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POTENCIAL BIOTECNOLÓGICO DE UMA PROTEASE CISTEÍNICA DO LÁTEX DE *Calotropis procera* PARA PRODUÇÃO DE QUEIJO

FORTALEZA
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Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas.

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A Deus

À minha família

Ao meu orientador

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“O aspeto mais triste da vida de hoje é que a ciência ganha em conhecimento mais rapidamente que a sociedade em sabedoria.”

Isaac Asimov

RESUMO

Embora várias proteases vegetais com atividade de coagulação do leite tenham sido descritas, a maioria delas produz queijos com sabores amargos. Em contraste, diferentes extratos proteolíticos da planta *Calotropis procera* têm sido utilizados com sucesso na fabricação de queijos. No entanto, existem várias desvantagens no uso de extratos proteolíticos. Os objetivos deste trabalho foram caracterizar e avaliar o potencial biotecnológico de uma protease cisteína purificada do látex de *C. procera*, chamada CpCP3. Assim como estudar o processo de coagulação do leite pela quimosina comercial e CpCP3 por microscopia de força atômica (AFM). CpCP3 foi altamente estável na presença de diferentes íons metálicos e foi capaz de hidrolisar a κ-caseína do leite de forma semelhante à quimosina bovina. A microscopia de força atômica mostrou que todo o processo de agregação de micelas de caseína induzida por CpCP3 foi muito semelhante ao causado pela quimosina. Os queijos produzidos com CpCP3 apresentaram maior teor de umidade do que os produzidos com quimosina, porém teores de proteína, gordura e cinzas foram semelhantes. Análise *in silico* mostrou a presença de quatro peptídeos alergênicos em CpCP3, mas apenas dois deles estavam presentes em sua superfície tridimensional. CpCP3 foi altamente suscetível a hidrólise por enzimas digestivas, pepsina e tripsina, inclusive seus peptídeos alergênicos. Mesmo usando altas doses, CpCP3 não apresentou toxicidade em embriões de peixe-zebra, um modelo animal amplamente utilizado para testes de toxicidade *in vivo*. Além disso, CpCP3 foi altamente expressa em células de *Escherichia coli*, mas em forma insolúvel e sem atividade proteolítica. Todos os resultados corroboram com o potencial biotecnológico do CpCP3 como uma enzima alternativa para substituir a quimosina, todavia, mais estudos devem ser realizados para otimizar a expressão heteróloga de CpCP3 e assim obter o seu enovelamento correto com sua atividade proteolítica.

Palavras chaves: Coagulação do leite. Microscopia de força atômica. Protease de planta. Expressão heteróloga.

ABSTRACT

Although various plant proteases with milk-clotting activity have been described, most of them produces cheeses with bitter tastes. In contrast, different proteolytic extracts from *Calotropis procera* plant have been used successfully for cheesemaking. Because there are several disadvantages for using proteolytic extracts, the aims of this work were to characterize and evaluate the biotechnological potential of a cysteine protease purified from *C. procera* latex, named CpCP3. Thus, with the study of the milk coagulation process by the commercial chymosin and CpCP3 by atomic force microscopy (AFM).This enzyme was highly stable to different metal ions and was able to hydrolyse κ-casein similarly to bovine chymosin. Atomic force microscopy showed that the whole process of casein micelles aggregation induced by CpCP3 was very similar to that caused by chymosin. The cheeses made using CpCP3 showed higher moisture content than those made with chymosin, but protein, fat, and ash were similar. *In silico* analysis predicted the presence of only four allergenic peptides in CpCP3, but only two of these were present on its three-dimensional surface. CpCP3 was highly susceptible to digestive enzymes, pepsin and trypsin, including its allergenic peptides. Even using high doses, CpCP3 did not show toxicity on zebrafish embryos, an animal model widely used for in vivo toxicity test. In addition, CpCP3 was highly expressed in *Escherichia coli* cells, but in the insoluble form and without proteolytic activity. All results support the biotechnological potential of CpCP3 as an alternative enzyme to replace chymosin. Further studies should be performed to optimize the heterologous expression of CpCP3 to reach its proteolytic activity.

Keywords: Milk coagulation. Atomic force microscopy. Plant protease. Heterologous expression.

SUMÁRIO

1 INTRODUÇÃO

1.1 Proteases

Proteases (peptidases, enzimas proteolíticas e proteinases, EC 3.4) são enzimas que atuam em reações catalíticas de hidrólise. Segundo o Comitê de nomenclatura enzimática (EC), as enzimas são classificadas em seis grupos (oxirredutase, transferases, hidrolase, liases, isomerase e ligase), enquanto as proteases pertencentes à classe 3 (hidrolases), possuem como característica fundamental a capacidade de utilizar uma molécula de água em reações de catalise enzimática que clivam ligações peptídicas entre aminoácidos de proteínas.

As proteases constituem uma grande família e são divididas em endopeptidases ou proteinases e exopetidases, de acordo com a posição da ligação peptídica a ser clivada na cadeia peptídica. Endopeptidases atuam preferencialmente nas regiões internas da cadeia polipeptídica, entre as regiões amino-terminal e carboxi-terminal. As exopeptidases atuam somente nas extremidades das cadeias polipeptídicas na região N- ou C-terminal. Enquanto as que atuam na região amino terminal podem liberar um único resíduo de aminoácido (aminopeptidases), um dipeptídeo (dipeptidil-peptidases) ou um tripeptídeo (tripeptidil-peptidases). As exopeptidases que atuam na região carboxi-terminal livre liberam um único aminoácido (carboxipeptidases) ou um dipeptídeo (peptidil-dipeptidases) (MCDONALD, 1985).

As enzimas proteolíticas são classificadas ainda em seis classes, baseada na natureza química do sitio de catálise: proteases serínicas, proteases cisteínicas, proteases treonicas, aspártil-proteases, metalo-proteases e glutamil-proteases. Cada classe possui um resíduo de aminoácido característico, agrupado em uma configuração própria para formar o sítio ativo (SHA; MIR; PARAY; 2014). Nas classes cisteínicas, serínicas e treonínicas, o nucleófilo é um resíduo de aminoácido (cisteína, serina ou treonina, respectivamente) que se localiza no sítio ativo enzimático. Os resíduos de aminoácidos presentes nos sítios ativos encontram-se ligados com aminoácidos que irão doar prótons. Esse mecanismo de catálise resulta na formação

de um produto intermediário covalente entre enzima e substrato (LÓPEZ-OTÍN; BOND, 2008).

1.2 Aplicações de proteases

Um número crescente de pesquisas com proteases está aumentado de acordo com o banco de dados MEROPS release 12.0 (acessível em https://merops.sanger.ac.uk/), que utiliza critérios evolutivos e estruturais para classificar as proteases (RAWLINGS; BARRETT; THOMAS, 2018). No total já foram depositadas 908.326 sequências de proteases. Essas enzimas, são largamente utilizada na indústria, uma vez que são compostos naturais e apresentam alta especificidade. Além disso, são capazes de alterar as características de variados tipos de resíduos, contribuindo para reduzir a poluição ambiental e os custos para o tratamento de dejetos industriais (JEGANNATHAN; NIELSEN, 2013). Entre as enzimas utilizadas, mais de 70% são proteases, obtidas principalmente a partir de microrganismos (bactérias e fungos) (GUPTA e SHUKLA, 2016). Assim, o interesse por novas enzimas, capazes de atuar em diversos processos, vem aumentando.

As proteases apresentam uma diversidade de mecanismos de reação, o que proporciona uma variedade de aplicações em vários campos industriais (detergentes, cosméticos, medicamentos, têxtil, alimentícia), tornando-as moléculas relevantes do ponto de vista biotecnológico, possibilitando a utilização em larga escala. Desde a antiguidade, as proteases obtidas de vegetais e tecidos animais já eram usadas, mesmo que de forma empírica. Ultimamente, as proteases vêm sendo largamente utilizadas em substituição a produtos químicos que são danosos ao meio ambiente (ELHOUL *et al.,* 2015).

Na indústria alimentícia, as proteases apresentam-se como eficientes biocatalisadores, principalmente, na hidrólise de substratos de elevada massa molecular. Essas moléculas são ainda capazes de melhorar o processamento de alimentos, pela hidrólise específica de aminoácidos, promovendo mudanças em algumas características nutricionais e propriedades funcionais. Um exemplo disso são os amaciantes de carnes, que promovem uma melhor digestibilidade do alimento, e os hidrolisados proteicos (LI, YANG, ZHU e WANG, 2012). Uma protease que se

destaca é a papaína (EC 3.4.22.2), uma protease cisteínica extraída do látex de *Carica papaya* que é largamente utilizada na indústria alimentícia como amaciantes de carnes, coagulante de leite para produção de queijos e em bebidas para aumentar a solubilidade de proteínas antes e depois da fermentação (FERNANDEZ-LUCAS, CASTANEDA, HORMIGO, 2017).

As proteases também podem causar alterações na qualidade sensorial, como a textura e o sabor dos alimentos, retardar a degradação, remover fatores tóxicos ou inibitórios, melhorar a capacidade antioxidante, reduzir a formação de compostos alergênicos, entre outros inúmeros efeitos benéficos na indústria alimentícia (TAVANO, 2013; 2018).

1.3 Proteases e produção de queijos

Dentro do segmento de uso de enzimas em alimentos e bebidas, o mercado de leite e produtos lácteos demostraram vendas que se destacaram com uma arrecadação de U$ 401,8 milhões em 2009 (PATEL; SINGHANIA; PANDEY, 2016). A indústria de queijo no Brasil supera 18 bilhões de reais por ano (EMBRAPA, 2018). Na indústria de alimentos, uma das aplicações mais importantes é o uso de proteases na coagulação do leite para produção de queijos. Uma das fontes encontradas é a protease quimosina (E.3.4.23.4) (renina), extraída dos estômagos de bezerros lactentes, que é usada como fonte para coagulação de leite na fabricação do queijo, entretanto, apresenta a desvantagem de ter um elevado preço. Outra enzima muito utilizada nesse processo é a pepsina (EC. 3.4.23.1), extraída do estômago de bovinos adultos.

No entanto, estes coalhos de origem animal sofrem resistência a sua comercialização por conceitos religiosos do judaísmo e islamismo (EGITO *et al.,* 2007). Neste contexto, busca-se a utilização de proteases de origem vegetal. Esse fato ainda se torna mais pertinente, devido à abrangência de um mercado de consumidores que possui dieta restritiva, como os vegetarianos, mais de 14% das pessoas no Brasil declaram-se vegetarianos (Fonte: IBOPE, 2018). Assim, as proteases de origem vegetal têm se apresentado como uma robusta ferramenta de

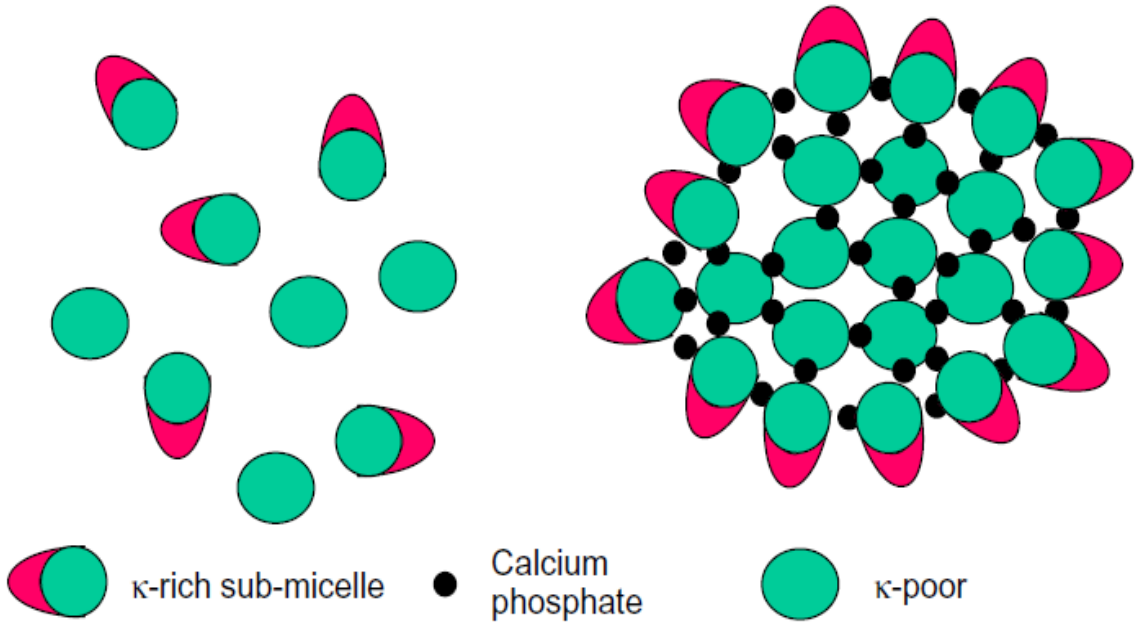
aplicação biotecnológica, possibilitando a preparação de queijos com características aceitáveis pelo marcado consumidor.

A produção de queijo é um hábito culinário bastante antigo e difundido, antes de Cristo já havia descrição da sua utilização. Há relatos de que o leite era transportado em sacos feitos de pele de estômago de animais e que, devido aos movimentos e às temperaturas elevadas da região do Oriente Médio, o leite coagulava formando uma coalhada a partir da fermentação dos açúcares do leite. O leite de origem bovino é o mais utilizado na fabricação do queijo, porém leite obtido de ovelhas, cabras e búfalos também é usado (PULINA *et al*., 2018).

O leite, matéria prima para produção de queijo, é definido como uma secreção produzida pelas glândulas mamárias e apresenta-se como uma rica fonte nutricional, além de ser usado para fabricação de laticínios (iogurtes, manteigas, entre outros). O leite é constituído por água, lactose, uma mistura de triglicerídeos e minerais (cálcio, potássio, sódio, cloro e magnésio), além de proteínas. Dentre estas, encontram-se as caseínas, uma mistura de quatro proteínas (α1, α2, β e k-caseínas), e as beta-lactoglobulinas, entretanto em menor quantidade do que as caseínas que constituem cerca de 80% do leite bovino (WALSTRA *et al.,* 2006). As caseínas são típicas do leite e possuem propriedades específicas como a presença de fosforilações, são facilmente precipitadas em pH ácido, em torno de 4,6, e diferem entre si quanto à massa molecular e composição de aminoácidos (JOHANSSON *et al.,* 2009).

As caseínas contêm um alto número de resíduos do aminoácido prolina. Além disto, tais moléculas apresentam poucas estruturas globulares e não possuem ponte dissulfeto. São moléculas anfipáticas e apresentam baixa solubilidade em água. No leite, são encontradas como uma emulsão de partículas de caseína, chamadas de micelas de caseína que permanecem unidas por íons cálcio e interações hidrofóbicas. A região apolar (hidrofóbica) está voltada para o interior da micela, enquanto a região polar (hidrofílica) posiciona-se na superfície e interage com moléculas de água (HORNE, 2006) (Figura 1).

Figura 1: Micelas de caseína.



Fonte: HORNE, 2006.

Micelas de fosfocaseinato de cálcio podem ser degradadas por enzimas proteolíticas. Essa propriedade é utilizada para a coagulação do leite com a finalidade de fabricar queijo. O processo de coagulação ocorre pela atuação de enzimas proteolíticas nas micelas da caseína sobre a k-caseína, originando dois peptídeos, chamados de para-k-caseína e o glicomacropeptídeo. Em seguida, ocorre a precipitação da para-k-caseína pelos íons de cálcio (FOX e BRODKORB, 2008). Portanto, a escolha do agente coagulante é de grande importância, visto que uma protease com baixa especificidade pode gerar um queijo de sabor amargo, reduzir a vida-útil e o rendimento do queijo, devido à alta degradação proteica.

Na fabricação de queijos, além do tipo de ação proteolítica de enzimas, a textura e características sensoriais também são afetadas pelas concentrações de sais, água, gorduras, temperatura, pressão, acidez, entre outros fatores (NAJERA, DE RENOBALES e BARRON, 2003; AREMA, LOWE e LEE, 2004; AREMA, LEE e

KLOSTERMEYER, 2007; VOIGT *et al*., 2012). A origem do leite utilizado também pode interferir nas características do queijo. Em adição, existe uma variedade de tipos de queijos, que depende do processo de fabricação e de seus constituintes (coalho, prato, parmesão, etc.). Além de uma variedade de queijos enriquecidos com antioxidantes, frutas, farinha de quinoa, chia, ácidos probióticos, etc. (PEREIRA *et al*., 2016; BEZERRA *et al*., 2016; LEMES *et al*., 2016).

Ainda no que diz respeito a fatores que influenciam nas características do queijo, uma diminuição moderada do pH do leite (por exemplo, até pH 6,4) resulta na diminuição da solubilização do cálcio, a partir das micelas de caseína, o que leva à formação mais rápida de uma coalhada mais firme. No entanto, um grau mais elevado de solubilização do cálcio ocasiona a grande desmineralização das micelas de caseína, o que resulta em géis de coalhada mais fracos e flexíveis (CHOI, HORNE e LUCEY, 2007). Para alguns queijos de pasta mole, é necessário um passo de desmineralização extensa antes da adição de coagulante para se obter a estrutura desejada do corpo do queijo maduro. As taxas de hidrólise, agregação e sinérese (expulsão do soro do leite pela contração do gel) aumentam com a elevação da temperatura até chegar em um ponto no qual a enzima passa a ser inativada pelo calor. A temperatura ótima à formação de coalho a pH 6,5 (pH do leite) é em torno de 34 a 38 ºC para a maioria dos coagulantes comerciais. Na prática, a coagulação geralmente é de 30 a 35 ºC para se ter o controle adequado sobre a firmeza da coalhada em corte (LAW e TAMIME, 2010).

A quimosina (E.3.4.23.4) é a principal enzima usada na obtenção do coalho. Ela é classificada como uma protease aspártica e sintetizada como pró-quimosina (inativa), contendo 16 resíduos de aminoácidos a mais que a enzima ativa. Esta protease hidrolisa a ligação da k-caseína do leite. A clivagem ocorre especificamente na ligação Phe105-Met106 da molécula de caseína, levando à formação de dois fragmentos: para-k-caseína e caseinomacropeptídeo (CMP). A perda deste glicopeptídeo, altamente hidrofílico, impede a k-caseína de assegurar a estabilidade adequada de manter a micela da caseína, de modo que quando ocorre a clivagem as proteínas precipitam e formam um gel (BEPPU*,* 1983). Assim, pode-se separar o líquido (soro do leite) das proteínas precipitadas, usadas para fazer o queijo, e o soro do leite pode ser aproveitado em outros processos de produtos lácteos.

Existem várias isoformas da quimosina, mas duas prevalecem: a quimosina A e a quimosina B, diferenciadas apenas pela substituição de um resíduo de asparagina por glicina na quimosina B (FOLTMANN *et al.,* 1977; 1979). A quimosina tem sido obtida a partir da inserção do seu gene em células de levedura. A expressão heteróloga dessa protease proporcionou uma melhor ferramenta para atender as necessidades do mercado, além de aumentar o rendimento proteico e ser um método rápido que facilita a purificação e obtenção desta proteína (CHITPINITYOL e CRABBE, 1998). Assim as proteases recombinantes podem ser promissoras na aplicação em vários tipos de processos no mercado, inclusive na fabricação de queijos, uma vez que são produzidas com alta eficiência, diminuindo custos e favorecendo sua obtenção (LANE e SEELIG, 2016).

1.4 Proteases de plantas na produção de queijo

Proteases de plantas são fontes promissoras de enzimas a serem aplicadas na fabricação de queijos, em substituição a quimosina e pepsina extraída de estômago de bezerros. Vários trabalhos já foram descritos com a utilização de proteases extraídas de frutos, flores, sementes e látex na coagulação de leite (SHAH, MIR e PARAY *et al*., 2014, AMIRA *et al.,* 2017). Entretanto, estes estudos ainda são insuficientes, visto a demanda e procura por novos agentes coagulantes.

Entre estes agentes coagulantes de origem vegetal, as proteases aspárticas são uma das proteases mais utilizadas na fabricação de queijos. Elas possuem dois resíduos aspárticos no seu sítio catalítico e são mais ativas em pH ácido. Em relação à especificidade, a clivagem ocorre entre os resíduos de aminoácidos hidrofóbicos responsáveis pela atividade catalítica (DOMINGOS *et al*., 2000). Proteases aspárticas com atividade de coagulação do leite foram relatadas em alcachofra (*Cynara scolymus* L.) (LLORENTE *et al*., 1997; 2004), arroz (*Oriza sativa*) (ASAKURA *et al.,* 1997) e *Centaurea calcitrapa* (DOMINGOS *et al*., 2000). Entre as partes das plantas, as enzimas de flores são bastantes usadas na fabricação de queijos. Estudos mostraram que as proteases aspárticas se acumulam nas flores maduras (pétalas e pistilos), mas não nas folhas ou sementes (CORDEIRO, PAIS e BRODELIUS, 1998). Três proteases aspárticas abundantes de flores de *Cynara*

cardunculus, com atividade coagulante no leite, foram purificadas e caracterizadas (HEIMGARTNER *et al*., 1990). As proteases aspárticas de flores, anteriormente citadas, compartilham parâmetros cinéticos e de especificidade com quimosina e pepsina (VERISSIMO *et al*., 1995; 1996).

As proteases serínicas de origem vegetal também possuem aplicabilidade como agentes coagulantes. Nas plantas, elas estão difundidas entre vários grupos taxonômicos e presentes em quase todas as partes do vegetal entretanto são mais abundantes nos frutos. Essas proteases têm sido encontradas e extraídas de látex, sementes, flores, caules, folhas e raízes. Neriifolin S, uma protease serínica dimérica de massa molecular de 94 kDa, purificada do látex de *E. neriifolia,* apresentou atividade de coagulação do leite (YADAV; PATEL; JAGANNADHAM, 2012). As proteínas Religiosina (43,3 kDa), Religiosina B (63 kDa) e Religiosina C (80 kDa) foram isoladas do látex de *Ficus religiosa* e apresentaram atividade de coagulação do leite (KUMARI, SHARMA e JAGANNADHAM, 2010; 2012; SHARMA, KUMARI e JAGANNADHAM, 2012).

Além das proteases aspárticas e serínicas, as proteases cisteínicas, também conhecidas como proteases de tiol pelo seu mecanismo de catálise, mostram-se com grande potencial biotecnológico na indústria alimentícia e farmacêutica, devido serem ativas em uma ampla faixa de temperatura e pH. As plantas são uma alternativa atrativa para obtenção de proteases cisteínicas, pois além de serem encontradas naturalmente em diferentes tecidos, estão, em alguns casos, presentes em quantidade excessiva (GONZALEZ-RABADE *et al.,* 2011). Algumas proteases cisteínicas como a ficina, isolada do látex de *Ficus racemosa*, mostraram capacidade de digerir caseína, o que sugere que estas enzimas possam apresentar a propriedade de coagulação do leite (DEVARAJ, GOWDA e PRAKASH, 2008).

Outras fontes promissoras de proteases de origem vegetal com possível aplicação na fabricação de queijos são as proteínas extraídas das sementes de *Albizia lebbeck* e *Helianthus annuus*. Essas proteínas foram obtidas através de precipitação com sulfato de amônio, apresentaram atividade de coagulação do leite e atividade proteolítica semelhante à quimosina sobre as proteínas do leite κ-caseína, alfa-caseína e β-caseína. Análises por espectrometria de massas mostraram que o extrato da semente de girassol hidrolisou a κ-caseína especificamente na ligação Phe105-

Met106, assim como a quimosina. O extrato de *A. lebbeck* também exibiu atividade proteolítica sobre κ-caseína, entretanto sua ação ocorreu entre os resíduos de aminoácidos Lys116-Thr117 (EGITO *et al*., 2007).

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

Ao tratar das proteases de origem vegetal e suas aplicações, as proteases extraídas de plantas laticíferas têm se sobressaído, visto que apresentam um vasto potencial como agente coagulante e que seus extratos são ricos em proteases cisteínicas. Entre elas está incluída a planta laticífera *Calotropis procera*, da qual várias proteases cisteínicas foram identificadas e purificadas da fração proteica do látex: procerain, procerain B, CPCP-1, CPCP-2 e CPCP-3 (DUBEY e JAGANNADHAM, 2003; SINGH *et al*., 2010, e RAMOS *et al*., 2013). CPCP-1, CPCP-2 e CPCP-3 foram purificadas por dois passos cromatográficos e apresentam atividade enzimática semelhante entre si e sequências N-terminal idênticas. Essas proteases possuem ponte dissulfeto em sua estrutura e apresentam atividade coagulante do plasma sanguíneo mediada por um mecanismo semelhante à trombina (RAMOS *et al*., 2013).

Calotropis procera é uma planta laticífera pertencente à família Apocinaceae e é encontrada em regiões tropicais e subtropicais, sendo originária do oriente médio e da África. Essa espécie é caracterizada como uma planta arbustiva, podendo atingir de 3 a 4 metros de altura, e pela sua grande capacidade em produzir látex. Devido essa última característica, ela é conhecida popularmente como leiteiro. Contudo, também pode ser conhecida como: algodão-de-seda, flor-de-seda, ciúme, hortência, entre outros, dependendo da região onde se encontra (Figura 2).

Figura 2: Planta *Calotropis procera* (Ait) R. Br.

Fonte: próprio autor.

O látex da planta *C. procera* é conhecido popularmente por ter atividades farmacológicas, apresentando efeitos fisiológicos desejáveis no combate a lepra, úlceras, hemorróidas, tumores, etc. (KUMAR E ARYA, 2006; SINGH *et al.,* 2010). Relatos demostraram que proteínas do látex de *C. procera* exercem toxicidade às larvas de *Anticarcia gemmatalis* (Lepidoptera: Noctuidae) e o besouro bruquídeo, *Callosobruchus maculatus* (Coleoptera: Bruchidae) (RAMOS *et al.,* 2007), assim como foi demonstrado possuir atividade contra fungos fitopatogênicos: *Fusarium oxysporum*, *Fusarium solani*, *Colletotrichum gloeosporioides* e *Rhizoctonia solani* (SOUSA *et al.,* 2011).

Essa fração proteica de *C. procera* também apresentou atividade de coagulação do leite, hidrolisando a k-caseína e produzindo um peptídeo de 16 kDa, processo semelhante à quimosina comercial. Os queijos produzidos apresentaram rendimentos, massas secas e proteínas solúveis semelhantes aos queijos preparados

com quimosina comercial (FREITAS *et al.,* 2016). Entretanto, o uso de extratos proteicos dessa planta contem inibidores de proteases, quitinases, osmotinas (DUBEY e JAGANNADHAM, 2003; RAMOS *et al.,* 2010; FREITAS *et al*., 2011; FREITAS *et al*. 2016) e enzimas relacionadas ao estresse anti-oxidativo (FREITAS *et al.,* 2007), as quais são consideradas como proteínas responsáveis pela atividade defensiva de látex e que poderia causar uma toxicidade ao queijo e não aceitação pelo mercado. Diante disto, torna-se necessário mais estudos sobre as proteases cisteínicas purificadas dessa fração proteica do látex de *C. procera* e sua aplicação na fabricação de queijos.

Um modo de se obter uma protease purificada seria com o uso de ferramentas biotecnológicas como clonagem e expressão heteróloga. Isso evitaria problemas como sazonalidade, mudanças climáticas e deste modo um controle na produção em escala industrial. Estudos já foram realizados sobre as proteases de *C. procera* como a proceraína B que, além de isolada do látex, purificada e caracterizada, teve sua sequência de aminoácidos elucidada, assim como foram realizadas a clonagem cDNA e a expressão heteróloga dessa proteína (SINGH *et al.,* 2013; NANDANA *et al*., 2014). Os transcritos de *C. procera* foram sequenciados e encontrado 20 isoformas de proteases cisteínicas. Dessas isoformas, a sequência de SnuCalCp03, apresentou similaridade de 99% com o N-terminal de CpCP3 de *C. procera*. A partir disso, essa protease foi expressa em forma de zimogênio em estipes de *E. coli,* no entanto a protease sem o zimogênio não foi expressa. E mesmo na forma de zimogênio a protease foi obtida em corpo de inclusão, solubilizada com ureia 8 M e o zimogênio ativado por autocatálise, porém o desdobramento da proteína não foi adequado e por esse fato a protease não apresentou atividade proteolítica (KWON *et al*., 2015).

1.6 *Escherichia coli*

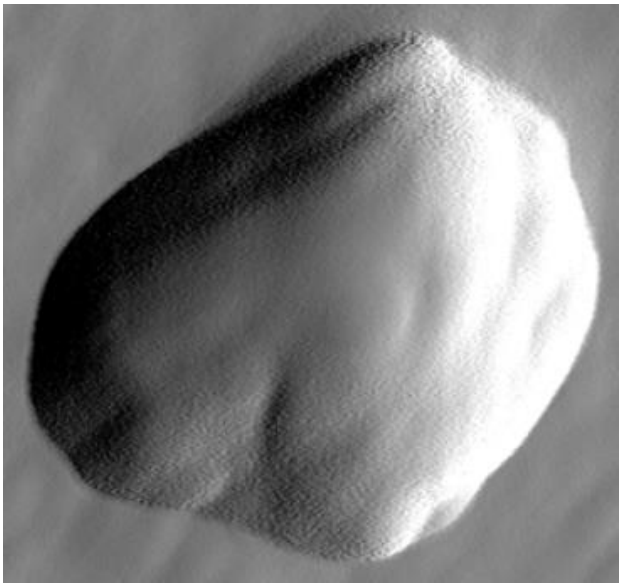
Microrganismos procariotos como a *Escherichia coli* são rotineiramente utilizados para a produção de proteínas por requerer um menor custo, ter uma alta velocidade de produção, ser fácil a manipulação, ter um alto rendimento e um grande número de sistemas geneticamente modificados (GUPTA e SHUKLA, 2016; SINGHA

et al., 2017). Além disso, este sistema possui facilidade e rapidez de transformação bacteriana através de inserção de plasmídeos, sendo fácil a preparação de células eletrocompetentes para receber o DNA plasmidial. O cultivo das células bacterianas não requer condições tão especificas de nutrientes e pH, sendo assim um sistema barato. Além de ser um organismo que crescem rapidamente em meio nutritivo e chega a dobrar o número de células a cada 20 minutos, esta rapidez favorece um menor custo de produção e agilidade de resultados (VINCENTELLI e ROMIER, 2013).

As características citadas anteriormente são essenciais, pois atualmente a *E. coli* é utilizada para produção dos principais medicamentos recombinantes como insulina, hormônio do crescimento, e até mesmo vacinas (LADISCH e KOHLMANN, 1992; BAESHEN *et al*., 2015; SANCHEZ-GARCIA *et al*., 2015). Estudos também têm mostrado seu potencial através da produção de combustível renovável e armazenamento de dados na área de bioinformática (KALSCHEUER, STÖLTING e STEINBÜCHEL, 2006). Portanto a utilização desse sistema de produção de proteínas heterólogas tem se mostrado eficiente para produzir proteases recombinantes para produção de queijos.

Entretanto *E. coli* apresenta desvantagens ao expressar proteínas que necessitem de modificações pós-traducionais como N-glicosilação e pontes dissulfeto. Proteínas que requerem pontes dissulfeto necessitam de um meio celular mais redutor no citosol da bactéria para formação dessas pontes. Para solucionar isso, a bactéria *E. coli* SHuffle foi geneticamente modificada e o seu citosol apresenta vias redutoras, permitindo a formação dessas pontes dissulfeto (LOBSTEIN *et al*., 2012). Algumas proteínas ainda são expressas e acumuladas em corpo de inclusão em decorrência desse fato. Corpo de inclusão consiste em acumulo de agregado de proteínas insolúveis que não apresentam sua conformação estrutural regular (VILLAVERDE e CARRIO, 2001; DE GROOT *et al*., 2008; CAPITINI *et al*., 2014) (Figura 3).

Figura 3: Corpo de inclusão visualizado por microscopia de força atômica



Fonte: próprio autor.

Uma maneira de obter proteínas recombinantes de corpos de inclusão é a solubilização por solventes caotrópicos como ureia e cloridrato de guanidina em altas concentrações. Outros agentes usados são os detergentes SDS (Dodecil Sulfato de Sódio), sarcosyl (Lauroil Sarcosinato de Sódio) e CTAB (Brometo de Cetiltrimetilamônio) (HE e OHNISHI, 2017), agentes redutores como 2-mercaptoetanol, álcool, entre outros (SINGH *et al*., 2012; HAMIDI 2019; WURM, SAFDARI e ARABI, 2018; KAUR, KUMMAR e KAUR, 2018). Deste modo estes agentes possibilitam a obtenção de proteínas solúveis.

2 OBJETIVOS

2.1 Objetivos gerais

Caracterizar e avaliar o potencial de uma protease cisteínica do látex de *Calotropis procera*, chamada de CpCP3, na coagulação do leite para fabricação de queijo. Assim como obtê-la por expressão heteróloga em *Escherichia coli*.

2.2 Objetivos específicos

1-Obter a protease purificada da fração proteica de *Calotropis procera* (CpCP3);
2-Analisar a estabilidade da atividade proteolítica de CpCP3 na presença de íons e na coagulação do leite, assim como na hidrólise da k-caseína;
3-Estudar o processo de coagulação do leite pela quimosina comercial e CpCP3;
4-Analisar o potencial alergênico de CpCP3 *in silico* e *in vitro* pela degradação de enzimas digestivas (pepsina e tripsina) e a toxidade em embriões de peixe zebra;
5-Produzir um queijo pela coagulação do leite por CpCP3 e analisar teores de umidade, proteínas, gorduras e cinzas do queijo em comparação com queijos produzidos com quimosina comercial;
6-Obter CpCP3 por expressão heteróloga em *Escherichia coli*.

Study of milk coagulation induced by chymosin using atomic force microscopy

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Abstract

Caseins (α_{s1}-, α_{s2}, β-, and κ-caseins) form the major protein fraction of milk, irrespective of their origins. They are able to form well-ordered colloidal structures in association with colloidal calcium phosphate, named casein micelles. Chymosin-mediated milk coagulation takes place through loss of casein micelles' stability by hydrolyzing κ-caseins. This process is critical for the quality of cheese and other milk derivatives. Therefore, many microscopy techniques have been used to understand the structural aspects underlying the integrity of casein micelles during chymosin action. However, these technologies can be costly and laborious. In this study, atomic force microscopy (AFM) and dynamic light scattering (DLS) were used to study milk coagulation by chymosin. Following 15 min of chymosin action, the AFM images showed the start of the formation of casein micelle aggregates. After 30-45 min, the micelles continued aggregating, forming structures such as bunches of grapes. Finally, after 45-60 min, these structures formed large clusters of casein micelles. After 60 min, the zeta potential did not drop to zero when the milk clotted, but still had a negative value, suggesting that the κ-caseins on the micelle surface were not totally hydrolyzed. The results corroborate those previously described and suggest this tool as an alternative to study the milk-clotting process at the ultrastructural level induced by proteases.

Keyword: AFM; cheese; coagulation; milk; protease.

1. Introduction

Milk is a rich source of nutrients such as proteins, fats, carbohydrates, vitamins and minerals. Therefore, it is regarded as a complete meal and has been recognized as an important ingredient of the daily diet over the centuries (Singh and Gallier, 2017). About four-fifths of milk proteins are caseins, which are classified as α_{s1}, α_{s2}, β-, and κ-caseins (Dalgleish, 2011). These proteins naturally associate with each other and with colloidal calcium phosphate, forming highly stable structures called casein micelles (de Kruif *et al*., 2012; Horne, 2002).

Although casein micelles are very stable, they can be readily destabilized by acidification or addition of specific proteases, resulting in curd formation, which is the basis of many foods, such as cheese and yogurt (Walstra *et al*., 2006). For that reason, some studies of the ultrastructure of casein micelles have been done to understand the dynamics of curd formation and some models have been proposed (Fox and Brodkorb, 2008; Horne, 2006; Mellema *et al*., 2002). In general, casein micelles are colloids formed by phosphorylated caseins (α- and β-caseins), which interact with calcium phosphate. Their size and stability are controlled by the layer of κ-casein on their surfaces (Dalgleish, 2011).

Chymosin (EC 3.4.23.4), the most extensively used protease in cheese making, is responsible for the specific cleavage between the Phe_{105}–Met_{106} bond of bovine κ-casein. This cleavage releases caseino-macropeptides (residues 106-169 of κ-casein) and results in the destabilization of casein micelles, and consequently milk coagulation (Egito *et al*., 2007). Some studies have shown that the quality of cheese (flavor and texture) can be affected by the specific hydrolysis of κ-casein by protease (Freitas *et al*., 2016). Thus, how this hydrolytic process affects the casein micelles and forms the milk coagulum needs more study (Dwyer *et al*., 2005; Mellema *et al*., 2002).

These studies need suitable technologies to monitor the ultrastructural changes of casein micelles at all stages of milk gelation by protease. Atomic force microscopy (AFM) is a powerful tool capable of recording nanostructural details of native and modified samples. AFM is a nonintrusive technology that needs minimal sample preparation and avoids metallic coating, fixation or an additional dye (Braet and Taatjes, 2018). AFM operates by scanning the surface of the sample with a probe

consisting of a tip mounted at the end of a small cantilever. During probing, the vertical deflection of the cantilever is obtained by a photodiode that detects the reflection of a laser beam positioned on the backside of the cantilever. These data are then converted into topological images of the sample surface with a nanometer scale (Shi *et al*., 2018). In addition, AFM can also investigate the mechanical, electrical and magnetic properties of a wide spectrum of materials, ranging from single molecules to living cells (Jalili and Laxminarayana, 2004).

Although AFM has been used successfully to study the role of acidification on casein micelles (Ouanezar *et al*., 2012), it has not been used to study the structural changes of casein micelles resulting from chymosin's action. In this study, some aspects of the dynamics of bovine casein micelle processing by chymosin were measured by both AFM and accessory techniques over time-course perspective. The results suggested that AFM is an alternative technology to study the milk-clotting process at the ultrastructural level.

2. Material and methods

2.1. Milk-clotting activity

The milk-clotting activity of a commercial bovine chymosin (Coalhopar®, Fortaleza, Ceará, Brazil) was assayed as described by Ahmed *et al*. (2010). The enzyme (20 µl, 2 mg/ml in water) was mixed with 2 ml of commercial bovine skimmed milk (Leite Betânia®, Fortaleza, Ceará, Brazil). This proportion (enzyme:milk) was used according to the manufacturer's instructions (Coalhopar®). Milk-clotting activity was obtained visually at different times at 25°C and the final result was the formation of a gel.

2.2. Atomic force microscopy (AFM)

The AFM measurements were done with an MFP3D-BIO microscope from Asylum Research (Oxford Instruments, Santa Barbara, California, USA). Images were acquired using tapping mode with average sizes of 5 µm and resolution of 256 x 256

points, where a scan frequency of 0.7 Hz and a set point voltage of 0.8 V were used, for a free amplitude oscillation of 1.0 V. Econo-LTESP-Au cantilevers from Oxford Instruments were used with nominal spring constant of 5 N/m. The maximum force applied to casein micelles was approximately 15 nN (Ramos et al., 2015). To capture the micelles' aggregation, 5 different samples were imaged: the control, to which no enzyme was added (time = 0), and 4 samples that were imaged at 15, 30, 45, and 60 min after enzyme addition. At each time point, the samples were immediately diluted (1:20, v/v) in 50 mM Tris-HCl buffer (pH 6.5), containing 10 mM $CaCl_2$, placed on glass surfaces (10 µl), spread to obtain a homogenous layer and dried in a desiccator at 25ºC for 5 min. The size (diameter and height) distribution of particles was analyzed with the AFM native software from 5 acquired images of each sample (around 1000 micelles). In addition, cross-section analyses of the topological images were also used to provide further insights into the growth dynamics of the casein micelles. The experiments were repeated twice with independent samples.

The volume (V) of casein micelles was estimated using the formula (Ouanezar et al., 2012):

$$V = \{[(\pi.h)/6].[3(w/2)^2] + h^2\} \quad (1)$$

where h = height and w = width of casein micelles.

2.3. Size distribution and zeta potential of casein micelles

The size and zeta potential (ζ) of casein micelles after chymosin addition were calculated from the measured diffusion coefficient introduced in equations (for size and electrical mobility) derived from the Einstein-Smoluchowski relation (Silva et al., 2015). The zeta or surface potential has been used to measure the net negative charge of the surfaces of casein micelles (Darling and Dickson, 1979). The chymosin was added to skimmed milk as described in section 2.2, and after each time point, the mixture was diluted (1:20, v/v) with 50 mM Tris-HCl buffer (pH 6.5), containing 10 mM $CaCl_2$, and filtered using a membrane with pore size of 0.45 µm (Milex®, São Paulo, Brazil). The measurements were done at 25°C during different time intervals at a scattering angle of 173°. The experiments were repeated twice with independent

samples. The diffusion coefficients were measured using dynamic light scattering (DLS), in a Zetasizer Nano ZS (Malvern Instruments, Swavesey, Cambridge, UK). DLS is a well-established technique used to study the diffusion behavior of macromolecules in solution. The diffusion coefficient of the particles can be determined since DLS typically measures fluctuations in scattered light intensity due to diffusing particles. Hence the hydrodynamic radii are calculated from the diffusion coefficient, and the size of macromolecules can be estimated (Stetefeld *et al*., 2016).

3. Results and discussion

3.1. General aspect of milk coagulation

When chymosin was added to the milk samples, nothing visually seemed to happen initially until 30 min. However, after this period, the milk-clotting effect could be observed with the formation of small aggregates (45 min), which became bigger (around 45-60 min), resulting in the formation of a gel (data not shown). Milk coagulation occurs because the hydrolysis of κ-casein destabilizes the casein micelles that are in equilibrium within the milk as colloidal particles. This reduces the steric and electrostatic repulsion between the micelles and the suspension becomes unstable (Dalgleish, 2011). If this hydrolysis is not specific, it can generate bitter peptides. In many such cases, the resulting cheese is commonly rejected by consumers (Freitas *et al*., 2016). Therefore, studies of enzymatic kinetics and specific hydrolysis of κ-casein by proteases are important and still need improvement.

3.2. Action of chymosin on casein micelle aggregation

Before chymosin addition (time = 0), the amplitude and topography images from AFM analyses showed individual casein micelles with a mean width (*w*) of 200 nm and height (*h*) of 100 nm (n = 1000 micelles) (Fig. 1). Using equation 1 and values of "*w*" and "*h"* of the casein micelles, the volume (V) was 1.57 x 10^6 nm^3, which was very close (V = 1.60 x 10^6 nm^3) to the volume of a sphere with a diameter of 230 nm, measured by dynamic light scattering (Fig. 2a). These values are in agreement with the results described by Ouanezar et al. (2012), who studied the structural rearrangement of casein micelles upon acidification using AFM and DLS.

Although the average size of casein micelles can differ widely (80-550 nm), in general their diameters are around 200 nm (de Kruif and Holt, 2003). Since micelles are generally spherical (when suspended in the colloidal solution), these differences between the width and height can be explained because they are organized and stabilized by non-covalent bonds among the hydrophobic cores of α-, β- and κ-caseins, making them more susceptible to deformations during AFM experiments (where samples are deposited on a flat surface). Accordingly, this flattening of casein micelles has also been observed previously by other authors (Ouanezar *et al*., 2012; Silva *et al*., 2015). Additionally, Olivares *et al.* (2010) observed that casein micelles were affected by the presence of the highly ordered pyrolytic graphite (HOPG) and mica surfaces, having a shape closer to a thick “pancake” than a sphere. Helstad *et al*. (2007) also suggested that the casein micelles deformed after immobilization on graphite and took the shape of a spherical cap.

Following 15 min of chymosin action, the AFM images showed the start of formation of casein micelle aggregates (Fig. 1). However, the main changes that characterizes clotting had not yet occurred. After 15 min most of the micelles were still individually maintained and their shapes and sizes were preserved (Fig. 1). The DLS assays also confirmed that the size distributions of micelles were not altered and aggregates were not observed after 15 min (Fig. 2a). The first step of micelle aggregation is the κ-casein hydrolysis, which is responsible for generating electrostatic repulsion among micelles. Studies by Dwyer *et al.* (2005), using ultrasonic analysis, suggested that at this moment there is an initial decrease in diameter of casein micelles resulting from κ-casein surface proteolysis. They proposed a reduction of around 20 nm in the diameter of the micelles, which was not detected by AFM and DLS in this study (Figs. 1 and 2). Presumably, after this period (15 min), the micelles randomly collide in a close-packed system due to Brownian motion, in such an orientation that they show a surface without κ-casein, since this protein has negative net charge at pH 6.5 (Dalgleish, 2011). Thus, when chymosin is added to milk, the κ-casein hydrolysis begins releasing caseino-macropeptides (residues 106-169 of k-casein), which are responsible for the negative charge of micelles (Dalgleish, 2011; Darling and Dickson, 1979). Fig. 2b supports these results, since the zeta potential was reduced by chymosin's action. After 15 min, the zeta potential was -24.9 ± 0.4 mV, in comparison with -26.6 ± 0.4 mV for milk without chymosin (time= 0) (Fig. 2b).

After 30-45 min, removal of κ-casein progressed and the micelles continued aggregating (aggregation phase, Fig. 1), forming structures that looked like bunches of grapes (Walstra *et al.*, 2006). DLS showed that the diameter of these structures, obtained at 30 min, increased to 304 nm (versus 230 nm, time = 0) (Fig. 2a). Although the zeta potential decreased 5 min after chymosin addition, it was apparently constant during 5-30 min, suggesting an overall uniform degradation of κ-casein on casein micelles before their aggregation, further indicating that this point is critical to milk coagulation (lag phase, Fig. 2b).

Finally, after 45-60 min, these structures, similar to a bunch of grapes, formed large clusters of casein micelles, when the charges on the micelles declined enough to allow hydrophobic interactions between the caseins of neighboring micelles, creating large individual macromolecular structures with a size distribution of about 640-860 nm (Fig. 1 and 2a). This process continued, forming larger structures and then a three-dimensional gel. It is hypothesized that this phenomenon of aggregation and coagulation of casein micelles is spontaneous and is driven by the gain in entropy of the system, when the ordered water molecules are removed from non-polar surfaces (without κ-casein). A similar effect has been observed when amphipathic compounds are mixed with water: the non-polar regions of the molecule cluster together to show the smallest hydrophobic area to the aqueous solvent, and the polar areas are arranged to maximize their interaction with the solvent (Walstra *et al*., 2006). A very similar size (850 nm) was also found as a result of micelle aggregation by chymosin in the study of Dwyer *et al*. (2005). However, because they used ultrasonic analysis, they were not able to distinguish the three-dimensional structure of casein micelles. Thus, they supported the formation of a three-dimensional gel network, which has been reported in the literature. That gel has been described as being formed by large clusters of casein micelles, forming large pores of about 10 μm in width (Walstra *et al*., 2006). In that model, all or almost all κ-caseins on casein micelles are hydrolyzed and the zeta potential is near zero (Darling and Dickson, 1979). Nevertheless, Fig. 2b shows that the zeta potential did not drop to zero when the milk clotted (60 min), but still had a negative value (-21.0 ± 0.2 mV) (Fig. 2b), suggesting that the κ-caseins on the micelle surface were not totally hydrolyzed.

3.3. Advantages and disadvantages of atomic force microscopy

The atomic force microscopy showed important results: 1) No casein micelle artifact was observed. Thus, the dilution and spread of micelles onto the glass surface did not alter their general structures. However, some flattening of casein micelles was observed. 2) Glass can be used as an efficient surface for casein micelle analysis using AFM, since the results were similar to those obtained using more expensive substrates such as HOPG, mica or Au-coated surfaces (Olivares et al., 2010; Ouanezar *et al*., 2012). 3) The images obtained in air on desiccated casein micelles are very close to those described in liquid (Ouanezar *et al*., 2012). 4) The assays were fast and no additional dye, fluorophore or reagent was necessary. 5). The interpretation of the results is straightforward. 6) The experimental setup can be modified to do real-time investigation of individual micelle aggregation/dissociation. 7) In terms of costs, only two AFM probes were used in the present study, with a cost of approximately $20/probe. The costly part is the AFM itself. Another disadvantage is that the AFM is not automated. Thus, it can be very difficult to handle, making it unsuitable for integration in industrial production lines.

3.4. The model for milk coagulation by chymosin

Fig. 3 shows the model for the milk coagulation process after chymosin addition. First, the casein micelles, with intact κ-casein hairy layer, undergo a homogeneous action with chymosin and this process continues for approximately 30 min, when the zeta potential is critical in all casein micelles (lag phase, Fig. 2b). The process of aggregation begins where the κ-casein layer was removed, but the casein micelles are still individually separated, forming structures which appear like bunches of grapes (aggregation phase, Fig. 2b). After around 45 min, these aggregates start to form bigger casein micelles, even containing κ-caseins on their surfaces. Finally, these large micelles form a three-dimensional gel (coagulation phase, Fig. 2b).

4. Conclusion

With the data reported here, the model for the milk coagulation process was reinforced using atomic force microscopy (AFM). The results suggested this tool as an alternative to study the effect of other proteases on casein micelles and, consequently milk coagulation. Considering that AFM is not a stand-alone technology and that it does not provide identification of milk samples, it is very important to associate the aggregated aspect with the type of enzyme used in the process. Therefore, perspectives are now open for the study of different proteases on casein micelles using AFM. This would bring further insights into cheese technologies in terms of taste and consistency, since these parameters are critically dependent on the caseins and enzymes used in the milk coagulation process.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Ethical approval

This study did not involve any experiments with humans or animals.

Contributions

All authors equally participated in all assays, contributing to data analysis, discussion and writing of the manuscript.

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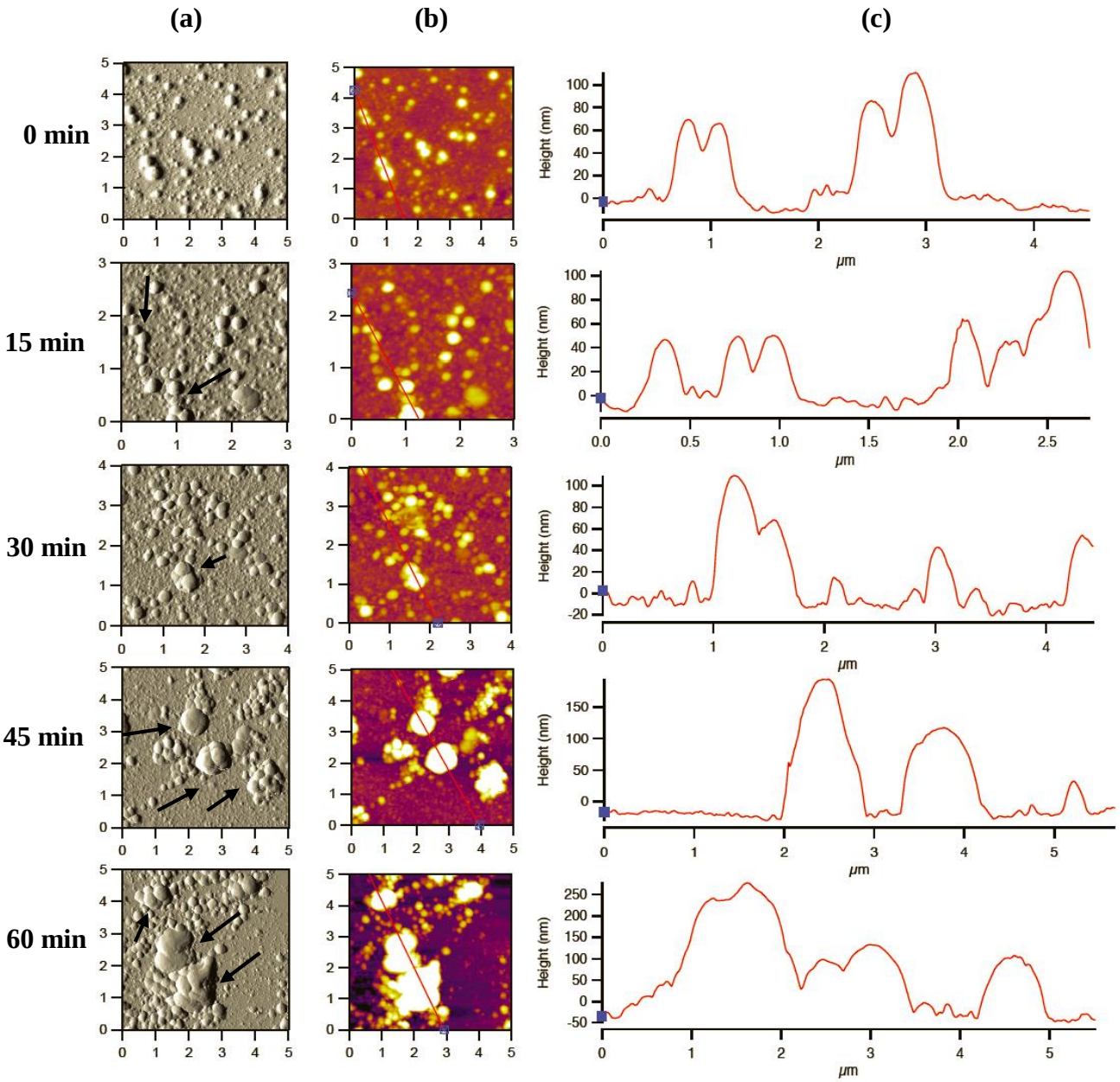
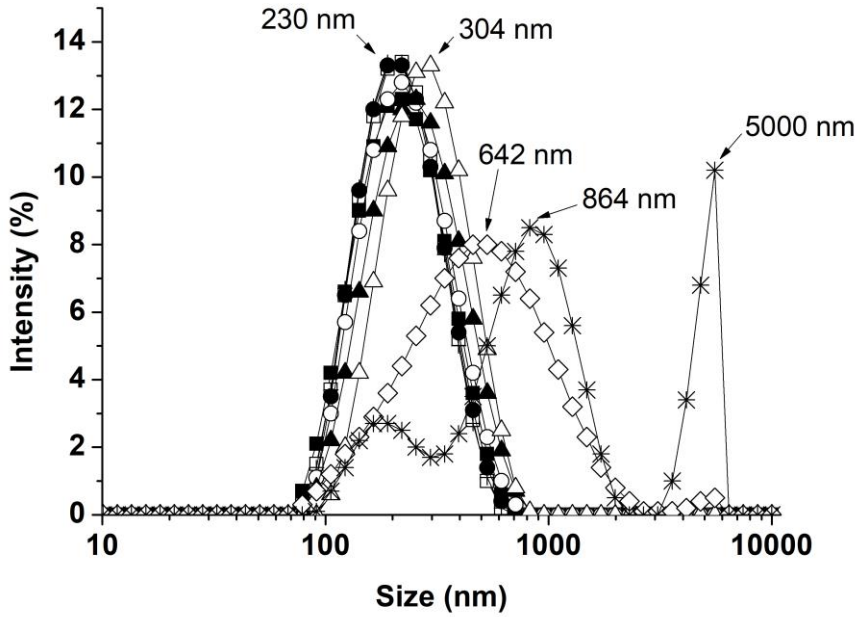


Fig. 1. Tapping mode AFM images of milk casein micelles after incubation with chymosin for different times. **(a)** topography images, **(b)** amplitude images and **(c)** height profile analysis of the cross-section highlighted in B. The enzyme (20 µl, 2 mg/ml) was dissolved in water and added to 2 ml of skimmed milk. Then, the samples were diluted with 50 mM Tris-HCl buffer pH 6.5 (1:20, v/v) containing 10 mM $CaCl_2$. The assays were done during 60 min and at 25°C. Arrows indicate the formation of aggregates and the coagulation of casein micelles.

(a)



(b)

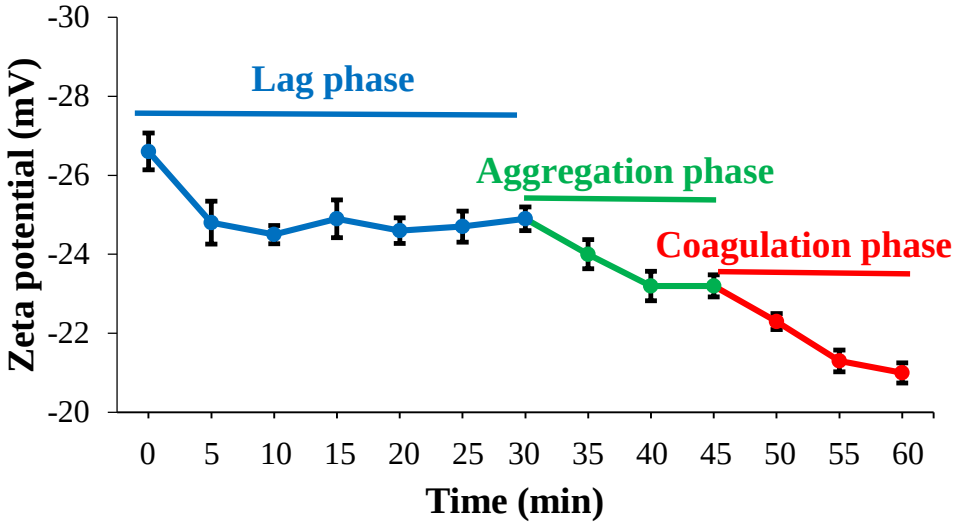


Fig. 2. (a) Particle size distribution and **(b)** zeta potential of milk casein micelles after addition of commercial bovine chymosin for different times measured by dynamic light scattering (DLS). The enzyme (20 µl, 2 mg/ml) was added to 2 ml of skimmed milk, and then the mixture was diluted with 50 mM Tris-HCl buffer pH 6.5 (1:20, v/v). The assays were done at 25°C. **(a)** Legend: Milk without chymosin (■) and at intervals after adding chymosin of 5 min (□), 10 min (+), 15 min (●), 20 min (○), 25 min (▲), 30 min (Δ), 45 min (◊) and 60 min (✱).

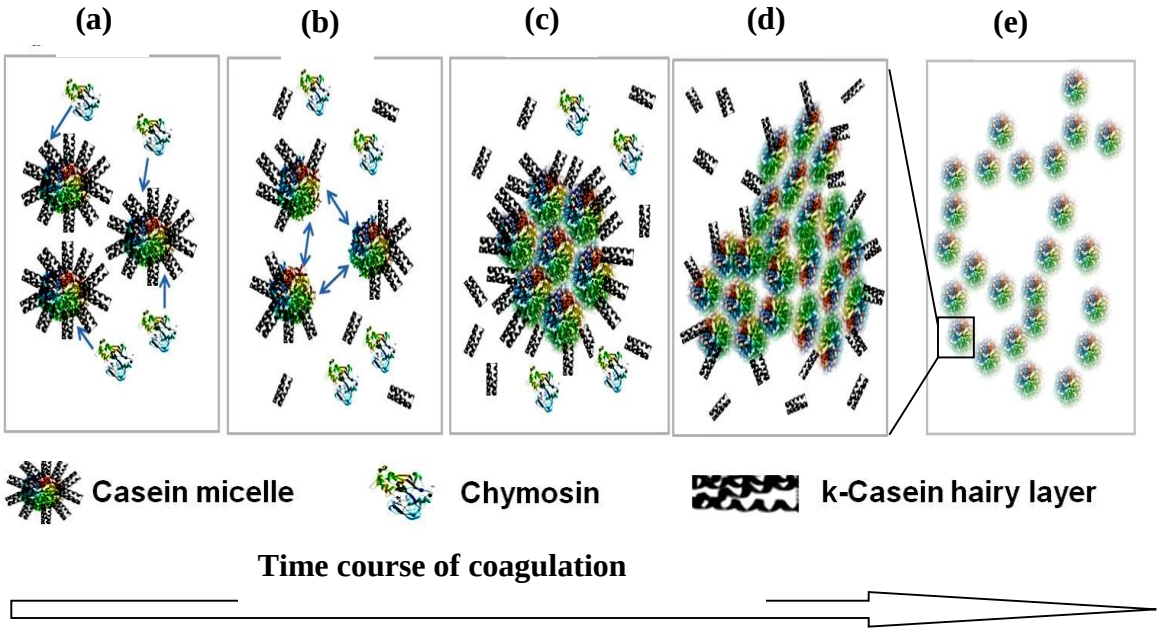


Fig. 3. Schematic figure showing the model for the milk coagulation process after chymosin addition. The three-dimensional model was proposed by Dwyer et al. (2005) with slights modifications: **(a)** Casein micelles with intact κ-casein hairy layer; **(b)** Action of chymosin on κ-Casein; **(c)** κ-casein is partially hydrolyzed and the aggregation process starts, where the κ-casein layer was removed; **(d)** The processes B and C restarts, forming larger

Biotechnological potential of a cysteine protease (CpCP3) from *Calotropis procera* latex for cheesemaking

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Abstract

Although various plant proteases with milk-clotting activity have been described, most of them produces cheeses with bitter tastes. In contrast, different proteolytic extracts from *Calotropis procera* plant have been used successfully for cheesemaking. Because there are several disadvantages for using proteolytic extracts, the aims of this work were to characterize and evaluate the biotechnological potential of a cysteine protease purified from *C. procera* latex, named CpCP3. This enzyme was highly stable to different metal ions and was able to hydrolyse κ-casein similarly to bovine chymosin. Atomic force microscopy showed that the whole process of casein micelles aggregation induced by CpCP3 was very similar to that caused by chymosin. The cheeses made using CpCP3 showed higher moisture content than those made with chymosin, but protein, fat, and ash were similar. *In silico* analysis predicted the presence of only four allergenic peptides in CpCP3, but only two of these were present on its three-dimensional surface. CpCP3 was highly susceptible to digestive enzymes, pepsin and trypsin, including its allergenic peptides. Even using high doses, CpCP3 did not show toxicity on zebrafish embryos, an animal model widely used for in vivo toxicity test. In addition, CpCP3 was highly expressed in *Escherichia coli* cells, but in the insoluble form and without proteolytic activity. All results support the biotechnological potential of CpCP3 as an alternative enzyme to replace chymosin. Further studies should be performed to optimize the heterologous expression of CpCP3 to reach its proteolytic activity.

Keywords: Cheese; Heterologous expression; Plant protease; Toxicity, Zebrafish.

1. Introduction

Cheese is among the most important milk-derived food products. It is an essential ingredient for many different types of foods, as well as it is rich in nutrients, such as proteins, vitamins, essential minerals, fat, and calcium. Besides this, it can offer several health benefits, which include prevention of osteoporosis, protective effect for dental caries, weight reduction or anti-obese effects, and anti-hypertensive properties (Lucas, Chamba, Verdier-Metz, Brachet & Coulon, 2006; Walther, Schmid, Sieber & Wehrmuller, 2008; Gul, Akgul & Seven, 2018). Cheesemaking is a complex procedure, since it involves many steps and biochemical transformations. Depending on the milk origin or applied coagulation process, a wide range of color, textures, tastes, firmness, and aromas can be obtained (Santiago-López, Aguilar-Toalá, Hernández-Mendoza, Vallejo-Cordoba, Liceaga & González-Córdova, 2018). Therefore, it is estimated that there are over 2,000 cheese varieties in the world. Some statistical studies have shown that the world cheese market is massive and valued at around 90 billion U.S. dollars in 2016, and it is forecasted to reach to more than 100 billion by 2022 (https://www.statista.com/). For all these reasons, researches for new enzymes able to produce cheeses with novel characteristics, aromas and flavors are still very relevant topics, deserving continuous studies.

Milk coagulation is the foremost phase for producing cheese. This process can be reached using coagulating enzymes, such as animal, microbial and plant-based proteases (Li, Scott, Hemar & Otter, 2018; Meng et al., 2018). Chymosin (EC 3.4.23.4), extracted from calf stomach, is the protease most extensively used for cheesemaking (Kumar, Grover, Sharma & Batish, 2010). Increasing on demand for chymosin combined to limited availability of calf stomachs, as well as religious (Islam and Judaism) and dietary (vegetarianism) aspects are some factors that have stimulated novel searches for alternative/substitute milk-clotting sources (Shah, Mir & Paray, 2014). Although several plant proteases have been described with milk-clotting activity, many of them are unsuitable for cheesemaking because they do not achieve most industrial requirements, such as cheese yield, process time and flavor (Jacob, Jaros & Rohm, 2011).

On the other hand, different parts of the *Calotropis procera* plant have been identified with milk-clotting activities (Aworh & Muller, 1987; Omotosho, Oboh & Iweala,

2011; Freitas et al., 2016; Rayanatou et al., 2017). Therefore, the main hypothesis of the present work is that proteases purified from *C. procera* are able to clot the milk and produce cheeses with desirable characteristics by industry. As works of biotechnological prospection, those previous studies were very important because they fashioned the perspective of new plant samples for cheesemaking. However, they used proteolytic fractions, which are constituted for several protease isoforms (Ramos et al., 2013). Thus, the main drawback of using a proteolytic fraction can be the difficulty of sample standardization and cheeses made at industrial level, besides the presence of possible toxic compounds within the extracts. Therefore, the main goals of the present work were to characterize a protease purified from *C. procera* by means of its stability against different ions, specific hydrolysis on κ-casein and effects on structure of casein micelles, to describe its toxic and allergenic potentials, as well as to characterize the cheese produced with this protease. Moreover, the biotechnological potential of protease was also evalueted through its cloning and expression in *Escherichia coli* cells.

2. Material and methods

2.1. Chemicals

Azocasein, κ-casein, papain, L-cysteine, pepsin, trypsin, Dithiothreitol (DTT), Isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin sulfate, Phenylmethylsulfonyl Fluoride (PMSF), urea, Sodium dodecyl sulfate (SDS), Triton X-100, and lysozyme were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). HisTrap FF affinity column, CM-Sepharose fast-flow column, RESOURCE-S column, and molecular mass markers were purchased from GE Healthcare (São Paulo, SP, Brazil). Commercial bovine chymosin (Coalhopar®) was obtained at local markets (Fortaleza, CE, Brazil). All other chemicals were of analytical grade.

2.2. Latex processing and protein content

The latex of *C. procera* was collected from healthy and non-cultivated plants growth in Fortaleza, Ceará, Brazil, as described by Freitas et al. (2007). Briefly, the fresh latex was collected in distilled water (1:1 ratio) and centrifuged at 10,000 x *g* for 10 min at 10 °C for rubber elimination and other insoluble materials. After, the supernatant was dialyzed for three days against distilled water using 10 kDa cut-off membranes, and then centrifuged again, as described above. The new supernatant, named as *Calotropis procera* latex proteins (CpLP), was lyophilized and used for purification of a cysteine protease (CpCP3). The Bradford method was used to quantify the protein content of all samples (Bradford, 1976).

2.3. Purification of Calotropis procera *cysteine peptidase (CpCP3)*

CpCP3 was purified according to the Ramos et al. (2013). CpLP, when chromatographed in a CM-Sepharose fast-flow column equilibrated with 50 mM sodium acetate buffer (pH 5.0), was fractioned in three distinct protein peaks (PI, PII, and PIII). PII, eluted with 200 mM NaCl, was dialyzed against distilled water, lyophilized, and then submitted to ion-exchange chromatography using a Resource-S column, equilibrated in 20 mM sodium phosphate buffer (pH 6.0), coupled to an AKTA chromatography system (GE healthcare). CpCP3 was eluted with a linear salt gradient from 0 to 500 mM NaCl during 30 min (1 mL/min flow rate) (Ramos et al., 2013)

2.4. Effect of different ions on proteolytic activity of CpCP3

The proteolytic activity was measured according to Freitas et al. (2007), using 1% azocasein as substrate at pH 6.5 (pH of milk). CpCP3 (20 µL, 2 mg/mL in 50 mM Tris-HCl buffer containing 1 mM L-cysteine, pH 6.5) was incubated with different ions at 10 mM (NaCl, MgCl, LiCl, KCl, or $CaCl_2$) for 30 min at 37 ºC. Afterward, 1% azocasein substrate was added (200 µL) and the reaction was permitted occur for 60 min at 37 ºC. The reaction was stopped with 300 µL of 10% trichloroacetic acid and the reaction mixture was centrifuged at 10,000 x *g* for 10 min at 25 °C. Finally, 400 µL of the supernatants were mixed with 400 µL of 2 M NaOH. The absorbance was

measured at 420 nm and one unit of specific proteolytic activity (SPA) was defined as the amount of enzyme (mg) required for increasing the absorbance in 0.01 per minute.

2.5. Milk-clotting activity

Milk-clotting assay was performed as described in Freitas et al. (2016). Different amounts of CpCP3 (2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg), dissolved in 200 µL of 50 mM Tris-HCl buffer containing 1 mM L-cysteine (pH 6.5), were incubated with 2 mL of milk containing 50 mM $CaCl_2$ at 37 °C. The clotting time or milk clotting activity (MCA) was assigned after observation of discrete particles in the milk when the tubes were gently inclined. Commercial bovine chymosin (Coalhopar®) was used as control. The specific milk-clotting activity (SMCA) was defined by the amount of enzyme (mg) able to clot the milk in 40 min, according to the formula:

SMCA (MCA/mg) = [(2400/clotting time (s)) x dilution factor] / mg (enzyme).

2.6. In vitro hydrolysis of κ-casein

Hydrolysis of bovine κ-casein was evaluated by SDS-PAGE as described by Freitas et al. (2016). CpCP3 (50 µL, 0.1 mg/mL in 50 mM Tris-HCl buffer containing 1 mM L-cysteine, pH 6.5) was incubated with 450 µL of κ-casein (10 mg/mL in 50 mM Tris-HCl buffer, pH 6.5) for different time intervals (0, 1, 5, 10, 15, 20, 25, and 30 min) at 37 °C. The reactions were stopped mixing each aliquot with SDS-PAGE sample buffer, followed by heating at 100 °C for 5 min. The extensiveness of the hydrolysis was observed by 15% SDS-PAGE (Laemmli, 1970). Bovine chymosin (Coalhopar®) was used as control (50 µL, 0.1 mg/mL in 50 mM Tris-HCl buffer pH 6.5).

2.7. Atomic force microscopy (AFM)

The overview of casein micelles treated with CpCP3 were evaluated using AFM technology (MFP3D-BIO microscope from Asylum Research, Oxford

Instruments, USA), as described by Freitas et al. (2019). CpCP3 (30 µg) or chymosin (30 µg), dissolved in 200 µL of 50 mM Tris-HCl buffer, were incubated with 2 mL of milk containing 50 mM $CaCl_2$ at 37 °C. The micelles' aggregation was studied using five different samples: the control (no enzyme, time = 0), and four samples imaged after 15, 30, 45, and 60 min of CpCP3 or chymosin addition. After, the samples were immediately diluted (1:20, v/v) in 50 mM Tris-HCl buffer (pH 6.5), spread on glass surfaces (10 µL), and dried at 25 ºC. The experiments were repeated twice with independent samples. AFM was performed using tapping mode and Econo-LTESP-Au cantilevers from Oxford Instruments, with nominal spring constant of 5 N/m and frequency of 150 KHz.

2.8. Manufacture and partial characterization of cheese

CpCP3 was used for cheese manufacturing in laboratory scale as described by Freitas et al. (2016). Briefly, CpCP3 (12.5 mg) was dissolved in 10 mL of distilled water containing 1 mM L-cysteine and incubated for 5 min at 25 °C. The enzymatic solution was added to 500 mL of pasteurized integral milk containing 50 mM $CaCl_2$ and kept undisturbed for 40 min at 37 °C. After clotting, the curd was cut, whey was removed for heating at 80 °C for 10 min, added again to the curd, and kept under constant stirring for 10 min. Finally, the curd was pressed during 6 h at 25 °C using a handmade machine. Similarly, cheeses made using bovine chymosin (Coalhopar®) were used as controls.

Texture profile analysis (TPA) was conducted according to Lashkari, Asl, Madadlou & Alizadeh (2014) using TA-XT2 Texture Analyser (Stable Micro Systems, UK). A flat probe of 35 mm diameter was attached to the moving crosshead. Cylindrical cheese samples (12 × 10 mm), taken from a depth of 5 mm in the cheese block at 8 °C with a cork borer, were placed in air-tight plastic bags, kept refrigerated at 4 °C for 4 h to equilibrate and then set aside at 21 ± 1 °C for 45 min. Samples were compressed in two-cycle tests at a speed of 1.2 mm/s with 33% deformation from the initial height of the sample. Textural parameters such as hardness, adhesiveness, cohesiveness, gumminess, chewiness, and springiness were determined. Each sample was analysed

in triplicate. Statistical significance was calculated by the paired t-test ($p < 0.05$) using the GraphPad Prism 6 program.

Cheeses were also analyzed for moisture, protein, ash, fat, and carbohydrate using the AOAC procedures (AOAC., 2016). Crude protein content (N × 6.38) was estimated by the Macro-Kjeldahl method, using an automatic distillation and titration unit (model Pro-Nitro M Kjeldahl Steam Distillation System, Barcelona, Spain). Ash was determined by sample incineration (550°C) and crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus. Total carbohydrates were calculated by difference according to the equation:

$$100 - (m_{moisture} + m_{proteins} + m_{fats} + m_{ashes}).$$

2.9. Molecular modelling and in silico *evaluation of allergenic epitopes*

The three-dimensional model of CpCP3 was obtained by molecular modelling using X-ray crystal structure of two latex cysteine proteases: Ervatamin B (PDB ID: 1IWD) and papain (PDB ID: 9PAP). The complete amino acid sequence of CpCP3 was obtained from the work of Kwon et al. (2015). The analyses were performed using the programs GalaxyWEB (http://galaxy.seoklab.org), M4T Server (http://manaslu.fiserlab.org/M4T/) and Phyre2 Server (http://www.sbg.bio.ic.ac.uk/phyre2/html/help.cgi?id=help/faq). The models were analyzed for their geometric and stereochemical quality using the ProSA, ERRAT2, Verify 3D, and Molprobity programs, as described by Cruz et al. (2019). The PyMOL software (http://pymol.org/) was used to analyze and visualize the three-dimensional model generated.

The possible allergenicity of CpCP3 was estimated comparing its primary sequence with other allergenic protein sequences using the program Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP/). CpCP3 sequence was searched for allergenic epitopes by matching six contiguous amino acids residues with all the sequences of allergenic proteins deposited in the SDAP database (Ivanciuc, Schein & Braun, 2003). In order to compare CpCP3 with other

proteases, the same study was performed using papain, which is a latex cysteine protease widely used in food industry. The predicted allergenic epitopes were labelled in the three-dimensional structures of CpCP3 and papain using PyMOL program (http://pymol.org/).

2.10. Digestibility assay

The *in vitro* susceptibility to sequential digestion of CpCP3 was developed as described by Farias et al. (2015), with slight modifications. The sample was sequentially incubated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at a concentration of 1 mg/mL. CpCP3 was incubated in SGF [34 mM NaCl, 0.07% HCl (pH 2.0), and 1 mg/mL of pepsin] at 37 ºC under stirring, and aliquots of 100 µL were collected at different times of incubation and then transferred to appropriate stopping buffer (2% SDS, 10% glycerol, 6% β-mercaptoethanol, 0.01% bromophenol blue, 200 mM DTT and 500 mM Tris-HCl, pH 8.0) in ratio of 1:1 (v/v). Sequentially, SIF was added [50 mM potassium phosphate (pH 8.0), and 1 mg/mL trypsin]. The mixture was incubated again at 37 ºC under stirring, and aliquots of 100 µL were collected and then transferred to an appropriate stopping buffer (3% SDS, 17% glycerol, 8.5% β-mercaptoethanol, 0.01% bromophenol blue, 170 mM DTT, 6 mM PMSF, 200 mM Tris-HCl, pH 7.2). The aliquots were collected after 30 min, 1 and 2 h of incubation in SGF and SIF. The digestibility of CpCP3 was monitored by 15% SDS-PAGE (Laemmli, 1970). The cleavage sites by proteases pepsin and trypsin in the CpCP3 sequence was performed using the ExPASy PeptideCutter tool (https://web.expasy.org/peptide_cutter/).

2.11. In vivo *toxicity*

2.11.1. Zebrafish embryos

The zebrafish (*Danio rerio*) embryos were provided by the zebrafish facility stablished at department of Molecular Biology, Federal University of Paraíba (João Pessoa, Brazil). Zebrafish adults (wild type strain) were kept at 26 ± 1 ºC under a 14:10 h (light:dark) photoperiod. The water quality was maintained by activated-carbon

filtration, conductivity at 750 ± 50 µS, and dissolved oxygen above 95% saturation. Fishes were fed daily with commercial food (Tropical Gran Discus, Sarandi, Brazil) and *Artemia* sp. nauplii, being also monitored for abnormal behavior or disease development.

To obtain embryos, an egg trap was placed overnight in a tank containing male and female specimens (1:1 ratio) one day prior to testing. One hour after the beginning of the light cycle, eggs were collected with a Pasteur pipette and rinsed with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) for subsequent selection of embryos by using a stereomicroscope (80x magnification). Viable fertilized eggs were selected for further assays of Fish Embryo Acute Toxicity (FET) test (section 2.11.3). All experiments conducted with zebrafish in this study were approved by the Ethic Commission in Animal Use in Research (CEUA), certified by number 6743030518 by Federal University of Paraíba, Brazil.

2.11.2. Stability of CpCP3 to the conditions of the embryotoxicity test

CpCP3 was dissolved in E3 medium at 2 mg/mL. Then, the protein solution was transferred for a 24-well plate and left for 96 h in the same conditions that would be run the FET test with zebrafish embryos. In this test 8 wells were filled, each one with 2 mL of the protein solution. Every 24 h were collected aliquots of 2 mL from two wells and rapidly frozen in a -20 ºC. Just before the assays, the aliquots were thawed and performed for analysis of total proteolytic activity and SDS-PAGE. The proteolytic activity of CpCP3 in the exposure conditions of the FET test was determined by colorimetric measurement using azocasein as the substrate (Section 2.4). The autolysis of CpCP3 was evaluated by 15% SDS-PAGE according to Laemmli (1970).

2.11.3. Acute toxicity test using zebrafish embryo

The Fish Embryo Acute Toxicity (FET) test was conducted with CpCP3 according to OECD's guideline number 236 (OECD, 2013). Twenty fertilized eggs were placed in 24-well plates (1 embryo per well) and exposed to 100 mg/L of CpCP3. This concentration is recommended by the FET test as the limit concentration for

embryotoxicity analysis of any chemical (OECD, 2013). Eggs, exposed only to E3 medium, were used as internal controls. Eggs with up to 3 h post fertilization were exposed to CpCP3 for 96 h, and embryos were analyzed every 24 h for the apical endpoints: egg coagulation; lack of somite formation; lack of detachment of the tail-bud from the yolk, sac and lack of heartbeat. In the presence of any of these endpoints the embryo/larva was considered dead. The exposures were under static conditions (without renovation of CpCP3 and E3 medium). Observations were performed in a stereomicroscope (80x magnification) and photographed (Zeiss). After 96 h, surviving larvae were euthanized with eugenol and discarded according to the Ethic Commission in Animal Use in Research of Federal University of Paraíba, Brazil.

2.12. Heterologous expression, purification, and proteolytic activity

The pET-SUMO expression vector (Supplementary Fig. 1), containing the CpCP3 sequence, was synthetized by GenOne Biotechnologies company (http://www.geneone.com.br/) and used to transform *E. coli* SHuffe cells by electroporation. To overcome the codon biasing problem, synthetic gene of CpCP3 was optimized to contain the codons more frequently used in *E. coli*. To help in solubilization and purification of recombinant protein, the SUMO tag, containing six histidines (6xHis), was added in N-terminal sequence of CpCP3.

The transformed *E. coli* cells were cultivated at 37 °C for 16 h in Luria-Bertani (LB) solid-medium containing kanamycin (50 µg/mL), and then an isolated colony was inoculated into 5 mL of liquid LB medium containing kanamycin and kept at constant stirring (200 rpm) at 37 °C for 16 h. Afterward, 5 mL of the medium was inoculated into 500 mL of another kanamycin-supplemented LB medium. When the OD_{600} was between 0.4-0.6, the expression was induced with different concentrations of IPTG (0.01, 0.1, 0.3, 0.5, 0.8 and 1 mM) at 160 rpm and 22 °C. Aliquots were taken at different incubation time (0, 6 and 18 h) and then centrifuged at 8,000 x *g* for 10 min at 4 °C. The cells were disrupted with 50 mM Tris-HCl buffer containing 300 mM NaCl, 100 µg lysozyme, 1 mM PMSF, 10% glycerol and 0.5% Triton X-100, for 30 min at 37 °C, followed by sonication for seven cycles of 30 s. The samples were centrifuged at 12,000 x *g* for 30 min. The precipitate (insoluble fraction) and the supernatant (the

soluble fraction) were analyzed by 12.5% SDS-PAGE. The precipitates were solubilized in 50 mM Tris-HCl buffer (pH 8.0) containing 8 M urea for 30 min at 37 °C or in 50 mM Tris-HCl buffer, containing 500 mM NaCl and 1% SDS. Samples were then centrifuged at 12,000 x *g* for 30 min at 4 °C. Besides the SDS detergent, the sarkosyl and CTAB detergents were tested under the same conditions. The solutions were kept at 55 °C for 2 h and then centrifuged at 12,000 x *g* for 30 min at 25 °C. The supernatants and precipitates were analysed by SDS-PAGE.

The recombinant protein (His_6-SUMO-CpCP3) was purified using a HisTrap FF affinity column coupled to an AKTA chromatography system (GE healthcare). The column was previously equilibrated with 50 mM Tris-HCl (pH 8.0), containing 500 mM NaCl and 2 M urea and the recombinant His_6-SUMO-CpCP3 was eluted with gradient of imidazole (0-500 mM) at a flow rate of 0.5 mL/min for 30 min. The chromatographic peaks were dialyzed (50 mM Tris-HCl buffer pH 8.0, 100 mM NaCl) and concentrated in Vivaspin ™ tubes with 10 kDa cut-off and then analysed by 12.5% SDS-PAGE. To remove the His_6-SUMO fusion tag, His_6-SUMO-CpCP3 was incubated with Ubl-specific protease 1 (ULP1) from *Saccharomyces cerevisiae* for 16 h at 4 °C. The digested material was analysed by 12.5% SDS-PAGE.

The proteolytic activity of recombinant CpCP3 (rCpCP3) was evaluated by zymogram containing 0.1% gelatin as substrate, according to Freitas et al. (2007). The recombinant protein without SUMO tag was separated by electrophoresis through 12.5% SDS-PAGE containing 0.1% gelatin at 25 ºC. After, the gels were incubated in 2.5% Triton X-100 (renaturing solution) and gently shaken for 30 min at 25 ºC to wash out SDS and allow protein rearrangement. The gels were then incubated in 50 mM Tris-HCl buffer (pH 6.5) containing 1 mM L-cysteine for 24 h at 37 ºC and stained with 0.2% Coomassie Brilliant Blue R-350. Enzymatic activity was detected as transparent bands. Native CpCP3 and papain were used as positive controls for proteolytic activity.

3. Results and discussion

3.1. Effect of ions on CpCP3 activity

In a previous study, Freitas et al. (2016) showed that a proteolytic fraction obtained from the *Calotropis procera* latex (CpLP) exhibited milk-clotting activity and an interesting potential for cheesemaking. CpLP was already characterized and five cysteine peptidases were described: procerain, procerain B, CpCP1, CpCP2, and CpCP3 (Dubey & Jagannadham, 2003; Singh, Shukla, Jagannadham & Dubey, 2010; Ramos et al., 2013). As pointed out before, the use of a mixture of peptidases can have several disadvantages for cheesemaking, such as extensive hydrolysis of caseins causing a low yield and bitter taste to cheeses. To overcome these drawbacks, it is recommended to work with purified enzymes. In this aspect, the most abundant peptidase (CpCP3) from *C. procera* latex fraction (CpLP) was purified, partially characterized, and its milk-clotting activity assessed.

Firstly, the effect of different metal ions on the proteolytic activity of CpCP3 was evaluated. Any of the ions tested had effects on CpCP3 activity (Supplementary Table 1). Accordingly, Freitas et al. (2016) showed that 1 M NaCl or $CaCl_2$ had no inhibitory effect on CpLP. In addition, similar results were observed by other milk-clotting enzymes (Silva et al., 2013). Study the effect of NaCl and $CaCl_2$ on the proteolytic activity is crucial, since both salts are widely used during cheesemaking. NaCl has been used to improve stability of casein micelles (Guinee & Fox, 1993), whereas $CaCl_2$ is essential to aggregation process of casein micelles and then the milk coagulation (Pires, Orellana & Gatti, 1999). Regarding to Li^+, K^+, and Mg^{2+} lake of effect on CpCP3 activity, He, Ren, Guo, Zhang, Song and Gan (2011) reported equivalent result for Li^+ and Mg^{2+}, whereas K^+ exhibited only a slight inhibitory effect on the microbial coagulant peptidase.

3.2. Hydrolysis of κ-casein by CpCP3

Caseins (αs1-, αs2, β-, and κ-caseins) are the most abundant milk proteins and form well-ordered colloidal structures, which are named casein micelles (De Kruif, Huppertz, Urban & Petukhov, 2012). Any event that destabilizes the casein micelle

structure can result in milk coagulation. For instance, proteases coagulate milk because of the hydrolysis of κ-casein reduces the steric and electrostatic repulsion between the micelles promoting their aggregation (Dalgleish, 2011). However, extensive or unspecific hydrolysis of caseins can produce cheeses with low yields or bitter tastes. Therefore, it is very important to determine the specificity of the protease towards κ-casein (Jacob et al., 2011). Herein, CpCP3 was able to cleavage κ-casein after one minute of incubation (Fig. 1). The presence of a protein band of 16 kDa (para-κ-casein) suggests that CpCP3 cleaved κ-casein similarly to bovine chymosin. Accordingly, a protein band with same molecular mass was observed after hydrolysis of κ-casein by other plant proteases (Egito et al., 2007). Moreover, after 5 min, other protein bands from κ-casein were observed after action of CpCP3 (Fig. 1). These results suggest that CpCP3 has a preference over the same chymosin cleavage site. However, after cleavage of all these bonds, CpCP3 can hydrolyse other ones, producing peptides of various sizes (Fig. 1). Interestingly, the proteolytic fraction of *C. procera* (CpLP) also showed the same pattern of cleavage on κ-casein and even so the cheeses exhibited good taste and yield (Freitas et al., 2016). These results reinforce that the time of coagulation as well as the concentration of enzyme are parameters that must be controlled to avoid the extensive hydrolysis of caseins, which is the main drawback found for most plant proteases for cheesemaking.

3.3. Milk-clotting activity

The supplementary table 2 shows that 30 μg of CpCP3 was the lowest dose able to coagulate 2 mL of milk, in approximately 40-50 min (time recommended to the enzymatic action during cheese manufacturing). The specific milk-clotting activity (SMCA) and proteolytic activity (SPA) of CpCP3 were 277.4 ± 72 and 33.7 ± 0.5, respectively. Although these two parameters are used to evaluate the potential of an enzyme in cheesemaking, SMCA/SPA ratio is the value more useful because a good coagulant should exhibit a high SMCA and a low SPA (Arima, Yu & Iwasaki, 1970). CpCP3 exhibited a SMCA/SPA ratio of around 8.4. In contrast, the SMCA/SPA ratio for bovine chymosin was 742.4, almost 90 times higher than CpCP3. This discrepancy is the resulted of low proteolytic activity of chymosin, since its SMCA was similar to that of CpCP3 (Supplementary table 2). These results are in accordance with other

studies, which showed that chymosin usually has higher SMCA/SPA ratio than other plant proteases (Mazorra-Manzano et al., 2013; Hashim, Mingsheng, Iqbal & Xiaohong, 2011). Despite the difference in SMCA/SPA ratio, the overall aspect of the curd produced by CpCP3 and chymosin were very similar (data not shown).

3.4. Effect of CpCP3 on casein micelles

Recently, AFM was used as an alternative to study the milk-clotting process induced by chymosin at casein micelle level (Freitas et al., 2019). The authors claimed that this technology has several advantages compared to other ones, because it is fast and no additional dye, fluorophore or reagent are necessary as well as the interpretation of the results is easy. Therefore, the action of CpCP3 on casein micelles during the coagulation process was evalueted using AFM and compared with bovine chymosin (Fig. 2). AFM showed that the whole aggregation process of the casein micelles induced by CpCP3 was very similar to that caused by chymosin. The casein micelles and their aggregates exhibited widths, heights, and areas very alike (Supplementary Fig. 2). Interestingly, the widths of casein micelles were greater than heights. These results are in agreement with those described by Freitas et al. (2019), who discussed that this flattening occurs because the casein micelles are stabilized by non-covalent bonds, making them more susceptible to deformations during AFM experiments. Accordingly, this flattening of casein micelles during the AFM analysis has also been described by other authors (Ouanezar, Guyomarc'h & Bouchoux, 2012; Silva, Bahri, Guyomarc'h, Beaucher & Gaucheron, 2015).

After 30 min, casein micelle aggregates were observed in both samples (Fig. 2). This step occurs after the κ-casein hydrolysis because it is responsible for generating electrostatic repulsion among micelles (Dalgleish, 2011). Fig. 1 corroborates these results, since the CpCP3 hydrolyzed κ-casein producing para-κ-casein peptides, similarly to chymosin. After 45-60 min, casein micelles continued aggregating, forming structures such as bunches of grapes, according to the Freitas et al. (2019). Finally, after 60 min, large clusters of casein micelles were formed (Fig. 2). Although it was not possible to be visualized by AFM, it is expected the formation of a

three-dimensional gel as final step of milk coagulation (Dwyer, Donnelly & Buckin, 2005).

3.5. Characterization of cheeses made using CpCP3

Some characteristics of cheeses obtained using CpCP3 and chymosin are reported in Fig. 3. In general, cheeses exhibited similar colour and yield, but those ones produced using CpCP3 were softer than those made with chymosin (49%) ($p < 0.05$). This was confirmed for higher moisture (54%) in the cheese made with CpCP3. In terms of composition, both cheeses presented equivalent protein, fat and ash percentage, which were 19, 3 and 5%, respectively (Fig. 3). However, the carbohydrate content was lower in cheeses made with CpCP3 (19% versus 25%) ($p < 0.05$), which can be related to softness of these chesses. Likely, cheeses were softer because of the larger spaces between casein micelle aggregates, which form the three-dimensional gel. Thus, the process of curd pressing resulted in higher loss of small carbohydrates, such as lactose (Logan et al., 2014). All these results are in agreement with those that used total extracts of *C. procera*. Aworh et al. (1987) reported moisture and protein contents of 49% and 20% against 54% and 19% measured in this study. Accordingly, Omotosho et al. (2011) reported a protein content of 25% and moisture of 50%.

Texture profile analysis of cheeses are also shown in Fig. 3. Cheese hardness was significantly decreased using CpCP3 (30%) compared with chymosin (3.5 N versus 5.0 N) ($p < 0.05$). Similar results were described by Mazorra-Manzano et al. (2013). They reported that cheeses made with kiwi, ginger, and melon proteases showed hardness of 6.1, 4.1, and 1.9 N, respectively. These low values were associated with the high moisture content of cheeses (62–67%), corroborating with our results. The cohesiveness, gumminess, and chewiness were significantly higher for cheeses made with CpCP3, whereas adhesiveness and springiness values were not statistically different (Fig. 3).

3.6. Evaluation of allergenicity, digestibility and toxicity of CpCP3

Only four allergenic epitopes (EKGALV; GSCWAFSAV; LISLSEQ, and YWIVRNSWG) were identified in CpCP3 sequence, which showed cross-reaction with allergens of insects, mites or foods (papaya, kiwi, pineapple) (Supplementary Table 3). In contrast, papain, a latex protease, widely used at industrial level (Fernández-Lucas, Castañeda & Hormigo, 2017), exhibited several allergenic epitopes (Supplementary Table 4). Interestingly, the three-dimensional model of CpCP3 showed that only two allergenic peptides (EKGLV and LISLSEQ) were present in the surface of the molecule, that is, accessible to a possible antibody recognition and induction of allergy. On the other hand, all allergenic peptides of papain were displayed on its surface, covering around 70% of protein (Fig. 4). Although papain has a high allergenic potential, there is only a few cases of papain allergy (Quarre, Lecomte, Lauwers, Gilbert & Thiriaux, 1995).

CpCP3 was highly susceptible to pepsin and trypsin digestion, already after 30 min (Fig. 5A). Thus, a possible intake of this protein by oral route, fragments with high molecular mass are not expected to reach the final stage of digestion. Even that this occurs, peptides would be degraded by other proteases such as chymotrypsin. These results corroborate with the *in silico* digestion performed by pepsin and trypsin (Fig. 5B). Remarkably, the four allergenic peptides of CpCP3 (EKGALV; GSCWAFSAV; LISLSEQ, and YWIVRNSWG) exhibit catalytic sites by trypsin and pepsin. This high susceptibility to pepsin and trypsin digestion supports the hypothesis of the low allergenic (Codex, 2009) and toxic potential of CpCP3 by oral via.

In the context of safety assessment of novel proteins, toxicity tests in animal models are often employed to gather information about the risks of these molecules to potential consumers (Delaney et al., 2008). Although the gold standard for toxicity testing has traditionally been rodents, in recent years they have been gradually replaced by alternative animal models, for ethical or economic reasons (Doke & Dhawale, 2015). In this direction, zebrafish embryos are considered as an emerging alternative to the use of mammals in several toxicological studies, such as for investigation of acute toxicity of diverse substances (Doke et al., 2015; Falcão, Souza, Dolabella, Guimarães, & Walker, 2018). The use of zebrafish embryos in toxicology presents innumerable advantages, such as low cost of production, availability in large

numbers, optical transparency, rapid development, easy manipulation and the possibility of obtaining a large number of toxicological information (e.g. teratogenicity, lethality) in a single experiment (Raghunath & Perumal, 2018). More important, results obtained in toxicity tests performed on zebrafish embryos are equivalent to those found in mammalian models, pointing out the reliability of zebrafish toxicity studies (Falcão et al., 2018).

The acute toxicity of CpCP3 was assessed on dechorionated zebrafish embryos at a concentration of 100 mg/L (OECD FET's limit test). Before performing the test, the protein sample was tested for its stability, including proteolytic activity and autolysis. CpCP3 retained all its proteolytic activity as well as maintained the same protein profile in the electrophoresis even after 96 h of incubation (data not shown). This ensured that the zebrafish embryos were exposed to a fully active protease and rule out the needing for daily renewal of the test solution during the assays. Thus, newly fertilized zebrafish eggs were exposed to activated CpCP3 for 96 h. Even at the exposure conditions, CpCP3 caused no mortality or any other morphological or developmental alteration in the embryos/larvae of zebrafish (Supplementary Fig. 3), similarly to the negative control.

Our group has shown that the proteolytic fraction of *C. procera* (CpLP) was not allergenic or toxic in other animal models. CpLP was totally digested by digestive proteases and was not detected in fecal material of rats even after 35 consecutive days of consumption (Ramos et al., 2006). Besides, no death or toxic effects were described. In another study, CpLP did not induce allergy in animals by oral route (Ramos et al., 2007). More recently, *in vivo* toxicity and oral immunological tolerance of CpLP were reported (Bezerra et al., 2017). Animals receiving orally a high dose of CpLP (5000 mg/kg) exhibited only slight behavioral alterations and alteration of monocytes. No death was observed after 14 days of assay.

All those results suggest that CpCP3 has a very low or no acute toxic and allergenic effect on vertebrate models. In the perspective of environmental risk assessment, the results also show that CpCP3 does not represent a potential risk to aquatic vertebrates. Moreover, the cheeses made with proteolytic extract of *C. procera* latex (CpLP) did not show proteolytic activity and nor residual proteins (Freitas et al., 2016). This is important because the risk of toxicity is very low as well as there will not

be hydrolysis of the milk proteins during the cheese ripening process, avoiding changes in texture, taste, and flavor of the product (Llorente, Obregón, Avilés, Caffini, & Vairo-Cavalli, 2014).

3.7 Heterologous expression, purification, and proteolytic activity

The best conditions for expression of recombinant CpCP3 (His_6-SUMO-CpCP3) were 0.1 mM IPTG and after 6 h at 22 ºC of induction (Fig. 6A). However, it was expressed mainly into inclusion body (Fig. 6B), recurrent factor in several heterologous proteins (Maseko *et al*., 2016; Quaas, Burmeister, Li, Nimtz, Hoffmann & Rinas, 2018). Similarly, two cysteine proteases from *C. procera* latex were also expressed in inclusion body even using different vectors and host cells (Singh, Yadav & Dubey, 2013; Kwon et al., 2015). Urea and SDS detergent showed to be the best agents to solubilize the inclusion bodies compared to the other chemicals used (Fig. 6C). Other studies also reported successfully these methods for solubilization of insoluble proteins (He & Ohnishi, 2017). Because, SDS inhibits the SUMO protease activity, the material solubilized with urea was used in further assays for purification of protein. Using a HisTrap FF affinity column, His_6-SUMO-CpCP3 was purified after elution with 300 mM imidazole, containing 0.5 M NaCl (Fig. 6D). Afterwards, His_6-SUMO-CpCP3 was digested with SUMO protease to remove the His_6-SUMO tag (Fig. 6E). Unfortunately, the rCpCP3 was not active as assessed by zymogram containing 0.1% gelatin (Fig. 6 F). Similarly, even using three different *E. coli* strains (Origami, C41, C43) and a pelB leader sequence to induce proper folding, Kwon and collaborators (2015) were not able to express an active cysteine protease from *C. procera*. In the same way, Singh et al., (2013) tried to express active procerain B (cysteine protease from *C. procera*) in BL21 (DE3) *E. coli*. They also used other modified *E. coli* strains [Rosetta-gami and Rosetta 2-(DE3)] but all proteins were expressed again in inclusion bodies and without proteolytic activity.

Genetic engineering in combination with recombinant DNA technology have been used to provide large-scale proteins for industry, avoiding problems of yield and homogeneity of samples. A practical example is the chymosin itself (Ulusu, Şentürk, Kuduğ & Gökçe, 2016). There are several types of host organisms, but bacteria,

including *Escherichia coli*, have been the most widely used, because they grow rapidly and in inexpensive growth media (Kaur, Kumar & Kaur, 2018). However, the major drawback is the formation of aggregates or inclusion bodies, as observed here. To overcome this problem, CpCP3 was constructed containing the SUMO-tag that has been used to promote the solubilization of several proteins in *E. coli* (Kaur et al., 2018). Moreover, the vector (pET-SUMO) was inserted into *E. coli* SHuffle, which is an expression system dedicated to producing correctly disulfide bond into proteins (Lobstein, Emrich, Jeans, Faulkner, Riggs & Berkmen, 2012). Although all the efforts, rCpCP3 was not recovered in its active form. The lack of proteolytic activity could be attributed to misfolding of protein or because of the presence of pro-peptide region is essential to the correct folding of proteases (Singh et al., 2013; Kwon et al., 2015).

4. Conclusion

The present study supports our hypothesis of the biotechnological potential of CpCP3 for cheesemaking, since it showed several characteristics appreciated by industry: (1) excellent stability to various metal ions; (2) it hydrolyses κ-casein and induces casein micelle aggregation similarly to chymosin; (3) it makes cheeses with yield, protein, fat and ash contents equivalents to cheeses made with chymosin; (4) it has a very low allergenic and toxic potential; (5) it is fully active even after 96 h at 25 ºC and does not suffer autolysis; (6) it can be successfully expressed in *E. coli* cells, thus it can be produced in high quantity. As perspective, further assays should be performed to enhance the refolding of CpCP3 and reach its proteolytic activity.

Conflict of interest

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

Contributions

MZRS, SRS, and JPBO performed purification of CpCP3, proteolytic activity and milk-clotting assays as well the hydrolysis of k-casein. AFBS and JSS performed the AFM assays. DFF, CAS and JACR performed all assays of digestibility and toxicity using zebrafish. RAZ and ACS conduced the assays of characterization of chesses.

GPF and TBG helped in heterologous expression of CpCP3. CDTF and MVR are the main researches of project, supporting, designing and analysing all assays and results. All authors contributed to data analysis, discussion and writing of the manuscript.

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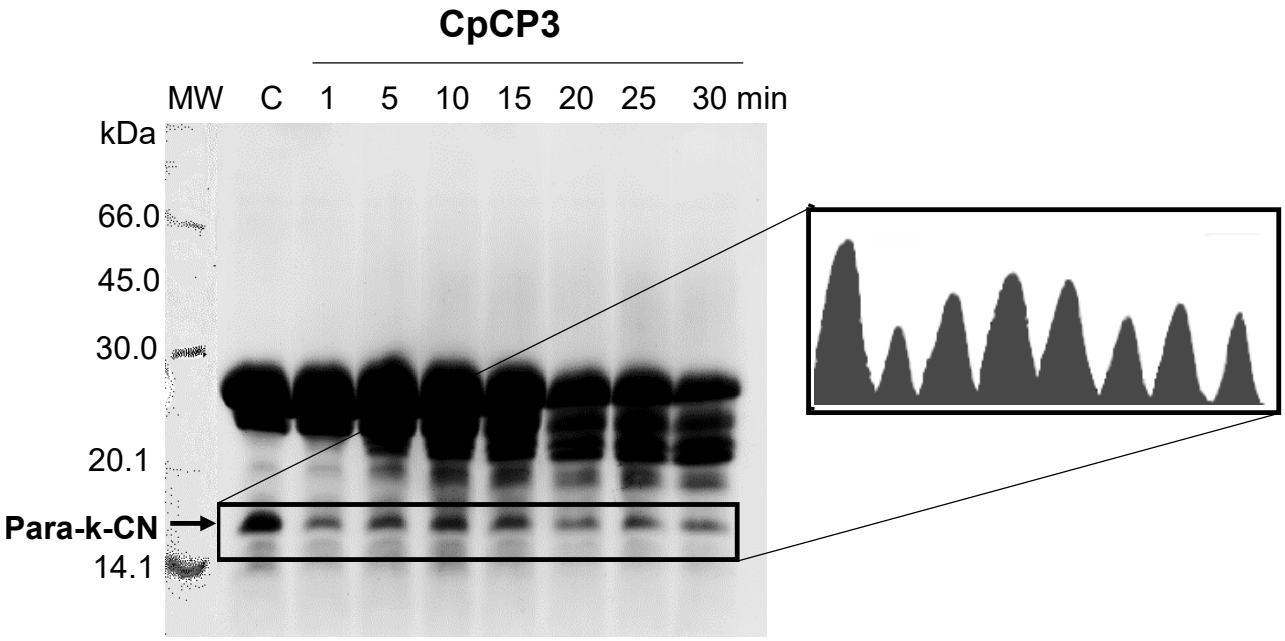


Fig. 1. SDS-PAGE (15%) of κ-casein hydrolyzed by CpCP3 at different time points (1, 5, 10, 15, 20, 25 and 30 min). The assay was performed at 37 °C and pH 6.5 using an enzyme:substrate ratio of 1:900 (mass:mass). Legend: C (Control): κ-casein hydrolyzed by bovine chymosin (Coalhopar®). MW: molecular weight markers. The arrow shows the para-κ-casein peptide produced after κ-casein hydrolysis. Panel represents the densitometry of the para-k-CN band.

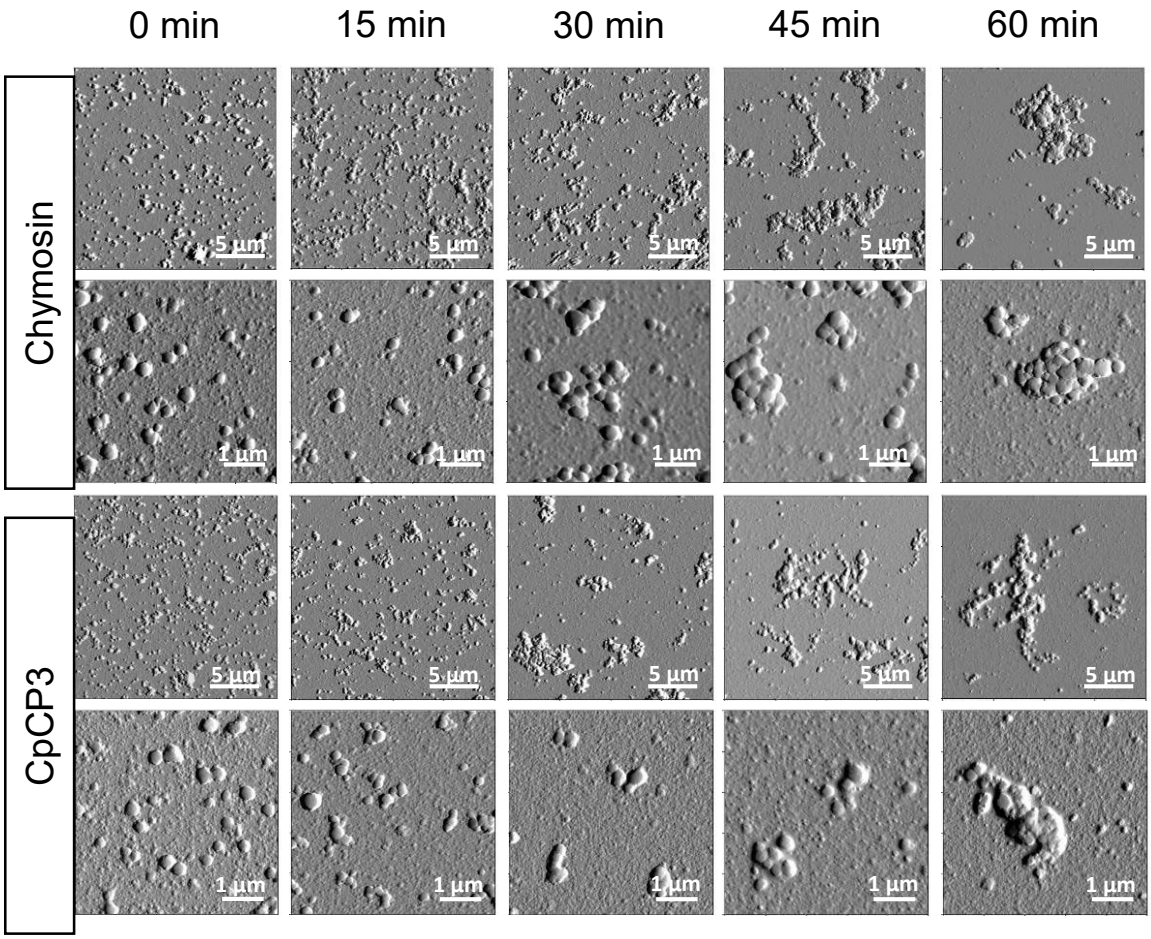
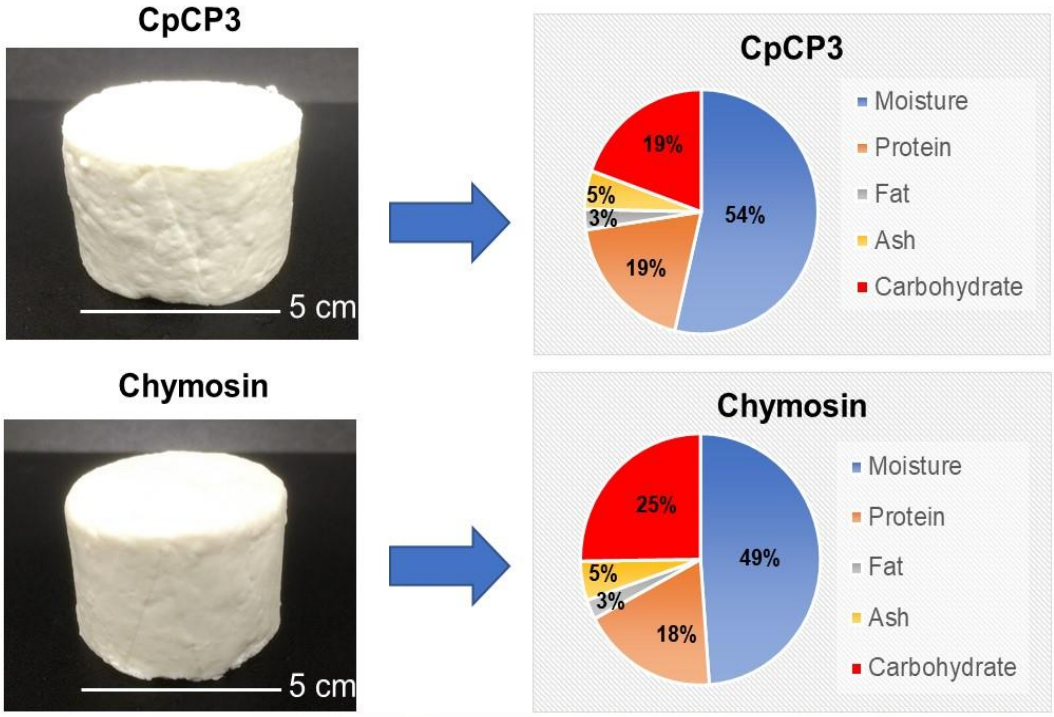


Fig. 2. Tapping mode AFM images of milk casein micelles after incubation with CpCP3 or commercial chymosin during 60 min and at 25 °C.



Samples	Hardness (N)	Adhesiveness	Springiness	Cohesiveness	Gumminess (N)	Chewiness (N)
CpCP3	$3.5.3^{b} \pm 0.08$	$-0.84^{a} \pm 0.34$	$0.84^{a} \pm 0.01$	$0.50^{b} \pm 0.04$	$18.04^{b} \pm 2.02$	$16.19^{b} \pm 2.11$
Chymosin	$5.0.0^{a} \pm 0.85$	$-0.92^{a} \pm 0.33$	$0.82^{a} \pm 0.02$	$0.22^{a} \pm 0.07$	$6.52^{a} \pm 0.87$	$5.24^{a} \pm 0.60$

Fig. 3. Overview of cheeses made using CpCP3 and commercial chymosin (control), percentage of moisture, protein, fat, ash, and carbohydrate, and texture profile analysis. The values represent a mean of three replicates with their respective standard deviation. Different letters show non-statistical significance ($p>0.05$). Statistical analysis was calculated by the paired *t*-test using the GraphPad Prism 6 program.

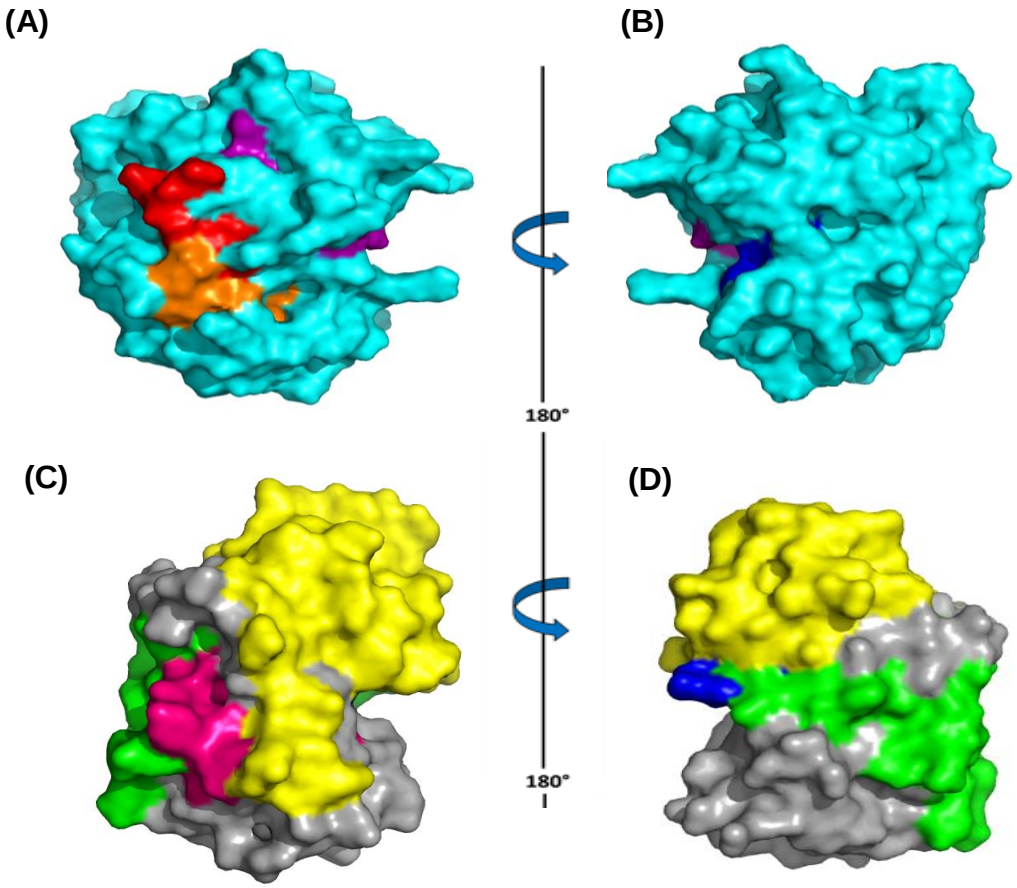
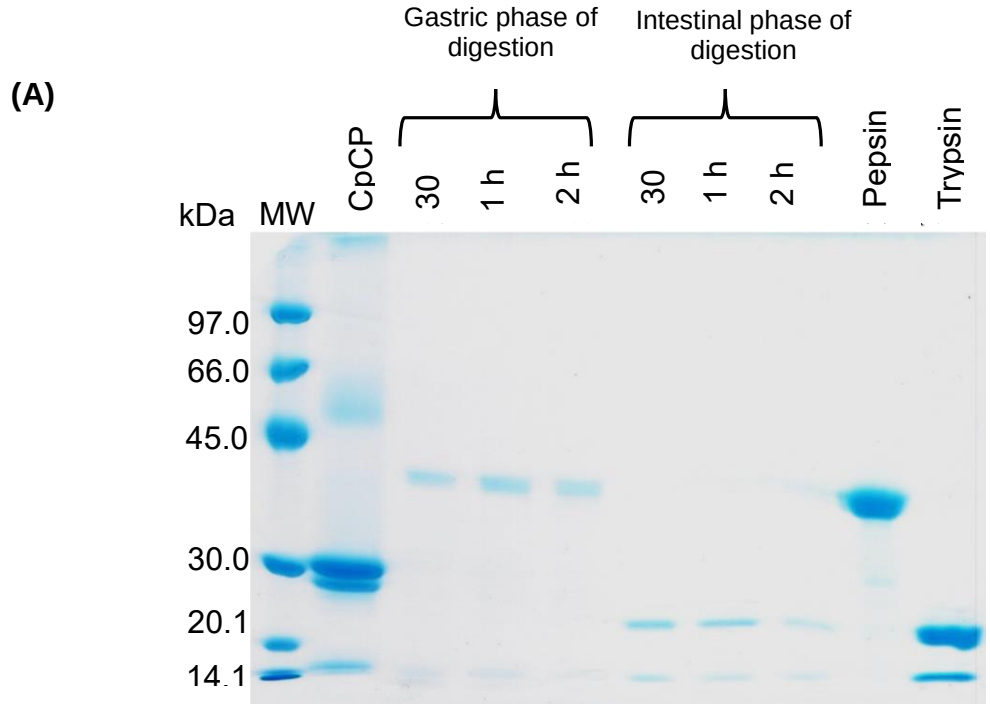


Fig. 4. Predicted allergenic peptides in the CpCP3 and papain sequences. Three-dimensional models of CpCP3 (A and B): putative allergenic peptides were marked in red (EKGALV), dark blue (GSCWAFSAV), orange (LISLSEQ), and purple (YWIVRNSWG). Papain (C and D): yellow (IPEYVDWRQKGAVTPVKQGSCGSCWAFSAVVTIEGIIKIRTGNLN), green (YSEQELLDCDRRSYGCNGGYPWSALQLVAQYGIHYRNTYPYEVQRYCRSREKGPYAAKTDGRQVQPYN), pink (GALLYSIANQPVSVVL). In dark blue was represented the allergenic peptide (GSCWAFSAV) shared between CpCP3 and papain. Light blue (CpCP3) and gray (papain) represents the non-allergenic amino acid sequences.



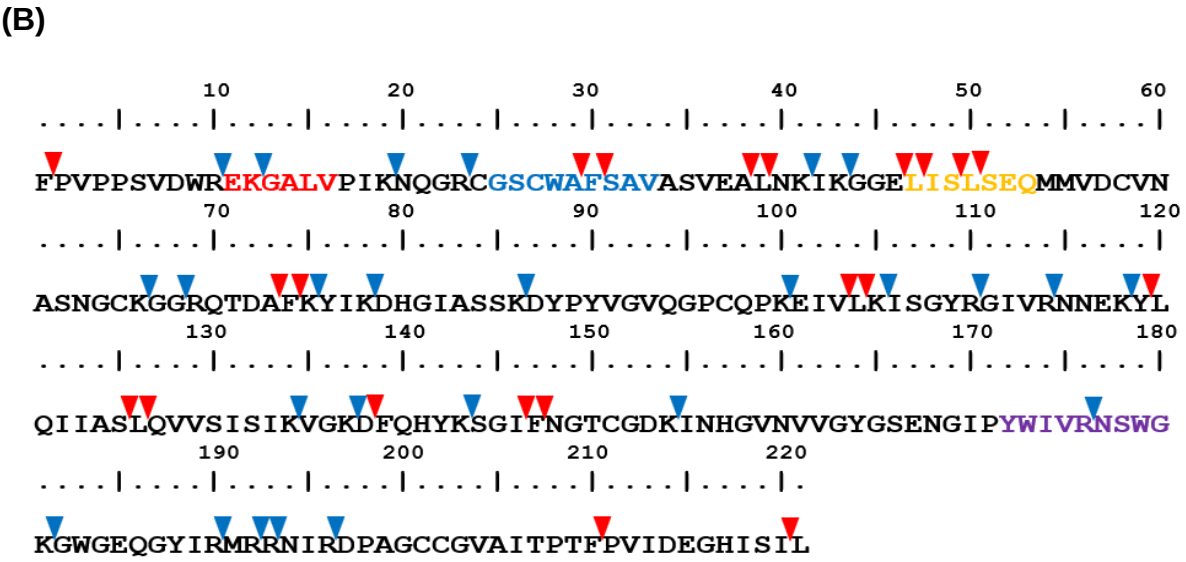


Fig. 5. (A) SDS-PAGE (12.5%) of CpCP3 after incubation with simulated gastric fluid followed by simulated intestinal fluid after 30 min, 1 and 2 h. MW: molecular mass markers. Controls: CpCP3, Pepsin and Trypsin. (B) Predicted cleavage sites of CpCP3 by pepsin (▼) and trypsin (▼) using the PeptideCutter program. Allergenic peptides from figure 4 were highlighted in red (EKGALV), blue (GSCWAFSAV), orange (LISLSEQ), and purple (YWIVRNSWG).

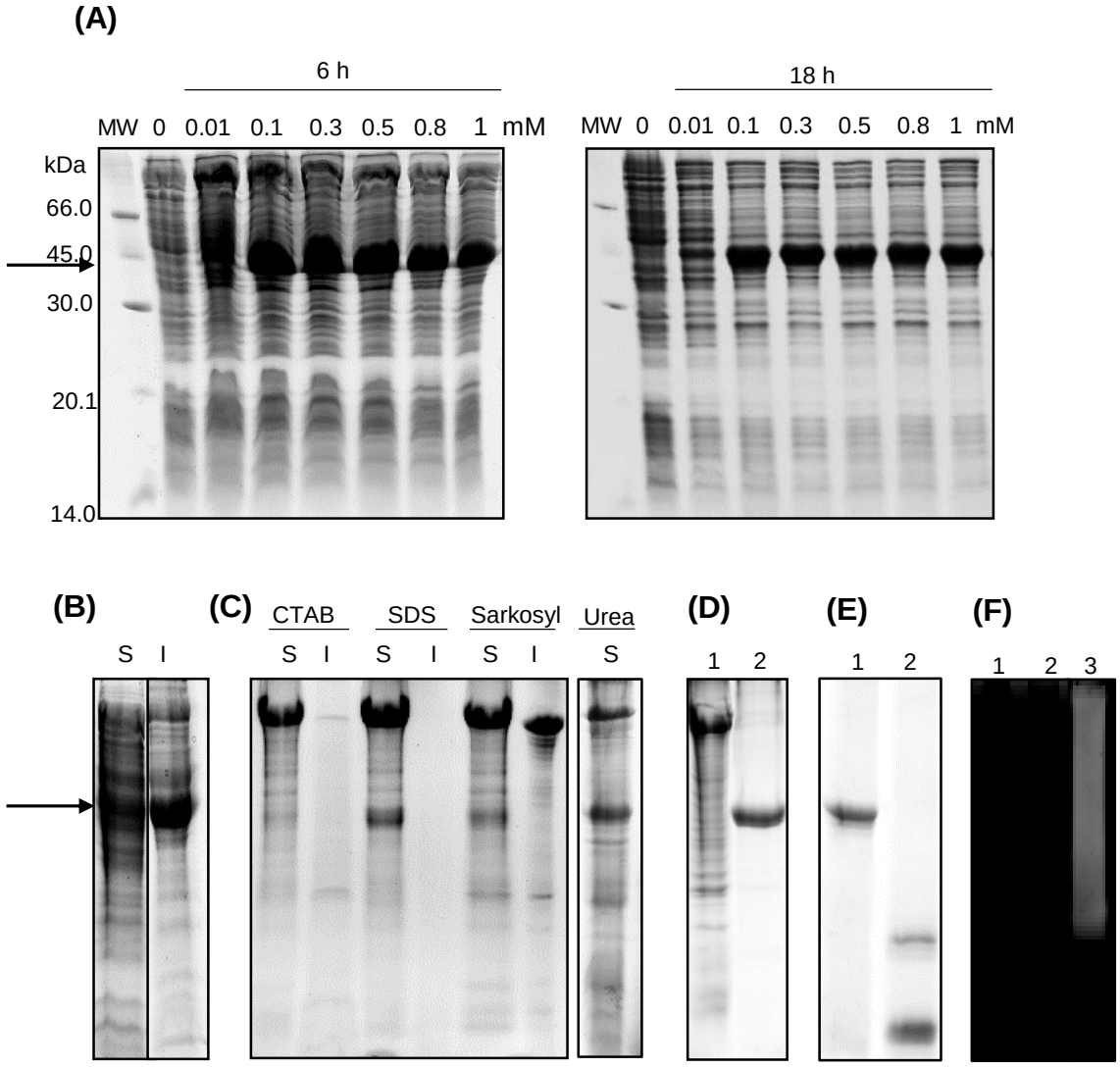
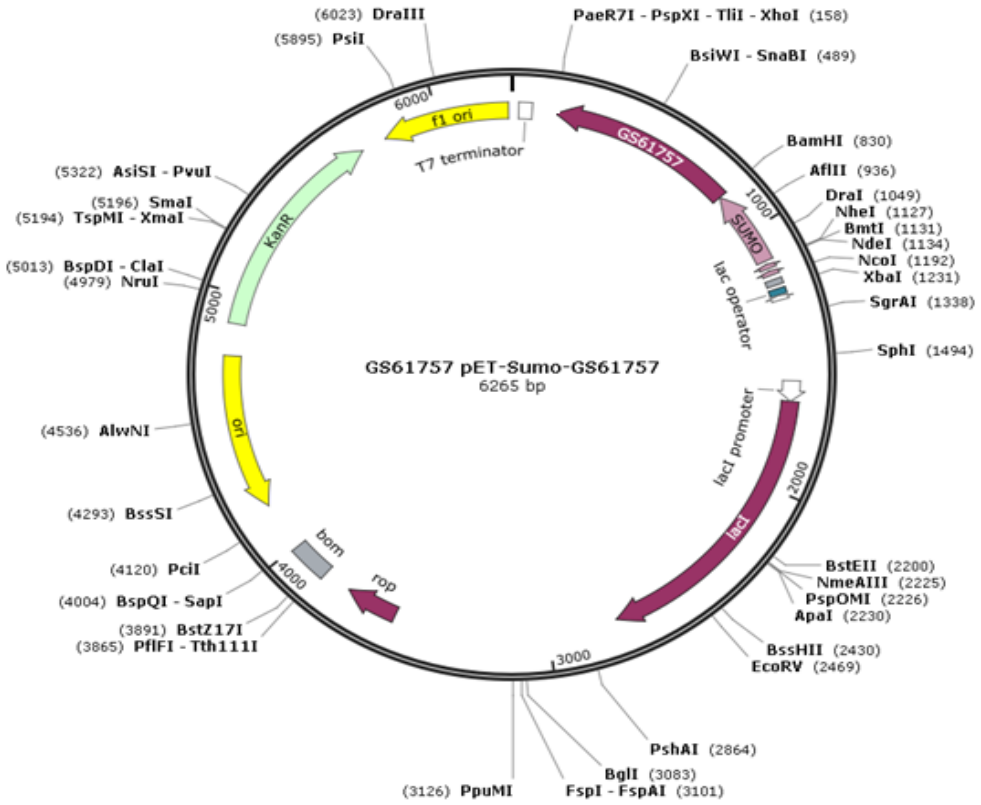
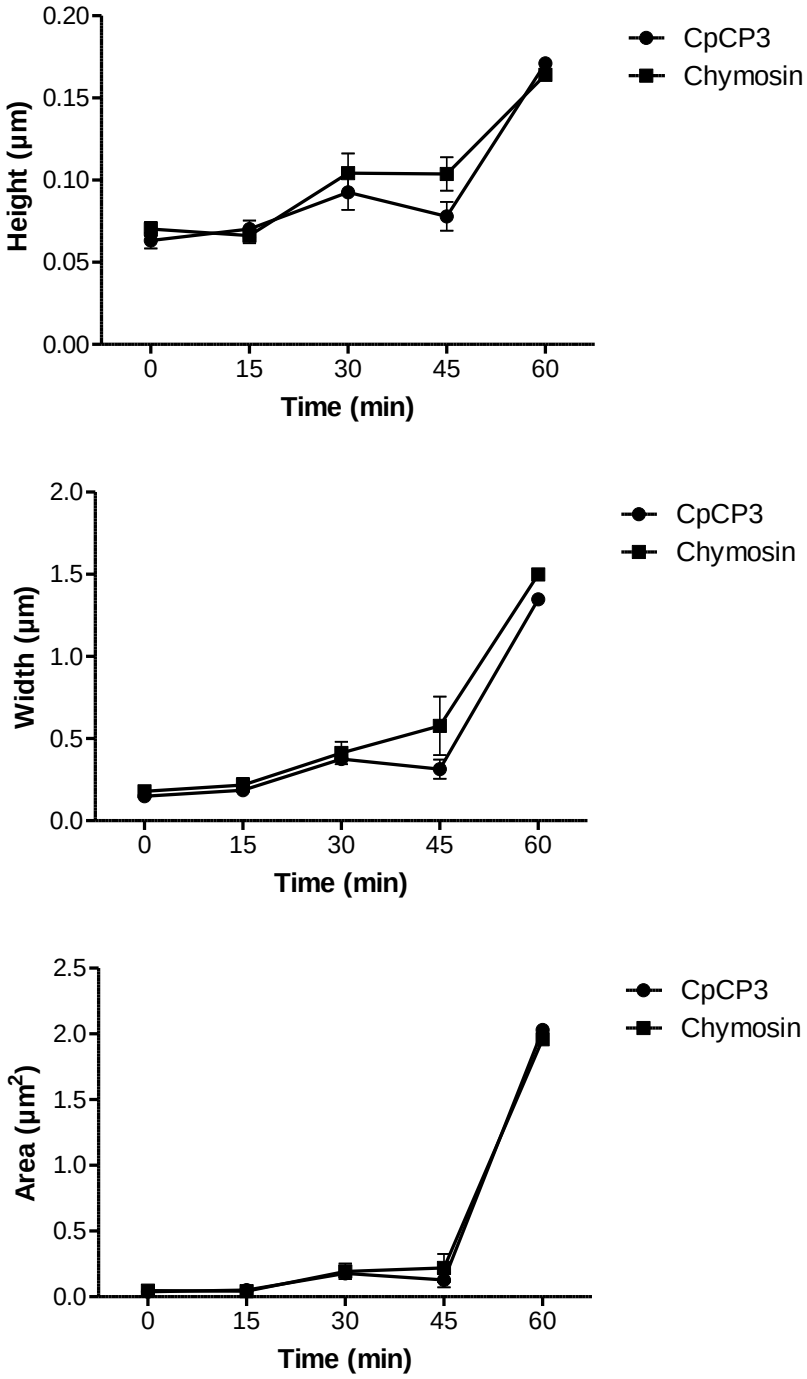


Fig 6. Expression, solubilization, purification, and proteolytic activity of recombinant CpCP3 (rCpCP3). **(A)** Effect of IPTG concentration (0.01 – 1 mM) and induction time (6 or 18 h) on rCpCP3 expression. **(B)** Protein profile of soluble (S) and insoluble (I) fractions of total cell lysates after 6 h and using 0.3 mM IPTG. **(C)** Effect of different detergents (CTAB, SDS and Sarkosyl, all at 1%) and urea (8 M) on solubility of the insoluble fraction (rCpCP3). The lanes show the soluble (S) and insoluble (I) proteins after detergent addition. **(D)** Purification of rCpCP3 using a HisTrap affinity column. Lane 1: non-retained proteins; lane 2: purified rCpCP3. **(E)** Digestion with UPL1. Lane 1: before digestion of protein with UPL1; lane 2: After digestion of protein. **(F)** Gelatin zymogram analysis of proteolytic activity. Lane 1: before digestion of protein with UPL1; lane 2: After digestion of protein UPL1; Line 3: CpCP3. Legend: MW molecular weight. Arrows represent recombinant CpCP3.



Supplementary Fig. 1. pET-SUMO vector used for CpCP3 expression. The pET-SUMO vector was designed to contain a N-terminal 6xHis and SUMO fusion protein in frame with CpCP3 (represented as GS61757).



Supplementary Fig 2. Height, width and area of casein micelles measured by AFM during milk-clotting process. The height was obtained from the micelle centre and width was considered as the longest path between two points of the micelles. The data were taken using three independent analysis. There is no statistical difference between casein micelles incubated with CpCP3 and bovine chymosin ($p > 0.05$).

Supplementary Fig. 3. Lateral view of zebrafish larvae after exposure to 100 mg/L CpCP3 (A) and E3 medium (B) for 96 h. No morphological or developmental alterations were observed in embryos and larvae during 96 h of exposure.

Supplementary Table 1. Effect of different ions on the proteolytic activity of CpCP3.

Ions	Concentration	Residual activity* (%)
NaCl	10 mM	103.14 ± 0.6
$CaCl_2$	10 mM	107.93 ± 6.8
LiCl	10 mM	99.02 ± 2.6
KCl	10 mM	98.64 ± 4.7
$MgCl_2$	10 mM	96.58 ± 2.3

* Each value represents the mean ± SD of three replicates. The assays were performed using azocasein as substrate (pH 6.5) at 37 °C.

Supplementary Table 2. Milk-clotting and proteolytic activity of CpCP3 and chymosin.

	MAE[1] (µg)	**MCT** [2] (min)	**SMCA**[3] (AU/mg)	**SPA**[4] (AU/mg)	**Ratio** (SMCA/SPA)
CpCP3	30.0	48.8 ± 1.7[a]	277.4 ± 72.1[a]	33.70 ± 0.50[a]	8.4
Commercial Chymosin	30.0	54.0 ± 1.1[b]	245.4 ± 32.8[a]	0.33 ± 0.03[b]	742.4

Each value represents the mean ± SD of three replicates. [1] MAE: Minimum amount of enzyme able to clot 2 mL of milk. [2] MCT: milk-clotting time. [3] SMCA: specific milk-clotting activity. [4] SPA: specific proteolytic activity using azocasein as substrate, pH 6.5, at 37 °C. Different letters show statistical difference ($p<0.05$). Statistical analysis was calculated by the paired t-test using the GraphPad Prism 6 program.

Supplementary Table 3: Allergenic peptides predicted in the CpCP3 sequence using Structural Database of Allergenic Proteins.

Allergens	Species	Peptides	Source
Api m 1	*Apis mellifera*	EKGALV	Bee
Der p 1, Car p 1	*Dermatophagoides pteronyssinus, Carica papaya*	GSCWAF	Mite, Plant
Der p 1, Eur m	*D. pteronyssinus, Euroglyphus maynei*	SCWAFS	Mite
Car p 1, Act d 1	*C. papaya, Actinidia deliciosa*	CWAFSA	Plant
Car p	*C. papaya*	WAFSAV	Plant
Act d 1	*A. deliciosa*	LISLSE	Plant
Act d 1	*A. deliciosa*	ISLSEQ	Plant
Eur m, Der p, Der f, Ana c 2	*E. maynei, D.pteronyssinus, D. farinae, Ananas comosus*	YWIVRN	Mite, Plant
Eur m, Der p, Ana c 2, Der f	*E. maynei, D. pteronyssinus, A. comosus, D. farinae*	IVRNSW	Mite, Plant
Ana c 2	*A. comosus*	VRNSWG	Plant

Supplementary Table 3: Allergenic peptides predicted in the CpCP3 sequence using Structural Database of Allergenic Proteins.

Allergens	Species	Peptides	Type
Car p papain	*Carica papaya*	IPEYVD	food
Car p papain	*Carica papaya*	PEYVDW	food
Car p papain	*Carica papaya*	EYVDWR	food
Car p papain	*Carica papaya*	YVDWRQ	food
Car p papain	*Carica papaya*	VDWRQK	food
Car p papain	*Carica papaya*	DWRQKG	food
Car p papain	*Carica papaya*	WRQKGA	food
Car p papain	*Carica papaya*	RQKGAV	food
Car p papain	*Carica papaya*	QKGAVT	food
Car p papain	*Carica papaya*	KGAVTP	food
Car p papain	*Carica papaya*	GAVTPV	food
Car p papain	*Carica papaya*	AVTPVK	food
Car p papain	*Carica papaya*	VTPVKN	food
Car p papain	*Carica papaya*	TPVKNQ	food
Car p papain	*Carica papaya*	PVKNQG	food
Car p papain	*Carica papaya*	VKNQGS	food
Car p papain	*Carica papaya*	KNQGSC	food
Car p papain	*Carica papaya*	NQGSCG	food
Blo t 1, Car p papain	*Blomia tropicalis*	GSCGSC	mites
Car p papain	*Carica papaya*	QGSCGS	food
Blo t 1, car p papain	*Blomia tropicalis, Carica papaya*	SCGSCW	mites, food
Der p 1, Eur m 1 Der f 1, Blo t 1, car p papain	*Dermatophagoides pteronyssinus, Euroglyphus maynei, Dermatophagoides farinae, Blomia tropicalis, Carica papaya*	GSCWAF	mites, food
Der p 1, Eur m 1, Der f, Car p papain	*Dermatophagoides pteronyssinus, Euroglyphus*	SCWAFS	mites, food

	maynei, Dermatophagoides farinae, Carica papaya		
Car p papain, Act d 1	*Carica papaya, Actinidia deliciosa*	CWAFSA	food
Car p papain	*Carica papaya*	WAFSAV	food
Car p papain	*Carica papaya*	AFSAVV	food
Car p papain	*Carica papaya*	FSAVVT	food
Car p papain	*Carica papaya*	SAVVTI	food
Car p papain	*Carica papaya*	AVVTIE	food
Car p papain	*Carica papaya*	VVTIEG	food
Car p papain	*Carica papaya*	VTIEGI	food
Car p papain	*Carica papaya*	TIEGII	food
Car p papain	*Carica papaya*	IEGIIK	food
Car p papain	*Carica papaya*	EGIIKI	food
Car p papain	*Carica papaya*	GIIKIR	food
Car p papain	*Carica papaya*	IIKIRT	food
Car p papain	*Carica papaya*	IKIRTG	food
Car p papain	*Carica papaya*	KIRTGN	food
Car p papain	*Carica papaya*	IRTGNL	food
Car p papain	*Carica papaya*	RTGNLN	food
Car p papain	*Carica papaya*	YSEQEL	food
Blo t 1,Car p papain	*Blomia tropicalis, Carica papaya*	SEQELL	mite, food
Blo t 1, Car p papain	*Blomia tropicalis, Carica papaya*	EQELLD	mite,food
Blo t 1, Car p papain	*Blomia tropicalis, Carica papaya*	QELLDC	mite, food
Car p papain	*Carica papaya*	ELLDCD	food
Car p papain	*Carica papaya*	LLDCDR	food
Car p papain	*Carica papaya*	LDCDRR	food
Car p papain	*Carica papaya*	DCDRRS	food
Car p papain	*Carica papaya*	CDRRSY	food

Car p papain		*Carica papaya*	DRRSYG	food
Car p papain		*Carica papaya*	RRSYGC	food
Car p papain		*Carica papaya*	RSYGCN	food
Car p papain		*Carica papaya*	SYGCNG	food
Car p papain		*Carica papaya*	YGCNGG	food
Car p papain,	Act d 1	*Carica papaya, Actinidia deliciosa*	GCNGGY	food
Car p papain		*Carica papaya*	CNGGYP	food
Car p papain		*Carica papaya*	NGGYPW	food
Car p papain		*Carica papaya*	GGYPWS	food
Car p papain		*Carica papaya*	GYPWSA	food
Car p papain		*Carica papaya*	PWSALQ	food
Car p papain		*Carica papaya*	WSALQL	food
Car p papain		*Carica papaya*	SALQLV	food
Car p papain		*Carica papaya*	QLVAQY	food
Car p papain		*Carica papaya*	VAQYGI	food
Car p papain		*Carica papaya*	QYGIHY	food
Car p papain		*Carica papaya*	YGIHYR	food
Car p papain		*Carica papaya*	GIHYRN	food
Car p papain		*Carica papaya*	IHYRNT	food
Car p papain		*Carica papaya*	HYRNTY	food
Car p papain		*Carica papaya*	YRNTYP	food
Car p papain		*Carica papaya*	RNTYPY	food
Car p papain		*Carica papaya*	NTYPYE	food
Car p papain		*Carica papaya*	TYPYEG	food
Car p papain		*Carica papaya*	YPYEGV	food
Car p papain		*Carica papaya*	PYEGVQ	food
Car p papain		*Carica papaya*	YEGVQR	food
Car p papain		*Carica papaya*	EGVQRY	food
Car p papain		*Carica papaya*	GVQRYC	food

Car p papain	*Carica papaya*	VQRYCR	food
Car p papain	*Carica papaya*	QRYCRS	food
Car p papain	*Carica papaya*	RYCRSR	food
Car p papain	*Carica papaya*	YCRSRE	food
Car p papain	*Carica papaya*	RSREKG	food
Car p papain	*Carica papaya*	SREKGP	food
Car p papain	*Carica papaya*	REKGPY	food
Car p papain	*Carica papaya*	EKGPYA	food
Car p papain	*Carica papaya*	KGPYAA	food
Car p papain	*Carica papaya*	GPYAAK	food
Car p papain	*Carica papaya*	PYAAKT	food
Car p papain	*Carica papaya*	YAAKTD	food
Car p papain	*Carica papaya*	AAKTDG	food
Car p papain	*Carica papaya*	AKTDGV	food
Car p papain	*Carica papaya*	KTDGVR	food
Car p papain	*Carica papaya*	TDGVRQ	food
Car p papain	*Carica papaya*	DGVRQV	food
Car p papain	*Carica papaya*	GVRQVQ	food
Car p papain	*Carica papaya*	VRQVQP	food
Car p papain	*Carica papaya*	RQVQPY	food
Car p papain	*Carica papaya*	QVQPYN	food
Car p papain	*Carica papaya*	GALLYS	food
Car p papain	*Carica papaya*	ALLYSI	food
Car p papain	*Carica papaya*	LLYSIA	food
Car p papain	*Carica papaya*	LYSIAN	food
Car p papain	*Carica papaya*	YSIANQ	food
Car p papain	*Carica papaya*	SIANQP	food
Car p papain	*Carica papaya*	IANQPV	food
Car p papain	*Carica papaya*	ANQPVS	food
Car p papain	*Carica papaya*	NQPVSV	food

Car p papain	*Carica papaya*	QPVSVV	food
Car p papain	*Carica papaya*	PVSVVL	food
Car p papain	*Carica papaya*	AAGKDF	food
Car p papain	*Carica papaya*	AGKDFQ	food
Car p papain	*Carica papaya*	GKDFQL	food
Car p papain	*Carica papaya*	KDFQLY	food
Car p papain	*Carica papaya*	DFQLYR	food
Car p papain	*Carica papaya*	FQLYRG	food
Car p papain	*Carica papaya*	QLYRGG	food
Car p papain	*Carica papaya*	LYRGGI	food
Car p papain	*Carica papaya*	YRGGIF	food
Car p papain	*Carica papaya*	RGGIFV	food
Car p papain	*Carica papaya*	GGIFVG	food
Car p papain	*Carica papaya*	GIFVGP	food
Car p papain	*Carica papaya*	IFVGPC	food
Car p papain	*Carica papaya*	FVGPCG	food
Car p papain	*Carica papaya*	VGPCGN	food
Car p papain	*Carica papaya*	GPCGNK	food
Car p papain	*Carica papaya*	PCGNKV	food
Car p papain	*Carica papaya*	CGNKVD	food
Car p papain	*Carica papaya*	GNKVDH	food
Car p papain	*Carica papaya*	NKVDHA	food
Car p papai	*Carica papaya*	KVDHAV	food
Car p papain	*Carica papaya*	VDHAVA	food
Car p papain	*Carica papaya*	DHAVAA	food
Car p papain	*Carica papaya*	HAVAAV	food
Tri a TAI, Hor v 1, Carica p papain	*Triticum aestivum, Hordeum vulgare, Carica papaya*	AVAAVG	food
Car p papain	*Carica papaya*	VAAVGY	food
Car p papain	*Carica papaya*	AAVGYG	food

Car p papain	*Carica papaya*	AVGYGP	food
Car p papain	*Carica papaya*	VGYGPN	food
Car p papain	*Carica papaya*	GYGPNY	food
Car p papain	*Carica papaya*	YGPNYI	food
Car p papain	*Carica papaya*	GPNYIL	food
Car p papain	*Carica papaya*	NYILIK	food
Car p papain	*Carica papaya*	YILIKN	food
Car p papain	*Carica papaya*	ILIKNS	food
Car p papain	*Carica papaya*	LIKNSW	food
Car p papain	*Carica papaya*	IKNSWG	food
Car p papain	*Carica papaya*	KNSWGT	food
Blo t 1, Carica p papain	*Blomia tropicalis, Carica papaya*	NSWGTG	mite, food
Car p papain	*Carica papaya*	GTGWGE	food
Car p papain	*Carica papaya*	TGWGEN	food
Car p papain	*Carica papaya*	GWGENG	food
Car p papain	*Carica papaya*	WGENGY	food
Car p papain	*Carica papaya*	GENGYI	food
Car p papain	*Carica papaya*	ENGYIR	food
Car p papain	*Carica papaya*	NGYIRI	food
Car p papain	*Carica papaya*	GYIRIK	food
Car p papain	*Carica papaya*	YIRIKR	food
Car p papain	*Carica papaya*	IRIKRG	food
Car p papain	*Carica papaya*	RIKRGT	food
Car p papain	*Carica papaya*	IKRGTG	food
Car p papain	*Carica papaya*	KRGTGN	food
Car p papain	*Carica papaya*	RGTGNS	food
Car p papain	*Carica papaya*	GTGNSY	food
Car p papain	*Carica papaya*	TGNSYG	food
Car p papain	*Carica papaya*	GNSYGV	food

Car p papain	*Carica papaya*	NSYGVC	food
Car p papain	*Carica papaya*	SYGVCG	food
Car p papain	*Carica papaya*	YGVCGL	food
Car p papain	*Carica papaya*	GVCGLY	food
Car p papain	*Carica papaya*	VCGLYT	food
Car p papain	*Carica papaya*	CGLYTS	food
Car p papain	*Carica papaya*	GLYTSS	food
Car p papain	*Carica papaya*	LYTSSF	food
Car p papain	*Carica papaya*	YTSSFY	food
Car p papain	*Carica papaya*	TSSFYP	food
Car p papain	*Carica papaya*	SSFYPV	food
Car p papain	*Carica papaya*	SFYPVK	food
Car p papain	*Carica papaya*	FYPVKN	food

5 CONCLUSÃO

A protease purificada de *C. procera*, CpCP3, possui potencial biotecnológico para produção de queijos semelhante a quimosina comercial, mas tendo a vantagem de ser uma protease de origem vegetal. Isso deve-se ao fato da protease ter uma ótima estabilidade na presença dos íons (Na^+ e Ca^{2+}), hidrolisar a k-caseína do leite semelhante a quimosina comercial, assim como agregação das micelas de caseínas ser semelhante à quimosina. O queijo obtido apresentou rendimento, teor de proteína, gordura e cinzas equivalentes ao queijo produzido pela quimosina bovina. A CpCP3 foi degradada pelas enzimas digestivas pepsina e tripsina, portanto, possíveis epítopos alergênicos não poderiam ser reconhecidos, deste modo apresentado baixíssimo potencial alergênico. CpCP3 não mostrou toxicidade a embriões de peixe-zebra. A obtenção de CpCP3 recombinante em *E. coli* foi obtida com sucesso através da solubilização de corpo de inclusão, entretanto não teve atividade proteolítica, necessitando de mais estudos para obtê-la em sua conformação estrutural adequada.

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