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DISSERTAÇÃO DE MESTRADO

APLICAÇÃO DE PROTEASES DE FLORES DE *Moringa oleifera* COMO COAGULANTE NA MANUFATURA DE QUEIJO

BELANY EMANUELE ALVES DE CARVALHO

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ORIENTADORA: Profa. Dra. PATRÍCIA MARIA GUEDES PAIVA
CO-ORIENTADORA: Profa. Dra. LUANA CASSANDRA B. B. COELHO

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Pernambuco.

Aprovada por:

Profa. Dra. Patrícia Maria Guedes Paiva - Presidente

Prof. Dr. Ranilson de Souza Bezerra

Prof. Dr. Thiago Henrique Napoleão

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À memória da minha avó,
Adalgiza, meu maior exemplo de
bondade e generosidade.

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“Tudo no mundo começou com um sim.
Uma molécula disse sim a outra molécula e
nasceu a vida.”

(Clarice Lispector)

RESUMO

Quimosina bovina é o principal agente coagulante utilizado na manufatura de queijos. Entretanto, a redução da oferta do coalho bovino e a incidência de doenças como a encefalopatia espongiforme (“mal da vaca louca”) tem estimulado a busca por novos agentes coagulantes de leite, tais como preparações contendo proteases de origem vegetal ou microbiana. Este trabalho avaliou uma preparação proteica (PP) obtida de flores de *Moringa oleifera* quanto às atividades caseinolítica e coagulante de leite, bem como sua aplicação como coagulante para produção de queijo. PP (480 mg de proteínas) apresentou atividade caseinolítica sobre azocaseína (37,5 U) e atividade coagulante de leite (1,9 U). Maior atividade caseinolítica foi detectada após incubação da preparação em pH 7,0 (35,5 U) e pré-aquecimento de PP a 50 ºC (94 U). Avaliação da atividade caseinolítica utilizando caseínas bovinas α_s, β e κ revelou que PP promoveu extensa clivagem da caseína κ e baixa hidrólise das demais, apresentando perfil similar à quimosina (controle positivo). A atividade coagulante de leite foi detectada apenas na presença de $CaCl_2$. Maior atividade foi observada em pH 4,0 (2,0 U) e essa atividade permaneceu estável após pré-aquecimento de PP até 50 ºC, sendo perdida após aquecimento a 70 ºC. O aquecimento prévio do substrato a 70 ºC resultou em aumento da atividade coagulante de PP (3,6 U). Ensaios enzimáticos na presença de inibidores de protease indicaram a presença de aspártico, cisteíno e serino proteases. Aspártico proteases são provavelmente as principais enzimas envolvidas na atividade coagulante de leite. A maior especificidade de PP pela caseína κ estimulou sua avaliação como coalho vegetal para a produção de queijo. Queijo coalho produzido utilizando quimosina foi utilizado como controle positivo. O queijo obtido utilizando PP apresentou conteúdo de sal similar e teor de cinzas menor que no queijo feito com quimosina. Ainda, o queijo produzido com PP se mostrou menos firme e apresentou maior umidade (72,34%) e menor conteúdo de proteínas (12,33 g/100 g) que o queijo coalho produzido com quimosina (46,37% e 23,11 g/100g, respectivamente). Com relação às propriedades organolépticas, o queijo produzido com PP foi classificado, predominantemente, como uniforme, branco, levemente ácido, levemente salgado, não-amargo e pouco duro. Em teste de apreciação as notas atribuídas pelos voluntários indicaram o queijo como bom em relação à sabor, textura e odor. Em conclusão: 1) flores de *M. oleifera* contêm uma mistura de aspártico, cisteíno e serino proteases com atividade caseinolítica; 2) preparação enzimática de flores de *M. oleifera* apresentou atividade coagulante de leite dependente de cálcio, provavelmente relacionado com a ação de aspártico proteases; 3) as proteases de flores foram mais específicas para caseína bovina κ, o que provavelmente está relacionado com a ausência de sabor amargo no queijo produzido utilizando PP; 4) o queijo produzido com PP apresentou propriedades distintas do queijo coalho produzido com quimosina.

Palavras-chave: coagulação de leite, proteases, *Moringa oleifera*, caseínas, manufatura de queijos.

ABSTRACT

Bovine chymosin is the main clotting agent used in cheese manufacture. However, the reduction in supply of calf rennet and the incidence of diseases such as the spongiform encephalopathy ("mad-cow disease") have stimulated the search for new milk-clotting agents, including preparations containing proteases from plant and microbial origin. This work evaluated a protein preparation (PP) from *Moringa oleifera* flowers for caseinolytic and milk-clotting activities as well as its application as coagulant to be used for cheese production. PP (480 mg of protein) showed caseinolytic activity on azocasein (37.5 U) and milk-clotting activity (1.9 U). The highest caseinolytic activity was detected after incubation of the preparation at pH 7.0 (35.5 U) and pre-heating of PP at 50 °C (94 U). Evaluation of caseinolytic activity using bovine α_s, β and κ-caseins revealed that PP promoted extensive cleavage of κ-casein and low hydrolysis of the others, showing a profile similar to chymosin (positive control). The milk-clotting activity was detected only at presence of $CaCl_2$. Highest activity was observed at pH 4.0 (2.0 U) and this activity remained stable after pre-heating of PP until 50 °C being neutralized after heating at 70 °C. The previous heating of the substrate at 70 °C resulted in increase of the PP coagulant activity (3.6 U). Enzyme assays in the presence of protease inhibitors indicated the presence of aspartic, cysteine and serine proteases. Aspartic proteases are probably the main enzymes involved in milk-clotting activity. The highest specificity of PP for κ-casein stimulated its evaluation as vegetable rennet for cheese production. Coalho cheese produced using chymosin was used as positive control. The cheese obtained using PP showed similar salt content and lower ash content than cheese made with chymosin. In addition, the cheese produced with PP showed to be less firm and showed higher moisture (72.34%) and lower protein content (12.33 g/100 g) than coalho cheese produced with chymosin (46.37% and 23.11 g/100 g, respectively). In regard to the organoleptic properties the cheese produced with PP was classified predominantly as uniform, white, slightly acid, slightly salted, non- bitter and low hard. In the appreciation assay the scores attributed by the volunteers indicated the cheese as good in respect of taste, texture and smell. In conclusion: 1) *M. oleifera* flowers contain a mixture of aspartic, cysteine and serine proteases with caseinolytic activity; 2) enzyme preparation from *M. oleifera* flowers showed calcium-dependent milk-clotting activity which is probably linked to the action of aspartic proteases; 3) flower proteases were more specific for bovine κ-casein, which is probably related with the absence of bitter taste in the cheese produced using PP; 4) the cheese produced with PP showed distinct properties from coalho cheese produced with chymosin.

Keywords: Milk-clotting activity, proteases, *Moringa oleifera*, caseins, cheese manufacture.

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Proteases são enzimas que degradam proteínas por catalisarem a hidrólise de ligações peptídicas (LÓPEZ-OTÍN & OVERALL, 2002). Em plantas, participam de diversos processos biológicos promovendo a remobilização de aminoácidos e a manutenção da homeostase, particularmente durante o desenvolvimento ou em resposta a estímulos externos (BAUMEISTER *et al*., 1998). Essas enzimas possuem diversas aplicações biotecnológicas e representam cerca de 60% das enzimas utilizadas nas indústrias de detergentes, farmacêutica, e alimentícia, dentre outras (KUMAR & BHALLA, 2005; SILVA *et al*., 2009).

A coagulação de leite é a clivagem ou desnaturação das proteínas que resulta na separação de uma fase sólida (coágulo ou coalhada) e uma fase líquida (soro). Esse processo pode ser promovido pela acidificação do meio ou pela ação de proteases com atividade caseinolítica, tais como tripsina e quimosina (KUMAR *et al.*, 2006). O coalho bovino, preparação enzimática obtida do intestino de bezerros, é o coagulante mais utilizado na manufatura de queijos. Entretanto, nos últimos anos, a oferta desse coalho tem diminuído (SHIEH *et al*., 2009). Além disso, a incidência de doenças como a encefalopatia espongiforme (“mal da vaca louca”) também resultou na diminuição da oferta e procura do coalho bovino. Tudo isso tem estimulado a busca por novos agentes coagulantes de leite, tais como preparações contendo proteases de origem vegetal ou microbiana (AHMED *et al*., 2009; SHIEH *et al*., 2009). Vários estudos têm avaliado preparações contendo proteases obtidas de diversos tecidos de plantas como potenciais agentes coagulantes a serem utilizados na manufatura de queijos (LO PIERO *et al*., 2002; AHMED *et al*., 2009).

Nesse contexto, o presente trabalho avaliou uma preparação protéica obtida de flores de *Moringa oleifera* como fonte de enzimas com atividades caseinolítica e coagulante de leite, bem como sua aplicação como coalho vegetal na manufatura de queijo.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Proteases

2.1.1 Generalidades

As proteases, ou enzimas proteolíticas, catalisam a hidrólise de ligações peptídicas em soluções aquosas, podendo também catalisar a reação no sentido contrário quando em soluções não-aquosas (GUPTA *et al.*, 2005). As proteases representam um dos grupos enzimáticos de maior relevância acadêmica e industrial e são classificadas dentro de seis grupos: aspártico, cisteíno, glutamato, metalo, serino e treonino proteases, baseando-se nos resíduos de aminoácidos presentes no sítio ativo e envolvidos no processo catalítico, bem como nos mecanismos de clivagem característicos de cada grupo (RAO *et al.*, 2009; LI *et al.*, 2013).

Representando cerca de 2% do genoma humano, as proteases regulam a maior parte dos processos fisiológicos, desempenhando papel fundamental na fertilização, homeostase, apoptose, replicação de DNA, digestão, degradação lisossomal e cascatas de sinalização (TURK, 2006; FEAR *ET al.*, 2007). Sua participação na regulação do sistema imunológico é crucial em mecanismos como detecção de antígenos, migração e ativação celular durante processos inflamatórios e resposta imune (PUENTE *ET al.*, 2003). Diferentes tipos de proteases atuam nesses diferentes processos de acordo com o seu mecanismo de ação (SHEN & CHOU, 2009).

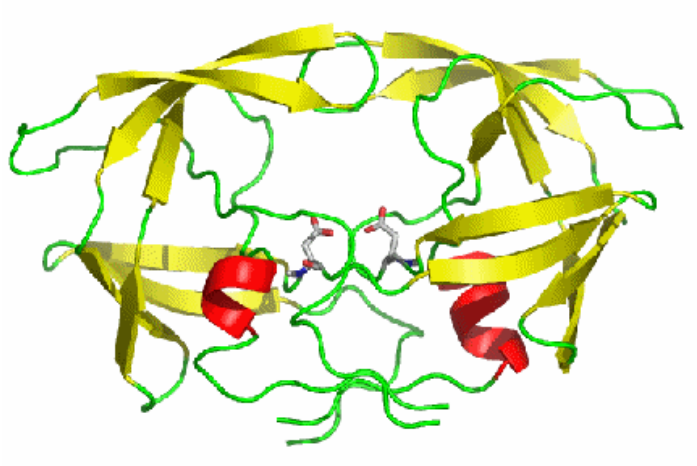
As proteases são sintetizadas na forma de precursores inativos – zimogênios - para prevenir a degradação protéica indesejada, promovendo uma regulação espacial e temporal da atividade proteolítica. Após triagem ou compartimentalização, a conversão desses precursores

em enzima ativa ocorre por proteólise e remoção do segmento de ativação. Esses mecanismos de conversão são bastante diversificados, variando desde co-fatores, enzimáticos ou não, que disparam a ativação, ou uma simples mudança no pH que resulta na conversão através de um mecanismo autocatalítico (KHAN & JAMES, 1998).

Em plantas, a proteólise é um passo essencial em muitos processos biológicos, como a degradação, remobilização de proteínas, reação ao ataque de patógenos e mobilização de proteínas de reserva da semente durante a germinação e crescimento primário das plantas. As proteínas de reserva são clivadas por classes específicas de enzimas, dando origem a intermediários que são susceptíveis à digestão e, assim, fornecerão os aminoácidos necessários para que o novo indivíduo em formação sintetize as proteínas que precisa (CHRISPEELS & BOULTER, 1975; ESTELLE, 2001). Em sistemas vegetais, essas enzimas são reguladas pela manutenção na forma de zimogênios ou, ainda, por combinação com inibidores, preservando-as em sua forma inativa (KULKARNI & RAO, 2009).

As aspártico proteases (APs) representam um grupo de enzimas amplamente distribuído na natureza. Essas enzimas são usualmente formadas por dois lobos, separados por uma fenda que contém o sítio catalítico formado por dois resíduos de aspartato (Figura 1) (BENES *et al.*, 2008). APs possuem papéis fisiológicos vitais nos mais diversos tipos de organismos, promovendo modificações químicas nas caudas das histonas durante o ciclo celular, participando dos processos de infecção e desenvolvimento viral, bem como atuando no envelhecimento, espermatogênese e esporulação (TESAR & MARQUARDT, 1990). Também estão relacionadas com o desenvolvimento de doenças importantes como Alzheimer, Síndrome da Imunodeficiência Adquirida (SIDA) e infecção por *Candida albicans*, entre outras (SKRBEC & ROMEO, 2002). Em vegetais, são encontradas principalmente em flores, sementes e folhas. A maioria dessas enzimas possui pH ótimo ácido e é inibida pela pepstatina (SOUSA, 2002).

Figura 1 – Vista lateral de aspártico protease do HIV-1, destacando os resíduos catalíticos de aspartato (em cinza).



Fonte: http://chemistry.umeche.maine.edu/CHY431/Peptidase15.html

Alguns compostos podem se ligar especificamente aos resíduos de aminoácidos presentes nos sítios catalíticos das proteases, inibindo a atividade catalítica. Devido a essa capacidade, inibidores de proteases já conhecidos podem ser utilizados na classificação dessas enzimas, indicando a qual grupo elas pertencem.

2.1.2 Aplicações biotecnológicas e industriais

As proteases representam grande parte das enzimas utilizadas no setor industrial, sendo amplamente empregadas na indústria química, como constituintes de detergentes e solventes orgânicos, como agente surfactante no tratamento de resíduos, na síntese de peptídeos. Na indústria de biocombustíveis as proteases de origem microbiana são utilizadas nos processos fermentativos. Guo *et al.* (2011) demonstraram que a otimização da produção de proteases ácidas por *Neurospora crassa* resultou em um aumento significativo na produção

de etanol. Além disso, enzimas proteolíticas também podem ser utilizadas no tratamento de dores, inflamações, artrite e em processos de cicatrização.

Uma protease produzida por *Saccharopolyspora* sp., apresentou propriedades importantes, como uma maior estabilidade na presença de diferentes solventes orgânicos, sal, pH alcalino e alta temperatura. Estas propriedades podem sugerir sua aplicação na indústria de detergentes e em reações de biossíntese de proteínas em meios não aquosos. A mesma enzima demonstrou propriedades cicatrizantes quando aplicada na forma de gel tópico, podendo também ser considerada como componente para este tipo de medicamento (RAUT *et al.*, 2012).

Na indústria alimentícia, destaca-se a aplicação de proteases na coagulação de leite para produção de queijos, na hidrólise do glúten, na panificação e no processamento de carnes, conservas e peixes, na tenderização e maturação de carnes, fermentação de farinha e na hidrólise de proteínas de soja que podem ser usadas como antioxidantes naturais melhorando a vida-útil de alimentos ricos em gordura (BON & PEREIRA, 1999; NAVEENA *et al.*, 2004; KUMAR *et al*., 2005; WANG *et al.*, 2005; BHASKAR *et al.*, 2007; RAUT *et al*., 2012).

2.2. Produção de queijos

2.2.1 Coagulação do leite

O leite é um alimento de alto valor nutricional e composição complexa. Segundo Torres *et al.* (2000), os principais componentes do leite integral são água, gorduras, carboidratos (lactose), proteínas (caseínas e proteínas do soro), minerais e vitaminas. O teor de cada um desses componentes é apresentado na Tabela 1.

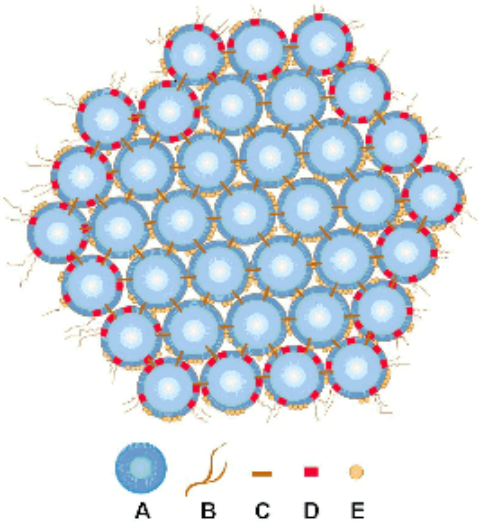
As caseínas são fosfoproteínas que correspondem a 80% de todas as proteínas contidas no leite bovino. A caseína total corresponde ao grupo de fosfoproteínas que são precipitáveis no leite desengordurado em pH 4,6 à temperatura de 20 ºC, pois temperaturas inferiores não permitem a precipitação total das caseínas (ANTUNES, 2003). Os diferentes tipos de caseínas diferem entre si quanto à massa molecular, composição aminoacídica e solubilidade em água e se apresentam associadas sob a forma de micelas de fosfocaseinato de cálcio (Figura 2), sendo facilmente degradadas por enzimas proteolíticas. A fração caseínica coagula pela ação de enzimas e não coagula pelo calor, enquanto as proteínas do soro coagulam pelo calor e não pela ação de enzimas coagulantes (VALSECHI, 2001).

Tabela 1 – Composição química do leite integral.

Constituinte	Teor (g/Kg)	Variação (g/Kg)
Água	873	855-887
Lactose	46	38-53
Gordura	39	24-55
Proteínas	32,5	23-44
Minerais	6,5	5,3-8,0
Ácidos orgânicos	1,8	1,3-2,2
Outros	1,4	-

Adaptado de: Walstra & Jenness (1984).

Figura 2 - Micela de Caseína. (A) submicela, (B) cadeias proteicas, (C) fosfato de cálcio, (D) caseína κ, (E) grupos fosfato.



Fonte: www.food-info.net/images/caseinmicelle.jpg

A coagulação do leite é um passo básico da produção de queijo. A coagulação acontece com o rompimento das micelas de caseína, quando as caseínas-κ são clivadas pelo agente coagulante liberando do interior da micela as caseínas α e β, além de partículas de para-κ-caseinato, que precipitam formando o coágulo. Nessa fase, a escolha do coagulante é crucial, pois o uso de proteases pouco específicas pode resultar num alto nível de degradação proteica, conferindo sabor amargo e reduzindo a vida-útil e o rendimento do queijo (FOX & LAW, 1991; FOX, 1997).

Devido à sua alta especificidade por κ-caseína e baixa atividade proteolítica geral, a quimosina continua sendo o principal agente coagulante de leite utilizado na manufatura de queijo (VISHWANATHA *et al.*, 2010; TAVARES & MALCATA, 2012). No entanto, o aumento da produção tem demandado grande quantidade desse coagulante, levando à escassez e ao aumento do preço (SOLORZA-FERIA *et al.*, 2011). Embora a produção de quimosina recombinante tenha diminuído o custo desse produto, alguns setores da sociedade

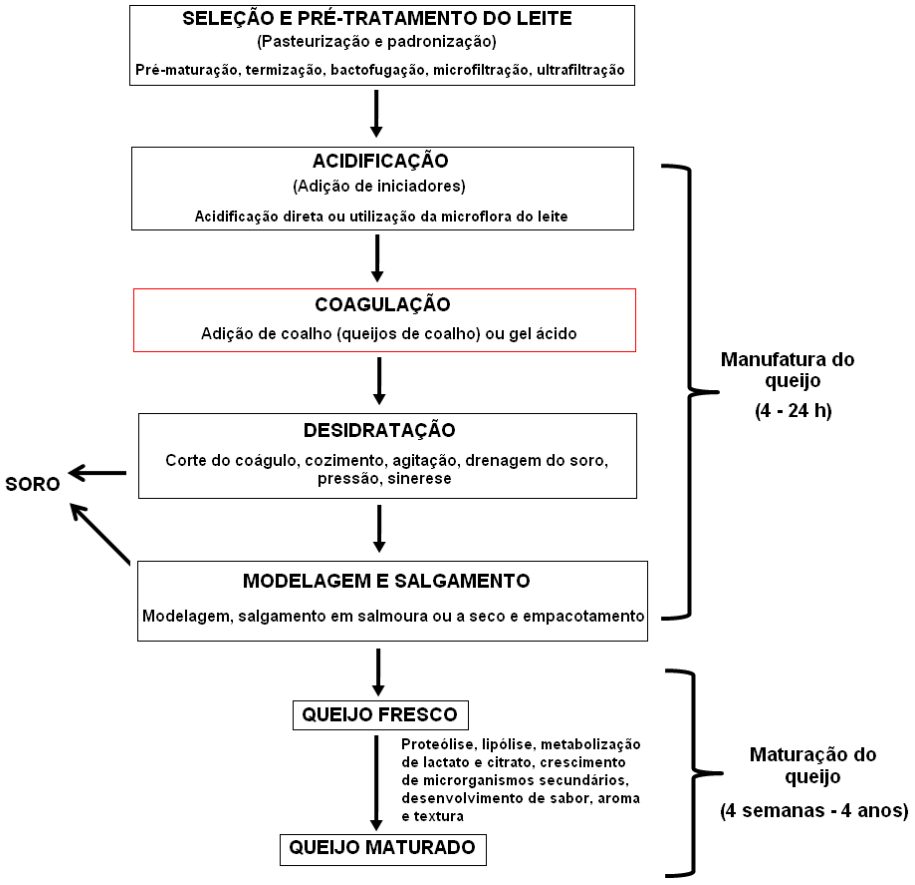
têm procurado por alternativas naturais; dessa forma, a busca por novos coagulantes de origem vegetal e microbiana tem se intensificado (CORRONS, 2012).

A utilização das proteases vegetais como coagulante de leite tem despertado grande interesse, uma vez que são enzimas naturais que podem substituir o coalho de origem bovina e a quimosina recombinante (GOMEZ *et al.*, 2001). Em alguns países, proteases extraídas de vegetais como *Ananas comosus* (abacaxi), *Calotropis procera* (algodão-de-seda), *Carica papaya* (mamão), *Cucumis melo* (melão), *Ficus glabra* (figueira), *Lactuca sativa* (alface) e *Oryza sativa* (arroz) vêm sendo utilizadas no processamento de queijos (UCHIKOBA & KANEDA, 1996; ASAKURA *et al*., 1997; LO PIERO *et al*., 2002). O látex obtido da raiz de *Jacaratia corumbensis* (mamãozinho-de-veado) é também uma fonte de enzimas cuja atividade permite sua utilização na manufatura de produtos lácteos (DUARTE *et al*., 2009). Testes com extratos brutos e enzimas parcialmente purificadas a partir de flores de *Cynara scolymus* (alcachofra) também demonstraram atividade caseinolítica e coagulante de leite (CHAZARRA *et al*., 2007).

2.2.2 Manufatura de queijo

O processo de produção de queijos envolve diferentes etapas de processamento e maturação, que variam também quanto ao tempo de duração, dependendo do tipo de queijo a ser produzido. Ainda, variações na origem do leite podem resultar em queijos com diferentes propriedades. A Figura 3 apresenta um fluxograma da produção de queijo do tipo coalho.

Figura 3 - Fluxograma do processamento do queijo de coalho.



Fonte: www.cheesescience.net

O uso de coagulantes na produção de queijo data de aproximadamente 6000 a.C. Tradicionalmente, a manufatura de queijo é feita através da coagulação do leite pela ação do coalho animal, que pode ser obtido de pedaços do estômago de pequenos animais, como cabrito e bezerro, sendo então chamados de abomaso ou coagulador (AQUINO, 1983). Contudo, sabe-se que o uso de proteases de origem vegetal de *C. cardunculus* como coagulante de leite é registrado em Portugal desde a época da ocupação romana (MACEDO *et al.*, 1993; FOX & MCSWEENEY, 1996; FREITAS & MALCATA, 2000).

O papel da proteólise durante a manufatura e maturação do queijo é essencial para o desenvolvimento de sua textura e sabor. Esse processo promove a quebra da matriz proteica e aumento do pH, o que facilita a liberação de compostos químicos durante a mastigação. Esse conjunto de processos contribui diretamente para o desenvolvimento do sabor e aroma do queijo através da formação de peptídeos e liberação de substratos (aminoácidos) para transformações metabólicas secundárias, tais como transaminação, desaminação, descarboxilação, desulfuração, catabolismo de aminoácidos aromáticos e reações com outros compostos (JOHNSON, 1988; SOUSA *et al.*, 2001).

Silva *et al.* (2012) realizaram uma ampla pesquisa que avaliou as propriedades de peptídeos ativos do queijo coalho, demonstrando que esses peptídeos, obtidos durante a hidrólise da caseína do leite utilizado na fabricação do queijo, conferem ao alimento propriedades antioxidantes, antimicrobianas e carreadoras de zinco, mineral que tem função de ativar enzimas que multiplicam as células de defesa.

2.3. *Moringa oleifera* Lam.

A família Moringaceae, pertencente à Ordem Brassicales, possui um único gênero (*Moringa*), com 14 espécies descritas, e é bastante conhecida e estudada devido à clarificação que ocorre em águas turvas quando tratadas com sementes de *Moringa* (McCONNACHIE *et al.*, 1999; JUDD *et al*., 2002).

Moringa oleifera, conhecida popularmente como moringa, lírio branco ou quiabo de quina, é uma árvore de pequeno porte (Figura 4A) originária da Índia e amplamente cultivada em regiões tropicais (BEZERRA *et al*., 2004). Apresenta folhas grandes e flores branco-amareladas (Figura 3C), cresce em terras quentes e secas e sobrevive em solos pobres, sendo pouco afetada pela aridez (JOLY, 1979; MORTON, 1991).

Figura 4 - *Moringa oleifera*. (A) árvore, (B) inflorescência e (C) flor.

Fotos: Thiago H. Napoleão.

Introduzida no Brasil por volta da década de 50 para fins ornamentais (MATOS, 2002), é atualmente bastante cultivada no país devido a suas diversas aplicações, sendo considerada uma espécie multiuso (MAKKAR & BECKER, 1996; BEZERRA *et al*., 2004; KARADI *et a*l., 2006; KWAAMBWA *et al*., 2010). Por ser um vegetal altamente nutritivo, *M. oleifera* é largamente utilizada na alimentação humana, principalmente em países como Índia, Paquistão, Filipinas, Havaí e em muitas partes da África (D'SOUZA & KULKARNI, 1993; ANWAR & BHANGER, 2003; ANWAR *et al*., 2005). Suas folhas destacam-se como uma rica fonte de β-caroteno, proteínas, vitamina C, cálcio e potássio, além de conter antioxidantes naturais como ácido ascórbico, compostos fenólicos e flavonóides (DILLARD & GERMAN, 2000; SIDDHURAJU & BECKER, 2003). O óleo produzido pelas sementes de *M. oleifera* apresenta composição de gorduras monoinsaturadas bastante similar a do óleo de oliva e pode ser utilizado para consumo humano (TSAKNIS *et al*., 1999).

As flores de *M. oleifera* são ricas em aminoácidos, sacarose, glicose, cálcio, potássio e antioxidantes como α-tocoferol, γ-tocoferol e flavonóides (RAMACHANDRAN *et al*., 1980;

FAIZI *et al*., 1994; MAKKAR & BECKER, 1996; RUCKMANI *et al*., 1998; GUEVARA *et al*., 1999; SIDDHURAJU & BECKER, 2003; SÁNCHEZ-MACHADO *et al*., 2006). As flores também contêm pterigospermina, um alcalóide com atividades fungicida e bactericida (LIZZY *et al*., 1968; ONG, 2008) e compostos com ação larvicida contra *Aedes aegypti*, vetor da dengue (PONTUAL *et al.*, 2012). A utilização medicinal das flores como agente colagogo, diurético, hipoglicêmico e tônico têm sido descrita (PARROTTA, 2009; KHARE, 2007).

Raízes, folhas, casca e sementes de moringa também apresentam uso medicinal como agente antitumoral, antipirético, antiinflamatório, antihipertensivo, antidiarréico, antiepilético, antiespasmódico, diurético e hepatoprotetor, e estimulante cardíaco e circulatório (FAIZI *et al*., 1994; GUEVARA *et al*., 1999; BENNETT *et al*., 2003; ANWAR *et al*., 2007).

Kalavathy & Miranda (2010) observaram que precursores de celulose e lignocelulose presentes no tronco de moringa podem ser utilizados na produção de carvão ativado para remoção de metais pesados de águas residuais. Sementes de *M. oleifera* são úteis para o tratamento de água porque contêm proteínas coagulantes, incluindo as lectinas cMoL e WSMoL (SANTOS *et al*., 2009; FERREIRA *et al.*, 2011). WSMoL apresenta potencial uso como agente inseticida, tendo apresentado atividades larvicida, ovicida e estimulante de oviposição contra *A. aegypti* (COELHO *et al*., 2009; SANTOS *et al.*, 2012).

O biodiesel obtido a partir do óleo de sementes de *M. oleifera* apresenta propriedades combustíveis importantes, como estabilidade oxidativa, viscosidade cinemática e o alto nível de cetano. Esta última torna o biodiesel de *M. oleifera* um possível substituto para outros combustíveis de origem vegetal que possuam menores níveis de cetano, já que altos índices desse composto estão relacionados à capacidade de auto-ignição e qualificam os biodieseis como potenciais substitutos ao petrodiesel (RASHID *et al.*, 2008).

3. OBJETIVOS

3.1. Geral

- Investigar as atividades caseinolítica e coagulante de leite em preparações protéicas de flores de *M. oleifera*, utilizar a preparação mais ativa como coalho vegetal para a produção de queijo e avaliar propriedades físico-químicas e organolépticas do queijo obtido.

3.2. Específicos

- Extrair proteínas de flores de *M. oleifera* e obter preparação de proteínas precipitadas (PP) através do fracionamento com sulfato de amônio.
- Avaliar a presença de atividade caseinolítica e coagulante de leite no extrato, PP e fração sobrenadante utilizando azocaseína e leite desnatado como substratos, respectivamente.
- Determinar o efeito do pré-tratamento térmico do leite na atividade coagulante de leite de PP.
- Investigar o efeito do pH, temperatura e de inibidores de proteases nas atividades caseinolítica e coagulante de leite de PP.
- Avaliar o perfil de hidrólise de caseínas α, β e κ por PP.
- Utilizar PP como agente coagulante na manufatura de queijo.
- Avaliar propriedades físico-químicas (conteúdo de proteínas, teor de umidade/voláteis, conteúdo de sal e teor de cinzas) e organolépticas (aparência, sabor, odor e textura) do queijo obtido.

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5. ARTIGO 1

Caseinolytic and milk-clotting activities from *Moringa oleifera* flowers

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Caseinolytic and milk-clotting activities from *Moringa oleifera* flowers

Emmanuel V. Pontual [1], Belany E.A. Carvalho [1], Ranilson S. Bezerra, Luana C.B.B. Coelho, Thiago H. Napoleão, Patrícia M.G. Paiva *

Departamento de Bioquímica-CCB, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420 Recife, Pernambuco, Brazil

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ABSTRACT

This work reports the detection and characterization of caseinolytic and milk-clotting activities from *Moringa oleifera* flowers. Proteins extracted from flowers were precipitated with 60% ammonium sulphate. Caseinolytic activity of the precipitated protein fraction (PP) was assessed using azocasein, as well as α_s-, β- and κ-caseins as substrates. Milk-clotting activity was analysed using skim milk. The effects of heating (30–100 °C) and pH (3.0–11.0) on enzyme activities were determined. Highest caseinolytic activity on azocasein was detected after previous incubation of PP at pH 4.0 and after heating at 50 °C. Milk-clotting activity, detected only in the presence of $CaCl_2$, was highest at incubation of PP at pH 3.0 and remained stable up to 50 °C. The pre-treatment of milk at 70 °C resulted in highest clotting activity. Enzyme assays in presence of protease inhibitors indicated the presence of aspartic, cysteine, serine and metallo proteases. Aspartic proteases appear to be the main enzymes involved in milk-clotting activity. PP promoted extensive cleavage of κ-casein and low level of α_s- and β-caseins hydrolysis. The milk-clotting activity indicates the application of *M. oleifera* flowers in dairy industry.

1. Introduction

Plant proteases, enzymes that catalyse the hydrolysis of peptide bonds, participate in several biological processes, including mobilisation of storage proteins, degradation of light-damaged chloroplast proteins, defense against phytopathogen attack, tissue differentiation, and floral senescence (Estelle, 2001). Different industrial processes utilise proteases such as papain, bromelain, and ficin, and new enzymes with appealing physicochemical properties have been investigated for that purpose (Feijoo-Siota & Villa, 2001).

Clotting of milk is a result of the action of proteases that destabilize casein micelles, which are particles present in fresh milk dispersed in a continuous phase comprising water, salt, lactose and whey proteins (Kruif, 1999). The caseins α_s and β are localised within the micelle, whose structure is maintained in solution by the κ-casein hydrophilic domain (Lo Piero, Puglisi, & Petrone, 2002). The hydrolysis of κ-casein results in the collapse of micelles and exposure of α_s- and β-caseins to calcium, leading to separation of milk into a solid (clot or curd) and liquid (whey) phases (Abreu, 2005).

In cheese production, milk-clotting by calf rennet is the procedure most commonly used. However, the low supply of calf rennet and the incidence of bovine spongiform encephalopathy are incentives in the search for enzymes from microorganisms and plants (Ahmed, Morishima, Babiker, & Mori, 2009; Barbano & Rasmussen, 1992; Cavalcanti, Teixeira, Lima Filho, & Porto, 2004; Shieh, Thi, & Shih, 2009).

An early study showed that the cheese produced using extract from *Calotropis procera* leaves was harder, less cohesive and gummier than that obtained using acidic pH as clotting agent (Aworh & Muller, 1987). Bruno, Lazza, Errasti, López, Caffini, and Pardo (2010) reported that the cheese produced using extract from *Bromelia hieronymi* fruits was acceptable in appearance, body, texture, and flavour. The *Albizia julibrissin* seed extract was also used as milk-clotting agent, and the resulting cheese did not develop bitterness after three months of ripening (Otani, Matsumori, & Hosono, 1991). Extract from *Cynara cardunculus* flowers containing proteases (cyprosins) is traditionally used in artisanal production of cheeses, and the recombinant form of cyprosin B is available for large-scale use (Sampaio, Fortes, Cabral, Pais, & Fonseca, 2008).

Milk-clotting activities from plant preparations have been associated with serine and aspartic proteases. A serine protease of *Cucumis melo* fruit exhibited a more stable milk-clotting activity, when compared to that of papain (Uchikoba & Kaneda, 1996). Additionally, it has been reported that a serine protease from *Lactuca sativa* leaves promoted clotting of skim milk as well as of milk with different fat contents (Lo Piero et al., 2002; Uchikoba & Kaneda, 1996). Aspartic protease from *Oryza sativa* seeds promoted cleavage of κ-casein, in a pattern similar to that obtained with chymosin and pepsin (Asakura, Watanabe, Abe, & Arai, 1997), and aspartic proteases from extract of *Silybum marianum* flowers

* Corresponding author. Tel.: +55 8121268540; fax: +55 8121268576.
E-mail address: ppaivaufpe@yahoo.com.br (P.M.G. Paiva).
[1] These authors equally contributed to this study

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hydrolysed caprine and ovine milk caseins (Cavalli, Silva, Cimino, Malcata, & Priolo, 2008).

Flowers of *Moringa oleifera* (Moringaceae family) are rich in calcium, potassium and antioxidants (α and γ-tocopherol), and are used in human diet, mainly in the Philippines (Makkar & Becker, 1996; Ramachandran, Peter, & Gopalakrishnan, 1980; Sánchez-Machado, López-Cervantes, & Vázquez, 2006). This work reports the detection in *M. oleifera* flowers of caseinolytic and milk-clotting activities using azocasein and skim milk as substrates, respectively. The effects of pH, temperature and protease inhibitors on these enzyme activities are also reported. Additionally, the caseinolytic and milk-clotting activities were assayed using α_s-, β- and κ-caseins or heated skim milk as substrates, respectively.

2. Materials and methods

2.1. Plant material

M. oleifera Lam. (Eudicots, Eurosids II, Order Brassicales, Family Moringaceae) has the vernacular names "moringa" in Portuguese, "árbol del ben" in Spanish and horseradish tree in English. Flowers were collected in Recife City, State of Pernambuco, northeastern Brazil. A voucher specimen is archived under number 73,345 at the herbarium Dárdano de Andrade Lima (Instituto Agronômico de Pernambuco, Recife, Brazil). The flowers were detached from the inflorescence rachis at the pedicel and dried at 27 ± 2 °C, relative humidity of 70 ± 5%, for 7 days before use. The extraction procedure is described below.

2.2. M. oleifera flower preparations

Powder (20 mesh) of *M. oleifera* dried flowers (50 g) was suspended in 0.15 M NaCl (500 ml) and homogenised in magnetic stirrer (4 h at 4 °C). After filtration through gauze and centrifugation (9,000 g, 15 min, 4 °C), the flower extract (clear supernatant) was treated with ammonium sulphate at 60% saturation (Green & Hughes, 1955). The precipitated protein fraction (PP) collected by centrifugation and the 60% supernatant fraction were dialysed (10 ml; 3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (2 h) using a volume of 2 L for dialysis fluid.

2.3. Protein content

Protein concentration was determined according to Lowry, Rosebrough, Farr, and Randall (1951) using serum albumin (31–500 μg/ml) as standard.

2.4. Caseinolytic activity

Caseinolytic activity was determined using azocasein (Sigma–Aldrich, USA) as substrate, according to Azeez, Sane, Bhatnagar, and Nath (2007). Flower extract (100 μl, 3.0 mg of protein), PP (100 μl, 3.2 mg of protein) or 60% supernatant fraction (100 μl, 3.0 mg of protein) was mixed with 300 μl of 0.1 M sodium phosphate pH 7.5 containing 0.6% (w/v) azocasein. The mixture was supplemented with 100 μl of 0.1% (v/v) Triton X-100 and incubated at 37 °C for 3 h. The reaction was stopped by adding 200 μl of 10% (w/v) trichloroacetic acid, and after incubation (4 °C, 30 min) the mixture was centrifuged at 9,000 g for 10 min. Next, the absorbance at 366 nm of the supernatant was determined.

Caseinolytic activity was also determined according to Sousa and Malcata (1998) using bovine α_s-, β-, and κ-caseins purchased from Sigma–Aldrich, USA. PP (50 μl, 1.7 mg of protein) was added to α_s-, β- or κ-casein solutions (1 ml, 10 mg of protein) in 0.1 M sodium phosphate buffer, pH 6.5 and reaction was allowed to proceed at 37 °C. Aliquots of 10 and 900 μl from the reaction mixtures were retrieved within 10, 30, 60, 120 min and 24 h of incubation. The aliquots of 10 μl were heated at 100 °C for 5 min and submitted to SDS–PAGE as described in Section 2.5. The aliquots of 900 μl were evaluated for absorbance at 366 nm after addition of 10% (w/v) trichloroacetic acid (200 μl) and centrifugation (9,000 g, 10 min, 4 °C). One unit of caseinolytic activity was defined as the amount of enzyme that promoted a 0.01 increase in absorbance. Chymosin (50 μl, 10 μg; Chy-Max® Liquid, Chr. Hansen, Denmark) and 0.15 M NaCl were used as positive and negative controls, respectively.

2.5. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS–PAGE)

Hydrolysis of α_s-, β- or κ-caseins by PP and chymosin (positive control) were evaluated by SDS–PAGE using 15% (w/v) polyacrylamide gels (Laemmli, 1970). Aliquots (10 μl) from reaction mixtures described in the Section 2.4, and molecular mass markers (SigmaMarker™ kit from Sigma–Aldrich, USA, containing the standard proteins: bovine serum albumin, 66,000 Da; glutamic dehydrogenase from bovine liver, 55,000 Da; ovalbumin from chicken egg, 45,000 Da,; glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle, 36,000 Da; carbonic anhydrase from bovine erythrocytes, 29,000 Da; trypsinogen from bovine pancreas, 24,000 Da; trypsin inhibitor from soybean, 20,000 Da; α-lactalbumin from bovine milk, 14,200 Da; and aprotinin from bovine lung, 6,500 Da) were applied on gel. After running and staining with 0.02% (v/v) Coomassie Brilliant Blue in 10% acetic acid, the gels were dehydrated and scanned. The densitograms were obtained using the software Scion Image Beta 4.02.2 (Scion Corporation, Frederick, MD, USA) and indicated the intensity of polypeptide bands.

2.6. Milk-clotting activity

The substrate (10% skim milk, Molico®, Nestlé, Brazil) was prepared in distilled water or in 10 mM $CaCl_2$ in water, and pH was adjusted at 6.5. The milk (2.0 ml) was incubated with flower extract (0.3 ml, 9.0 mg of protein), PP (0.3 ml, 9.8 mg of protein) or 60% supernatant fraction (0.3 ml, 9.0 mg of protein) at 37 °C, and curd formation was observed. The end point was recorded when the full separation between whey and curd was observed. One milk-clotting unit was defined as the amount of enzyme that clots 2 ml of the substrate within 180 min. Chymosin and 0.15 M NaCl were used as positive and negative controls, respectively. Milk-clotting activity was also determined using skim milk (10% w/v) heated at 30, 50 and 70 °C.

2.7. Effect of heating, pH and protease inhibitors on caseinolytic and milk-clotting activities

Caseinolytic (on azocasein) and milk-clotting activities were determined after heating (30 min) of PP at 30, 40, 50, 60, 70, 80, 90 and 100 °C under the same conditions described in Sections 2.4 and 2.6, respectively.

To determine the effect of pH on the enzyme activities, PP was previously incubated in 0.1 M citrate phosphate buffer (pH 3.0 to 6.0, 24 h, 37 °C), 0.1 M sodium phosphate pH 7.0, 0.1 M Tris–HCl (pH 8.0 and 9.0) or 0.1 M sodium borate buffer (pH 10.0 and 11.0). Next, assays were performed as described in Sections 2.4 and 2.6.

Inhibitors (8 mM, 1 ml) of serine proteases (phenylmethylsulfonyl fluoride, PMSF), cysteine proteases (transepoxy-succinyl-leucyl-amido-(4-guanidino)-butane; E-64), metallo proteases (ethylenediaminetetracetic acid, EDTA), and aspartic proteases (pepstatin A) were added to PP (1 ml, 32 mg of protein) and the

mixture was incubated at 37 °C for 30 min. Subsequently, the incubation mixtures were evaluated for caseinolytic (on azocasein) and milk-clotting activities. Inhibition percentages were calculated as follows: % inhibition = 100 − [100 × (residual activity/activity in control without inhibitor)].

2.8. Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as a mean of replicates ±SD. Significant differences between treatment groups were analysed by the Studenťs t-test (significance at $p < 0.05$) using the Origin 6.0 program.

3. Results and discussion

3.1. Caseinolytic and milk-clotting activities from M. oleifera flowers

Flower extract (2,940 mg of protein) was not able to hydrolyse azocasein, and it did not show milk-clotting activity using milk supplemented or not with $CaCl_2$. Differently, Satish, Sairam, Ahmed, and Urooj (2012) reported that aqueous extracts from *M. oleifera* leaf and roots showed caseinolytic activity and were also able to hydrolyse human plasma clot. Although proteolytic activity was not detected in flower extract, PP (480 mg of protein) showed caseinolytic (37.5 U, using azocasein) and milk-clotting (1.9 U, using milk supplemented with $CaCl_2$) activities. Fig. 1 shows the aspect of milk-clotting activity in the assay tubes. The 60% supernatant fraction (2,460 mg of protein) hydrolysed azocasein (1.4 U), but it did not show milk-clotting activity. The data reveal that ammonium sulphate concentrated the caseinolytic and milk-clotting activities from *M. oleifera* flowers in PP. Milk-clotting enzymes of extracts of *Albizia lebbeck*, *Helianthus annus* and *Solanum dubium* seeds were also precipitated using ammonium sulphate (Ahmed, Babiker, & Mori, 2010; Egito et al., 2007). According to Kent (1999) protein concentration using ammonium sulphate has three main advantages: it is a rapid and inexpensive method, it does not affect the structure and function of proteins, and the salt can be easily removed from the protein solution by dialysis.

Fig. 1. Milk-clotting activity of positive control chymosin (1), negative control 0.15 M NaCl (2) and PP (3).

Milk-clotting activity from PP was $CaCl_2$-dependent, similarly to what has been reported for *Solanum dubium* and *Withania coagulans* seeds, *Bromelia hieronymi* fruits and *Cynara scolymus* flowers (Ahmed et al., 2010; Bruno et al., 2010; Chazarra, Sidrach, López-Molina, & Rodríguez-López, 2007; Naz, Masud, & Nawaz, 2009). $CaCl_2$ forms bridges between positive and negative charges on casein micelles, causing them to break and releasing α_s- and β-caseins; curd is formed due to association between these proteins and calcium (Abreu, 2005; Ahmed et al., 2010; Anema, Lee, & Klostermeyer, 2005; Ishak et al., 2006).

Milk-clotting activity exerted by PP did not change when milk was heated up to 30 and 50 °C. However, the activity using milk heated up to 70 °C as substrate was higher (3.6 U) than when non-heated milk (1.8 U) was used. Similarly, the milk-clotting activities from goat (*Capra hircus*) chymosin and *C. scolymus* flower extracts have been reported to reach the highest value when the milk was heated up to temperatures above 50 °C (Chazarra et al., 2007; Kumar, Sharma, Mohanty, Grover, & Batish, 2006). Protein aggregation by heating of milk has been related to the increasing of milk clotting activity (Nájera, Renobales, & Barron, 2003).

Bovine α_s-, β-, and κ-caseins were used as substrates to determine the specificity of caseinolytic activity from PP. The enzyme reactions were monitored by absorbance at 366 nm. Fig. 2A shows that hydrolysis of κ-casein by PP started after 30 min of incubation, while degradation of α_s- and β-casein could only be detected after 60 min. Incubation for longer periods (120 min and 24 h) did not lead to any considerable improvement in degradation of α_s- and β-caseins by PP, though hydrolysis of κ-casein increased over 4 times (Fig. 2A). Oppositely, milk-clotting enzymes from *C. cardunculus* flowers have been reported to hydrolyse α_s-casein better than β-casein, and was less effective in cleaving κ-casein (Ordiales et al., 2012).

Chymosin is the major enzyme of calf rennet, and it has been extensively used in the dairy industry to produce a stable curd with good flavour due to its high specificity for the κ-casein (Rao, Tanksale, Ghatge, & Deshpande, 1998). Thus, this enzyme was used as a benchmark positive control. Specificity of PP for bovine caseins was similar to that of chymosin, which extensively cleaved κ-casein and promoted very slight hydrolysis of α_s- and β-caseins (Fig. 2B). On the other hand, the time course of κ-casein hydrolysis by PP was slower than that by chymosin (Fig. 2). However, unlike chymosin, PP is a partially purified protease preparation and thus the protein concentration reflects the amount of flower extract proteins that were precipitated with ammonium sulphate.

The molecular masses of bovine α_s-, β-, and κ-caseins on SDS–PAGE were between 20 and 25 kDa (Fig. 3), values that were similar to those reported by Dalgleish (1990). The degrees of casein hydrolysis by PP and chymosin were also evaluated by the reduction of α_s-, β-, and κ-caseins bands on SDS–PAGE, since peptides from casein proteolysis can be quantified by gel scanning, followed by densitometry (Cavalli et al., 2008; Franco, Prieto, Urdiales, Fresno, & Carballo, 2001).

The densitogram revealed that the intensities of α_s-casein bands (Fig. 3A, lanes 1 and 2) did not fall after incubation with PP for 10 to 120 min. The intensities of α_s-casein bands were reduced only after 24 h (Fig. 3A, lanes 1 and 2), indicating the occurrence of hydrolysis with the generation of a remarkably intense 17-kDa polypeptide band (Fig. 3A, lane 3). The peak in the densitogram for α_s-casein band after incubation with positive control chymosin was higher than that obtained after incubation with PP (Fig. 3A, lanes 1 and 2), indicating that α_s-casein was more hydrolysed by PP than by chymosin. Low reduction of β-casein band intensity was observed only after 24-h incubation with PP and chymosin (Fig. 3B, lane 1). Hydrolysis by PP generated several polypeptides with molecular mass between 7 and 19 kDa (Fig. 3B,

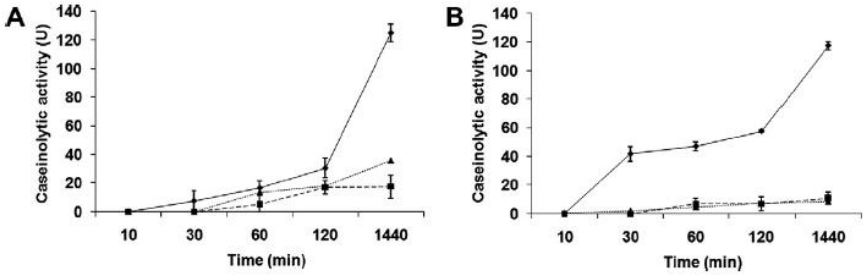

Fig. 2. Time course of hydrolysis of bovine α_{s}- (■), β- (▲) and κ- (♦) caseins by PP (A) and positive control chymosin (B).

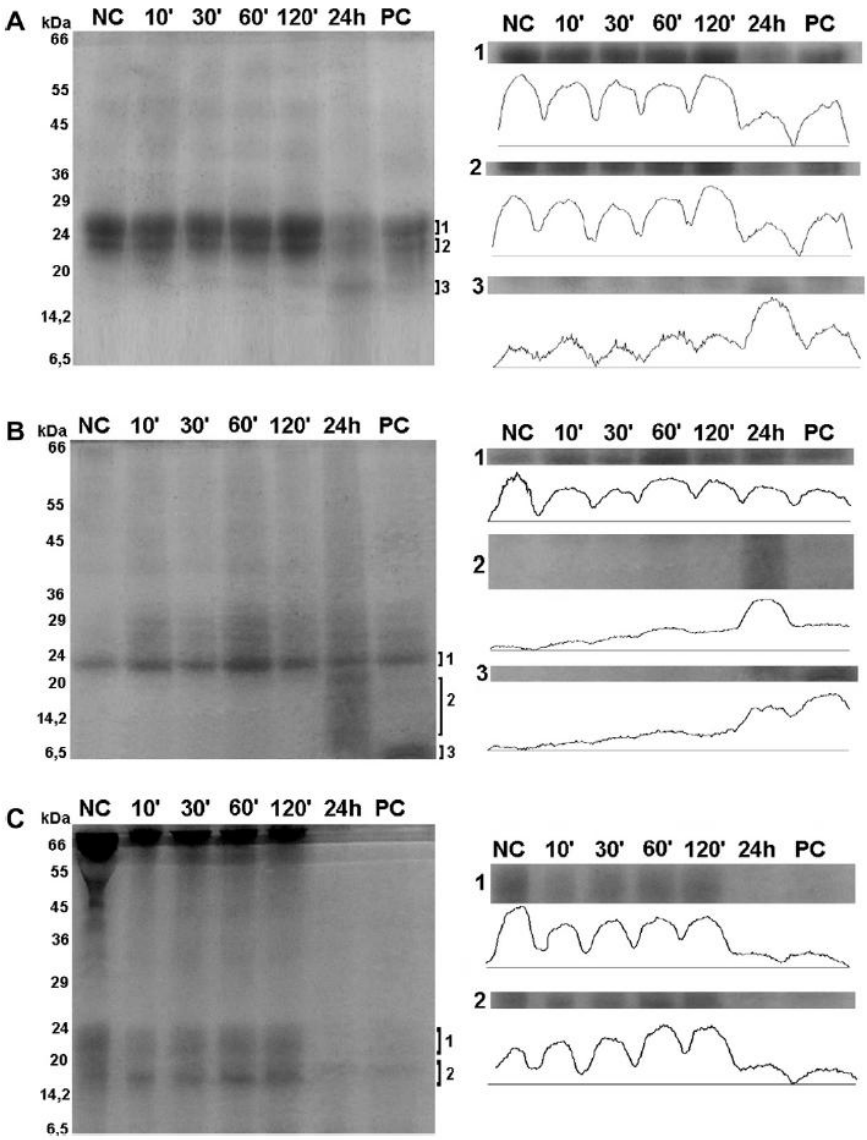

Fig. 3. SDS–PAGE patterns of hydrolysis of α_{s}- (A), β- (B) and κ- (C) caseins by PP for 10, 30, 60, 120 min and 24 h or by chymosin (PC, positive control) for 24 h. 0.15 M NaCl was used as negative control (NC). The gels were stained with Coomassie Brilliant Blue and the intensities of the polypeptide bands are represented in the densitograms obtained after gel scanning and analysis using Scion Image Beta 4.02.2 software.

lane 2), while cleavage by chymosin resulted mainly in polypeptides with very low molecular masses (Fig. 3B, lane 3). Reduction in intensity of κ-casein band due to hydrolysis by PP after 10, 30, 60 and 120 min (Fig. 3C, lane 1) was accompanied by an increase in the intensity of a 16-kDa polypeptide band, which probably corresponds to para-κ-casein (Fig. 3C, lane 2). No other peak of intensity in the region of κ-casein band was detected after incubation with PP and chymosin for 24 h, revealing total degradation of protein (Fig. 3C, lane 1). In addition, the para-κ-casein band intensity was strongly reduced after 24-h incubation with PP and chymosin (Fig. 3C, lane 2). Chymosin cleaves a single peptide bond in κ-casein, producing insoluble para-κ-casein and a C-terminal glycopeptides (Fox & Stepaniak, 1993; Rao et al., 1998). Extracts from sunflower (*Helianthus annuus*), as well as from albizia (*Albizia lebbeck*) and *S. dubium* seeds, have been proved to hydrolyse κ-casein to para-κ-casein (Ahmed et al., 2010; Egito et al., 2007).

Curd constituents include α_s-, β- and para-κ-caseins (Abreu, 2005). The detection of para-κ-casein on SDS–PAGE after casein hydrolysis by PP and the fact that milk-clotting activity of PP was detected only in the presence of calcium suggests that milk coagulation was probably due to the degradation of κ-casein, leading to the collapse of the micellar structure and aggregation of α_s- and β-caseins under the influence of calcium, resulting in gel formation (Merin, Talpaz, & Fishman, 1989).

PP from *M. oleifera* flowers is a potentially useful tool in cheese production processes, since it did not promote extensive hydrolysis of α_s- and β-caseins. The speed of hydrolysis of caseins influences the yield, consistency as well as flavour of cheese, and slow degradation of α_s- and β-caseins is guarantee of production of a firm curd, which is what occurs when chymosin is used, as mentioned above (Bruno et al., 2010; Fox, 1989). Plant rennets which promote extensive proteolysis of caseins are inappropriate for cheese production, because the generated peptides confer a bitter taste (Lo Piero et al., 2002; Macedo, Faro, & Pires, 1996).

3.2. Effect of heating, pH and protease inhibitors on caseinolytic and milk-clotting activities

Caseinolytic activity on azocasein significantly increased after heating of PP at 50 °C, while loss of this activity was detected after heating of PP at 60 °C (Table 1). Caseinolytic activities from *Lactuca sativa* leaves, *Opuntia ficus-indica* fruits and *S. dubium* seeds were also shown to be highest at 50, 55 and 70 °C, respectively (Ahmed et al., 2009; Lo Piero et al., 2002; Teixeira, Santana, Pais, & Clemente, 2000). Molecular rearrangements in protein structure can lead to increase of enzyme activity (Purich, 2010).

Caseinolytic activity was higher when PP was previously incubated at pH 4.0 and 7.0 (Table 2). A partially purified enzyme from *S. dubium* seeds also showed proteolytic activity towards azocasein at pH 4.0 but, unlike *M. oleifera* activity, the enzyme was highly active up to pH 11.0 (Ahmed et al., 2009). It is known that pH affects the shape, charge properties, the correct positioning of the substrate and the ionisation of side chains of amino acids, in both the active site and in the whole enzyme (Purich, 2010).

Heating of PP from 30 to 40 °C did not interfere in milk-clotting activity, which increased significantly after heating at 50 °C and was neutralised at 70 °C (Table 1). Milk-clotting enzymes from *Bromelia hieronymi*, *W. coagulans*, *Solanum esculentum* and *Solanum macrocarpon* are stable proteins, remaining active after heating to 45, 70 and 70 °C, respectively (Bruno et al., 2010; Guiama et al., 2010; Naz et al., 2009). A milk-clotting enzyme called religiosin B, purified from *Ficus religiosa* stem latex, showed highest milk-clotting activity at temperatures of 55 and 60 °C (Kumari, Sharma, & Jagannadham, 2012).

Milk-clotting activity from *M. oleifera* flowers was highest after previous incubation of PP at pH 3.0 (Table 2) and lost of activity was detected when PP was previously incubated at pH values higher than 8.0. Calf rennet showed similar behaviour, acting better in acid than in alkaline reaction medium (Richardson, Nelson, Lubnow, & Schwarberg, 1967). Differently, the milk-clotting enzyme religiosin B showed highest clotting ability at pH 6.0 (Kumari et al., 2012).

High thermal stability and ability to work in a wide pH range are important criteria for the choice of proteases to be used in industrial processes (Vieille & Zeikus, 1996). In this sense, the

Table 1
Effect of heating of PP (precipitated protein fraction from *M. oleifera* flowers) on caseinolytic and milk-clotting activities.

Temperature (°C)	Activity (U)	
	Caseinolytic	Milk-clotting
Control	37.5 ± 2.5^{a}	1.9 ± 0.2^{A}
30	38.2 ± 2.3^{a}	1.9 ± 0.3^{A}
40	39.4 ± 1.1^{a}	1.8 ± 0.2^{A}
50	94.0 ± 1.0^{b}	2.1 ± 0.0^{B}
60	0	0.3 ± 0.1^{C}
70	0	0
80	0	0
90	0	0
100	0	0

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$. Control treatment corresponds to incubation of PP for 24 h at 28 °C.

Table 2
Effect of incubation of PP (precipitated protein fraction from *M. oleifera* flowers) at different pH values on caseinolytic and milk-clotting activities.

pH	Activity (U)	
	Caseinolytic	Milk-clotting
3.0	22.4 ± 1.8^{a}	2.1 ± 0.4^{A}
4.0	30.8 ± 1.0^{b}	1.9 ± 0.2^{B}
5.0	10.1 ± 1.2^{c}	1.75 ± 0.2^{B}
6.0	3.4 ± 1.0^{d}	1.8 ± 0.1^{B}
7.0	35.5 ± 2.8^{e}	1.8 ± 0.1^{B}
8.0	19.6 ± 1.0^{a}	1.7 ± 0.3^{B}
9.0	3.5 ± 0.0^{d}	0
10.0	7.1 ± 1.2^{f}	0
11.0	3.0 ± 1.0^{d}	0

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$.

Table 3
Effect of protease inhibitors on enzyme activities from PP (precipitated protein fraction from *M. oleifera* flowers).

Inhibitor	Activity (U)	
	Caseinolytic	Milk-clotting
Control	39.0 ± 1.2^{a}	2.0 ± 0.2^{A}
Pepstatin A	30.0 ± 1.1^{b}	0.8 ± 0.0^{B}
EDTA	37.7 ± 3.6^{a}	1.9 ± 0.2^{A}
E-64	36.0 ± 1.8^{a}	1.3 ± 0.1^{C}
PMSF	35.2 ± 4.1^{a}	1.5 ± 0.1^{C}

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$. Control treatment corresponds to PP activity in abscence of inhibitors.

milk-clotting enzymes present in PP are promising candidates for application in milk-clotting at an industrial large scale. Additionally, the traditional use of *M. oleifera* flowers in human diet, being eaten raw or after lightly blanched (Makkar & Becker, 1996), is an indicative of PP safety for use in cheese production.

The evaluation of enzyme activities from *M. oleifera* flowers in presence of protease inhibitors (Table 3) showed that the caseinolytic activity on azocasein was not significantly ($p > 0.05$) altered in presence of PMSF, while milk-clotting activity was significantly ($p < 0.05$) reduced, by as much as 25%. E-64 significantly ($p < 0.05$) inhibited only milk-clotting activity (by 30%), while pepstatin A significantly reduced ($p < 0.05$) caseinolytic and milk-clotting activities, by 25% and 57.5%, respectively. The results reveal that milk coagulation promoted by PP can be due to serine, cysteine and aspartic proteases. Milk-clotting agents belonging to these three classes of enzymes have been reported. Corrons, Bertucci, Liggieri, López, and Bruno (2012) reported the presence of serine proteases with caseinolytic and milk-clotting activities in latex of *Maclura pomifera* fruits. Also, it has been shown that religiosin B is a serine protease (Kumari et al., 2012). Cysteine proteases from *B. hieronymi* fruits with milk-clotting ability were also described (Bruno et al., 2010). Chymosin and milk-clotting enzymes from *C. cardunculus* flowers and *Strebus aspler* twigs are aspartic proteases (Heimgartner et al., 1990; Llorente, Brutti, & Caffini, 2004; Senthilkumar, Ramasamy, & Subramanian, 2006).

4. Conclusions

M. oleifera flowers contain caseinolytic and milk-clotting activities. The data showed that PP contains a mixture of aspartic, cysteine, serine and Ca^{2+}-dependent proteases. Caseinolytic and milk clotting activities showed slightly different sensitivities to pH treatment. A heat dependent activation of proteolytic activities from PP was also demonstrated. From the perspective of food treatment and engineering, PP is a new source of proteases with potential use for cheese production, since it promotes extensive hydrolysis of κ-casein and low degradation of α_s- and β-caseins.

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6. ARTIGO 2

Cheese production using vegetable rennet from *Moringa oleifera* flowers

ARTIGO A SER SUBMETIDO AO PERIÓDICO "LWT – Food Science and Technology"

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Qualis B1 (Ciencias Biológicas I)

Research Note

Cheese production using vegetable rennet from *Moringa oleifera* flowers

Belany Emanuele Alves de Carvalho, Emmanuel Viana Pontual, Luana Cassandra Breitenbach Barroso Coelho, Thiago Henrique Napoleão, Patrícia Maria Guedes Paiva*

Departamento de Bioquímica-CCB, Universidade Federal de Pernambuco, 50670-420, Recife, Pernambuco, Brazil.

*Corresponding author. Tel: +558121268540. Fax: +558121268576.

E-mail: ppaivaufpe@yahoo.com.br

Abstract

Moringa oleifera flowers have been reported to be source of milk-clotting proteases with higher activity on κ-casein than α_s- and β-caseins. This work evaluated a protein preparation (PP) from *M. oleifera* flowers as vegetable rennet for cheese production. Cheese produced using PP was submitted to physicochemical characterization and evaluated for organoleptic properties. Cheese produced employing chymosin was used as positive control. The cheese obtained using PP as coagulant showed similar salt content and lower ash content than cheese made with chymosin. Also the cheese produced with PP showed lower firmness as well as higher moisture (72.34%) and lower protein content (12.33 g/100 g) than chymosin cheese (46.37% and 23.11 g/100 g, respectively). In the organoleptic assessment volunteers classified the cheese produced with PP as uniform, white, slightly acid, slightly salted, non-bitter and low hard. The cheese was predominantly scored as good in regard to taste, texture and smell but regular for appearance. In conclusion, PP was effective as vegetable rennet for cheese manufacture and the cheese obtained showed distinct properties from cheese produced using chymosin.

Keywords: Milk-clotting activity, proteases, *Moringa oleifera*, cheese manufacture.

1. Introduction

Milk clotting agents are conventionally employed in cheese manufacture and, despite the similarities in general procedure, variations in milk origin as well as in processing and maturing steps may result in different types of cheese (Perry, 2004). Chymosin, the major enzyme in calf rennet, shows high specificity for κ-casein regarding to α_s- and β- caseins, and therefore produces a stable curd with good flavour (Rao, Tanksale, Ghatge, & Deshpande, 1998). However, the increase in demand of calf rennet for cheese production has not accompanied its supply which, consequently, increases its price. This scenario has encouraged the search for new milk-clotting enzymes and many studies have focused in preparations from plants containing proteases with caseinolytic activity (Gomez et al., 2001; Lo Piero et al., 2002; Ahmed et al., 2009; Corrons, 2012 and Kumari 2012).

Pontual *et al.* (2012) described the milk-clotting ability of proteases of *Moringa oleifera* flowers. Caseinolytic activity was detected using azocasein as well as α_s-, β- and κ-caseins as substrates. The milk-clotting activity, detected only in presence of $CaCl_2$, was highest at pH 3.0 and after heating at 70 ºC. Study on the effect of protease inhibitors on enzyme activity revealed that aspartic proteases appear to be the main enzymes involved in milk-clotting ability. Furthermore, the authors demonstrated the specificity of *M. oleifera* flower proteases to cleave the κ-casein, in comparison with α_s- and β-caseins, which is an indicative for application in cheese manufacture.

In this sense, this work describes the production of cheese using a protein preparation from *M. oleifera* flowers as well as the evaluation of the physicochemical and organoleptic characteristics of the cheese produced.

2. Materials and Methods

2.1. Plant material

M. oleifera flowers were collected in Recife City, State of Pernambuco, northeastern Brazil. A voucher specimen (number 73,345) is deposited at the herbarium "Dárdano de Andrade Lima" from the *Instituto Agronômico de Pernambuco* (Recife, Brazil). The flowers were detached from the inflorescence rachis at the pedicel and dried at 27 ± 2 °C for 7 days before use.

2.2. Protein preparation from M. oleifera flowers

Protein preparation (PP) from *M. oleifera* flowers was obtained as described by Pontual et al. (2012). Dried flowers were powdered (20 mesh), suspended (50 g) in 0.15 M NaCl (500 ml) and homogenized in magnetic stirrer (4 h at 4 ºC). After filtration through gauze and centrifugation (9,000 g, 15 min, 4 ºC), the supernatant was treated with ammonium sulphate at 60% saturation (Green & Hughes, 1955). The precipitated proteins were collected by centrifugation, ressuspended in 0.15 M NaCl and dialysed (10 ml; 3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (2 h) using a volume of 2 L for dialysis fluid. The dialyzed sample corresponded to the PP.

2.3. Protein content

Protein concentration was determined according to Lowry, Rosebrough, Farr & Randall (1951) using serum albumin (31-500 µg/ml) as standard.

2.4 Caseinolytic and milk-clotting activities

Caseinolytic activity was determined using azocasein (Sigma–Aldrich, USA) as substrate, according to Azeez, Sane, Bhatnagar and Nath (2007). PP (100 µl, 3.2 mg of protein) was mixed with 300 µl of 0.1 M sodium phosphate pH 7.5 containing 0.6% (w/v) azocasein. The mixture was supplemented with 100 µl of 0.1% (v/v) Triton X-100 and incubated at 37 ºC for 3 h. The reaction was stopped by adding 200 µl of 10% (w/v) trichloroacetic acid, and after incubation (4 ºC, 30 min) the mixture was centrifuged at 9,000 g for 10 min. Next, the absorbance at 366 nm of the supernatant was determined. One unit of caseinolytic activity was defined as the amount of enzyme that promoted a 0.01 increase in absorbance.

Milk-clotting activity was determined according to Pontual et al. (2012). The substrate (10% skim milk, Molico®, Nestlé, Brazil) was prepared in 10 mM $CaCl_2$ in water, and pH was adjusted at 6.5. The milk (2.0 mL) was incubated with PP (0.3 mL, 9.8 mg of protein) at 37 ºC, and curd formation was observed. The end point was recorded when the full separation between whey and curd was observed. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of the substrate within 180 min.

2.5. Cheese manufacture

Cheeses were produced using PP (test cheese) or chymosin (positive control) as milk-clotting agents. PP (2 g of protein; 6 mL) was added to two liters of fresh cow milk supplemented with $CaCl_2$ (10 mM). Cheese produced with chymosin (1 mL, 0.2 g; Chy-Max® Liquid, Chr. Hansen, Denmark) was obtained by adding the enzyme to two liters of milk supplemented with $CaCl_2$. After 24 h the whey was drained and the curd cut and pressed

against a strainer until the lowest possible humidity. The physicochemical analysis of the produced cheeses was performed at the *Laboratório de Análises de Alimentos Nonete Barbosa* (LEAAL) from the *Universidade Federal de Pernambuco* according to the *Instrução Normativa nº 68 de 12/12/2006 do Ministério da Agricultura, Pecuária e Abastecimento (MAPA).* The organoleptic characterization was made with ten volunteers using a form divided in two parts: 1) classification (appearance, uniformity, color, flavour and texture) being the cheese classified according to Table 1; and 2) appreciation (appearance, taste, texture and smell) being the cheese scored according to the grade 1 = bad, 2 = regular, 3 = good, 4 = very good, 5 = excellent.

2.6. Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as a mean ± SD.

3. Results and discussion

PP showed caseinolytic (36 U) and milk-clotting (1.8 U) activities similar to that reported by Pontual et al. (2012) and was then used as vegetal rennet for production of cheese. After 24-h incubation, the aspect of curd obtained using PP showed to be more grain and less firm in comparison with that obtained with chymosin. After draining, the cheeses were submitted to physicochemical evaluation.

The physicochemical characteristics of cheeses are shown in Table 2. The salt content was similar between the two cheeses. The ash content of PP cheese was lower than that of

chymosin cheese and both values were below of reference for 'Coalho' cheese (Axiotes, 1991). This result is probably derived from the original composition of the milk. The ash content indicates the inorganic residue present in the food after full removal of the organic material and is correlated to the presence of impurities (such as dirt and sand) as well as used to quantify the level of micro and macronutrients (Silva *et al.*, 2009).

The moisture of cheese obtained with PP was high and associated with low protein content when compared to that prepared with chymosin, which showed characteristics similar to the reference values for 'Coalho' cheese (Axiotes, 1991). These results are in according with the aspects of curd observed and the lower firmness of cheese produced using PP may be linked to a loss of fat and protein particles during drainage. Similarly, Bruno *et al*. (2010) produced a cheese using an enzyme preparation from unripe fruits of *Bromelia hieronymi* and observed an association between low protein content and high water holding capacity.

The results of organoleptic assessment of cheese produced using PP revealed that it was predominantly classified by the volunteers as uniform(60%), white (50%), slightly acid (50%), slightly salted (90%), non-bitter (50%) and low hard (90%). The absence of bitterness is probably a result of the high specificity of *M. oleifera* flower proteases to κ-casein which is a differential in regard to other cheeses produced using vegetable rennet.

The results regarding the appreciation step are show in Table 3. The taste, texture and smell were predominantly classified as good while the appearance was regular as demonstrated by the average means of scores attributed by the volunteers. However the volunteers classified the cheese bearing in mind a 'Coalho' cheese and having the cheese produced with chymosin as reference. The PP cheese seems to be more similar in appearance and hardness to other types of cheese such as cottage and its moisture was similar to that of quarg cheese (79%) (von Hohendorff and Santos, 2006). Thus, in general the cheese produced using PP can be classified as acceptable.

In conclusion, PP acted as a milk-clotting agent on fresh cow milk being able to produce a cheese that showed distinct characteristics of 'Coalho' cheese produced using chymosin. Studies aiming to define the adequate storage and presentation for moringa cheese as well as the use of PP for production of other dairy products were started.

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Table 1. Classification panel provided to volunteers in evaluation of organoleptic properties of cheese produced using protein preparation (PP) from *M. oleifera* flowers as rennet.

Sensorial attribute	Evaluation
Appearance	
Uniformity	1: uniform; 2: not uniform
Color	1: white; 2: slightly yellow; 3: yellow
Taste	
Acidity	1: strongly acid; 2: slightly acid; 3: not acid
Salt	1: strongly salted; 2: slightly salted; 3: savorless
Texture	
Hardness	1: very hard; 2: low hard; 3: flaccid.

Table 2. Physicochemical characteristics of cheese produced using PP or chymosin as coagulant agent.

Parameter	Chymosin	PP	Reference values*
Moisture (%)	46.37	72.34	47.92
Protein (g/100g)	23.11	12.33	24.86
Ash content (g/100g)	1.36	0.8	3.74
Salt (g/100g)	0.12	0.14	-

*****According to Axiotes (1991)

Table 3: Mean scores for organoleptic characteristics of cheese produced using PP as coagulant attributed by volunteers in the appreciation step.

Characteristic	Score
Appearance	2.2 ± 0.63
Taste	3.2 ± 0.17
Texture	2.9 ± 0.52
Smell	3.0 ± 0.39

The scores were attributed by volunteers using the following classification: 1 = bad; 2 = regular; 3 = good; 4 = very good; 5 = excellent.

7. CONCLUSÕES

- Preparação proteica de flores de *M. oleifera* apresentou atividade caseinolítica e coagulante de leite, sendo esta última dependente de $CaCl_2$.

- As atividades caseinolítica e coagulante de leite de PP apresentaram diferente comportamento frente a mudanças de pH e aquecimento.

- Testes com inibidores de proteases revelaram que PP contém uma mistura de proteases do tipo aspártico, cisteíno e serino.

- PP apresentou maior especificidade para a caseína κ, em detrimento das caseínas α e β.

- O queijo produzido utilizando PP e leite bovino fresco apresentou alto teor de umidade e menor teor de proteínas que queijo coalho produzido utilizando quimosina.

- Na avaliação das propriedades organolépticas, o queijo produzido com PP foi predominantemente classificado como bom.

- Flores de *M. oleifera* constituem potencial fonte de proteases a serem usadas na indústria de laticínios.