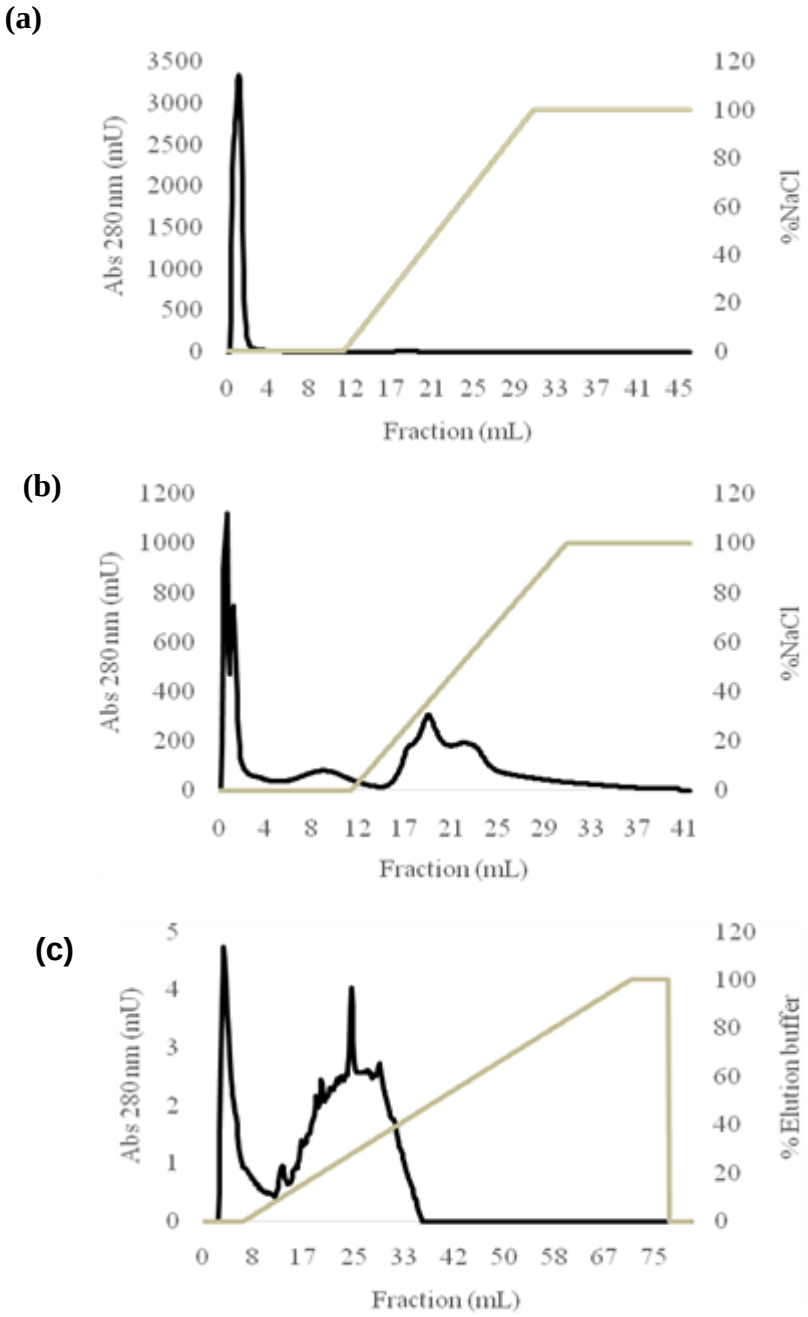


Fig. 5. Comparison between commercial hydrolyzed milk formulas with hydrolysate produced by DpGp (1:25 ratio).(**a**) Protein profile of all formulas was performed by 15% 1D-SDS-PAGE. The same amount of protein was added protein to each well (40 µL, 20 mg/mL). (**b**) ELISA comparing the residual immunogenicity of the milk formulas using anti-caseins or anti-whey protein antibodies. Itambé® milk (non-hydrolyzed milk) was incubated with DpGp (1:25 ratio) for 24 h at 37 ºC and pH 9.0. This sample represents the well 1:25 in (**a**) and milk hydrolyzed by gut peptidases in (**b**).

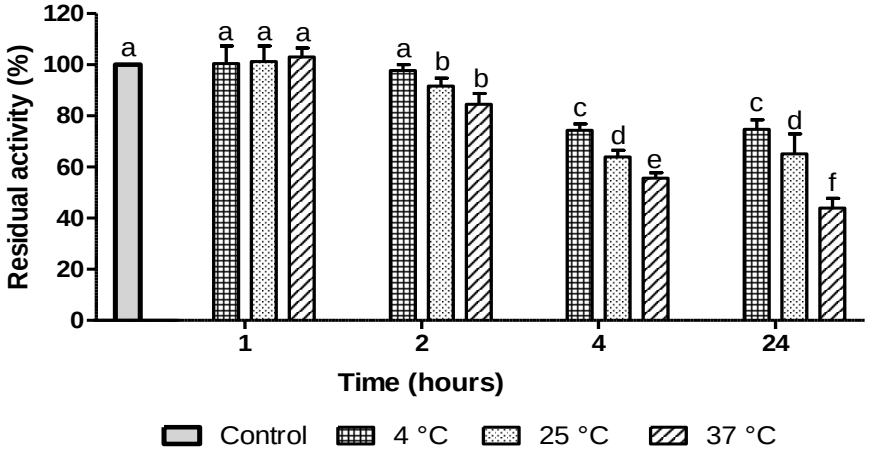


Supplementary Fig. 1. (a) Chromatographic profile on a HiTrap™ Benzamidine Sepharose 4 Fast Flow column of the gut protein extract from *Danaus plexippus.* (**b**) 1D-SDS-PAGE (15%) of the gut protein extract (GE) and fractions (PI and PII) obtained by affinity chromatography. Numbers represent the bands removed, destained and digested with sequencing-grade modified trypsin for identification by mass spectrometry. (**c**) Proteolytic activity of the purified peptidases (PII peak) at pH 6.5 or pH 9.0, using 1% azocasein as the substrate at 37 ºC. In (a), the arrow indicates where the 50 mM glycine-HCl (pH 3.0) buffer was added to elute the peptidases. In (**c**), the values represent the mean of three independent experiments and the standard deviation of the mean.

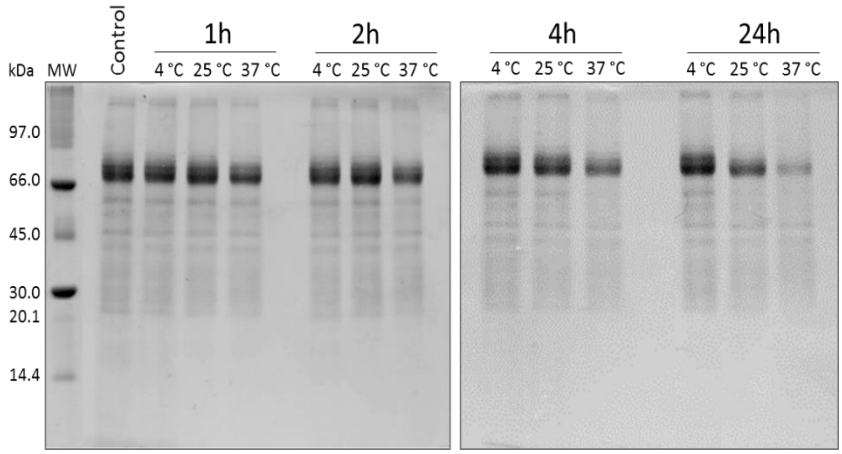


Supplementary Fig. 2. Chromatographic profiles of the PII fraction from HiTrap™ Benzamidine Sepharose 4 Fast Flow column on CM-Sepharose (A), DEAE-Sepharose (B), and C2/C18 columns (C). In A and B, the columns were equilibrated with 25 mM Tris-HCl buffer (pH 7.0) and the proteins were eluted from the column by using a linear gradient from 0 up to 1 M NaCl at 1 mL/min flow rate. In C, the proteins were submitted to reversed-phase high-performance liquid chromatography (RP-HPLC). The proteins were eluted using a linear gradient from 5% to 80% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate.

(a)

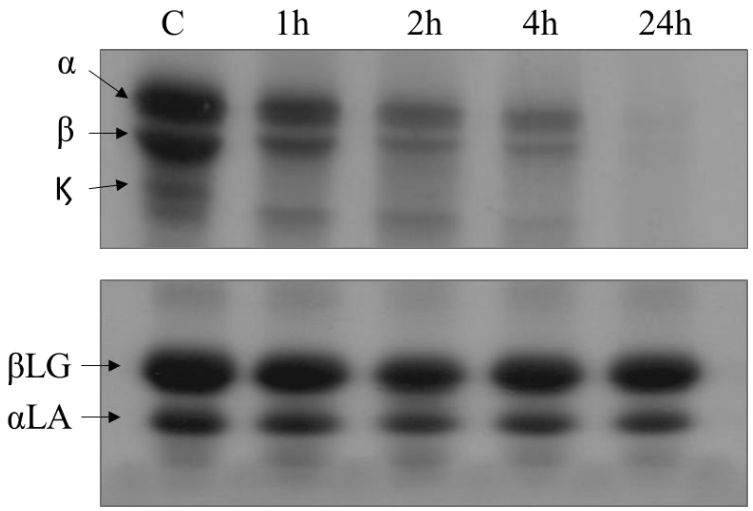


(b)

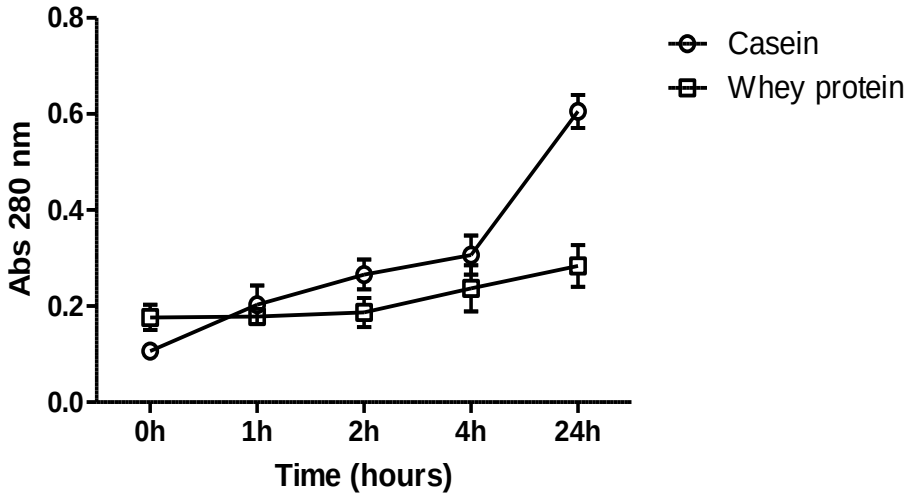


Supplementary Fig. 3. Effect of time and temperature on proteolytic activity and autolysis of *Danaus plexippus* gut peptidases (DpGp) analyzed by *in vitro* assays (**a**) and 15% 1D-SDS-PAGE (**b**). DpGp, dissolved at pH 9.0, were incubated at different temperatures and aliquots were taken at different time points. The residual proteolytic activity was assessed using 1% azocasein as the substrate at pH 9.0 and 37 ºC. Each value represents the mean of three independent experiments and the standard deviation of the mean. Different letters represent significant differences ($p<0.05$) between the indicate group and the control. The same samples from the *in vitro* assays (**a**) were analyzed by electrophoresis (**b**).

(a)



(b)



Supplementary Fig. 4. Milk protein hydrolysis by DpGp performed at 37°C and pH 6.5. (a) 1D-SDS-PAGE (15%) of the caseins and whey proteins hydrolyzed at different times and an enzyme:substrate ratio of 1:25. Each well received 50 µg of proteins. C: non-digested proteins. α: α-casein. β: β-casein. Ķ: Ķ-casein. βLG: β-lactoglobulin. αLA: α-lactalbumin. (b) The same samples in (a) were also used to quantify the degree of hydrolysis at 280 nm. Each value represents the mean of three independent experiments and error bars indicate the standard deviation of the mean.

Supplementary Table 1. List of proteins identified in gut extract from *Danaus plexippus* by ESI-QUAD-TOF.

[1] The indicated numbers were from 1D SDS-PAGE (Supplementary Fig. 1b). [2]The theoretical molecular masses and pI values were those of the proteins represented in the ID (NCBI) column

[1]Spots	[2]Theoretical		Protein Score	Sequences of all identified peptides	ID (NCBI)	Protein description [Organism]
	Molecular mass (kDa)	pI				
1	26.549	6.8	140	RLGEHNIDVLEGNEQFINAAKI KLSSPATLNSRV	NP_001156363.1	Trypsin precursor [*Sus scrofa*]
1	26.748	4.9	83	KLSSPATINSRV	XP_004661211.1	Anionic trypsin-like [*Jaculus jaculus*]
2	26549	6.8	140	RLGEHNIDVLEGNEQFINAAKI KLSSPATLNSRV	NP_001156363.1	Trypsin precursor [*Sus scrofa*]
2	26.748	4.9	83	KLSSPATINSRV	XP_004661211.1	Anionic trypsin-like [*Jaculus jaculus*]
3	27.749	8.2	99	RTSIPAQGSVIPANLPVVHAGWGTTSSGGQLSSVLRD	OWR45697.1	Serine protease [*Danaus plexippus plexippus*]
4	90.481	8.7	145	KLLTENLDWIIIPLANPDGYEYSINEDRL RNFDFDWGSRPDSNIACSIIYEGPSPFSEPETRI	OWR48083.1	Carboxypeptidase [*Danaus plexippus plexippus*]
5,6	46.251	4.7	70	KIVLYDPIFTTYGETVVYRS	KPJ10556.1	Carboxypeptidase [*Papilio machaon*]
5,6	47.436	3.7	65	KYVQYNDLIQPIRI	OWR55096.1	Trypsin serine proteinase [*Danaus plexippus plexippus*]
7	27.749	8.2	99	RTSIPAQGSVIPANLPVVHAGWGTTSSGGQLSSVLRD	OWR45697.1	Serine protease [*Danaus plexippus plexippus*]
7	31.952	6.5	130	RIITTDVVTHPLWIPQLTFNDIAMVRL KGDTGGPLVVMRN	OWR49146.1	Chymotrypsin protein [*Danaus plexippus plexippus*]

Allergenicity reduction of cow's milk proteins using latex peptidases

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Abstract

The present study evaluated four laticifer fluids as new source of peptidases able to hydrolyze cow's milk proteins. The latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) were able to perform total hydrolysis of caseins, after 30 min at pH 6.5, confirmed by a significant reduction in the residual antigenicity. Casein hydrolysis by *Plumeria rubra* latex peptidases (PrLP) was negligible. On the other hand, whey proteins were more resistant to proteolysis by latex peptidases. However, the heat pretreatment of the whey proteins enhanced the degree of hydrolysis and reduced the residual antigenicity of the hydrolysates. The *in vivo* assays showed that the cow's milk proteins hydrolyzed by CgLP and CapLP did not exhibit immune reactions in mice allergenic to cow's milk, similar to a commercial partially hydrolyzed formula. Thus, these peptidases are promising enzymes to develop novel hypoallergenic formulas for children with milk allergy.

Keywords: Allergy. Laticifer. Protein. Protease.

1. Introduction

Peptidases are used in a wide range of industrial processes. Currently, the world market for these enzymes is on the order of billions of dollars annually (Singh, Mittal, Kumar & Mehta, 2016). The growing interest in these proteolytic enzymes is driven by their versatility, specificity, stability, and high efficiency. Moreover, the use of peptidases represents an eco-friendly alternative compared to synthetic catalysts (Errasti, Caffini & López, 2018).

The hydrolytic action of peptidases has been applied to achieve chemical modifications in various foods, generating new products with improved sensory and nutritional quality, or even producing bioactive peptides (Giacometti & Buretic-Tomljanovic, 2017). Additionally, peptidases can be also used to reduce food allergenicity. Cow's milk proteins are among the primary cause of food allergy in infants and young children (Sicherer & Sampson, 2014). This immunological reaction can result in gastrointestinal, respiratory and dermatological problems, affecting children (Fiocchi et al., 2010). The only approved therapy for food allergy is diet restriction, where a dairy substitute should be employed. Therefore, hypoallergenic cow's milk formulas are widely used (Souroullas, Aspri & Papademas, 2018), and extensively hydrolyzed formulas or amino acid formulas are among the most effective alternatives employed in clinical practice. However, their usage is limited due to their high costs (Fiocchi et al., 2018). Although peptidases from microbial, insect, plant and animal sources have been studied to hydrolyze cow's milk proteins, searches for new proteolytic enzymes are still important since some peptidases have exhibited technical drawbacks, such as low yield and activity or very limited hydrolytic action on milk proteins.

Latex is a milky plant fluid composed of a complex mixture of molecules, including proteolytic enzymes. The peptidase content in some latex samples can reach 90% of all proteins (Zare, Moosavi-Movahedi, Salami, Mirzaei, Saboury & Sheibani, 2013). This feature has

enabled the prospection of these enzymes in different biotechnological approaches, such as dehairing of leather (Lopéz et al., 2017) and milk clotting (Freitas et al., 2016). In this respect, our hypothesis is that latex peptidases can be efficient molecules to hydrolyze cow's milk proteins, producing hypoallergenic formulas. Therefore, the present work studied the proteolytic action of different latex peptidases on cow's milk proteins as well as it was evaluated the *in vivo* allergencity of the hydrolyzed proteins in a validated IgE-mediated food allergy mouse model to cow's milk proteins.

2. Material and methods

2.1 Reagents

Acrylamide (17-1302-02), bis-acrylamide (17-1304-02), sodium dodecyl sulfate (SDS) (17-1313-01), a HiTrap Protein-A Sepharose high performance column (17-0402-01), and Superdex 75 Increase 10/300 GL (29148721) were acquired from GE Healthcare Life Sciences (São Paulo, SP, Brazil). Azocasein (A2765), bromelain (code B4882), L-cysteine (C7352), O-phthaldialdehyde (P0657), β-mercaptoethanol (M6250), Evans blue dye (E2129), cholera toxin from *Vibrio cholerae* (C8052), Freund's complete (F5881) and incomplete adjuvant (F5506), alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (A6066) and *p*-nitrophenylphosphate disodium (N2765) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). All other chemicals were of analytical grade.

2.2 Plant material and proteolytic activity

Latex proteins (LP) from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), *and Plumeria rubra* (PrLP) were collected by cutting the ending branches of each plant as described by Freitas et al. (2007; 2010), whereas *Carica papaya* (CapLP) latex proteins were obtained from the green fruits as reported by Souza et al. (2011). The latex fluids were collected in distilled water (1:1 ratio) and the rubber was separated by centrifugation (10,000 x g at 4 °C for 10 min). The supernatants were dialyzed against distilled water at 4 °C using membranes with 8 kDa cut-off for two days, lyophilized and stored for further analysis. The total proteolytic activity of each latex protein (LP) fraction was determined using 1% azocasein as non-specific substrate at pH 6.5 (milk pH) according to Freitas et al. (2007). For all experiments, bromelain (EC 3.4.22.32), a cysteine peptidase from pineapple stem, was used as positive control.

2.3 Cow's milk protein purification

Caseins and whey proteins were purified as described by Oliveira et al. (2018). Briefly, whole bovine milk obtained from a local market (Fortaleza, Ceará, Brazil) was skimmed by centrifugation (2,100 x g at 25 °C for 30 min), and the supernatant was acidified with 1 M HCl up to pH 4.6. The caseins and whey proteins were separated by centrifugation (1,500 x g at 20 °C for 20 min): the supernatant (whey proteins) was collected and separated, and the precipitate (sodium caseinate) was then washed three times with distilled water, followed by centrifugation. Both fractions were dialyzed against distilled water at 4 °C for 48 h using dialysis membranes with 8 kDa cut-off and then lyophilized.

2.4 Hydrolysis of milk proteins

2.4.1 Casein hydrolysis

The hydrolysis of the caseins was performed by incubating different aliquots (10, 15, 20, 25 and 30 µL) of each LP fraction (2 mg/mL in 50 mM Tris-HCl buffer pH 6.5 containing 1 mM L-cysteine) with 450 µL of caseins (10 mg/mL in Tris-HCl buffer pH 6.5) and the final volume was adjusted to 500 µL with the same buffer. The reactions were performed at 37 °C for 30 min and then aliquots were retrieved to measure the extensiveness of hydrolysis by 15% SDS-PAGE (Oliveira et al., 2018). The hydrolysis degree was also measured using O-phthaldialdehyde (OPA) reagent according to Church, Swaisgood, Porter and Catignani (1983). The OPA reagent was prepared as follows: 25 mL of 100 mM sodium tetraborate; 2.5 mL of 20% SDS; 40 mg of OPA dissolved in 1 mL of methanol; 100 µL of β-mercaptoethanol and final volume adjusted to 50 mL with distilled water. Aliquots of 50 µL of the casein hydrolysates were mixed with 1 mL of the OPA reagent and the absorbance was measured after two minutes at 340 nm (Church et al., 1983).

2.4.2 Whey protein hydrolysis

Because whey proteins have been reported to be more resistant to proteolysis, the assays were performed using the highest concentration of LP and during different incubation times. We mixed 30 µL of each LP fraction (2 mg/mL in Tris-HCl buffer pH 6.5 containing 1 mM L-cysteine) with 450 µL of whey proteins (10 mg/mL in 50 mM Tris-HCl buffer pH 6.5), and adjusted the final volume to 500 µL with the same buffer. The reactions were performed at 37 °C and the extensiveness of the hydrolysis was monitored at different time points (1, 2, 4 and 24 h) by 15% SDS-PAGE and size exclusion chromatography.

For SDS-PAGE analysis, the hydrolyzed proteins were mixed with sample buffer [0.0625 M Tris buffer (pH 6.8) containing 2% SDS]. Runs were performed at 25 mA at 25 ºC and 2 h, gels were stained with Coomassie brilliant blue (R-350) solution in water:acetic acid:methanol (7:1:2, v:v:v) and de-colored with the same solution without the dye (Oliveira et al., 2018).

For chromatographic assays, the whey protein hydrolysates (500 µL) were loaded in a Superdex-75 (10/300 GL) column previously equilibrated with 50 mM Tris-HCl buffer pH 6.5 coupled to a high-performance liquid chromatographic system (AKTA purifier, GE Healthcare). The proteins were eluted with 50 mM Tris-HCl buffer (pH 6.5) at a 0.3 mL/min flow rate and the peaks were monitored at 280 nm.

To improve the hydrolysis degree of whey proteins by latex peptidases, the whey proteins were also preheated at 85 °C for 30 min and incubated with each latex fraction for 24 h, as described before. Hydrolysis yield was analyzed by 15% SDS-PAGE and ELISA.

2.5 Polyclonal antibody production and enzyme-linked immunosorbent assay (ELISA)

Rabbit polyclonal antibodies against bovine caseins and whey proteins were produced according to Oliveira et al., (2018). Briefly, the animals were sensitized intramuscularly either with caseins or whey proteins (1 mg dissolved in 0.5 mL saline and 0.5 mL complete Freund's adjuvant) and booster injections (1 mg of caseins or whey proteins dissolved in 0.5 mL saline and 0.5 mL incomplete Freund's adjuvant) were administered subcutaneously after 21, 35 and 42 days. The immunoglobulins were purified using Protein A immobilized in a Sepharose 4B column as described by Freitas et al. (2017).

The residual antigenicity of the caseins and whey protein (heated or unheated) hydrolysates was measured using ELISA according to Oliveira et al. (2018). The samples were added to 96-well microplates and stored overnight at 4 °C. Unbound proteins were washed and the empty sites were blocked with gelatin (10 g/L). Anti-caseins and anti-whey protein polyclonal antibodies (1:20,000 dilution) were used as primary antibodies whereas alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000 dilution) was used as secondary antibody. The reaction was detected using *p*-nitrophenyl phosphate disodium as a substrate, and the absorbance was measured at 405 nm. Enfamil® Gentlease® formula (Mead Johnson Nutrition) was used as the control for partially hydrolyzed cow's milk (PHM) and whole cow's milk as non-hydrolyzed milk (NHM).

2.6 Mice sensitization and challenge

Six- to eight-week-old male mice were purchased from the School of Animal Sciences, University of La Plata, Argentina, and kept under pathogen-free conditions with water and commercial diet provided *ad libitum*, at 20 ºC, 70% relative humidity, and 12 h photoperiod. The sensitization protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals, of the School of Sciences of the UNLP (CICUAL-FCE, Argentina) (protocol #017-10-15) and performed according to Candreva, Smaldini, Curciarello, Fossati, Docena and Petruccelli (2016). Briefly, mice were grouped into sensitized (n=20) and control group (n=5). Sensitized groups received 6 weekly intragastric doses of cow's milk proteins (20 mg/dose) with cholera toxin (10 µg/dose) dissolved in 200 µL of 125 mM bicarbonate buffer. Mice from the control group received only cow's milk proteins (20 mg/dose). Ten days after the final boost, sensitized mice were intragastrically challenged with 20 mg of whole cow's milk proteins (NHM) or with 20 mg of whole cow's milk protein hydrolyzed samples by

different latex peptidases (CpLP, CgLP and CapLP). Sixty micrograms of each LP was used to hydrolyze 4.5 mg of whole cow's milk protein (casein + whey proteins) preheated for 30 min at 85 ºC. The reactions were performed for 24 h at 37 ºC, and the material was lyophilized. Enfamil® Gentlease® formula (Mead Johnson Nutrition) (PHM) was used as control for partially hydrolyzed cow's milk. Sensitization was controlled by measuring milk-specific IgE antibodies in serum by ELISA as described before (Smaldini et al., 2012).

2.7 In vivo evaluation of allergenic reactions

Symptoms were evaluated immediately after the oral challenge in sensitized and control mice and scored according to Table 1. The mice were observed 30 min after the oral challenge in blinded fashion, and two independent investigators assigned the scores. Cutaneous tests were performed according to Candreva et al., (2016).

Mice from sensitized and control groups received subcutaneous injection of the non-hydrolyzed (NHM) and cow's milk proteins hydrolyzed by CpLP, CgLP and CapLP in the footpad (1 µg/µL or 20 µg in sterile saline) and then they received an intravenous injection of 100 µL of 0.1% Evans blue dye. Saline buffer was injected in all animals in the contra lateral footpad as control. Blue color observed in the skin pad a few minutes after the injection, was considered a positive cutaneous test. The footpad swelling was quantified using a digital micrometer with a minimum increment of 0.01 mm.

2.8 Statistical analysis

The data were expressed as mean ± SD of three independent assays. Statistical analyses were performed using the GraphPad Prism 5 software, using ANOVA followed by multiple comparison by the Student–Newman–Keuls test. In all tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1 Hydrolysis of milk proteins by latex peptidases

Figure 1 shows the hydrolytic potential of different latex peptidases on bovine caseins. We found a differential hydrolysis of bovine caseins during 30 min, according to the peptidase employed. As can be seen in Fig. 1a, the peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP) and *Carica papaya* (CapLP) latex rendered extensive processing of caseins, even at the lowest concentration assessed (20 µg LP to 4.5 mg caseins; 1:225 ratio). Similar results were observed for bromelain, a peptidase used as a positive control. On the contrary, *P. rubra* (PrLP) latex peptidases were unable to hydrolyze casein even at the highest concentration (60 µg LP to 4.5 mg casein; 1:75 ratio). The degree of casein proteolysis by different latex peptidases was also quantified by the OPA method (Fig. 1b). We observed concentration-dependent hydrolysis for CpLP, CgLP, CapLP, and bromelain. The maximum hydrolysis was reached by using 60 µg of each fraction. CapLP exhibited the highest degree of casein hydrolysis followed by bromelain, CgLP, and CpLP. In concordance with the results depicted in Fig. 1a, PrLP showed no significant proteolysis, confirming its inability to hydrolyze the bovine caseins (Fig. 1b).

A similar analysis was carried out with whey proteins and it was found that this milk fraction was resistant to proteolysis using latex peptidases, even at the highest concentration (60 µg LP to 4.5 mg whey protein; 1:75 ratio). SDS-PAGE patterns showed that only CapLP exhibited substantial hydrolysis on β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA). The hydrolysis could be observed after 2 h of treatment and proceeded to almost complete hydrolysis after 24 h (Fig. 2). These findings were confirmed by size exclusion chromatography. We observed a reduction of the eluted components in the chromatographic peaks corresponding to β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA) hydrolyzed with

CapLP (around 90%). The other peptidases rendered no significant hydrolysis of whey proteins after 24 h of incubation. These results were similar to those observed with bromelain, which were better monitored by chromatography analysis than by SDS-PAGE. PrLP was unable to perform any hydrolysis of whey proteins (Fig. 2).

3.2. In vitro residual antigenicity

The *in vitro* residual antigenicity of the hydrolyzed caseins and whey proteins was evaluated by ELISA using anti-casein and anti-whey protein polyclonal antibodies, respectively (Fig. 3a and 3b). The immunological recognition of the remaining casein peptides after 30 min of treatment was significantly reduced when CpLP (2%), CgLP (1%), CapLP (2%) and bromelain (1%) were used (1:75 ratio), compared to untreated caseins (control). Treatments rendered a higher degree of hydrolysis compared to the commercial partial hydrolysate employed as control (PHM) ($p < 0.05$). In contrast, we found a high residual antigenicity when caseins were treated with PrLP (Fig. 3a).

Whey proteins were assessed with the same method and the residual antigenicity after 24 h of incubation (1:75 ratio, enzyme: substrate) was partially reduced. We observed a significant reduction of antigenicity with CpLP, CgLP, and bromelain compared to untreated whey proteins, similar to the commercial partially hydrolyzed milk formula (PHM). The residual antigenicity values after 24 h of proteolysis were: 78% for CpLP, 71% for CgLP, 31% for CapLP, 62% for bromelain and 50% for PHM, when compared to non-hydrolyzed whey proteins (100%) ($p < 0.05$). This assay again showed that PrLP did not reduce whey protein antigenicity (Fig. 3b) ($p > 0.05$).

3.3 Analysis of hydrolysis of heated whey proteins

Whey proteins were pre-treated (85 °C for 30 min) to enhance proteolysis by latex peptidases (Fig. 4). The hydrolysis of β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA) by CapLP and bromelain were greatly enhanced when compared with results obtained with unheated proteins (Fig. 2). SDS-PAGE showed a large number of peptides with reduced relative molecular weight (Fig 4a) and significantly reduced antigenicity when samples were treated with CpLP, CgLP and bromelain (Fig. 4b). PrLP did not cleave preheated whey proteins, as showed by SDS-PAGE. The ELISA data were consistent with SDS-PAGE patterns, thus suggesting that temperature enhanced the hydrolysis of whey proteins with CapLP, CpLP and CgLP.

3.4 In vivo residual allergenicity

The viability of different latex peptidases as enzymatic sources to hydrolyze the cow's milk proteins was also assessed using a milk-specific food allergy mouse model. IgE production was monitored by assessing milk-specific IgE in serum at days 0, 20 and following the oral challenge by ELISA (data not showed). Allergenicity of the residual hydrolysates was evaluated by oral challenge and skin testing in sensitized and control mice. Samples assessed were those of whole milk proteins hydrolyzed with peptidases that cleaved milk fractions (*in vitro* analysis).

Clinical scores of challenged mice were calculated according to symptoms elicited immediately after the gavage, according to Table 1. As observed in Fig. 5a, cow’s milk proteins hydrolyzed by CgLP and CapLP developed no clinical scores compared with oral challenge with non-hydrolyzed milk (NHM) in sensitized animals. The results were similar to commercial partially hydrolyzed milk formula (PHM). Control animals showed no symptom upon all

challenges (PBS). Milk samples treated with CpLP rendered intermediate clinical scores compared with NHM ($p < 0.05$). The animals showed scratching and rubbing around the snout and head (score 1), puffiness around the eyes and mouth, piloerection, reduced activity and/or decreased activity with increased respiratory rate (score 2), or no activity upon stimuli and convulsion (score 4). As controls, sensitized mice received by gavage NHM, PHM or PBS, and clinical scores of 4, 0 and 0 were observed, respectively.

Footpad swelling was quantified and we observed a significant reduction of inflammation when the skin test was performed with all hydrolysates compared to NHM (Fig. 5b). As can be observed, no critical adverse effect was observed with any of the hydrolysates assessed. Finally, the skin test results were consistent with the previous findings. An intense blue color was observed when sensitized animals were injected with non-hydrolyzed cow's milk proteins, representing the highest degree of inflammation. Cow's milk proteins hydrolyzed by CgLP and CapLP exhibited results similar to partially hydrolyzed milk formula (Enfamil®) (PHM) (Fig. 5c). All sensitized mice PBS injected in the contra lateral footpad rendered a negative skin test. Overall, *in vivo* results suggested that milk proteins treated with CpLP and CapLP showed the lowest reaction in the allergic mice to milk proteins.

4. Discussion

Enzymatic hydrolysis is the most promising strategy to decrease antigenicity and allergenicity of cow’s milk proteins. This enzymatic procedure disrupts lineal and conformational epitopes, preventing IgE-mediated responses (Bu, Luo, Chen, Liu & Zhu, 2013). The extensiveness of protein hydrolysis by peptidases is a critical point in the development of hypoallergenic milk formulas for allergic children. Although some plant

peptidases, such as bromelain and ficin, have been highlighted in this process (Abd El-Salan & El-Shibiny, 2017), the search for novel plant proteolytic enzymes is still the focus of numerous studies and patents. In the present study, the latex of four different species (*C. procera, C. grandiflora, C. papaya,* and *P. rubra*) was evaluated as potential enzymatic sources for cow's milk protein hydrolysis, because previous studies have reported their proteolytic potential (Freitas et al., 2007; 2010). The *C. procera, C. grandiflora* and *C. papaya* lattices are rich in cysteine peptidases, while *P. rubra* latex presents a mixture of cysteine and serine peptidases (Freitas et al., 2010). On the other hand, bromelain (a cysteine peptidase from pineapple) was used as the positive control, since previous studies have reported its capacity to hydrolyze cow's milk proteins (Medeiros, Rainha, Paiva, Lima & Baptista, 2014).

The extensiveness of protein hydrolysis can be affected by peptidase specificity, as well as the hydrolysis conditions, such as pH, temperature, ions, enzyme:substrate ratio and reaction time (Abd El-Salan & El-Shibiny, 2017). In contrast to some standard animal peptidases, such as trypsin, chymotrypsin and pepsin, the plant cysteine peptidases are able to hydrolyze proteins in several cleavage sites, promoting extensive proteolysis (Hedstrom, 2002; Choe et al., 2006). Therefore, from this standpoint, plant cysteine peptidases exhibit higher biotechnological potential to produce hypoallergenic milk formulas or other kinds of hydrolyzed foods. In hydrolysis reactions, the pH should be near to the optimum pH value of the enzyme to reach the maximum cleavage of the substrate. Previous studies have reported that *C. procera, C. grandiflora, C. papaya* and *P. rubra* peptidases have optimal enzymatic activities at pH values close to 6.5 (milk pH) (Freitas et al., 2007; 2010). Thus, proteolysis performed with latex peptidases has this advantage when compared to serine peptidases, which are more active at alkaline pH values, such as 9.0-10.0 (Oliveira et al., 2018). The presence of some ions can also represent an important element in enzymatic reaction because they can decrease the activity or even inactivate some enzymes. Calcium ions, abundant in milk, did not

affect the proteolytic activity of *C. procera*, *C. grandiflora* and *C. papaya,* even at 1 M $CaCl_2$ (Freitas et al., 2016). Time can be also limit enzymatic reactions catalyzed by peptidases, since these enzymes can be inactivated by autodigestion (Oliveira et al., 2018). Interestingly, *C. procera* and *C. grandiflora* latex peptidases did not undergo autolysis, and they were active even after 24 h of incubation at 37 ºC (Freitas et al., 2016). All these biochemical characteristics strengthen the evidence that peptidases from *C. procera*, *C. grandiflora* and *C. papaya* present high stabilities and have potential uses in the food industry.

Here, it was shown that latex peptidases from *C. procera*, *C. grandiflora* and *C. papaya* hydrolyzed bovine casein within 30 min, similar to bromelain. The residual antigenicity data confirmed that caseins were extensively hydrolyzed by these latex peptidases because anti-casein polyclonal antibodies only slightly detected the hydrolysis-resulting peptides. Furthermore, casein hydrolysates exhibited less immunoreactive peptides when compared to a commercial hypoallergenic formula (Enfamil®). A comparison between different peptidases showed that papain (peptidase from *C. papaya* latex) was more efficient than trypsin and pancreatin in the hydrolysis of bovine caseins (Luo, Pan, & Zhong, 2014). In another study, anti-casein polyclonal antibodies were used to detect the residual antigenicity of different hydrolyzed formulas, and casein components were detected even in extensively hydrolyzed formulas (Plebani et al., 1997). Since caseins are the most abundant cow's milk proteins (about 80%) and also comprise the major antigenic proteins in bovine milk (Docena, Fernandez, Chirdo & Fossati, 1996), their extensive hydrolysis is essential in the production of hypoallergenic formulas.

In contrast to caseins, whey proteins were much more resistant to proteolysis by latex peptidases. The resistance of both α-lactalbumin and β-lactoglobulin reflects their intrinsic structural features and compact structures, which are stabilized by disulfide bonds (Papiz et al.,

1986; Permyakov & Berliner, 2000). The whey protein hydrolysis by latex peptidases was only partial, and residual antigenicity was still detected in the hydrolysates, although to a lesser extent when compared to the commercial hypoallergenic formula (Enfamil®). Similar results were reported by Quintieri, Monaci, Baruzzi, Giuffrida, Candia and Caputo (2017). The authors showed by SDS-PAGE that papain hydrolyzed whey proteins, but residual antigenicity was still detected by ELISA. Other peptidases have been studied for the hydrolysis of whey proteins, but without success (Cheison, Leeb, Toro-Sierra & Kulozik, 2011). Therefore, attempts to improve the degree of hydrolysis of whey proteins are focused on changing the hydrolysis condition and/or pretreatment of the substrate (Cheison & Kulozik, 2017). Among different strategies, heat denaturation has been investigated aiming to improve their hydrolysis and consequently the decrease their residual antigenicity (Oliveira et al., 2018). Some of the whey proteins are thermolabile so heating can cause structural alterations that facilitate proteolysis (Reddy, Kella & Kinsella, 1988). In the present study, the degree of hydrolysis by latex peptidases increased after heat denaturation of whey proteins, with subsequent reduction in the residual antigenicity. Similarly, the heat treatment slightly enhanced the peptic and tryptic hydrolysis of the whey proteins (Adjonu, Doran, Torley, & Agboola, 2013). In addition, Kim and collaborators (2007) showed that reduction of the residual antigenicity of whey proteins was also improved when hydrolysis was combined with two enzymes. This result reinforces the potential of latex fluids, since they are rich in multiple peptidases. More than 100 different lattices are known to contain at least one peptidase (Domsalla & Melzig, 2008). In addition to papain, the latex of *C. papaya* (CapLP) contains three other cysteine peptidases (Mezhlumyan, Kasymova, & Yuldashev, 2003). Similarly, the latex of *C. procera* (CpLP), *C. grandiflora* (CgLP) and *P. rubra* (PrLP) possess multiple cysteine peptidases (Freitas et al. 2007; 2010).

Allergenicity of cow's milk hydrolysates was also studied using a milk-specific IgE-mediated food allergy mouse model. Studies with animal models are essential as a

biological tool to test the hypoallergenicity of processed foods or proteins. This mouse model was previously validated and we found that casein- and whey-specific IgE antibodies are elicited, although casein is the major allergen of milk. The existence of remaining IgE epitopes that could cross link IgE molecules on the surface of mast cells and basophils, and hence trigger hypersensitivity reactions immediately after the challenge, was evaluated. This is the only way to characterize the sensitizing capacity and residual immunological response of hydrolyzed formulas (Fritsché, 2003). Here, we showed that CpLP hydrolysates triggered hypersensitivity symptoms and cutaneous reaction in sensitized mice, probably caused by high residual antigenicity of whey proteins. Interestingly, cow's milk hydrolyzed by CgLP and CapLP generated weak immune responses and no symptom, similar to the commercial formula Enfamil®. There are several definitions of hypoallergenic formulas, but the most stringent says that hypoallergenic formulas must be tolerated by 90% of allergic infants, without showing any symptoms (Kleinman, Bahna, Powell & Sampson, 1991). Some studies have shown that several IgE epitopes can still remain intact after proteolysis. Based on that, even extensively hydrolyzed formulas can elicit immune reactions (Docena, Rozenfeld, Fernández & Fossati, 2002). Therefore, the implementation of complementary techniques is recommended, such as ultrafiltration, to eliminate antigenic peptides containing potential B epitopes and even intact proteins (Quintieri, Monaci, Baruzzi, Giuffrida, Candia & Caputo, 2017).

5. Conclusion

C. grandiflora (CgLP) and *C. papaya* (CapLP) latex peptidases were found to be the best enzymes to hydrolyze milk proteins. They exhibited the best performance in different *in vitro* and *in vivo* tests in terms of reduced antigenicity and allergenicity. Thus, these peptidases are promising enzymes to develop novel hypoallergenic formulas for children with milk allergy, confirming our initial hypothesis.

Conflict of interest

The authors confirm that the contents of this article pose no conflicts of interest.

Contributions

JPBO, MVR and CDTF performed the main research work, including latex peptidase purification, gel electrophoresis, proteolytic activity assays and hydrolysis assays. MBA, JSO and HDO produced polyclonal antibodies and performed ELISA. AMC and GR performed the *in vivo* assays, while GD supported and designed *in vivo* studies. All authors contributed to data analysis, discussion and writing of the manuscript.

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Figure Captions

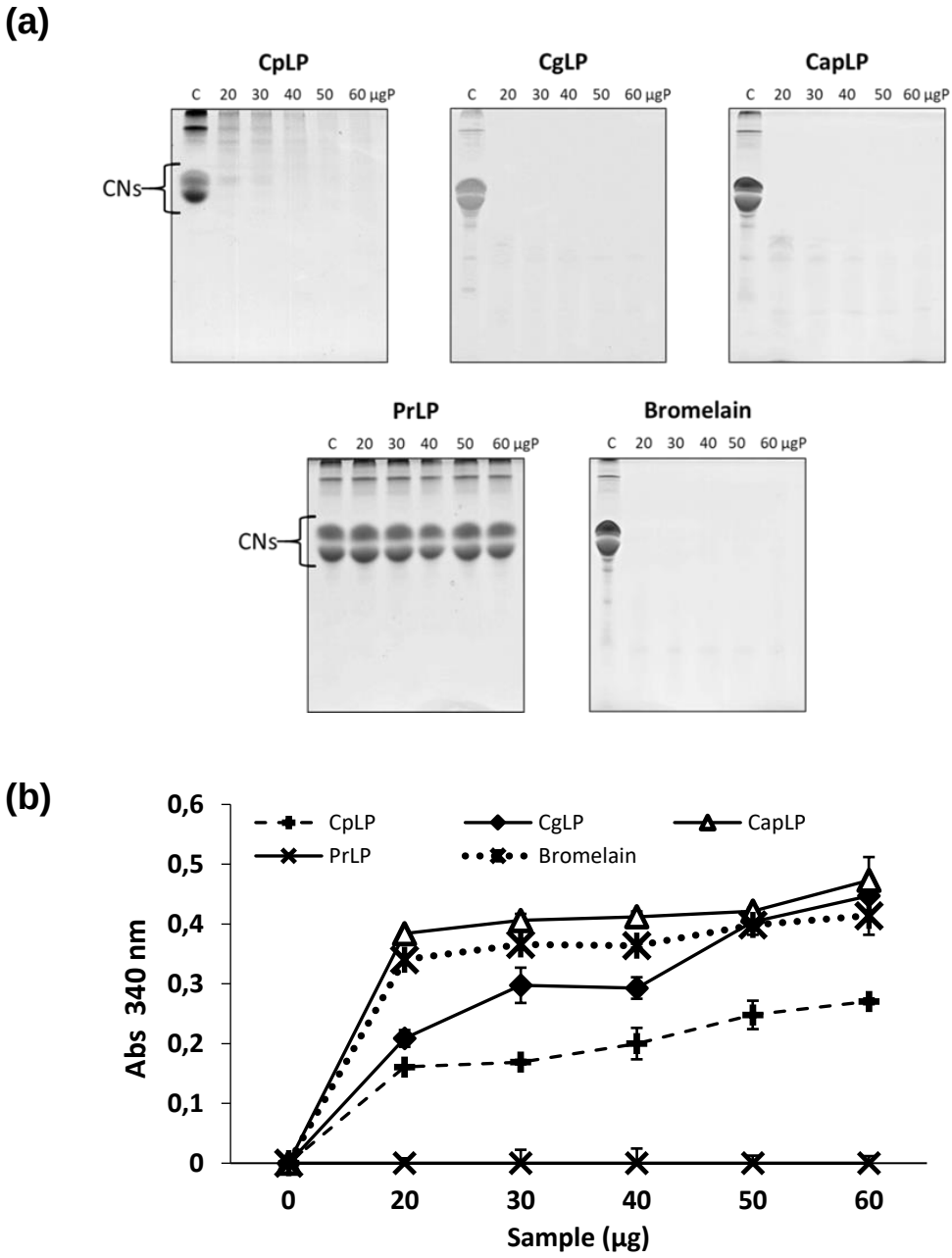

Fig.1. Bovine casein hydrolysis by different latex peptidases evaluated by 15% SDS-PAGE (a) and colorimetric assay (b). Legend: C (Control), non-hydrolyzed caseins. CNs, total casein fraction. CpLP, CgLP, CapLP and PrLP: latex peptidases from *C. procera*, *C. grandiflora*, *C. papaya* and *P. rubra*, respectively. Bromelain, a cysteine peptidase, was used as positive control. In "b", the hydrolysis degree was evaluated using the O-phthaldialdehyde (OPA) method. Each value represents the mean of three independent experiments ± SD. The hydrolysis assays were performed at 37 °C (pH 6.5) for 30 min using different concentrations of LP (20, 30, 40, 50 and 60 µg of proteins) for 4,500 µg of total casein fraction, corresponding to 1:225, 1:150, 1:112.5, 1:90 and 1:75 ratios, respectively.

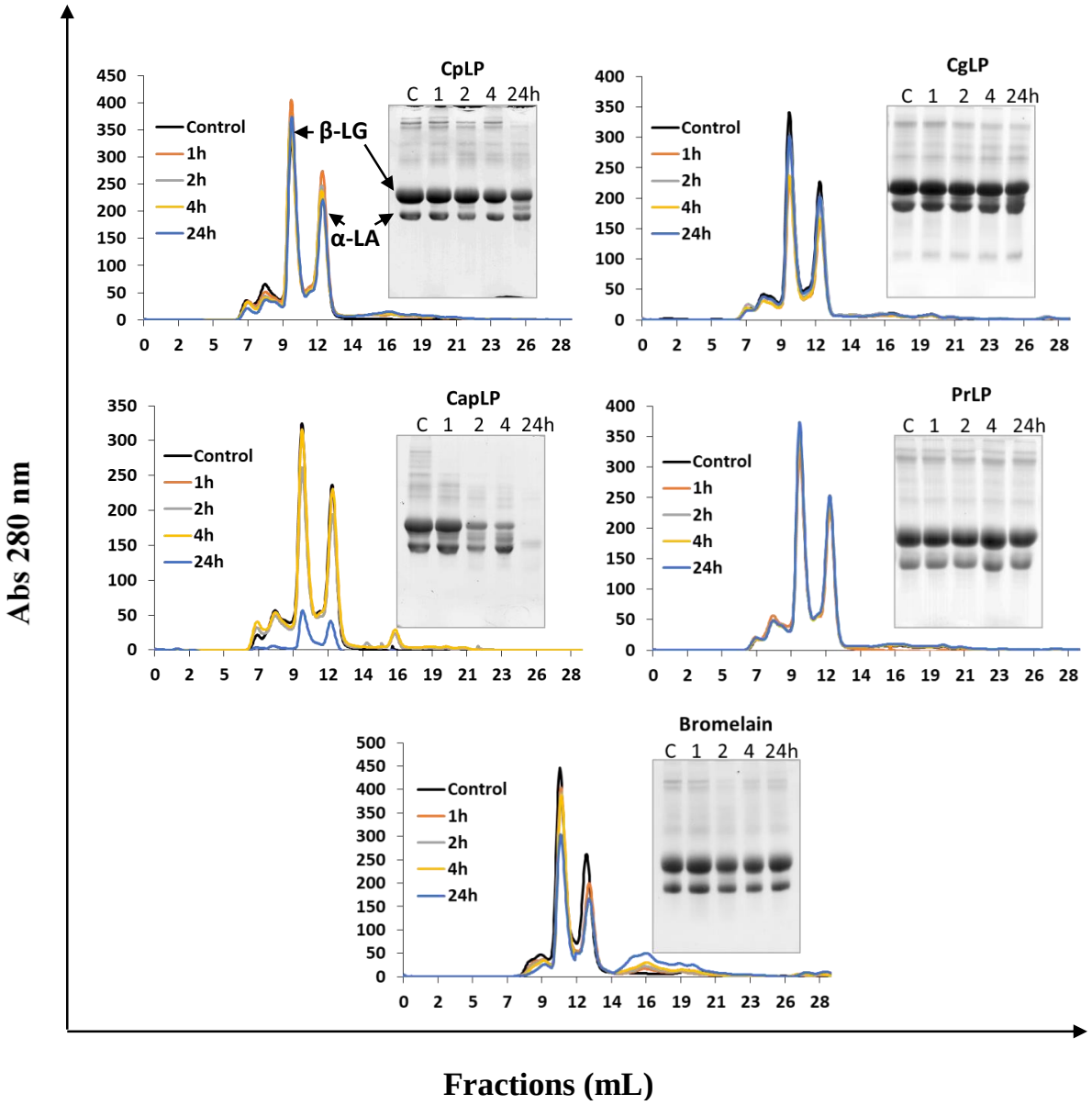

Fig. 2. Bovine whey protein hydrolysis by latex peptidases measured by 15% SDS-PAGE and size exclusion chromatography. Legend: C (Control), non-hydrolyzed whey proteins. CpLP, CgLP, CapLP and PrLP: latex peptidases from *C. procera*, *C. grandiflora*, *C. papaya* and *P. rubra*, respectively. β-LG: β-lactoglobulin. α-LA: α-lactoalbumin. Bromelain, a cysteine peptidase, was used as positive control. The assays were performed at 37 °C (pH 6.5) for different times (1, 2, 4 and 24 h) and enzyme:substrate ratio of 1:75, corresponding 60 µg of LP for 4,500 µg of whey protein.

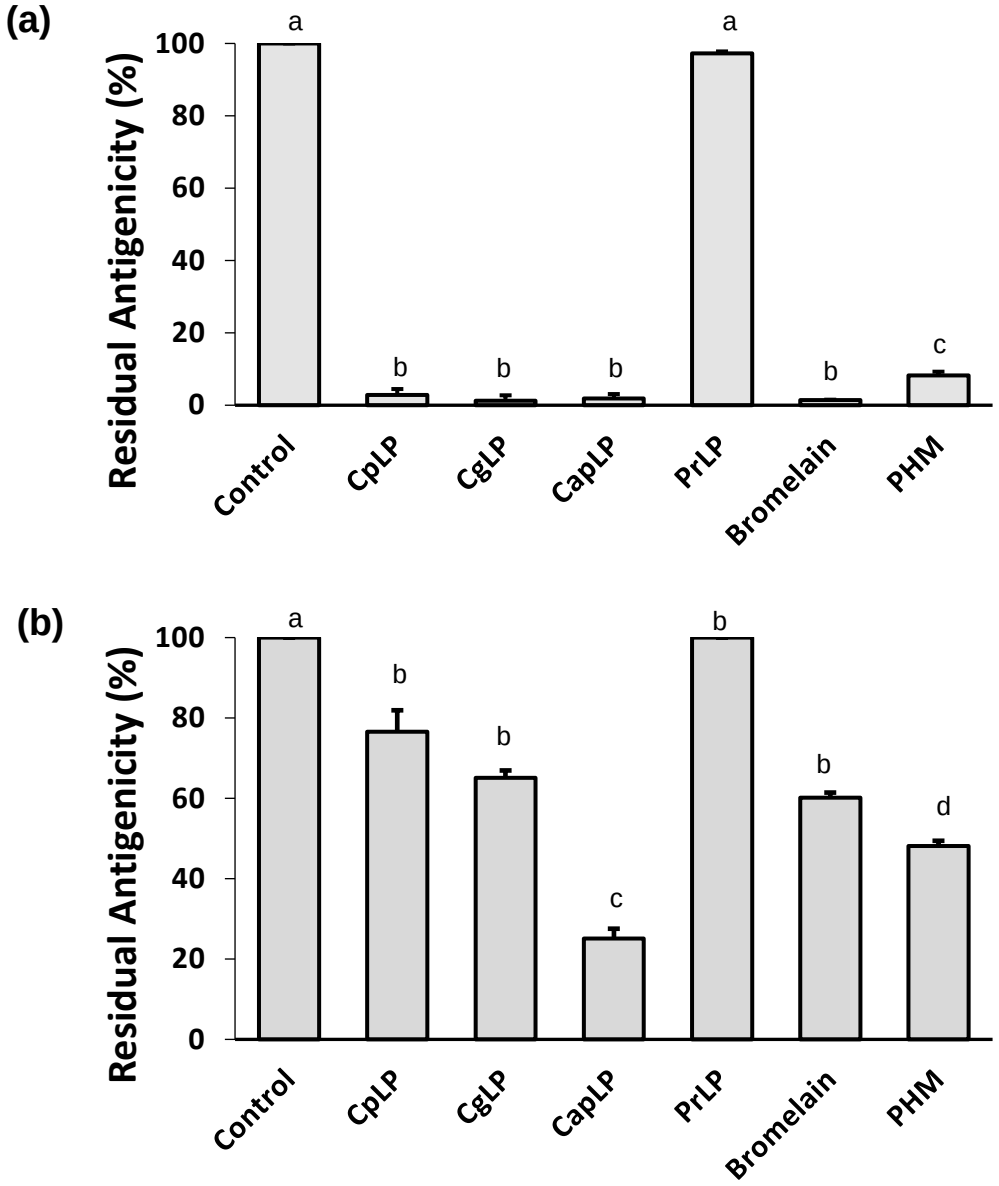

Fig. 3. Residual antigenicity of cow's milk protein measured by ELISA using polyclonal antibodies against caseins and whey proteins. (a) Casein fraction was hydrolyzed for 30 min using enzyme:substrate ratio of 1:75 (60 µg enzyme:4,500 µg caseins) at 37 ºC. (b) Whey proteins were hydrolyzed for 24 h using an enzyme:substrate ratio of 1:75. Each value represents the mean ± SD. Different letters represent significant differences ($p < 0.05$) between the indicated group and the control. Legend: CpLP, CgLP, CapLP and PrLP: milk proteins hydrolyzed by latex peptidases from *C. procera*, *C. grandiflora*, *C. papaya*, and *P. rubra*, respectively. Bromelain, a cysteine peptidase, was used as positive control. PHM: Enfamil® Gentlease® formula (Mead Johnson Nutrition) was used as control for partially hydrolyzed cow's milk.

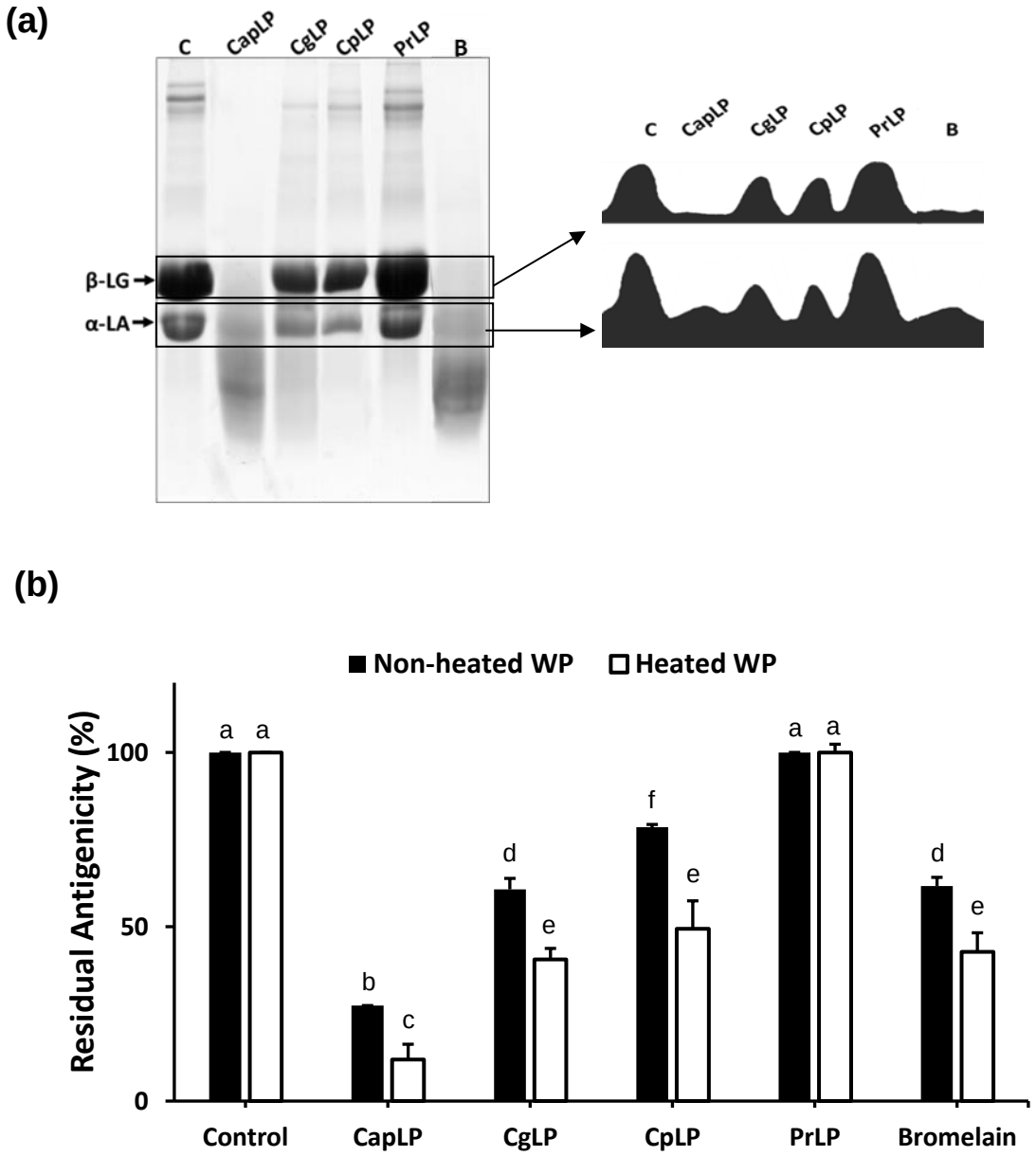

Fig. 4. Effect of preheating on hydrolysis degree of whey proteins measured by 15% SDS-PAGE (a) and ELISA (b). Whey proteins were preheated at 85 ºC for 30 min before enzyme addition. The assays were performed at 37 °C (pH 6.5) using enzyme:substrate ratio of 1:75 for 24 h. Densitometry of the protein bands in (a) were measured using the IMAGEJ software. Each value represents the mean ± SD. Different letters represent significant differences ($p < 0.05$) between the groups and the control. Legend: C (Control), non-hydrolyzed preheated whey proteins. Preheated whey proteins incubated with latex peptidases from *C. papaya* (CapLP), *C. grandiflora* (CgLP), *C. procera* (CpLP), *P. rubra* (PrLP) and (b) bromelain. β-LG: β-lactoglobulin. α-LA: α-lactoalbumin.

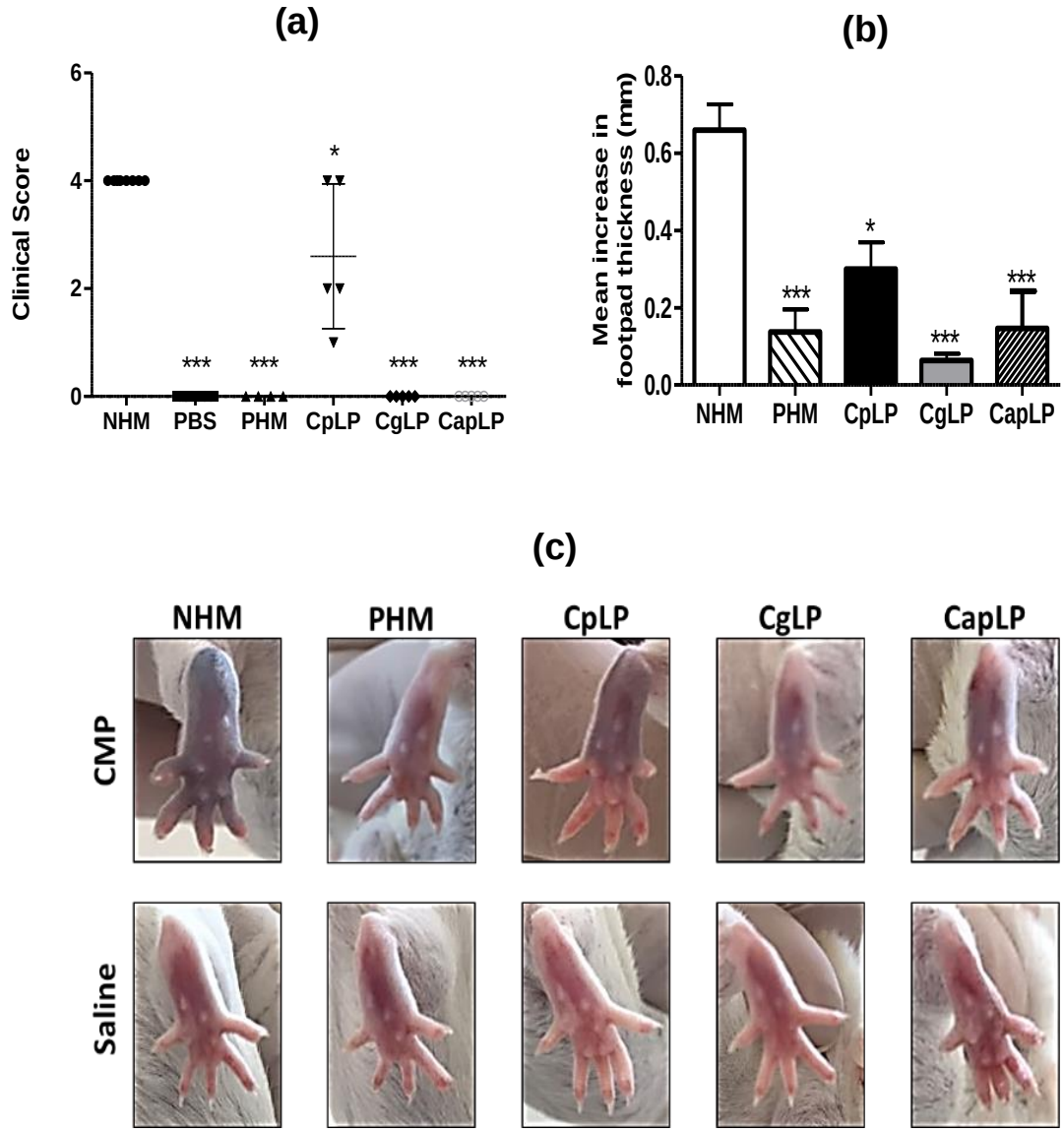

Fig. 5. *In vivo* responses of allergic mice after administration of cow's milk proteins hydrolyzed by latex peptidases. (a) Clinical scores corresponded to symptoms observed 30 min following oral challenge with milk proteins hydrolyzed by latex peptidases. The scores were assigned according to Table 1. (b) Increase in the footpad thickness and (c) Cutaneous test after injection in the footpad of milk proteins hydrolyzed by latex peptidases. Blue color observed in the skin after the injection was considered a positive cutaneous reaction. Data were expressed as mean ± SD. Statistical significant difference by ANOVA: ***$p < 0.01$, *$p < 0.05$. Legend: NHM, non-hydrolyzed milk; PHM, partially hydrolyzed milk; PBS, phosphate buffered-saline; Milk proteins hydrolyzed by latex peptidases of *C. procera*, *C. grandiflora* and *C. papaya:* CpLP, CgLP and CapLP, respectively.

Table 1. Clinical scores assigned to trigger the symptoms following the oral challenge.

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the snout and head
2	Puffiness around the eyes and mouth, piloerection, reduced activity and/or decreased activity with increased respiratory rate
3	Respiratory distress, cyanosis around snout and tail
4	No activity upon stimuli, convulsion
5	Death

5. CONCLUSÃO

As peptidases do látex de *C. grandiflora* e *C. papaya*, assim como as peptidases serínicas do intestino médio de *D. plexippus* foram capazes de reduzir de forma significativa a antigenicidade das proteínas do leite bovino. Os ensaios imunológicos *in vitro* e *in vivo* confirmaram a semelhança dos hidrolisados obtidos por DpGp, CgLP e CpLP com um leite comercial parcialmente hidrolisado, destacando assim a potencialidade das enzimas proteolíticas presentes nessas frações para o desenvolvimento de formulas hipoalergênicas. Foi observado que o pré-aquecimento do lactosoro foi uma etapa essencial para aumentar o grau de hidrólise e consequentemente, reduzir a antigenicidade das proteínas, contudo etapas adicionais como a ultrafiltração podem ser incluídas no preparo dos hidrolisados com o objetivo de eliminar antígenos residuais para a obtenção de fórmulas mais adequadas ao consumo por crianças alérgicas.

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