BRUNO RICARDO DE CASTRO LEITE JÚNIOR

APPLICATION OF HIGH PRESSURE HOMOGENIZATION TECHNOLOGY IN THE MODIFICATION ON MILK-CLOTTING ENZYMES

APLICAÇÃO DA TECNOLOGIA DE HOMOGENEIZAÇÃO À ALTA PRESSÃO NA MODIFICAÇÃO DE ENZIMAS COAGULANTES DO LEITE

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FACULDADE DE ENGENHARIA DE ALIMENTOS

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Dissertation presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Food Technology

Dissertação apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Tecnologia de Alimentos

Orientador: Prof. Dr. Marcelo Cristianini

Co-orientador: Profa. Dra. Alline Artigiani Lima Tribst

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Abstract

High pressure homogenization (HPH) is a process that can alter the conformation and functionality of enzymes. The objectives of this study were: (i) evaluate the influence of HPH up to 190 MPa on the proteolytic and milk-clotting activities and stability of four milk-clotting enzymes, (ii) monitor the coagulation process by rheological assays and (iii) evaluate the gel development for 24 hours analyzing proteolysis, syneresis, rheological and microstructural behavior. The evaluations were performed by comparing the results between the processed and non-processed enzymes. The calf rennet processed at 190 MPa decreased 52 % its proteolytic activity, increased the rate of milk-clotting and a more consistent gel was formed. The evaluation of the gel for 24 hours indicated the formation of a protein network with lower proteolysis, higher syneresis, higher consistency and lower porosity. After processing at 150 MPa adult bovine rennet showed a reduction proteolytic activity, increase activity and stability of milk-clotting, higher milk-clotting rate and formed more consistent gels. This gel was more compact, firm and higher whey separation of protein matrix during the 24 hours of evaluation. The fungal protease from *Rhizomucor miehei* was the most resistant enzyme to the HPH process, showing minimal or no change in proteolytic activity and milk coagulation when processed up to 190 MPa at different concentrations and multiple consecutive processes. However, in the rheological evaluation of milk coagulation using fungal protease homogenized to 190 MPa for up 3 cycles or when homogenized in a solution with a concentration of 20% observed increase in the consistency of the gel. For porcine pepsin, changes on proteolytic activity and milk coagulation were only observed during storage, with reduction of proteolytic activity and an increase on the milk-clotting activity for the enzyme processed at 150 MPa. However, this enzyme promoted a faster coagulation of milk forming more consistent gel immediately after the processing by HPH. During the observation of this gel for 24 hours, this was more compact, firm, less porous and more release of whey of the protein matrix. Overall it was concluded that the

highest applied pressures (150 MPa and 190 MPa) positively affected the enzymes with reduced nonspecific proteolytic activity and increased milk-clotting activity, with consequent formation of gels with lower levels of proteolysis, which favors the maintenance of a network of protein rigid, firm and cohesive. Thus, it is concluded that HPH is a promising process that can be applied as a technology to improve the hydrolytic characteristics of milk coagulating enzymes, especially to reduce proteolytic activity and increase the milk-clotting activity. Furthermore, the lower proteolysis in the gel may result in an extension of the shelf life of fresh cheese, by possibly reducing the formation of bitterness flavor.

Key-words

High pressure homogenization • Non-thermal processing • Milk enzymatic coagulation • Proteolytic activity • Milk-clotting activity.

Resumo

A homogeneização à alta pressão (HAP) é um processo capaz de alterar a conformação e funcionalidade de enzimas. Os objetivos deste trabalho foram: (i) avaliar a influência da HAP até 190 MPa nas atividades proteolítica e de coagulação do leite bem como na estabilidade de quatro enzimas coagulantes do leite, (ii) acompanhar o processo de coagulação por ensaios reológicos e (iii) avaliar o desenvolvimento dos géis por 24 horas por meio das análises de proteólise, sinérese, reologia e microscopia. As avaliações foram feitas comparando-se os resultados obtidos com as enzimas processadas e não processadas. O coalho de vitelo processado a 190 MPa apresentou redução de 52% na atividade proteolítica, aumento da taxa de coagulação do leite e gel formado mais consistente. A avaliação deste gel por 24h indicou a formação de uma rede proteica com menor proteólise, maior sinérese, maior consistência e menor porosidade. Após processamento a 150 MPa, o coalho de bovino adulto apresentou redução da atividade proteolítica, aumento da atividade e estabilidade de coagulação do leite, maior taxa de coagulação do leite e formação de gel com maior consistência. O gel se mostrou mais compacto, firme e com maior expulsão do soro da matriz proteica nas 24h em que foi avaliado. A protease fúngica do *Rhizomucor miehei* foi a enzima mais resistente ao processo de HAP, sofrendo mínima ou nenhuma alteração na atividade proteolítica e de coagulação do leite quando processada até 190 MPa em diferentes concentrações e em múltiplos processos consecutivos. Entretanto, na avaliação reológica da coagulação do leite utilizando-se a protease fúngica homogeneizada a 190 MPa por até 3 ciclos ou quando homogeneizada a 190 MPa em soluções com concetração de 20 % foi observado aumento da consistência do gel. Para pepsina suína, as alterações na atividade proteolítica e de coagulação do leite só foram observadas durante a estocagem, com redução na atividade proteolítica e um aumento na atividade de coagulação do leite para enzima processada a 150 MPa. No entanto, esta enzima processada promoveu uma coagulação do leite mais rápida formando um gel mais

consistente, mesmo imediatamente após o processamento por HAP. Durante a observação deste gel por 24h, este se mostrou mais compacto, firme, menos poroso e com maior liberação de soro da matriz proteica. De uma forma geral foi possível concluir que as maiores pressões aplicadas (150 MPa e 190 MPa) afetaram positivamente as enzimas avaliadas, com redução da atividade proteolítica inespecífica e aumento da atividade de coagulação de leite, com consequente formação de géis com menores níveis de proteólise, o que favorece a manutenção da rede proteica rígida, firme e coesa. Desta forma, conclui-se que a HAP é um processo promissor que pode ser aplicado como uma tecnologia para melhorar as características hidrolíticas das enzimas coagulantes do leite, especialmente quando se deseja diminuir atividade proteolítica e aumentar sua atividade de coagulação do leite. Além disso, a menor proteólise no gel pode resultar numa extensão da vida de prateleira de queijos frescos, por, possivelmente, reduzir a formação de compostos de sabor indesejável.

Palavras-chave:

Homogeneização à alta pressão • processo não térmico • coagulação enzimática do leite • atividade proteolítica • atividade de coagulação do leite.

Sumário

“Somos aquilo que fazemos de forma repetida. Por isso, a excelência não é um ato, mas um hábito.”

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Introdução e Justificativas

Enzimas são proteínas produzidas por organismos vivos e apresentam a função de catalisar reações bioquímicas necessárias para a sobrevivência dos mesmos (Olempska-Beer et al., 2006). Elas podem ser aplicadas industrialmente no processo produtivo de diversos alimentos (Haki e Rakshit, 2003; Krajewska, 2004; Jayani, Saxena e Gupta 2005), na fabricação de papel, de couro, no tratamento de águas residuárias (Haki e Rakshit, 2003; Jayani, Saxena e Gupta 2005), nas áreas médica (Krajewska, 2004), farmacêutica, nas indústrias têxteis e de detergentes (Iyer e Ananthanarayan, 2008). Na indústria de lácteos uma das aplicações mais importantes de enzimas ocorre na manufatura da maioria dos queijos, cuja etapa inicial de produção envolve a coagulação enzimática do leite.

A produção de queijo no Brasil registrou um crescimento de 18% em 2011 em comparação com o período de 2006-2010 com uma taxa de crescimento anual de 16% (Euromonitor International, 2012). Em 2011 a produção nacional de leite atingiu cerca de 32,2 bilhões de litros sendo que 40% foram destinados à produção de queijos, totalizando aproximadamente 800 mil toneladas/ano (Embrapa, 2012). Atualmente, o Brasil é o quarto maior produtor de queijos do mundo (Euromonitor International, 2012).

Desta forma, observa-se que a produção de queijos está crescendo, fazendo com que a etapa de coagulação se torne cada vez mais crítica em função da baixa disponibilidade de coalho de vitelo. Apesar dos avanços em pesquisas, ainda não se tem um coagulante substituto do coalho de vitelo adequado, considerando atividade e especificidade. A homogeneização à alta pressão (HAP) pode ser uma ferramenta interessante no processo de modificação de enzimas, pois estudos prévios indicaram que a tecnologia poderia promover aumento de atividade, especificidade e de estabilidade enzimática (Vannini et al., 2004; Dosualdo 2007; Lanciotti et al., 2007; Lucci et al., 2007; Welti-Chanes et al., 2009; Liu et al., 2009a,b; Tribst e Cristianini, 2012a,b; Tribst, Augusto e Cristianini, 2012a). Apesar de o processo de HAP ter sido bastante estudado sobre os constituintes do leite e seus derivados lácteos (Kheadr et al., 2002; Hayes e Kelly, 2003; Thiebaud et al., 2003; Sandra e Dalgleish, 2005; Serra et al., 2007; Escobar

et al., 2011), e também apresentar resultados efetivos no aumento de atividade e estabilidade de enzimas, o efeito da HAP especificamente sobre as enzimas coagulantes de queijo ainda não tinha sido estudado. Isso gerava uma lacuna nos conhecimentos sobre a aplicação deste importante processo não térmico.

Assim, esse projeto visou ser um estudo de base para avaliar (i) a influência da homogeneização à alta pressão na atividade proteolítica e na atividade de coagulação do leite de quatro enzimas utilizadas no processo de fabricação de queijo, além do acompanhamento do processo de coagulação por meio de ensaio reológico e (ii) suas estabilidades durante estocagem (reversibilidade e/ou manutenção das atividades). Para aquelas enzimas que apresentaram as modificações de maior relevância, foi estudado também (iii) o desenvolvimento do gel por 24 horas por meio das análises de proteólise por eletroforese capilar, separação de soro, perfil reológico e microscopia de confocal. A partir destes resultados, foi possível estabelecer a HAP como um processo alternativo para modificação de enzimas coagulantes, além de avaliar se estas mudanças melhoraram o coágulo de leite formado.

Os ensaios preliminares para determinação do pH e temperatura ótima para atividade proteolítica dentro da faixa de coagulação do leite estão descritos no capítulo 2. Os efeitos do processamento a alta pressão sobre as enzimas de coalho de vitelo, coalho de bovino adulto, protease fúngica obtida do *Rhizomucor miehei* e pepsina suína e a caracterização dos géis formados são apresentados nos capítulos de 3 a 7.

Capítulo 1. Revisão Bibliográfica e Objetivos

1.1 Revisão Bibliográfica

1.1.1 Micela de caseína

As caseínas são fosfoproteínas insolúveis em pH 4,6, corresponde a 80% das proteinas contidas no leite e podem ser subdivididas nas seguintes classes: α- (αs_1 e αs_2), β- e κ-caseínas (Walstra et al., 1999). Estão presentes na forma de estruturas coloidais, denominadas micelas (Farrell et al., 2006). As frações αs_1-CN contém de 7 a 9 resíduos fosfato-serina por mol, a αs_2-CN de 10 a 13 e a β-CN 5 (Farrell et al., 2004), assim possuem a capacidade de se ligarem fortemente ao cálcio pelo envolvimento dos resíduos fosfato-serina e precipitarem. Entretanto, a κ-caseína, que possui apenas um resíduo fosfato por mol, é solúvel em altas concentrações de Ca^{2+} e, por interagir hidrofobicamente com as frações α e β, consegue estabilizá-las mantendo as micelas estáveis em suspensão no leite (Law, 1997), além disso, devido à presença de glicomacropetídeos negativos limitam o tamanho da micela por repulsões eletrostáticas e impedimento de natureza estérica (Fox et al., 2000).

Por muitos anos, uma explicação muito utilizada para descrever a estrutura micelar foi o modelo de submicelas (Walstra, 1990; Varnam e Sutherland, 2001). Esse modelo evoluiu e Walstra (1999) apresentou uma nova figura, onde o fosfato de cálcio está presente em pacotes dentro das submicelas (*Figura 1.1*).

Apesar do modelo de submicelas, atualizado por Walstra (1999), ter aceitação na comunidade científica, modelos alternativos foram propostos por Holt em 1992 e por Horne em 1998 (Phadungath, 2005), os quais aceitam a existência da camada externa estabilizadora de κ-caseína e destaca o papel do fosfato de cálcio coloidal como “cimento” da rede proteica, mas não aceitam a organização das caseínas em submicelas. O modelo de Holt (*Figura 1.2A*) descreve a micela como um gel mineralizado composto por proteínas unidas por ligações cruzadas, onde *nanoclusters* de fosfato de cálcio coloidal são os agentes responsáveis por essas ligações cruzadas e por manter a rede unida (Holt, 1992; Horne, 1998).

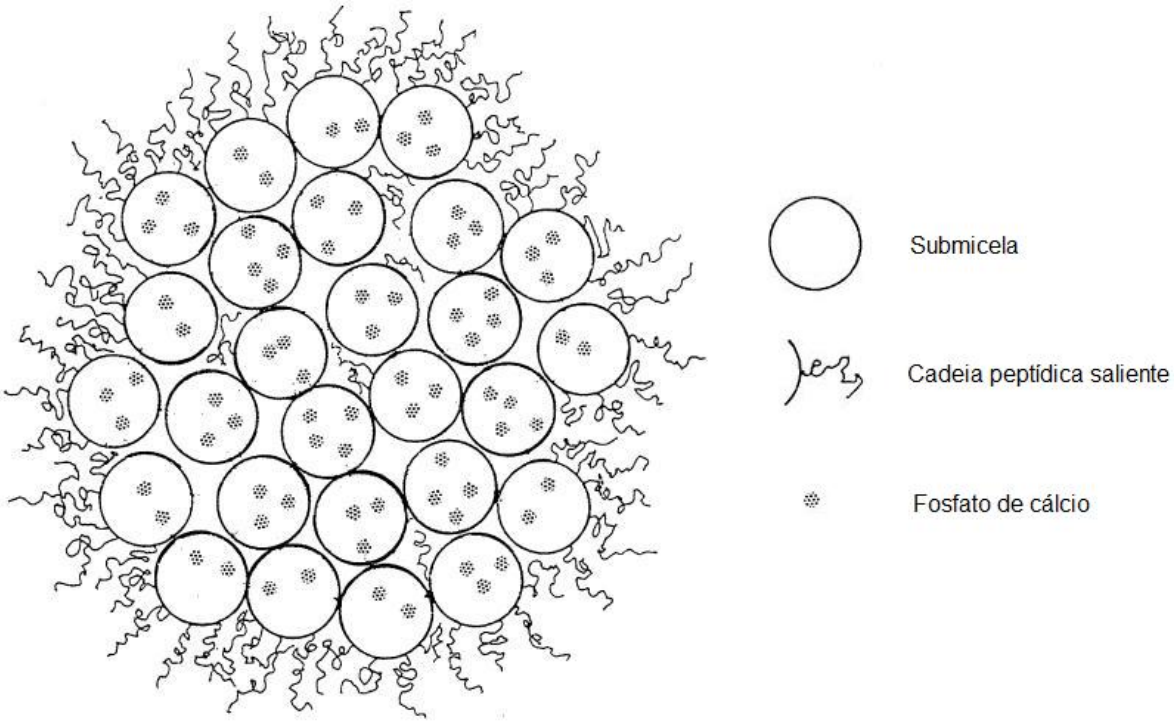

Figura 1.1 Corte transversal da micela de caseína no modelo de submicelas (Walstra, 1999).

O modelo proposto por Horne (1998) apresenta o fosfato de cálcio micelar não apenas como promotor de ligações cruzadas, mas também como agente neutralizante, o qual, sendo carregado positivamente, se liga aos *clusters* de fosfoserina negativamente carregados reduzindo a carga proteica até o nível em que interações atrativas entre as regiões hidrofóbicas das caseínas passam a dominar (Phadungath, 2005). Ainda de acordo Horne (1998), apesar das moléculas de κ-CN poderem interagir através dos seus domínios hidrofóbicos com as regiões hidrofóbicas de outras caseínas, o crescimento além da κ-CN não é possível já que ela não possui nenhum *cluster* de fosfoserina para ligar via fosfato de cálcio coloidal, nem outro ponto hidrofóbico para estender a cadeia. Assim a κ-CN age como um finalizador do crescimento da micela e faz parte da sua estrutura superficial. O modelo de Horne está ilustrado na *Figura 1.2B*.

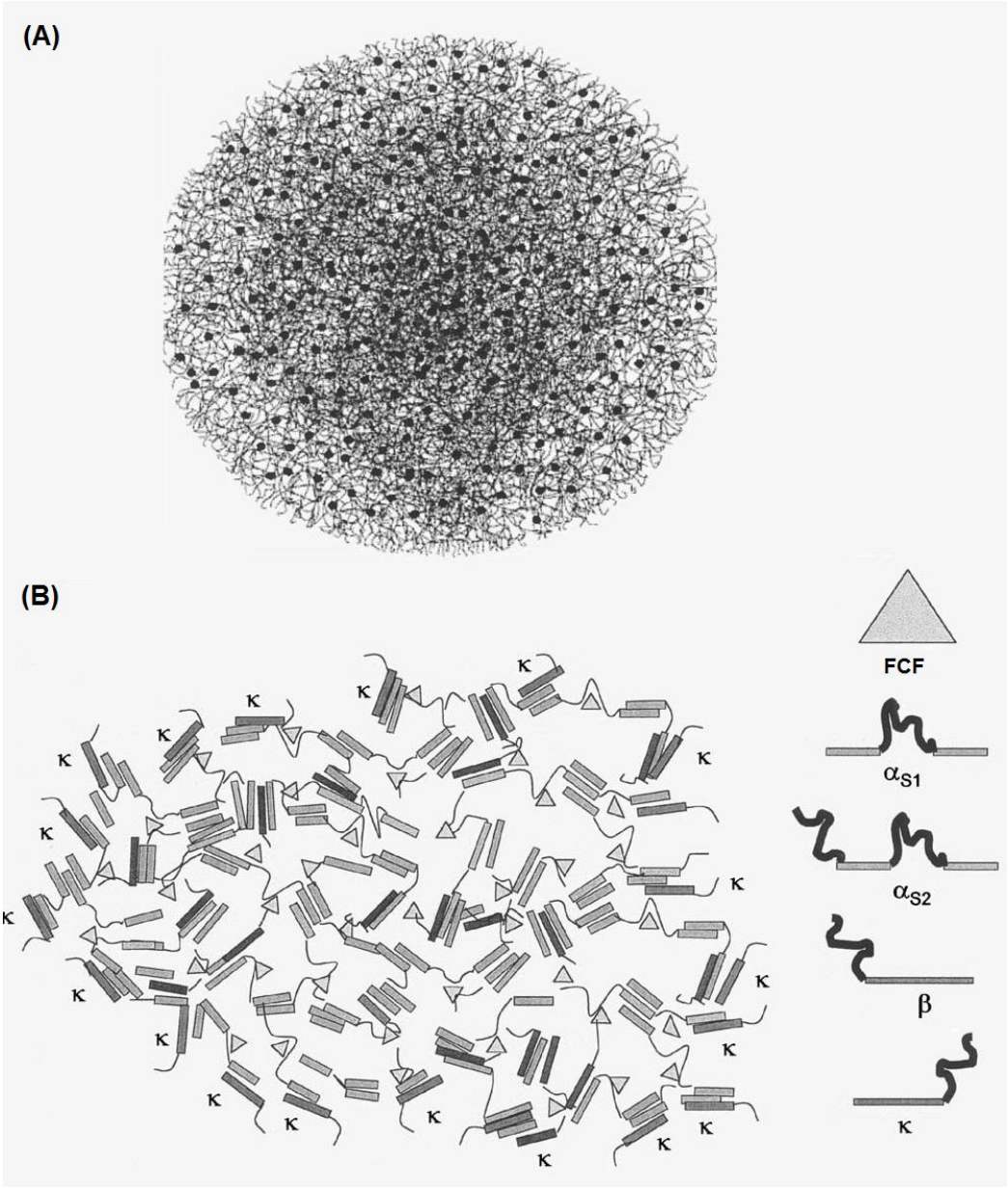

Figura 1.2 Modelo da micela de caseína proposto por Holt (A) onde os pontos pretos (•) são os nanoclusters de fosfato de cálcio e modelo da micela de caseína proposto por Horne (B) onde as proteínas interagem pelas regiões hidrofóbicas (barras pretas retangulares), e as regiões hidrofílicas das proteínas (alças) que contém os "clusters" de fosfoserina se ligam aos "clusters" de fosfato de cálcio (triângulos) sendo que as moléculas de κ-CN limitam o crescimento micelar (K) (Farrell et al., 2006).

Dalgleish, Spagnuolo e Goff (2004), a partir de micrografias obtidas de microscópio eletrônico de varredura reportaram que a micela de caseína é uma estrutura mais complexa e flexível do que apenas filamentos atados a uma esfera. Consistindo de pequenos canais de caseína, além de apresentar entradas ou fendas altamente porosas, sugerindo que o interior da micela é acessível a pequenas moléculas como as enzimas. Mais recentemente, McMahon e Oommen (2008) apresentaram imagens de microscopia eletrônica de transmissão de alta resolução da estrutura da micela, com base na interpretação destas imagens, um modelo da estrutura interligada foi desenvolvido, em que ambos os agregados de caseína associados ao fosfato de cálcio e as cadeias poliméricas de caseína agem em conjunto para manter a integridade das micelas. Assim, as caseínas estão apresentadas em cadeias lineares e cadeias ramificadas interligadas por *nanoclusters* de fosfato de cálcio. Este modelo sugere que ocorre a estabilização de *nanoclusters* de fosfato de cálcio por domínios fosfoserina de αs_1 -, αs_2- ou β-caseína, ou sua combinação, podendo orientar seus domínios hidrofóbicos para fora, permitindo a interação e ligação com outras moléculas de caseína.

Outras interações entre as caseínas, como a ponte de cálcio, também podem ocorrer, para estabilizar ainda mais a "supermolécula" (McMahon e Oommen, 2008; Fox e Brodkorb, 2008). Além disso, destaca-se a presença da água, na qual as micelas de caseína são altamente hidratadas e apresentam partículas coloidais semelhantes a esponjas. Foi estimado que de 4 g de água / g de proteína contida na micela coloidal, apenas 15% está ligado à proteína, sendo o restante simplesmente aprisionado dentro da partícula (de Kruif e Holt, 2003; Farrell et aL, 2003). A combinação de uma estrutura entrelaçada com múltiplas interações resulta então em uma "supermolécula" coloidal esponjosa e porosa que é resistente as alterações espaciais e de desintegração (McMahon e Oommen, 2008).

1.1.2 O fenômeno da coagulação do leite

A dispersão coloidal das micelas de caseína no leite é responsável pela estabilidade da suspensão de proteínas e ocorre devido às repulsões eletrostáticas e impedimento de natureza estérica. Esses fenômenos ocorrem primordialmente pela presença de glicomacropeptídeos negativos que são encontrados na superfície das micelas de caseína (Fox et al., 2000).

Na fabricação de queijos, a função primária das enzimas é iniciar a coagulação do leite (*Figura 1.3*). O processo de coagulação enzimática pode ser dividido em três fases: hidrólise enzimática da κ-caseína, agregação das micelas de caseína na presença de íons de cálcio e desenvolvimento do gel coagulado (Karlsson, Ipsen e Ardo, 2007). Entretanto, a maioria dos autores relata que a coagulação ocorre em dois estágios. Na primeira fase da coagulação ocorre a clivagem específica do glicomacropeptídio hidrofílico, preferencialmente no sítio Phe_{105}-Met_{106} da κ-caseína (κ-CN) que é altamente suscetível à hidrólise por proteases ácidas (Fox et al., 2000; Walstra et al., 2006). Na segunda fase da coagulação, a para-κ-caseína^{2+} (formada a partir das micelas clivadas), fica instável pela saída do glicomacropeptídeo e inicia-se um processo de agregação sob a influência dos íons cálcio do meio (Dalgleish, 1992; Walstra et al., 2006). Então, ligações cruzadas são progressivamente formadas entre cadeias de micelas floculadas para formar um gel final (Fox e McSweeney, 1998; Lucey, 2002a; Lagaude et al., 2004; Bönisch, Heidebach e Kulozik, 2008).

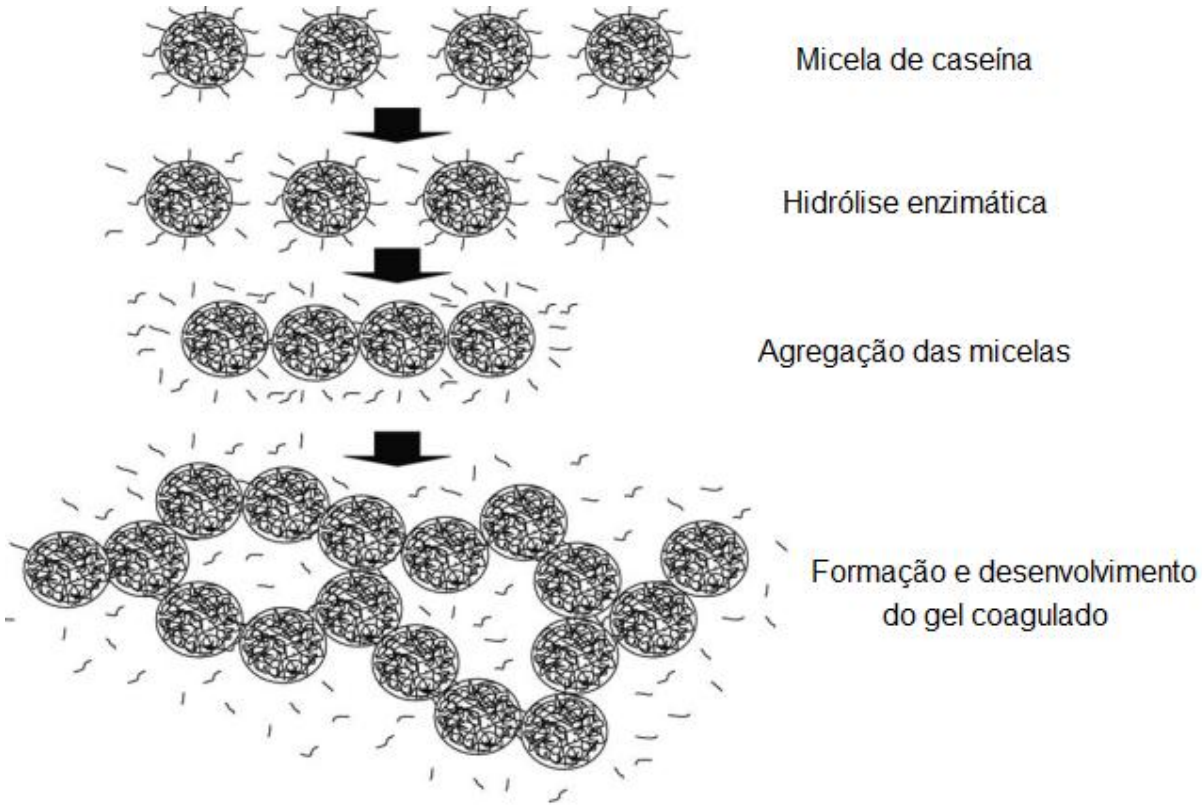

Figura 1.3 Representação esquemática das etapas que ocorrem durante a coagulação enzimática do leite (Adaptado de Lucey, 2011)

Após a coagulação, a formação da rede de coalhada continua por um tempo considerável após a obtenção de um gel visível, mesmo após o corte (Fox e McSWeeney, 1998; Walstra et. al., 1999). As características do gel formado por ação enzimática, tais como capacidade de retenção de água, sinérese e força, são importantes no processo de elaboração de queijos, pois afetam parâmetros como o rendimento, conteúdo de umidade e textura do produto (Pandey, Ramaswamy e St-Gelais, 2000).

1.1.3 Propriedades físicas, reológicas, microestruturais e bioquímicas do gel obtido por coagulação enzimática

A velocidade de clivagem da κ-caseína pela ação enzimática é o principal responsável para o início da agregação do gel. As progressivas ligações hidrofóbicas entre para-κ-caseína na presença de íons de cálcio, além da presença de fosfato de cálcio coloidal (FCC) desempenham um papel essencial para formação do gel final (Dalgleish, 1983; Fox e McSweeney, 1997; Lucey, 2002a,b). Neste contexto, a força de um gel obtido por ação enzimática é dependente do tipo e quantidade de enzima adicionada no leite, além das propriedades físicas e químicas das micelas de caseína, ou seja, o tamanho das micelas, o teor de proteína (isto é, de caseína), a concentração de cálcio, o pH, temperatura e a proporção entre caseína e proteínas do soro de leite (Lucey et al., 2003; Auldist et al., 2004; Amenu e Deeth, 2007).

Possivelmente a maneira mais direta de medir a formação do gel é a partir do monitoramento da evolução das propriedades reológicas (Horne, 1995; Horne, 1996; Horne e Banks, 2004; Sandra et al., 2011; Sandra, Alexander e Corredig, 2012; Hussain, Grandison e Bell, 2012; Sandra e Corredig, 2013; Hussain, Bell e Grandison, 2013). Nos ensaios dinâmicos aplica-se uma tensão de cisalhamento oscilatório que mede a resposta do gel (material viscoelástico) em desenvolvimento (Sandra, Alexander e Corredig, 2012; Hussain, Bell e Grandison, 2013). Como resposta obtém-se o módulo elástico ou de armazenamento (G'), que é uma medida da energia armazenada por ciclo de oscilação e reflete o quanto sólido é a amostra, e o módulo viscoso ou de perda (G''), que é uma medida da energia dissipada por ciclo e indica o quanto da amostra se comporta como um líquido viscoso (Horne e Banks, 2004). Outro parâmetro importante é *Loss tangent* obtida por meio da divisão entre G'' e G' (tan δ = G'' / G'), que pode ser expressa pela tangente do ângulo de fase da resposta da tensão aplicada, este parâmetro indica a preponderância de um gel viscoso ou elástico (Dejmek e Walstra, 2004).

Preferencialmente, as análises deverão ser feitas na região viscoelástica linear, isto é, a deformação deve ser proporcional à tensão aplicada (Horne e Banks, 2004). Quando o gel é muito fraco nos primeiros estágios da reação, é discutível se essa situação é possível. Forças mínimas devem ser aplicadas pelo instrumento para a produção de um movimento discernível na sua geometria. Esta tensão ou movimento mínimo pode ser suficiente para danificar o gel neste ponto durante o desenvolvimento. No entanto, o desenvolvimento do gel viscoelástico induzido por coagulação enzimática ocorre rapidamente e assim move-se em resposta linear rápida, porém, a aplicação de grandes tensões deve ser evitada (Horne e Banks, 2004).

Portanto, o processo de coagulação do leite pode ser monitorado por meio de ensaios dinâmicos reológicos que utilizem testes oscilatórios de baixa deformação em reômetro com temperatura, tensão e frequência controlada para realização de uma simulação real do processo de coagulação sem que haja destruição do gel. Os dados reológicos são importantes para avaliar o comportamento de coagulação do leite em diferentes condições, e determinar a consistência final do gel obtido (Sandra, et al., 2011; Sandra, Alexander e Corredig, 2012; Hussain, Grandison e Bell, 2012; Sandra e Corredig, 2013; Hussain, Bell e Grandison, 2013). O módulo de armazenamento (G') descreve o comportamento elástico (sólido) do produto, e começa a aumentar no início da segunda fase da coagulação, caracterizada pela agregação de micelas. Portanto o ensaio reológico é um método instrumental com alta sensibilidade capaz de monitorar o processo de coagulação.

A reologia final do queijo é determinada em função da sua composição, microestrutura (isto é, o arranjo estrutural dos seus componentes), o estado físico-químicos dos seus componentes, e sua macroestrutura, que reflete a presença de heterogeneidade, tais como empacotamento dos glóbulos na rede proteica (O'Callaghan e Guinee, 2004). As propriedades físico-químicas incluem parâmetros tais como a coalescência da gordura, a razão de sólido para líquido de gordura, grau de hidrólise e a hidratação da matriz proteína, aprisionamento da

água, nível de atrações intra-moleculares e entre as moléculas de para-k-caseína, além da solubilidade do fosfato de cálcio coloidal (Horne e Banks, 2004).

Géis de leite formados por adição de coalho podem apresentam sinérese, ou seja, expulsão de líquido (soro de leite), devido à contração do gel (coalhada) acompanhada por um rearranjo da rede (Lucey, 2001). Depois que o gel é formado, as micelas de caseína ainda possuem muitos locais reativos na sua superfície e, portanto, mais ligações entre para-κ-caseína podem ser formadas na presença de íons de cálcio (Walstra e Jenness, 1984). A formação de novas ligações conduz a uma rede mais compacta entre as micelas e a uma maior expulsão do soro de leite a partir do gel (Walstra e Jenness, 1984; Walstra, van Dijk e Geurts, 1985; Lodaite et al., 2000; Grundelius et al., 2000; Lucey, 2001).

A sinérese do gel é complexa e ainda não é bem compreendida (Lodaite et al., 2000). Uma compreensão mais profunda da sinérese e do impacto dos fatores relevantes podem ser investigados por meio de modelos baseados em parâmetros que possam ser medidos, como a porosidade, permeabilidade e coeficientes reológicos apropriados da matriz caseína que apresentem as condições de ensaio com parâmetros e limites bem definidos. Vários estudos fundamentais sobre a sinérese dos géis de caseína obtido por coalho foram relatados (Walstra, van Dijk e Geurts, 1985; van Dijk e Walstra, 1986; van Vliet et al., 1991; Lucey et al., 2000; Lucey, 2002a). Empiricamente, sinérese tem sido amplamente estudado. A taxa e extensão da sinérese dependem de vários fatores, tais como, a firmeza do gel para o corte, a área de superfície do gel, a pressão, o pH, a temperatura, a composição do leite e a quantidade e o tipo de coagulante (Lawrence, 1959; Patel, Lund e Olson, 1972; Lelievre, 1977; Marshall, 1982; Pearse et al., 1984; Pearse e Mackinlay, 1989; Walstra, van Dijk e Geurts, 1985; Lomholt e Qvist, 1999; Daviau, 2000).

Diferentes metodologias têm sido usadas para determinar a sinérese em função do tempo, tal como a quantificação do soro de leite expelido, a medição direta da contração da coalhada ou medindo a densidade da coalhada (Beeby, 1959; Lawrence, 1959; Zviedrans e Graham, 1981; Marshall, 1982; Pearse et al.,

1984; Nilsen e Abrahamsen, 1985; Walstra, van Dijk e Geurts, 1985; Renault et al., 1997). Desta forma, a partir da quantificação da expulsão de soro, pode-se determinar o índice de sinérese, o qual pode impactar no teor de umidade dos queijos, e consequentemente na textura e qualidade do produto (Castillo et al., 2006).

A microscopia confocal a laser (CLSM) foi inventada por Marvin Minsky em 1955 e patenteado em 1957, mas o refinamento dos lasers para permitir seu uso como uma técnica padrão só foi possível a partir do final da década de 1970 (Heertje et al., 1987). CLSM é uma ferramenta que tem um grande potencial para melhorar a compreensão da microestrutura de queijos incluindo as etapas envolvidas no processo de fabricação (Hassan et al., 1995; Lucey et al., 1997; Lucey et al., 1998; Lucey, 2001; Lucey, 2004; Ko e Gunasekaran, 2007; Ong et al., 2010; Ong et al., 2011; Ong et al., 2012; Hussain, Grandison e Bell, 2012; Hussain, Bell e Grandison, 2013; Nguyen et al., *in press*).

Na CLSM, a capacidade de corte óptico em alta resolução permite que as amostras sejam avaliadas com uma preparação da amostra rápida e simples sem afetar sua estrutura (Brooker, 1995). Desta forma, o feixe de laser pode penetrar na amostra e obter informações estruturais em diferentes profundidades, sem perturbar a estrutura interna do gel (Gunasekaran e Ding, 1999).

Assim, CLSM tem sido utilizado com sucesso para estudar as redes tridimensionais de gordura e proteína tanto em géis como em diferentes matrizes de queijo (Everett et al., 1995; Auty et al., 2001; Ko e Gunasekaran, 2007; Ong et al., 2010; Ong et al., 2011; Ong et al., 2012; Hussain, Grandison e Bell, 2012; Hussain, Bell e Grandison, 2013; Nguyen et al., *in press*). Além disso, as imagens de CLSM são analisadas em programas computacionais, uma vez que as imagens já estão em formato digital para determinação da porosidade, número e tamanho médio de poros (Lucey, 2004; Ko e Gunasekaran, 2007; Ong et al., 2010; Ong et al., 2011; Ong et al., 2012; Hussain, Grandison e Bell, 2012; Hussain, Bell e Grandison, 2013; Nguyen et al., *in press*).

Há uma série de trabalhos que utilizam outros microscópios para avaliar a microestrutura de queijos, como a microscopia eletrônica de varredura (MEV) ou microscopia eletrônica de transmissão (MET) (Kalab 1995; Schmidt e Buchheim, 1992; Tamime et al., 2007; Bermúdez-Aguirre, Mawson e Barbosa-Cánovas, 2008). No entanto, durante a etapa de preparação das amostras as microestruturas das amostras podem sofrer alterações físicas ou químicas, dentre as quais pode-se destacar à utilização de fixadores químicos, técnicas de desidratação e corte das amostras. Assim, uma vantagem da CLSM é a obtenção da informação estrutural de uma maneira não destrutiva.

Considerando que a maioria de coalho adicionado ao leite durante a produção de queijo é eventualmente perdida no soro de leite, uma fração menor que depende do tipo de queijo é mantida dentro da coalhada (Choisy et al., 2000; Upadhyay et al., 2004). Essa fração é responsável pela proteólise inicial (Silva e Malcata, 1998; Silva e Malcata, 2004; Silva e Malcata, 2005). Durante esta fase, as caseínas são hidrolisadas em grandes peptídeos, bem definidos, mediante a ação do coalho residual, que são posteriormente digeridos em menores e até mesmo aminoácidos por enzimas microbianas (Benfeldt e Sørensen, 2001).

As primeiras 24 horas após a coagulação do leite são as mais importantes para o perfil bioquímico dos queijos afetando diretamente a maturação, especialmente para sistemas modelos de queijos (que apresentam alta atividade proteolítica devido à alta concentração de enzima, elevada temperatura e umidade) (Picon et al., 1995; Silva e Malcata, 2004; Silva & Malcata, 2005). A agregação da caseína e a separação do soro continuam ocorrendo ao longo dos primeiros estágios de maturação; a caseína é compactada no interior da coalhada, a água é perdida, os glóbulos de gordura são englobados e comprimidos, todos os quais são determinantes para estrutura e composição final dos queijos (Green e Grandison, 1999; Choisy et al., 2000; Wilkinson e Kilcawley, 2005). A proteólise primária é, possivelmente, o evento mais importante para o desenvolvimento de sabor em queijos curados (Fox et al., 1999). Se a degradação das caseínas resultar na formação de peptídeos com aminoácidos hidrofóbicos na extremidade

N-terminal, especialmente sobre a fração da β-caseína, haverá produção de sabor amargo (Hassan et al., 2013).

1.1.4 Coalho e coagulantes

O coalho é o extrato obtido do abomaso de animais ruminantes. Este extrato é rico em proteinases ácidas que apresentam atividade coagulante sobre o leite. Os coagulantes, por sua vez, correspondem às demais proteinases de diferentes origens, capazes de coagular o leite sob condições adequadas de temperatura e pH (Fox, 1988; Fox e McSweeney, 1997).

As proteinases encontradas no abomaso de ruminantes são a quimosina e a pepsina. A quimosina caracteriza-se por ser uma enzima de atividade altamente específica (Phe_{105}-Met_{106} da κ-caseína) e com bom poder coagulante (Hyslop, 2003; Crabbe, 2004). Por outro lado, a pepsina apresenta menor especificidade, hidrolisando ligações que tenham aminoácidos como Phe, Tyr, Leu ou Val (Agudelo et al., 2004; Papoff et al., 2004), podendo desta forma ocasionar a liberação de peptídeos de cadeias médias com sabor desagradáveis, apesar de ter poder hidrolítico maior do que a quimosina (Fox, 1988; Fox et al., 2004). A concentração de quimosina e de pepsina varia em função da idade do animal, sendo que ela é encontrada na proporção 80% de quimosina e 20% de pepsina em bezerros e na proporção inversa para animais adultos.

O coalho extraído do abomaso de bezerros era considerado a melhor enzima para produção de queijos (Fox et al., 2004). A sua aplicação, entretanto, é cada vez mais restrita devido ao crescimento da produção mundial de queijos (cerca de 4% ao ano) e ao decréscimo da oferta de coalho de bezerros (uma vez que há uma tendência de redução de abate precoce de novilhos em função do baixo aproveitamento em termos de produção de carnes) (FAO, 2010).

Assim, é crescente a busca de enzimas alternativas para substituição do coalho (Walstra et al., 2006). Atualmente, apenas 20-30% dos queijos produzidos no mundo utilizam coalho de vitelo (Jacob, Jaros e Rohm, 2011). Substitutos

potenciais devem imitar as suas propriedades específicas apresentando alta atividade de coagulação do leite (ou seja, especificidade na hidrólise da κ-caseína) e baixa atividade proteolítica em pH e temperatura de fabricação de queijos. Além disso, devem ser inativados à temperatura de pasteurização, de forma que seja possível obter um soro com boa qualidade proteica, sem restos de coagulante ativo (Dalgleish, 1992; Fox e Kelly, 2004). Adicionalmente, devem cumprir as regras e regulamentos em vigor de cada país, restrições tecnológicas e econômicas, bem como o mercado-alvo (certificação kosher, aprovação orgânico ou vegetariano) (Rolet-Répécaud et al., 2013).

Nas últimas décadas, diversos coagulantes de origem animal, microbiana e vegetal foram considerados substitutos potenciais para o coalho (Dalgleish, 1992; Jacob, Jaros e Rohm, 2011). Contudo, a aplicação destas enzimas na coagulação do leite poderia resultar em inconvenientes como a redução do rendimento da fabricação (decorrente da intensa atividade proteolítica destas enzimas) e o aparecimento de defeitos de aroma e sabor (especialmente o gosto amargo). Há relatos de que as pepsinas de origem suína e avícola, esta última principalmente por questões religiosas (Andrén, 2011), eram consideradas aceitáveis e aplicadas como coagulantes de origem animal para a produção de queijo (Fox e McSweeney, 1997), entretanto, estas enzimas apresentam dificuldades específicas de aplicação como baixa especificidade hidrolítica (pepsina de aves), com consequente defeito no sabor e na textura dos queijos (Fox e McSweeney, 1997; Fox et al., 2004). Também podem apresentar baixa atividade de coagulação de leite em pH > 6,6 (pepsina suína), o que possibilita sua aplicação apenas para a produção de queijos frescos, por não exigir uma etapa de maturação (Chitpinityol e Crabbe, 1998; Fox et al., 2004).

As enzimas de origem vegetal, por sua vez, apresentam atividade proteolítica acentuada em relação ao seu poder coagulante. Com isso, provocam defeitos na massa e gosto amargo ou anormal. Desta forma, apesar de serem bastante estudadas e algumas tentativas de aplicação terem sido realizadas com ficina, bromelina e papaína, elas não são consideradas próprias para a fabricação

de queijo (Cattaneo et al., 1994; Teixeira et al., 2000; Fadyloglu, 2001; Patil et al., 2003; Llorente et al., 2004; Fox et al., 2004; Moharib, 2004; Libouga et al., 2006; Low et al., 2006; Senthilkumar et al., 2006; Chazarra et al., 2007; Egito et al., 2007; Raposo e Domingos, 2008; Vairo Cavalli et al., 2008; Duarte et al., 2009). Em contrapartida, os extratos de *Cynara cardunculus* L. têm sido usados durante séculos na produção artesanal de queijos a partir de leite de ovelhas, como o Serra da Estrela, Manchego, La Serena ou Serpa em Portugal e Espanha, alguns deles com Denominação de Origem Controlada (DOC) (Sousa e Malcata, 2002; Roseiro et al., 2003; Prados et al., 2007; Jacob, Jaros e Rohm, 2011).

Cynara cardunculus L. é uma variedade de cardo que cresce principalmente em áreas secas e pedregosas de Portugal e algumas outras partes da Península Ibérica (Sales-Gomes e Lima-Costa, 2008). Uma característica especial destes queijos é a acentuada proteólise, resultando em um queijo cremoso e amanteigado de textura macia (Chen et al., 2003; Prados et al., 2007; Galan et al., 2008; Pereira et al., 2008; Pino et al., 2009). No entanto, um problema para sua aplicação na fabricação de queijos em escala automática é a variabilidade de extratos não padronizados, sendo o pH um fator crucial para manutenção da atividade proteolítica (Sousa e Malcata, 1996). Assim, foi realizada a fabricação de cynarase recombinante que, como a quimosina, pode ser produzida por micro-organismos (Fernandez-Salguero et al., 2003; Sampaio et al., 2008).

Diversas proteases extracelulares de origem microbiana possuem ação semelhante à quimosina e são, em parte, apropriadas para a produção de queijo (Andrén, 2011). Tais coagulantes podem ser produzidos por fermentação em produção ilimitada, além disso, como essas enzimas não são derivadas de tecidos de ruminantes são aceitas pelos lacto-vegetarianos (Jacob, Jaros e Rohm, 2011).

Desde a década de 60 até os dias atuais mais de 100 fungos foram relatados com atividade de coagulação sobre o leite (Garg e Johri, 1994; Tubesha e Al-Delaimy, 2003; Jacob, Jaros e Rohm, 2011), o que reflete o interesse científico em coagulantes alternativos para a produção de queijo. Três espécies, *Rhizomucor miehei*, *Rhizomucor pusillus* e *Cryphonectria parasitica* foram

estabelecidas para a produção em larga escala (Walstra et al., 2006; Jacob, Jaros e Rohm, 2011). A protease aspártica produzida por *R. miehei* consiste de uma única cadeia polipeptídica com alta semelhança a quimosina na sua estrutura tridimensional (Chitpinityol e Crabbe, 1998). Esta protease é o coagulante microbiano mais comumente utilizado para a produção de queijo e comercialmente disponível em diferentes níveis de pureza e estabilidade térmica (Jacob, Jaros e Rohm, 2011). Na França, as preparações enzimáticas de fontes animais e fúngicas são as mais utilizadas. Em 2010, coalho de vitelo representou 33% da demanda para a produção de queijos, quimosina recombinante apenas 14% e coagulantes microbianos 53% sendo 35% para *R. miehei* e 18% para *C. parasitica* (SPPAIL, 2010).

A protease obtida a partir de *R. pusillus* apresenta atividade de coagulação do leite melhor a 50 ºC e sensibilidade ao pH o que reduziu sua aplicação em larga escala (Nouani et al., 2009). A protease de *Cryphonectria* é reconhecida por sua maior atividade proteolítica e, em contraste com as proteases de *Rhizomucor*, hidrolisa principalmente a β-caseína (Tam e Whitaker, 1972; Vanderporten e Weckx, 1972; Ustunol e Zeckzer, 1996; Awad et al., 1999; Trujillo et al., 2000; Broome et al., 2006), entretanto, é menos sensível a adição do Ca^{2+} do que as proteases do *Rhizomucor*. Como a quimosina, a ligação Phe_{105}-Met_{106} de κ-caseína é também preferencialmente hidrolisada pelas proteinases ácidas de *R. miehei* e *R. pusillus*, mas a proteinase ácida da *Cryphonectria parasítica* preferencialmente cliva a ligação Ser_{104}-Phe_{105} (Andrén, 2011).

No entanto, ao contrário de quimosina, as proteinases destes microrganismos também clivam outras ligações da κ-caseína e possuem uma atividade proteolítica mais acentuada (Chitpinityol e Crabbe, 1998). Além disso, essas enzimas fúngicas são produzidas em fermentação em estado sólido e após a purificação, as modificações químicas são geralmente aplicadas para reduzir suas termoestabilidade. Uma alta estabilidade térmica foi a grande desvantagem da primeira geração de proteases fúngicas, o que significa que mesmo após a pasteurização do soro a enzima mantinha-se intacta, o que inviabilizou a aplicação

deste subproduto (Jacob, Jaros e Rohm, 2011). Apesar disso, os queijos produzidos com essas enzimas têm apresentado boa aceitabilidade (Foltmann, 1993; Fox e McSweeney, 1997; Chitpinityol e Crabbe, 1998; Fox et al., 2000). Contudo, mais estudos sobre a maturação de queijo precisam ser realizados para verificar a utilidade destas enzimas para o processamento de queijo.

A baixa especificidade dos coagulantes disponíveis industrialmente e o custo do coalho alavancaram a busca de processos alternativos para obtenção de novas enzimas para aplicação no processo de coagulação do leite (Rooijen et al., 2008). Nesta busca, a engenharia genética se tornou uma ferramenta importante (Badiefar et al., 2009), permitindo a produção de quimosina recombinante por micro-organismos a partir do sequenciamento genético que leva à produção destas enzimas obtidas de vitelos e bovinos, além de animais como veados, búfalos, girafas, ovinos, caprinos, camelos e espécies de equídeos (Jacob, Jaros e Rohm, 2011).

A quimosina sintetizada é idêntica, química e funcionalmente, à quimosina obtida a partir coalho, com as mesmas condições ótimas de ação e tendo o mesmo desempenho durante a coagulação e maturação do queijo (Rampilli et al., 2005). De acordo com Johnson e Lucey (2006) estima-se que 70-80% das enzimas utilizadas na fabricação de queijos são coalhos recombinantes. Nos EUA e na Grã-Bretanha 80-90% de queijos são fabricados com quimosina recombinante (GMO Compass, 2010).

É importante ressaltar que o coalho é diferente da quimosina obtida por engenharia genética, uma vez que a primeira contém quimosina e pepsina bovina I (gastricsina) e II, encontradas na proporção aproximada de 80% de quimosina, 3% de gastricsina e 17% de pepsina bovina II em animais jovens (Dalgleish, 1992, Moschopoulou et al., 2007). Algumas pesquisas relataram a importância da pepsina bovina para uma melhor maturação controlada do queijo. Como consequência disto, alguns coagulantes comerciais são formulações que contém quimosina produzida por micro-organismos por meio de engenharia genética com adição de pepsina bovina (Fox et al., 2000).

Além disso, o coalho animal contém uma mistura de quimosinas A, B e C na proporção aproximada de 30:55:15. Essas frações apresentam atividades e especificidades diferentes e, para o coagulante obtido por recombinação, não ocorre a mistura de mais de um tipo de quimosina; ele é composto exclusivamente pelo tipo A ou B (Fox et al., 2000), o que pode reduzir um pouco as faixas de ação da enzima.

Novas pesquisas vêm sendo realizadas para obtenção de quimosina de outras espécies. Dentro deste contexto, trabalhos mais recentes destacam-se a utilização de quimosina obtida de camelo (*Camelus dromedarius*) expressa em *A. niger* var. *awamori* (Jacob, Jaros e Rohm, 2011). Kappeler et al. (2006) descreveram a produção e purificação em escala piloto, utilizando cromatografia de afinidade. Esta enzima apresenta uma atividade específica sobre a κ-caseína cerca de 70% superior comparada com a quimosina recombinante obtida do vitelo e uma atividade proteolítica inferior, sendo atualmente produzida em escala industrial, por Chr. Hansen A/S (Hoersholm, Dinamarca) e comercialmente disponível desde o final de 2009 (Jacob, Jaros e Rohm, 2011).

1.1.5 Desafios da produção de queijos: custo e atividade das enzimas responsáveis pelo processo de coagulação

A etapa de coagulação é crítica para a produção de queijos e ainda não se tem um coagulante substituto do coalho de vitelo adequado, considerando-se a atividade e especificidade. O uso de engenharia genética através da produção de coalhos recombinantes melhora o desempenho das enzimas microbianas para aplicação na produção de queijos, mas ainda assim, não representam uma solução final para o desafio da produção de coagulantes com custo e desempenho adequados.

Recentemente, novos processos, como ultrasom, micro-ondas e alta pressão isostática (API), se mostraram capazes de promover alterações de atividade e estabilidade em enzimas, melhorando o desempenho das mesmas

com consequente redução de seus custos (Cano, Hernández e Ancos, 1997; Barton, Bullock e Weir, 1996; Rajesse et al., 2007).

O ultrasom, utilizado durante a ação das enzimas sobre o substrato, resulta em aumento de atividade por melhorar a difusividade e transferência de massa de produtos e substratos (Lee et al., 2008), tornando os processos mais efetivos, principalmente quando existe algum tipo de inibição (Barton, Bullock e Weir, 1996) ou quando se trata de enzimas imobilizadas, onde o uso de ultrasom aumenta a superfície de contato (Mason, Paniwnyk e Lorimer, 1996). Além disso, o processo também pode promover alterações no substrato, como quebra de regiões helicoidais, favorecendo o acesso das enzimas (Jian, Wenyi e Wuyong, 2008).

O processamento por micro-ondas foi estudado por alguns autores que observaram aumento de atividade, estabilidade e/ou seletividade enzimática após aplicação de micro-ondas de baixa energia em meio não aquoso (Roy e Gupta, 2003). Isso pode ser explicado pela transferência direta de energia do campo magnético para as regiões polares das enzimas, aumentando a flexibilidade das mesmas, sua reatividade (Rajesse et al., 2007) e as colisões entre enzima e substrato (Yadav e Lathi, 2005). Resultados prévios obtidos para enzimas tratadas por micro-ondas também indicam aumento de atividade (Rajesse et al., 2007).

A alta pressão isostática também foi recentemente descrita como um processo capaz de alterar a atividade de enzimas. Os resultados obtidos indicaram que o processo é capaz de promover a ativação (Mozhaev et al., 1996; Sila et al., 2007; Eisnmenger e Reyes-de-Corcuera, 2009a,b) e estabilização de enzimas (Mozhaev et al., 1996; Eisnmenger e Reyes-de-Corcuera, 2009a,b), pela aplicação de baixas pressões (até 400MPa) e temperaturas moderadas (Kudryashova et al., 1998; Knnor, 1999; Sila et al., 2007). Além disso, o efeito nas enzimas depende do solvente e do substrato utilizado (Eisnmenger e Reyes-de-Corcuera, 2009a). O aumento de atividade foi relatado tanto em enzimas previamente processadas por API (Cano, Hernández e Ancos, 1997; Hernández e Cano, 1998; Katsaros et al., 2009) como nas reações enzimáticas conduzidas a

API (Mozhaev et al., 1996; Kudryashova et al., 1998; Sila et al., 2007; Eisnmenger e Reyes-de-Corcuera, 2009b).

O processo de pressurização, seguindo o princípio de "Le Chatelier", induz a redução de volume molecular (Knnor, 1999), acelerando, de forma exponencial, a ocorrência de reações favorecidas nessas condições (Mozhaev et al., 1996). Em termos moleculares, a API pode induzir a ativação pelo aumento da flexibilidade conformacional das enzimas gerado pela hidratação dos seus grupos carregados (Eisenmenger e Reyes-de-Corcuera, 2009a,b), aumento das interações físicas da molécula com o substrato (Eisenmenger e Reyes-de-Corcuera, 2009a) e aumento da concentração de grupos polares e carregados no complexo de Michaelis e no estado de transição (Kudryashova et al., 1998), com consequente aumento na taxa das reações.

Assim, a aplicação de algumas tecnologias não convencionais tem se destacado pela melhoria do desempenho de algumas enzimas. Nenhuma destas tecnologias, entretanto, foram aplicadas para enzimas comerciais destinadas a fabricação de queijos.

1.1.6 Uso da homogeneização à alta pressão em enzimas: Nova proposta para melhorar o desempenho de enzimas utilizadas na coagulação do leite

A homogeneização à alta pressão (HAP), também conhecida como alta pressão dinâmica (APD) é um processo não convencional desenvolvido com o primeiro propósito de substituir o processamento térmico de alimentos visando à inativação de micro-organismos com menores danos às suas características físicas e sensoriais (Franchi, Tribst e Cristianini, 2012). Uma vez que as respostas obtidas indicaram a HAP como um método promissor para o processamento de alimentos, estudos posteriores foram realizados para avaliar o efeito deste processo sobre os constituintes dos alimentos, como proteínas (Subirade et al., 1998; Bouauina et al., 2006; Gárcia-Juliá et al., 2008, Keerati-u-Rai, Corredig, 2009; Luo et al., 2010; Dong et al., 2011; Yuan et al., 2012), polissacarídeos

(Lagoueyete e Paquin, 1998; Floury et al., 2002; Lacroix, Fliss e Makhlouf, 2005; Modig et al., 2006; Kivelä et al., 2010; Villay et al., 2012) e lipídeos (Kheadr et al., 2002; Kielczewska et al. 2003; Hayes e Kelly, 2003; Serra et al., 2007) de forma a determinar o efeito do processo nos produtos.

Este processo é descrito para aplicação em alimentos fluidos (Torrezan, 2003) e surgiu a partir dos processos comuns para homogeneização de produtos lácteos e emulsões, tendo, o mesmo princípio de operação (Diels e Michiels, 2006), porém utilizando-se pressões da ordem de 10 a 15 vezes superiores às habitualmente aplicadas, ou seja, pressões de até 350 MPa (3500 bar). No equipamento, o fluido é impelido a passar por uma válvula de homogeneização a altas pressões (Torrezan, 2003). A passagem pelo orifício estreito da válvula (da ordem de micrometros) e a descompressão abrupta do fluido geram um aumento da sua velocidade (entre 150 e 300 $m.s^{-1}$ – Floury et al., 2004; Pinho et al., 2011) e também aumento de temperatura (em torno de 1,5 a 2,5 ºC a cada incremento de pressão de 10 MPa – Diels e Michiels, 2006) causado pelo atrito intenso na região da válvula de homogeneização (Floury et al., 2004). A pressão de operação é controlada pela distância entre a válvula de homogeneização e seu cabeçote (Diels e Michiels, 2006).

Recentemente, algumas pesquisas se dedicaram a avaliar o efeito do processo sobre enzimas (Vannini et al., 2004; Dosualdo 2007; Lanciotti et al., 2007; Lucci et al., 2007; Welti-Chanes et al., 2009; Liu et al., 2009a,b; Tribst e Cristianini, 2012a,b; Tribst, Augusto e Cristianini, 2012a), sendo observado ativação ou inativação das mesmas, o que é dependente do tipo de enzima avaliada, das condições em que o processo foi realizado e a atividade foi medida. Dentre as respostas obtidas, destacam-se as enzimas que apresentaram maior atividade e estabilidade em condições não ótimas, indicando que o processo altera a conformação das enzimas e, consequentemente, suas condições ideais de atividade (Liu et al., 2009a,b; Tribst e Cristianini, 2012b; Tribst, Augusto e Cristianini, 2012a).

O aumento de atividade é normalmente atribuído às alterações causadas pela HAP nas estruturas quaternária, terciária e secundária das enzimas (Lacroix, Fliss e Makhlouf, 2005; Liu et al., 2009b; Liu et al., 2010). Estas alterações envolvem:

1. Aumento na exposição dos grupos sulfidrilas superficiais e redução do número total de grupos SH disponíveis, indicando que o processo causa desnaturação e desdobramento molecular ao mesmo tempo em que favorece a formação de pontes de sulfeto (Liu et al., 2009b; Liu et al., 2010),
2. Aumento da exposição dos resíduos de tirosina e triptofano (Liu et al., 2009b; Liu et al., 2010) e alterações da configuração da vizinhança destes aminoácidos (Liu et al., 2009b),
3. Modificação da exposição de grupos hidrofóbicos (Liu et al., 2009b; Liu et al., 2010),
4. Alterações da exposição dos sítios ativos das enzimas (Vannini et al., 2004; Lanciotti et al., 2007; Iucci et al., 2007),
5. Redução das pontes de hidrogênio inter e intra moleculares (Liu et al., 2010),
6. Mudança nos percentuais de estruturas secundárias (α-hélice e β-pregueada) após a homogeneização (Liu et al., 2009b).

Segundo Vannini et al. (2004), Iucci et al. (2007) e Lanciotti et al. (2007), durante a pressurização há rompimento das estruturas tridimensionais das proteínas, permitindo que os grupos movam-se livremente, independentemente de sua configuração original e, durante a descompressão instantânea, há rearranjo molecular, mas os grupamentos não retornam às suas configurações originais.

Em estudos realizados por Tribst e Cristianini (2012a) foi verificado que a α-amilase é altamente estável ao processo de HAP, não sendo alterada quando homogeneizada até 150 MPa independente das temperaturas e pH em que a amostra foi tratada. Os resultados da β-galactosidase (Tribst, Augusto e Cristianini, 2012b), por outro lado, mostraram que a enzima é pouco estável ao processo de homogeneização, apresentando redução da atividade após

processamento em pressões de 150 MPa quando processadas em pH não ótimo de atividade da enzima.

Em outros estudos os resultados obtidos para a protease neutra (Tribst, Augusto e Cristianini, 2012a), amiloglicosidase (Tribst e Cristianini, 2012b) e glicose oxidase (Tribst e Cristianini, 2012c) indicaram que o efeito da HAP foi dependente dos parâmetros utilizados no processo (pH, temperatura e pressões de homogeneização) e das condições utilizadas na medida de atividade (pH, temperatura e tempo de estocagem). Para estas três enzimas, significativos ganhos de atividade e/ou estabilidade foram observados para pelo menos uma das condições avaliadas, sendo que os mais importantes foram: (i) redução da temperatura ótima de atividade da protease neutra de 55 para 20ºC após APD a 200 MPa, (ii) aumento da atividade a 75ºC da glicose-oxidase após homogeneização a 150 MPa, (iii) aumento da atividade residual entre 100 e 400% após armazenamento refrigerado de glicose-oxidase homogeneizada em diferentes pressões, (iv) aumento da atividade de amiloglicosidase a 80ºC após a APD a 100 MPa.

Os efeitos da HAP no leite têm sido intensivamente estudados visando à inativação de micro-organismos (Hayes e Kelly, 2003; Lanciotti et al., 2007) e avaliação dos efeitos sobre os seus macros constituintes (Thiebaud et al., 2003; Sandra e Dalgleish, 2005; Serra et al., 2007). Outros estudos focaram na produção de queijo com leite tratado previamente por HAP (Lanciotti et al., 2004; Lanciotti et al., 2006; Pareda et al., 2008; Vannini et al., 2008; Escobar et al., 2011).

Alguns autores afirmam que a aplicação da HAP no leite provoca redução do tamanho das micelas de caseína (Kheadr et al., 2002), com consequente aumento do número de micelas, o que reduz o tempo de coagulação pela interação mais rápida entre as proteínas. Além disso, sugere-se que a combinação do tratamento térmico e HAP promove a desnaturação térmica de proteína de soro de leite exercendo um efeito positivo que propicia um aumento do rendimento pela agregação parcial das proteínas do soro à matriz caseica (Escobar et al., 2011).

A redução dos glóbulos de gordura é função do nível de pressão aplicada no processo de HAP, porém utilizando-se pressões entre 50 e 300 MPa observa-se reduções maiores do que as obtidas pelo processo de homogeneização comum (Sandra e Dalgleish, 2005). Em pressões de homogeneização superiores a 300 MPa (Thiebaud et al., 2003; Serra et al., 2007), por outro lado, um efeito contrário é observado, visto que a intensa redução dos glóbulos modifica sua carga elétrica e favorece a coalescência (Serra et al., 2007; Thiebaud et al., 2003). Este fenômeno pode ser minimizado pela realização da homogeneização em 2 estágios (Hayes e Kelly, 2003).

Apesar do processo de HAP ter sido bastante estudado para leites, incluindo a produção de queijos e também apresentar resultados efetivos no aumento de atividade e estabilidade de enzimas, o efeito da HAP sobre as enzimas coagulantes de leite ainda não foi estudado. Isso gera uma lacuna nos conhecimentos sobre a aplicação deste importante processo não térmico.

Assim, esse projeto tem por objetivo ser um trabalho de base para avaliar a influência da homogeneização à alta pressão na atividade proteolítica, na atividade de coagulação de leite e estabilidade das enzimas utilizadas no processo de fabricação de queijo e avaliar as mudanças no coágulo de leite formado.

1.2 Objetivos

O objetivo geral do trabalho foi avaliar a influência da homogeneização à alta pressão na atividade proteolítica, na atividade de coagulação do leite e estabilidade de quatro enzimas utilizadas no processo de fabricação de queijo, bem como avaliar o desenvolvimento do coágulo de leite formado. Para uma avaliação global deste objetivo, o mesmo foi subdividido em alguns objetivos específicos:

1. Determinar o pH e a temperatura ótima para atividade proteolítica das enzimas comerciais coagulantes do leite: coalho de vitelo (extraída do abomaso

de vitelo), coalho de bovino (extraída do abomaso de bovinos adultos), protease fúngica ácida (obtida por fermentação de *Rhizomucor miehei*) e pepsina pura (de origem suína) (Capítulo 2).

2. Avaliar o efeito do processamento de homogeneização à alta pressão na atividade proteolítica, na atividade de coagulação do leite, e estabilidade de coalho de vitelo (Capítulos 3 e 4), coalho de bovino adulto (Capítulo 5), protease de *Rhizomucor miehei* (Capítulo 6) e pepsina suína (Capítulo 7) utilizadas na fabricação de queijos; caracterizar o fenômeno da coagulação do leite por meio de ensaio reológico e avaliar o desenvolvimento do gel a partir das enzimas selecionadas, por meio da avaliação da proteólise, sinérese, ensaio reológico e microscopia de confocal a laser.

1.3 Referências (Introdução, Justificativas e Revisão Bibliográfica)

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Capítulo 2. Ensaios Preliminares - Determination of optimal pH and temperature for proteolytic activity of selected milk-clotting enzymes

Resumo

Esse trabalho avaliou a atividade proteolítica de enzimas coagulantes do leite em diferentes pH (5,1; 5,6; 6,1; 6,6) e temperaturas (25, 30, 35, 40 e 45 °C). A faixa de pH e temperatura foram escolhidas considerando a faixa mais comumente usada em processos de fabricação de queijo. A maior atividade para o coalho de vitelo foi obtida no pH 5,1 a 35 ºC (64,66 $U.mL^{-1}$). Para esta enzima, a perda da atividade enzimática foi observada com aumento do pH, resultando numa atividade residual de ~18 % no pH 6,6 a 35 °C. Para o coalho de bovino adulto a maior atividade foi obtida a pH 5,6 a 35 ºC (11,58 $U.mL^{-1}$). A maior atividade para protease do *Rhizomucor miehei* foi obtida a 35 ºC num pH de 5,6 (22,21 $U.mL^{-1}$). Na mesma temperatura uma perda de 57% e 91% foram observados no pH de 5,1 e 6,1, respectivamente. Verificou-se que a maior atividade para pepsina suína foi obtido num pH de 5.6 a 35 °C (288,03 $U.mL^{-1}$), e uma redução de ~77% foi observado para atividade avaliada na mesma temperatura num pH de 6,6. Assim, o pH e temperatura ótima de atividade para o coalho de vitelo foi no pH de 5,1 a 35 ºC e para coalho de bovino adulto, protease fúngica e pepsina suína foi a 35 ºC no pH de 5,6.

Palavras-Chave: *Enzimas coagulantes de leite* • *Atividade proteolítica* • *pH* • *Temperatura*

Abstract

This work assessed the proteolytic activity of milk-clotting enzymes at different pH (5.1, 5.6, 6.1, 6.6) and temperatures (25, 30, 35, 40 and 45 °C). This range of pH and temperature were chosen considering the range most commonly used in cheese manufacturing processes. The highest activity for calf rennet was obtained at pH 5.1 and 35 °C (64.66 $U.mL^{-1}$). In addition, a loss in enzyme activity was observed with the increment of the pH, resulting in a residual activity of ~18% at pH 6.6 and 35 °C. For bovine rennet the highest activity was obtained at pH 5.6 and 35 °C (11.58 $U.mL^{-1}$). The highest activity for *Rhizomucor miehei* protease was obtained at pH 5.6 and 35 °C (22.21 $U.mL^{-1}$). At the same temperature, an activity loss of 57% and 91% were observed at pH 5.1 and 6.1, respectively. It was found that the highest activity for porcine pepsin was obtained at pH 5.6 and 35 °C (288.03 $U.mL^{-1}$), and reduction of ~ 77% was observed for activity evaluated at the same temperature at pH 6.6. Thus, the optimal pH and temperature of activity for calf rennet was at pH 5.1 at 35 °C and for bovine rennet, fungal protease and porcine pepsin was at pH 5.6 at 35 °C.

Key-words: *Milk-clotting enzymes* • *Proteolytic activity* • *pH* • *Temperature*.

2.1 Introduction

The aspartic proteases, commonly known as acid proteases, are endopeptidases that depend on aspartic acid residues for their catalytic activity and using a water molecule activated as the nucleophile to attack the peptide bond of the substrate (Lopez-Otin & Bond, 2008). All commercial clotting enzymes are aspartic proteases (EC 3.4.23.) which specifically cleave the Phe_{105}–Met_{106} bond of κ-casein with one exception, a protease from *Cryphonectria parasitica*, which cleaves the Ser_{104}–Phe_{105} bond (Jacob, Jaros, & Rohm, 2011).

By definition, rennet is the extract obtained from the abomasum (fourth stomach) of ruminant animals. The rennet extract of calf abomasum contains about 80% chymosin and 20% bovine pepsin. When rennet is extracted from adult animals, this ratio is reversed, and pepsin predominates to the detriment of the chymosin (Andrén, 2011). Due to its specificity for breaking Phe_{105}-Met_{106} bonds of κ-casein, chymosin becomes more suitable to use in milk coagulation and cheese production than pepsin, which has generalized proteolytic action (Fox & Kelly, 2004), affecting the yield and flavour of cheese.

Proteases from other origins, which also have the ability to coagulate milk under suitable conditions, are denominated coagulants (Andrén, 2011). The coagulants are represented by recombinant chymosin metabolised by genetically modified micro-organisms; different microbial coagulants, especially *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica* (Rolet-Répécaud et al., 2013); one of the earliest rennet substitutes used was porcine pepsin, an extract of pig stomach; chicken pepsin has also been used as a rennet substitute, mainly for religious reasons and by plant enzymes (Andrén, 2011), being the aqueous extract obtained from the flowers of *Cynara cardunculus* the most popular and successful in Portugal (Sousa & Malcata, 1998).

This chapter aimed to determine the optimal pH and temperature of some enzymes used in cheesemaking.

2.2 Material and Methods

2.2.1 Enzymes

A commercial calf rennet (freeze dried powder Carlina™ Animal Rennet 1650 - Danisco, Vinay, France, this enzyme is composed of 94% chymosin and 6% pepsin), adult bovine rennet (COALHO LÍQUIDO BV®, Bela Vista Produtos Enzimáticos Indústria e Comércio Ltda, Santa Catarina, Brazil), *Rhizomucor miehei* protease (Marzyme 150 MG Powder Microbial Rennet, Danisco, Vinay, France) and porcine pepsin protease (freeze dried powder Porcine Pepsin) PEPSINA SUINA TS (Bela Vista, Santa Catarina, Brazil) were used in the experiments.

2.2.2 Proteolytic activity (PA) determination at different pH values and temperatures

The proteolytic activity was evaluated at pH values of 5.1, 5.6 (0.2 M sodium acetate buffer), 6.1 and 6.6 (0.2 M sodium phosphate buffer) and at temperatures of 25, 30, 35, 40 and 45°C. This range of pH and temperature were chosen considering the range most commonly used in cheese manufacturing processes. The assays were evaluated according to Merheb-Dini et al. (2010), changing the buffer used to prepare the enzyme and casein solution and the reaction temperatures.

The enzyme solutions (1.5% of calf rennet, w/v; 3% of adult bovine rennet, v/v; 2% of *Rhizomucor miehei* protease, w/v; and 0.1% of porcine pepsin protease, w/v) were prepared in different buffers (0.2 M). 600 µL of the enzymatic solution was added to 400 µL of a 0.5% (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at different temperatures for 40 min in a shaken water bath (62 rpm) and 1 mL of 10% (w/v) trichloroacetic acid (TCA) was added to stop the hydrolysis. The samples were

centrifuged at 2300 *g*/5 min/10 ºC and the absorbance was measured at 280 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution, and the ΔAbs_{280nm} was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to *Equation 2.1*.

$U/mL = (\Delta Abs_{280nm} \times 10 \times \text{dilution factor} / 0.6 \times 40)$ (*Equation 2.1*)

2.3 Results and Discussion

The analytic method of proteolytic activity was chosen due its simplicity, specificity, speed of response and reproducibility of the data. It is widely used to characterize the proteolytic activity of milk-clotting enzymes using casein as substrate (Kembhavi, Kulkarni, and Panti, 1993; Zotos & Taylor, 1997; Raheem, Suri, & Saris, 2007; Merheb et al., 2007; Merheb-Dini et al., 2010, Bruno et al., 2010).

Figure 2.1 shows the results obtained for proteolytic activity of calf rennet at the different pH values and temperatures. The highest activity was obtained at pH 5.1 and 35 °C (64.66 $U.mL^{-1}$). In addition, a loss in enzyme activity was observed with increase in pH, resulting in a residual activity of ~18% at pH 6.6 and 35 °C.

The determination of the activity at different temperatures showed that almost no differences could be observed when the activity was evaluated at pH 5.1. On the contrary, for the other pH values evaluated, an increase in proteolytic activity was observed with an increment in temperature, showing maximum activity at 40 °C for pH 5.6 and 6.6 and for pH 6.1 the maximum activity was observed at 45 ºC (higher temperature evaluated), which possibly indicates that the activity could slightly increase at higher temperatures. It was observed that the activity at non ideal pH was at least 30% lower than the measured at optimum pH, independently of the temperature used in the proteolytic activity evaluation. These

results were similar to those previously obtained for the chymosin from cow, goat, camel and buffalo (Vallejo et al., 2012).

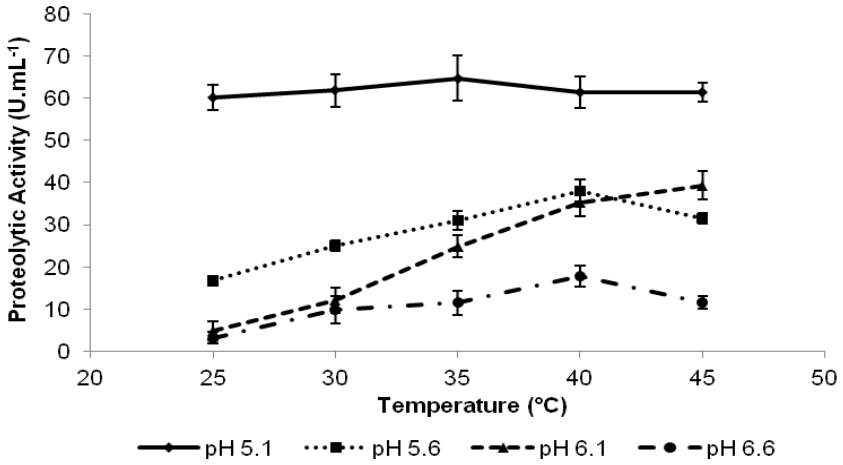

Figure 2.1 Proteolytic activity of the calf rennet as a function of pH and temperature

Figure 2.2 shows the results obtained for proteolytic activity of bovine rennet at the different pH values and temperatures. The highest activity was obtained at pH 5.6 and 35 °C (11.58 $U.mL^{-1}$). In addition, a loss in enzyme activity was observed with increase in pH, resulting in a residual activity of ~18% at pH 6.6 and 45 °C.

These results were expected, since the isoelectric point and optimum proteolytic pH of all aspartic proteinases are acidic. Chymosin has general an optimum pH at 4.0, and instability at pH <3.5, due to an irreversible conformational change and pepsin has general proteolytic pH optimum at about 3 (Fox et al., 2004). Despite the relatively low pH for maximum activity, the rennet is used at a more neutral pH for cheese production, due to the higher specific milk-clotting activity at the pH of milk (pH 6.7), the milk-clotting activity of 1 mg chymosin corresponds to that of about 5 mg pepsin at pH 6.7 (Andrén, 2011). Similar results were also obtained for camel chymosin (Kappeler et al., 2006) and milk clotting enzyme from goat (Kumar et al., 2006).

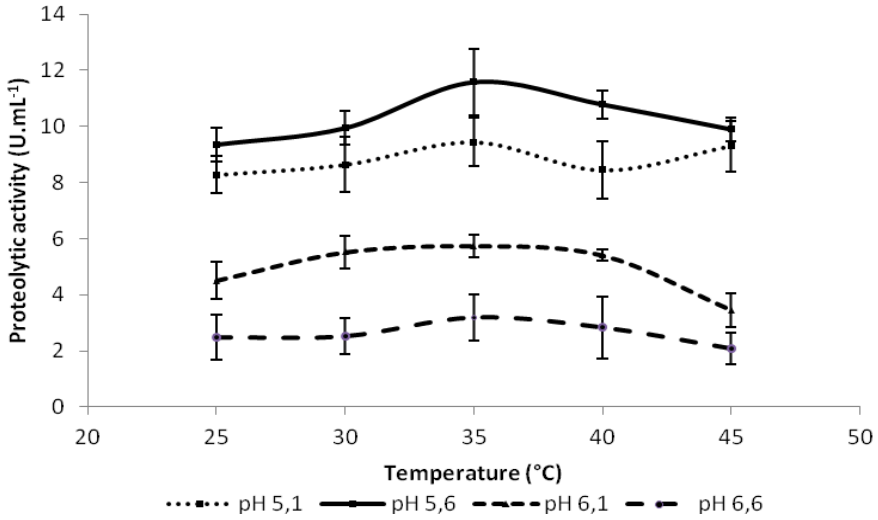

Figure 2.2 Proteolytic activity of the bovine rennet as a function of pH and temperature

Figure 2.3 shows the results of the proteolytic activity of *Rhizomucor miehei* protease at different pH and temperatures. The highest activity was obtained at pH 5.6 and 35 °C (22.21 $U.mL^{-1}$). At the same temperature, an activity loss of 57% and 91% were observed at pH 5.1 and 6.1, respectively. The pH increase to 6.6 did not affect the enzyme activity, which remained the same measured at pH 6.1, independent on the temperature evaluated. This behavior was expected, since fungi proteolytic enzymes generally have maximum catalytic activity at acidic pH, being classified as aspartic proteases. Similar results were found by Merheb-Dini et al. (2010) for fungal protease from *Thermomucor indicae-seudaticae* N31.

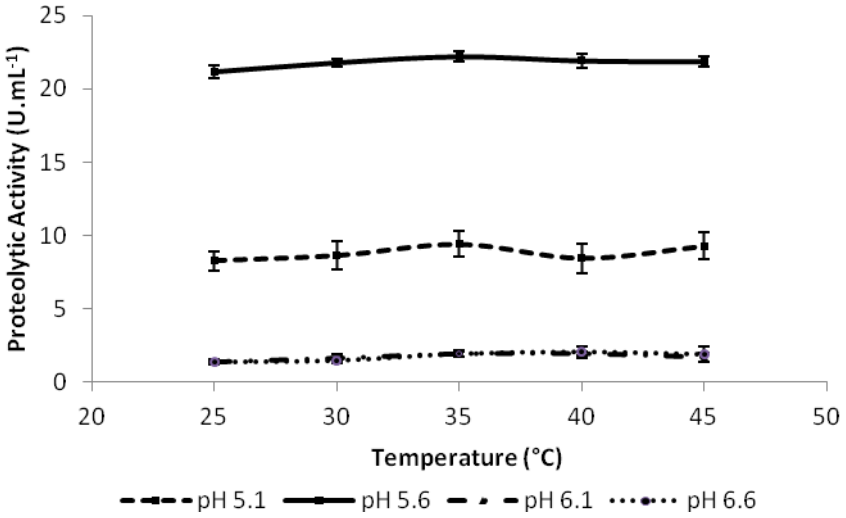

Figure 2.3 Evaluation of proteolytic activity of the Rhizomucor miehei protease as a function of temperature and pH

Figure 2.4 shows the results of the proteolytic activity of porcine pepsin protease as a function of pH and temperature. It was found that the highest activity was obtained at pH 5.6 and 35 °C (288.03 $U.mL^{-1}$), being that in pH 6.6 and 35 °C was verified a reduction of ~ 77%.

One of the earliest rennet substitutes used was porcine pepsin, an extract of pig stomach. Porcine pepsin was either used alone or mixed 50:50 with calf rennet. A disadvantage of porcine pepsin coagulants is that their milk-clotting activity is very pH-dependent making them sensitive to inactivation. At the pH (~6.5) and temperature (~30 °C) used for cheesemaking, porcine pepsin starts to denature and after 1 h only 50% of its milk-clotting activity remains. Today, porcine pepsin coagulants are seldom used (Andrén, 2011).

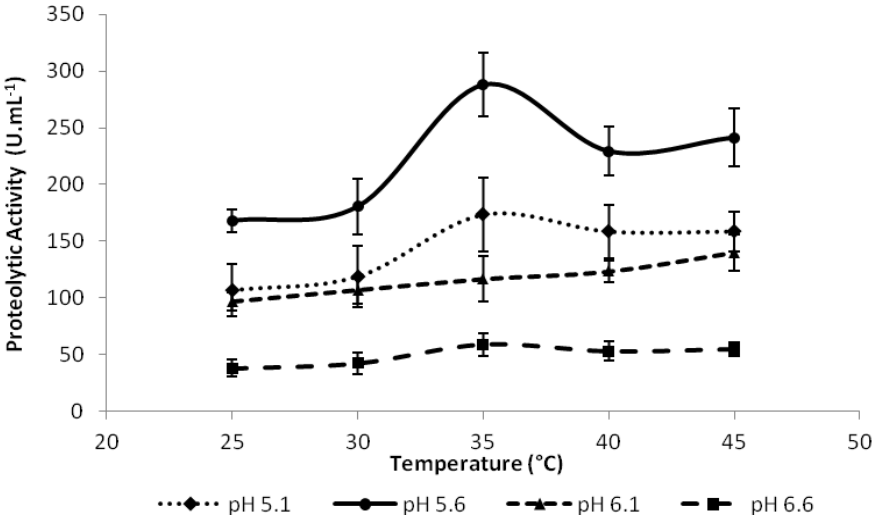

Figure 2.4 Evaluation of proteolytic activity of the porcine pepsin protease as a function of temperature and pH

2.4 Conclusions

The optimal pH and temperature for calf rennet activity was at pH 5.1 at 35 ° C and bovine rennet, fungal protease and porcine pepsin was at pH 5.6 at 35 ° C. Additionally, it was observed that the methodology was effective to determine the optimal pH and temperature of proteolytic activity within the range of choice.

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Capítulo 3. Proteolytic and milk-clotting activities of calf rennet processed by high pressure homogenization and the influence on the rheological behavior of the milk coagulation process

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Resumo

Esse trabalho estudou o efeito da homogeneização à alta pressão (HAP) no desempenho de coalho de vitelo comercial. A solução enzimática foi processada a 50, 100, 150 e 190 MPa e, em seguida, armazenada por até cinco dias a 4 °C. Os efeitos foram avaliados na atividade proteolítica e na atividade de coagulação do leite das enzimas e no comportamento reológico do processo de coagulação usando as enzimas processadas. HAP causou uma redução na atividade proteolítica com aumento da pressão (perda de até 52% da atividade a 190 MPa). Durante as primeiras 48 h de armazenamento, a enzima processada manteve sua atividade enquanto a amostra não processada apresentou uma contínua redução. Os resultados mostraram que o processamento de HAP não alterou a atividade de coagulação do leite e os testes reológicos mostraram maiores valores de G' para o leite coagulado com a enzima processada a 190 MPa (7% maior após 84 minutos de coagulação). Assim, HAP melhora a coagulação do leite por coalho bovino e reduz a atividade proteolítica da enzima, o que pode melhorar a qualidade de queijos frescos, especialmente, após a estocagem.

Palavras-Chave: *Alta pressão dinâmica* • *Coalho de Vitelo* • *Atividade proteolítica* • *Atividade de coagulação do leite*

Abstract

This work studied the effects of high pressure homogenization (HPH) on the performance of a commercial calf rennet. The enzyme solution was processed at 50, 100, 150 and 190 MPa and then stored for up to five days at 4 ºC. The effects were evaluated on the proteolytic and milk-clotting activities of the enzyme and on the rheological behavior of the milk gels obtained using the processed enzyme. HPH caused a reduction in proteolytic activity with increase in pressure (up to 52% loss of activity at 190 MPa). At the first 48h of storage, the processed enzyme maintained its activity while the non-processed sample showed a continuous reduction in activity. The results showed that HPH processing did not alter the milk-clotting activity and the rheological tests showed high G' values for the milk coagulated with the enzyme processed at 190 MPa (7% higher after 84 minutes of coagulation). Thus, HPH improves the milk coagulation by calf rennet and reduces the proteolytic activity of enzyme, which may improve the quality of fresh cheeses, especially after their storage.

Key-words: *Dynamic high pressure • Calf rennet • Proteolytic activity • Milk-clotting activity*

3.1 Introduction

Cheese production is growing annually at a rate of 4% throughout the world (Euromonitor International, 2012). However, the supply of calf rennet is decreasing year by year due to the tendency to reduce the early slaughter of steers because of low achievement in terms of meat production (Food Agriculture Organization [FAO], 2010). Currently, only 20-30% of the cheeses produced worldwide use calf rennet (Jacob, Jaros, & Rohm, 2011).

Potential substitutes should emulate their specific properties, with high specificity and good proteolytic activity at the pH and temperature of cheese manufacture (Fox & Kelly, 2004; Kumar et al., 2010). Several coagulants from animals, microorganisms and plants have been considered as potential substitutes for rennet (Walstra et al., 2006; Rolet-Répécaud et al., 2013). However, the application of these enzymes in the coagulation of milk could result in problems such as a reduction in manufacturing yield (due to the intense activity of these proteolytic enzymes) and the appearance of defects in flavor and texture (especially a bitter taste and crumbly texture) (Møller et al., 2012). Thus, it can be seen that the coagulation step is critical for cheese production and that there is a real demand for new coagulants with better activity and stability.

High pressure homogenization (HPH) or dynamic high pressure (DHP) is a non-thermal process primarily developed to ensure the microbiological quality of food without affecting its sensory and nutritional attributes (Tribst, Sant'Ana, & Massaguer, 2009). The effect of HPH on enzymes has been studied by some authors (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009; Liu et al., 2009 a,b; Liu et al., 2010a,b), who found that the process was capable of promoting the activation and stabilization (Liu et al., 2009 a,b; Liu et al., 2010a; Tribst & Cristianini, 2012b; Tribst, Augusto, & Cristianini, 2012a) or inactivation (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009) of enzymes, the effect being associated with the type of enzyme and the pressure applied (Liu et al., 2009 a,b). These changes

in enzyme performance are normally linked to changes in the tertiary and quaternary structures of the enzymes, with the exposure of hydrophobic amino acids to a less hydrophobic environment (Liu et al., 2009b).

Thus HPH may be an interesting tool for calf rennet modification, which can increase its milk-clotting activity and stability, and consequently reduce the amount of enzyme required for cheese production, affecting directly the enzyme relative costs. Therefore, this study aimed to evaluate the influence of high pressure homogenization on the proteolytic activity, milk-clotting activity and stability of the commercial calf rennet used in cheese manufacture.

3.2 Material and Methods

3.2.1 Calf rennet and high pressure homogenization processing

A commercial calf rennet was used in the experiments (freeze dried powder Carlina^{TM} Animal Rennet 1650 - Danisco, Vinay, France). This enzyme is composed of 94% chymosin and 6% pepsin.

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the assays. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

A volume of 2 L of the calf rennet solution was prepared at 1.5 % (w/v) in 0.2 M sodium acetate buffer (pH 5.1) and homogenized under pressures of 50, 100, 150 and 190 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately in an ice bath after exiting the homogenizing valve. A non-processed sample of calf rennet was evaluated as the control sample.

3.2.2 Relative proteolytic activity (RPA) determination

The proteolytic activity of calf rennet was measured according to Merheb-Dini et al. (2010): the enzyme solution (1.5% w/v) was prepared in a 0.2 M acetate

buffer (pH 5.1). 600 µL of the enzymatic solution was added to 400 µL of a 0.5% (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at 35 ºC/40 min in a shaken water bath (62 rpm) and 1 mL of 10% (w/v) trichloroacetic acid (TCA) then added to stop the hydrolysis. The samples were centrifuged at 2300 *g*/5 min/10 ºC and the absorbance was measured at 280 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution, and the ΔAbs_{280nm} was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to *Equation 3.1*.

$U/mL = (\Delta Abs_{280nm} \times 10 \times \text{dilution factor} / 0.6 \times 40)$ (*Equation 3.1*)

RPA assays were carried out immediately after processing (time 0 h) and after 12 h, 24 h, 48 h, 72 h, 96 h and 120 h. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed sample was used for a comparative evaluation. The relative proteolytic activity (RPA) was calculated considering the activity of the HPH processed and non-processed samples, according to *Equation 3.2.*

$RPA = (\text{enzyme activity}_{after\_HPH\_and/or\_storage} / \text{enzyme activity}_{non\text{-}processed\_sample\_at\_0h}) * 100$ (*Equation 3.2*)

3.2.3 Relative milk-clotting activity (RMCA) determination

The milk-clotting activity was determined according to Merheb-Dini et al. (2010). A volume of 5 mL of skimmed milk powder, reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) was added to a 0.01 M $CaCl_2$ solution and pre-incubated at 35 °C/10 min. An aliquot of 0.5 mL of the enzyme solution (0.003%, w/v, prepared in 0.1 M sodium acetate buffer, pH 5.1) was then added and the time count started. Clot formation was determined by manual tube rotation

and the time taken for the first particles to form measured. One milk-clotting activity unit (MCA) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 ºC. The MCA was calculated using *Equation 3.3*:

Unit of milk-clotting activity (MCA) = 2400/*t* x *S*/*E* (*Equation 3.3*)

where *t* is the time (seconds) necessary for clot formation, *S* is the milk volume and *E* is the enzyme volume.

MCA assays were carried out immediately after processing (time 0 h) and after 24 h, 48 h, 72 h, 96 h and 120 h. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed sample was also used for a comparative evaluation. The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to *Equation 3.4*:

$$RMCA = (MCA_{after\_HPH\_and/or\_storage} / MCA_{non\text{-}processed\_sample\_at\_0h}) * 100 \quad (Equation\ 3.4)$$

3.2.4 Rheological assays

The milk coagulation was evaluated by monitoring the milk coagulation process by way of a time sweep using a low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments, USA). These assays were carried out with the processed and non-processed calf rennet.

The experiments were determined according to Lee & Lucey (2003) using 60 mL of skimmed milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids) with the addition of a 0.01 M $CaCl_2$ solution. This mixture was pre-incubated at 35 °C/10 min, and subsequently 0.8 mL of enzyme solution (0.03%, w/v) prepared in 0.1 M acetate buffer (pH 5.1) was added. The mixture was immediately transferred to the rheometer cup (30 mm diameter and 80 mm height), which had a *vaned quarter* geometry (with 28mm of diameter and 42 mm of length) and a 4 mm *gap*. The stress was set at 0.1 Pa and frequency at 0.1 Hz. The parameter G' (storage modulus) was measured at 3 min intervals for 84 min of

the clotting process at 35 °C. The temperature was controlled by a *Peltier* system. The rheological assays were carried out immediately after processing (time 0 h) and after 120 h. The samples were stored under refrigeration (4 ºC) throughout this period.

Furthermore, the rate of milk-clotting was calculated as the variation of G' with variation in time as the log (dG'/dt) at three minute intervals, and expressed in $Pa.min^{-1}$. The storage modulus (G') describes the elastic (solid) behavior of the product, and consequently the energy stored and released in each oscillatory cycle.

3.2.5 Statistical analysis

The processes and analyses were carried out with three repetitions and each experimental unit was carried out in quadruplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and the Tukey test to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software– (StatiSoft, Inc., Tulsa, Okla., U.S.A.) and the results were presented as the mean ± standard deviation.

3.3 Results and Discussion

3.3.1 Effect of high pressure homogenization on the proteolytic activity

The fast decompression during HPH promotes intense shear and friction, with consequent heating of the product. Since enzymes can be affected by heating, the temperature reached under each set of process conditions was also measured. The residence time at those temperatures was less than 10 s, and *Equation 3.5* can be used to estimate the temperatures reached after homogenization. The increase in pressure promoted a linear increase in temperature of the enzyme

solution of around 1.8 ºC/10 MPa (R^2 = 0.99), the maximum temperature (57.2 ºC) being reached at 190 MPa. The thermal effect on the enzyme can be considered minimum once the temperature is bellow 68ºC (temperature normally used to inactivate calf rennet) and the residence time less than 10s. Consequently, all the effects observed after HPH can be attributed exclusively to the homogenization process.

Temperature (ºC) = 0.18 * Pressure (MPa) + 23.55 (*Equation 3.5*)

Figure 3.1 shows the results for relative proteolytic activity after HPH processing and during 120 h of storage. The HPH caused a reduction in the proteolytic activity of the calf rennet, reaching a minimum of 48.1% after processing at 190 MPa ($p<0.05$). During the storage, the activity of non-processed sample was higher than HPH processed ones. However, the evaluation of enzyme activity during the storage showed different profiles: the HPH treated enzyme showed no significant differences in activity during storage, while the non-processed enzyme showed a reduction in residual activity at the first 48 h of storage and reached a minimum of 75% after 120 h. Thus it can be concluded that, although the process reduces the enzyme activity, it improves enzyme stability (at lower activities).

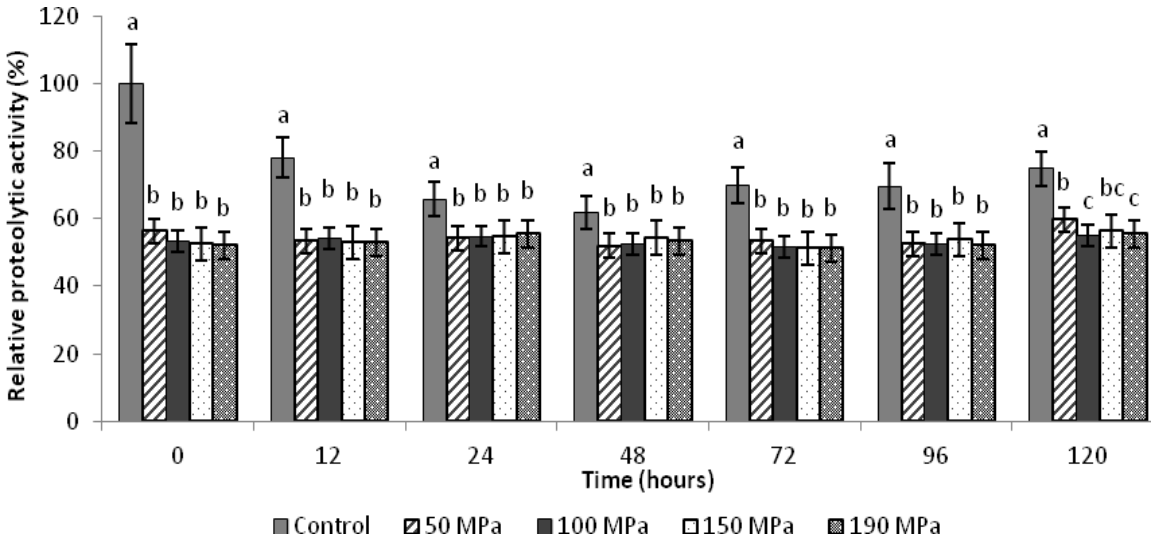

Figure 3.1 Evaluation of the proteolytic activity of the calf rennet subjected to the high pressure homogenization process. Different letters mean significant difference

(p<0.05) between the non-processed and processed calf rennet samples evaluated at each time.

Results obtained in previous studies showed a diversity of effects on enzymes caused by HPH processing. α-amylase is highly stable to the HPH process (Tribst & Cristianini, 2012a), while other enzymes, such as β-galactosidase, were severely affected, even at pressures of 50 MPa or lower (Tribst, Augusto, & Cristianini, 2012b). Studies evaluating other proteases showed that the process was able to shift the optimum temperature of a neutral protease from 55 to 20 ºC (Tribst, Augusto, & Cristianini, 2012a) and improve the thermal stability of trypsin (Liu et al., 2010a). The molecular evaluation of the homogenized enzymes indicated that the process affected quaternary, tertiary and secondary structures of the enzymes (Lacroix, Fliss, & Makhlouf, 2005; Liu et al., 2009b; Liu et al., 2010b), changing their tridimensional configuration and, consequently, their active sites.

3.3.2 Effect of high pressure homogenization on milk-clotting activity

The milk-clotting activity is an indirect way of evaluating the specificity of milk coagulants in the cleavage the site Phe_{105}-Met_{106} of κ-casein. This cleavage destabilizes the casein micelles, which coagulates in the presence of Ca^{2+} (Andrén, 2011). *Figure 3.2* shows the results for milk-clotting of the non-processed and processed calf rennet samples immediately after HPH processing up to 120 hours of enzyme processing.

The results showed that HPH processing did not alter the milk-clotting immediately after the process, with no significant differences in milk-clotting activities between the non-processed and processed samples up to 190 MPa (p<0.05). Therefore, considering the results for enzyme activity (section 3.3.1) and for milk-clotting, it was concluded that although HPH processing caused a reduction in enzyme activity (proteolytic performance) it was not linked to a

reduction in specific proteolysis (responsible for milk coagulation). From the industrial point of view, for the manufacture of fresh cheeses, these results are interesting, since they reduce proteolysis during the shelf life of the product without affecting the milk-clotting activity. Thus it is possible to extend the shelf life of cheeses made with enzymes processed by HPH.

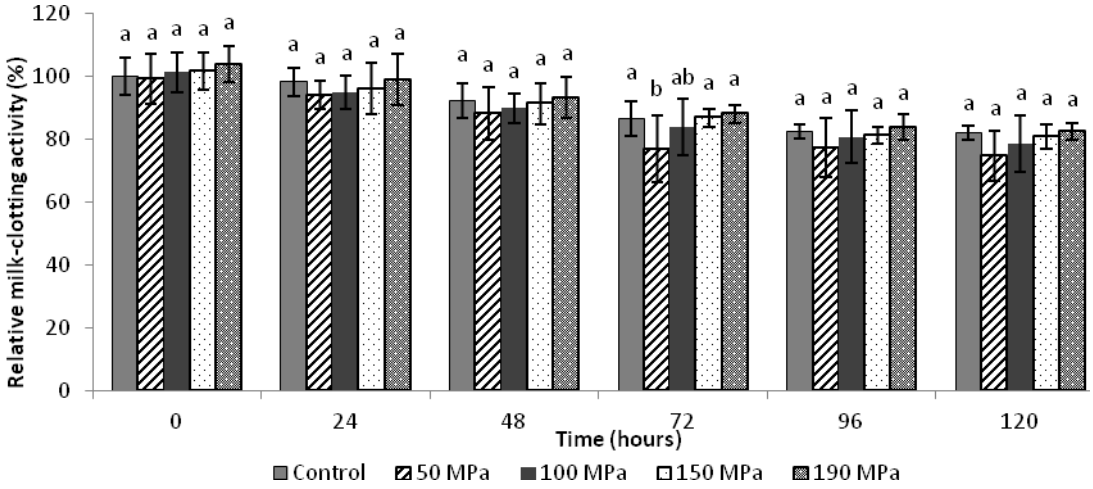

Figure 3.2 Evaluation of the milk-clotting activity of the calf rennet subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed calf rennet samples evaluated at each time.

The evaluation of the milk-clotting activity during 120 h of storage at 4 °C showed a continuous reduction on RMCA during the enzyme storage, but no differences were found among non-processed and homogenized samples. This indicates that the HPH process did not alter the stability of the calf rennet RMCA. Comparing the results for proteolytic activity (section 3.3.1) and RMCA during the 120 hours, it was supposed that the loss in activity observed for the processed enzyme (proteolytic performance) possibly was not linked to a reduction in proteolysis at the specific site Phe_{105}-Met_{106} of κ-casein, since RMCA remained the same for non-processed and high pressure homogenized samples.

Calf rennet is composed of chymosin and pepsin. Chymosin has a strong affinity for the Phe_{105}-Met_{106} region of κ-casein and has the highest specific milk-clotting activity of the aspartic proteinases (Rolet-Répécaud et al., 2013). Pepsin

does not cause specific hydrolysis of the peptide bonds involving aromatic amino acids (Phe, Tyr and Trp) (Jacob, Jaros, & Rohm, 2011). Thus, considering the results for RMCA and RPA, it was possible to suggest that the main effects of the HPH process occurred in the pepsin fraction (able to promotes unspecific hydrolysis), reducing its proteolytic activity with no effects on the enzyme milk-clotting activity (specific for chymosin hydrolysis).

3.3.3 Rheological evaluation

The rheological data are important to evaluate the behavior of milk coagulation using the HPH processed and non-processed enzymes, and gels consistency at the end of the coagulation process (*Figure 3.3*). In addition, the beginning of the aggregation can be better visualized using a log scale (*Figure 3.3, a2 and b2*). The storage modulus (G') describes the elastic (solid) behavior of the product, and starts to increase at the beginning of the coagulation process, characterized by micelle aggregation. The results showed that the aggregation process started at approximately 39 min for the sample that used the enzyme processed at 150 MPa, 190 MPa and the non-processed enzyme. For samples processed at 100 MPa and 50 MPa, aggregation started at 42 min (time 0 h). After 84 min of coagulation, it was found that the sample treated at 190 MPa produced the gel with G' values 7% higher than non-processed enzyme, being more consistent than the other samples ($p<0.05$). The differences between the G' values of the samples highlighted that each pressure increment possible cause slights changes in the enzyme conformation with consequent minimal alterations in the coagulation performance. The differences between the results of the rheological analysis and RMCA can be attributed to the sensibility of the methods, since RMCA is a visual evaluation while rheological is an instrumental method with high sensibility. By the rheological data, it was observed that 190 MPa positively changed the calf rennet, reducing the time of coagulation and improving the gel consistence.

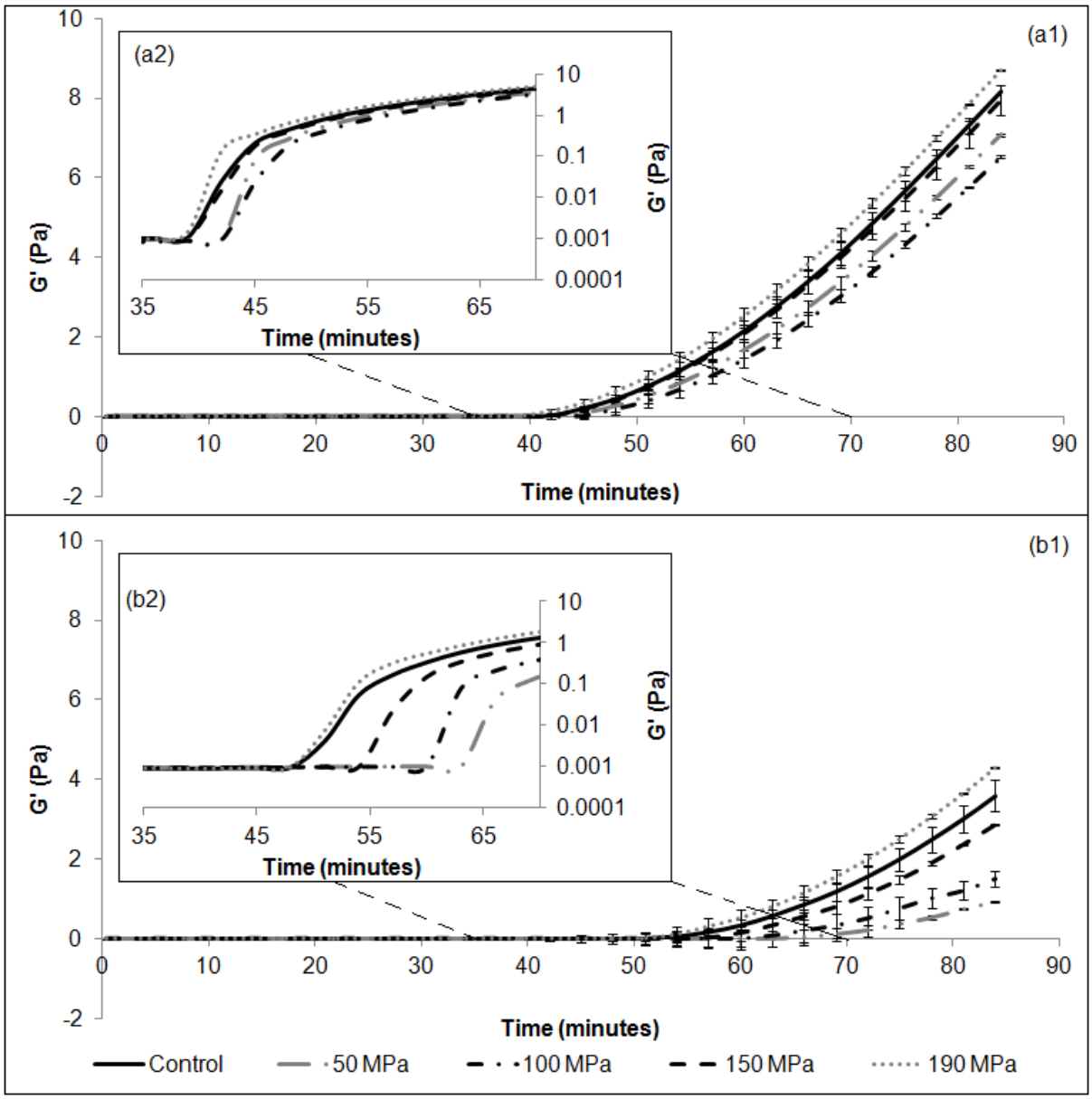

Figure 3.3 Evaluation of milk coagulation (a1) immediately after the process (0h) (a2) log scale, and (b1) after 120 hours stored at 4 °C (b2) log scale.

Another rheological evaluation was carried out after 120 h of storage of the calf rennet solutions at 4 °C, to determine if storage in solution affected the coagulation profile of the enzymes. The storage period caused a loss of milk-clotting activity for both processed and non-processed enzymes, which was evidenced by the increase in time spent to start the aggregation process. Comparing the results of 0 and 120 h of enzyme storage, similar G' values profiles were observed for samples. The G' value after 84 min of coagulation was 17% higher for enzyme processed at 190 MPa than for non-processed ones, which is possibly linked to improvements in enzyme stability during storage in aqueous solution.

Figure 3.4 shows the rate of milk-clotting, calculated as the variation in G' at the different times. The graph shows the moment at which aggregation started, represented by the higher values for log(dG'/dt) reached by each curve (higher speed). Thus it was observed that the enzyme processed at 190 MPa presented a faster start and achieved a greater initial velocity both after processing and after 120 hours of storage. Consequently, the protein aggregation was faster and the gel produced firmer when the enzyme processed at 190 MPa was used, decreasing the milk coagulation time and improving the consistency of the final product.

In addition, it was shown that the calf rennet changed with each pressure evaluated, indicating that even 50 MPa was able to promote some alterations in the enzyme structure, with consequent alterations in the enzyme coagulation behavior. These changes, however, are not always positive, highlighting the importance of studying the effects of the process under different conditions.

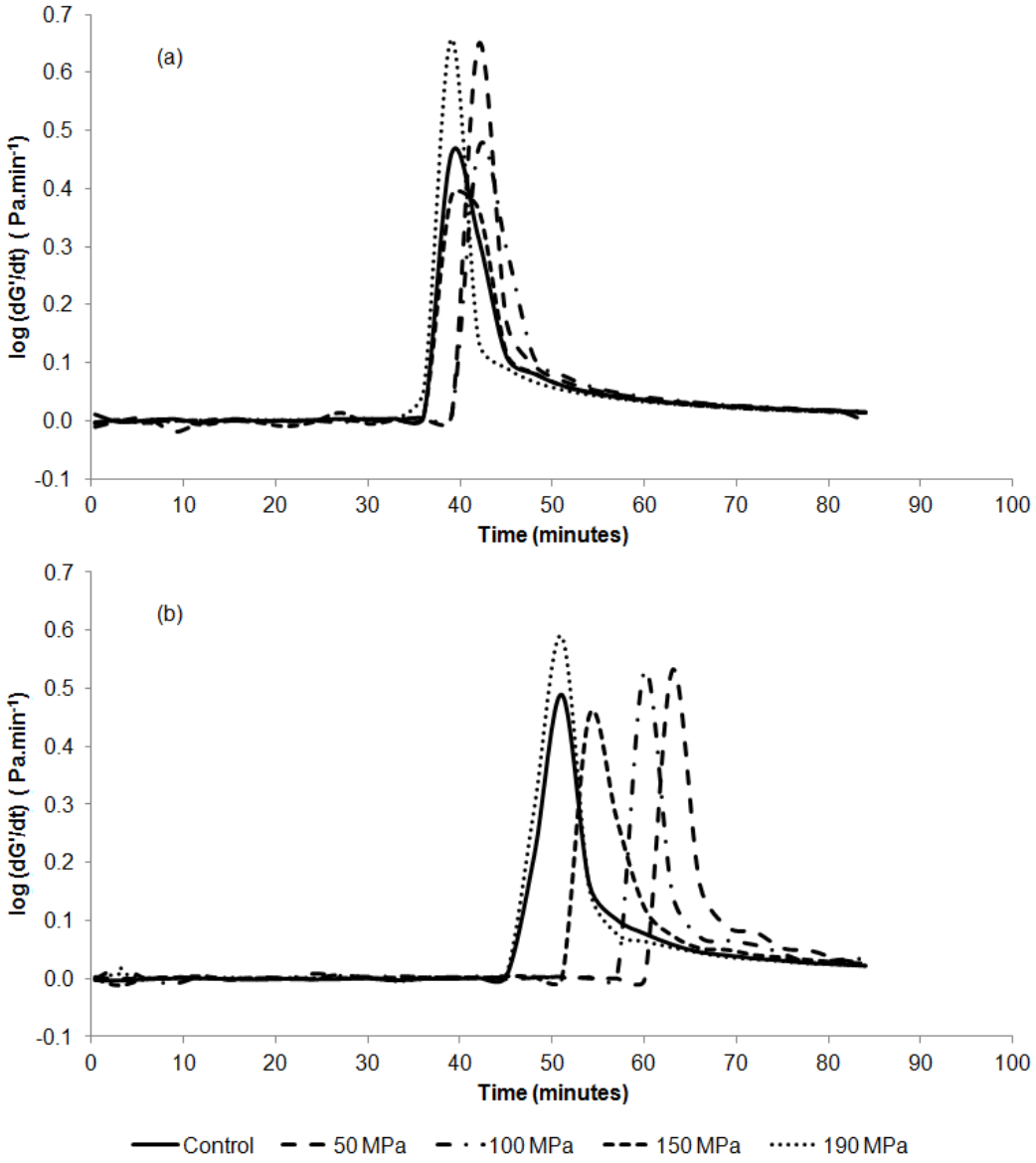

Figure 3.4 Rate of milk-clotting caused by the enzyme processed using high pressure homogenization, (a) immediately after the process (0h) and (b) after 120 h stored at 4 °C.

3.4 Conclusion

The high pressure homogenization process reduces the proteolytic activity of calf rennet (maximum reduction of 52% after processes at 190 MPa), however

no changes in the milk-clotting activity were observed. In addition, the gels produced with the enzyme processed at 190 MPa were more consistent (G' value 7% higher at 84 minutes) than those produced using the non-processed ones. This highlights the fact that HPH processing can be applied as tool to change the calf rennet performance and the curd characteristics. By the results, it was suggested that HPH of calf rennet might improve the quality of fresh cheese, reducing proteolysis during the product shelf-life.

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Capítulo 4. Characterization of rennet-induced gels using calf rennet processed by high pressure homogenization: Effects on proteolysis, whey separation, rheological properties and microstructure

Resumo

Esse estudo avaliou a coagulação do leite durante 24 horas usando coalho de vitelo submetido ao processo de homogeneização à alta pressão (HAP). A solução enzimática foi processada a 190 MPa e a formação e o desenvolvimento do gel foram acompanhados pelas análises de proteólise, sinérese, ensaio reológico e microscopia de confocal a laser (MSLC). Os resultados evidenciaram que HAP foi capaz de reduzir a atividade proteolítica inespecífica do coalho de vitelo (proteólise aleatória) aumentando a taxa de coagulação do leite. Os géis produzidos com a enzima processada a 190 MPa apresentaram maior sinérese formando uma rede mais compacta com maiores valores de G', sendo uma rede mais densa evidenciada por uma menor porosidade das imagens obtidas por MSLC com maior aprisionamento de água corroborado pelos maiores valores G''. Assim, queijos produzidos com as enzimas processadas a HAP pode permitir a obtenção de géis com maior rendimento (devido ao aumento da capacidade de ligação da água às proteínas) e aumento da matéria seca (devido à redução da perda de peptídeos pequenos no soro). Além disso, a baixa proteólise no gel pode ser interessante para a extensão da vida de prateleira de queijo fresco reduzindo a formação de sabores indesejáveis.

Palavras-chave: *Alta pressão dinâmica • Coagulação enzimática do leite • Coalho vitelo • Proteólise • Sinérese • Microscopia confocal*

Abstract

This study evaluated the coagulation of milk during 24 hours using calf rennet subjected to high pressure homogenization (HPH). The enzyme solution was processed at 190 MPa and gel formation was followed by analysis of proteolysis, syneresis, rheological assay and confocal microscopy (CSLM). The results evidenced that HPH was able to reduce the unspecific proteolytic activity of calf rennet (random proteolysis) increasing the rate of milk clotting. The gels produced with the enzyme processed at 190 MPa showed higher syneresis forming a more compact network with higher values of G', being a denser network evidenced by lower porosity of images obtained by CSLM with higher trapping of water corroborated by higher values of G''. Thus, cheese produced with HPH enzymes would allow to obtain gels with higher yield (due to the increment on water binding capacity of protein) and increased dry matter (due to the reduction of small peptides loss in the whey). Furthermore, the low proteolysis in the gel coud be interesting for the extension of shelf-life of fresh cheese avoiding bitterness flavour.

Key-words: *Dynamic high pressure* • *Enzymatic coagulation of milk* • *Calf rennet* • *Proteolysis* • *Syneresis* • *Confocal microscopy*

4.1 Introduction

The enzymatic coagulation involves the steps of hydrolysis of κ-casein, aggregation of the casein micelles in the presence of Ca^{2+} and the development of coagulated gel. Then, crosslinks are progressively formed between chains flocculated micelles to form a final gel (Fox & McSweeney, 1998; Walstra et. al., 1999; Lucey et al., 2000; Lucey, 2002; Lagaude et al., 2004; Karlsson, Ipsen, & Ardo, 2007; Bönisch, Heidebach, & Kulozik, 2008). After the coagulation, the gel network formation continues and the characteristics of the enzymatic gel, such as water retention capacity, syneresis and strength are important in the process of cheese production, once they affect parameters such as yield, moisture and texture of the product (Pandey, Ramaswamy, & St-Gelais, 2000).

The kinetics of syneresis is complex and still not well understood (Lodaite et al., 2000). A deeper understanding of syneresis and the impact of the relevant factors can be investigated using models based upon measured material parameters such as porosity, permeability and appropriate rheological coefficients of the casein matrix. Several fundamental studies on syneresis of rennet-induced casein gels have been reported (Walstra et al., 1985; Van Dijk & Walstra, 1986; van Vliet et al., 1991; Lucey et al., 2000; Lucey, 2002).

The rheology of cheese is a function of its composition, microstructure (i.e., the structural arrangement of its components), the physico-chemical state of its components, and its macrostructure, which reflects the presence of heterogeneities such as curd granule junctions, cracks and fissures. The physicochemical properties include parameters such as degree of hydrolysis and hydration of the *para-casein* matrix, and the level of inter-molecular attractions between *para-casein* molecules (O'Callaghan & Guinee, 2004).

The first 24 h after coagulation are the most important for the biochemical profile of cheese during the ripening, especially for cheese models systems (that have high proteolytic activity due to high enzyme concentration, high temperature and humidity) (Picon et al., 1995; Silva & Malcata, 2004, Silva & Malcata, 2005).

Casein aggregation and whey expression continue taking place throughout the early stages of ripening; casein is compacted within the curd, water is lost, and fat globules are entrapped and compressed, all of which are determinant of the final cheese structure and composition (Green & Grandison, 1999; Choisy et al., 2000; Wilkinson & Kilcawley, 2005). Primary proteolysis is possibly the most important set of events toward the development of flavor and texture in ripened cheeses (Fox et al., 1999).

It is observed that various factors affecting the cheese production and that the choice of coagulant can change these properties. Thus, the use of new enzymes with higher specific and different activity can affects the rheological and microstructural properties of cheese and its proteolysis. Previous results of Leite Junior, Tribst, & Cristianini, (*in press*) found that the high pressure homogenization (HPH) on calf rennet at 190 MPa caused reduction in proteolytic activity (52% loss of activity), did not alter the milk-clotting activity and improved the G' values obtained for milk coagulation applying this enzyme. Therefore, this study aimed to evaluate the formation of the rennet-induced gels during 24 hours at 35 ºC using calf rennet subjected to high pressure homogenization (HPH).

4.2 Material and Methods

4.2.1 Calf rennet and high pressure homogenization processing

A commercial calf rennet was used in the experiments (freeze dried powder Carlina^{TM} Animal Rennet 1650 - Danisco, Vinay, France). This enzyme is composed of 94% chymosin and 6% pepsin.

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the assays. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

A volume of 1 L of the calf rennet solution 1.5 % (w/v) prepared in sodium acetate buffer 0.2M (pH 5.1) was homogenized under 190 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately after exiting the homogenizing valve in an ice bath. A non-processed sample of calf rennet was evaluated as the control sample.

4.2.2 Capillary zone electrophoresis method of rennet-induced gels

The capillary electrophoresis analysis was determined according to the conditions described by Ortega et al. (2003) and Otte et al. (1997). Firstly, the samples preparation were carried out: an aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added by 0.01 M $CaCl_2$ and by 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 300 µL of enzyme solution (0.3 %, w/v) prepared in acetate buffer 0.1M (pH 5.1) was added and time started. After 40 min, 3 h, 6 h, 18 h and 24 h of coagulation at 35 ºC, 20 mg of samples were collected and dissolved in 1 mL of 10 mM sodium phosphate buffer solution containing 8 M urea (Merck, Darmstadt, Germany) and 10 mM of dithiothreitol (DTT, Sigma Chemical Co, St. Louis, USA) at pH 8, and left for 1 h at temperature of 23 ºC before filtration (0.22 µm Millex-GV_{13}, Millipore, Molsheim, France).

Then, Capilary zone electrophoresis (CZE) analysis was carried out using a Beckman P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) controlled by 32 Karat software (Beckman Coulter). The separations were performed using a fused-silica capillary (eCap™, Beckman Instruments Inc., San Ramon, CA, USA) of 57 cm (50-cm effective length to detector) x 75 µm I.D. The running buffer was prepared with 10 mM sodium phosphate containing 6 *M* urea and 0.05 % hydroxypropyl methylcellulose (HPMC, Sigma Chemical Co, St. Louis, USA); the pH was adjusted to 3.0 with 1 M HCl. Buffer solutions were filtered through 0.22 µm filters (Millipore, Molsheim, France) before use.

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 5 s at 0.5 psi. During sample analysis, a constant voltage (18.5 kV ~35 µA) was applied and the temperature of the separation was kept at 23 ºC circulating coolant surrounding the capillary. For all experiments, electrophoresis was carried for 70 min and detection was at 214 nm (data collection rate 5 Hz). In CZE, the capillaries were conditioned by washing with 0.5 M NaOH for 5 min, then with pure water for 5 min, and finally with running buffer for 5 min after each electrophoretic runs.

The first electropherogram in a series was always discarded. The repeatability of peak areas was assessed by replicate injections (n = 3). Standard curves were made with a mixture of α_s-casein (C-6780), β-casein (C-6905) and κ-casein (C-0406) at 10 $mg.mL^{-1}$ obtained from Sigma Chemical Co (St. Louis, MO, USA) in the ratio of 1:1:1. Assignment of peaks was based on previous reports by Ortega et al. (2003) and Albillos et al. (2006).

4.2.3 Determination of spontaneous syneresis of rennet-induced gels by the siphon method

The level of spontaneous whey separation in undisturbed rennet-induced gels was determined using a siphon method in according Amatayakul et al. (2006). An aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 300 µL of enzyme solution (0.3 %, w/v) prepared in acetate buffer 0.1M (pH 5.1) was added and time started. After 40 min, 3 h, 6 h, 18 h and 24 h coagulation at 35 ºC samples were cooled to 5 ºC and inclined at an angle of 45 º and maintained at rest for a period of 1 hour. Then, the serum exceeded was collected from the surface of the samples (using a syringe) and weighed. The syneresis was expressed as the percent weight of the whey divided the initial weight of the gels sample, according to *Equation 4.1*.

Whey separation (%) = $(S_F/P_T - P_E) \times 100$ (*Equation 4.1*)

Where, S_F is the weight of the separated whey, P_T is the weight of sample plus packaging and P_E is the weight of the empty package.

4.2.4 Rheological assays of coagulation process and rennet-induced gels

The milk coagulation was evaluated by monitoring the milk coagulation process through time sweep using low deformation oscillatory test in rheometer with controlled stress (AR2000ex, TA Instruments, USA).

The milk-clotting activity was determined according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany). This mixture was pre-incubated at 35 °C/10 minutes. Subsequently, 300 µL of enzyme solution (0.3 %, w/v) prepared in acetate buffer 0.1M (pH 5.1) was added. Immediately after enzyme addition, the sample was transferred to the rheometer cup (30 mm diameter and 80 mm height). It was used a *Vaned Quarter* geometry (with 28mm of diameter and 42 mm of length) and a *gap* of 4 mm. The stress was set at 0.1 Pa, frequency 0.1 Hz. The parameters G' (storage modulus) and G'' (loss modulus) were measured at 3 minute intervals (at first's 40 minutes) and then at 10 minute intervals (up to 24 hours) of clotting process at 35 ºC. The tan δ (loss tangent) was calculated by G''/G' ratio. The temperature was controlled by a *Peltier* system.

4.2.5 Three dimensional (3D) microstructure of coagulation process and rennet-induced gels by confocal scanning laser microscopy (CSLM)

An aliquot of 1 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 5 µL of enzyme solution (0.3 %, w/v) prepared in acetate

buffer 0.1M (pH 5.1) and 25 µL of fast-green FCF (0.1 % w/v, in distilled water, Sigma–Aldrich, Ireland) (used to observe the protein matrix in a confocal microscope) were added and time started. Immediately after addition, 200 µL of the solution was transferred to a coverglass (a cavity dish of 10 mm in depth), covered with a glass coverslip (0.17 mm thick) with 8 chambers (Lab-Tek® II Chambered Coverglass, USA) and the confocal imaging was performed using a Zeiss Upright LSM780-NLO microscope (Carl Zeiss AG, Germany) with temperature control set to 35 ºC.

CLSM is able to penetrate deeply but noninvasively through the sample to obtain a large number of sequential, thin optical sections that may then be assembled by image-analysis software to produce 3D reconstructions and projections. For each sample, 20 adjacent planes (2D layers 512×512 pixels in resolution from a 134.95 µm × 134.95 µm sample area) were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. Images were recorded at a distance of 5 µm and 20 µm from the surface of the coverglass (bottom). Images of representative areas of each sample were taken every 90 seconds for 40 minutes and after 3 h, 6 h, 18 h and 24 h using an oil immersion 63x objective lens (numerical aperture = 1.40) at excitation wavelengths of 633 nm to fast-green (He/Ne laser), in which the fast-green FCF stained protein appears red, in contrast, the serum phase appears black in these images. The images were acquired in RGB color (8 bits), of 512 x 512 pixels in size to give final resolutions of 0.26 µm/pixel (Ong et al., 2010).

4.2.6 Image analysis

Image analysis of CLSM micrographs was performed using image J software (Research Service Branch, National Institute of Health, Maryland, USA) equipped with “Pore Analysis” and “ComputeStats” plug-ins. The images were enhanced and flattened using a bandpass filter. The enhanced image was then transformed to a binary image using an Otsu threshold algorithm binarisation with

all structural features contributing black pixels and all backgrounds features contributing white pixels (Impoco et al., 2006).

The porosity was calculated as an average area of pores and as the fraction of pore area with respect to the total sample area (0.018 mm^2). The porosity calculated here is only equal to a two-dimensional pore area and fraction and not the absolute porosity of the sample (Ong et al., 2011). The average pore area is also calculated as total pore area with respect to total sample area divided by total numbers of pores and Image J analysis was used to quantify the porosity and total numbers of pores from CLSM micrographs (Hussain, Grandison, & Bell, 2012).

4.2.7 Statistical analysis

The processes and analyses were carried out with in three repetitions and each experimental unit was carried out in triplicate. The analysis of variance (ANOVA) was performed to compare the effects of the different treatments and the Tukey test was used to determine the difference of them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software–(StatiSoft, Inc., Tulsa, Okla., U.S.A.). The results were represented as mean ± standard deviation.

4.3 Results and Discussion

4.3.1 Proteolysis of rennet-induced gels by capillary electrophoresis

Electropherograms of casein standards are shown in *Figure 4.1* and *Figure 4.2* shows the electropherograms of the rennet-induced gels by using calf rennet during 24 hours at 35 ºC. The peaks were indicated on the electropherograms with serial numbers and the identification of peaks is detailed in the figure caption.

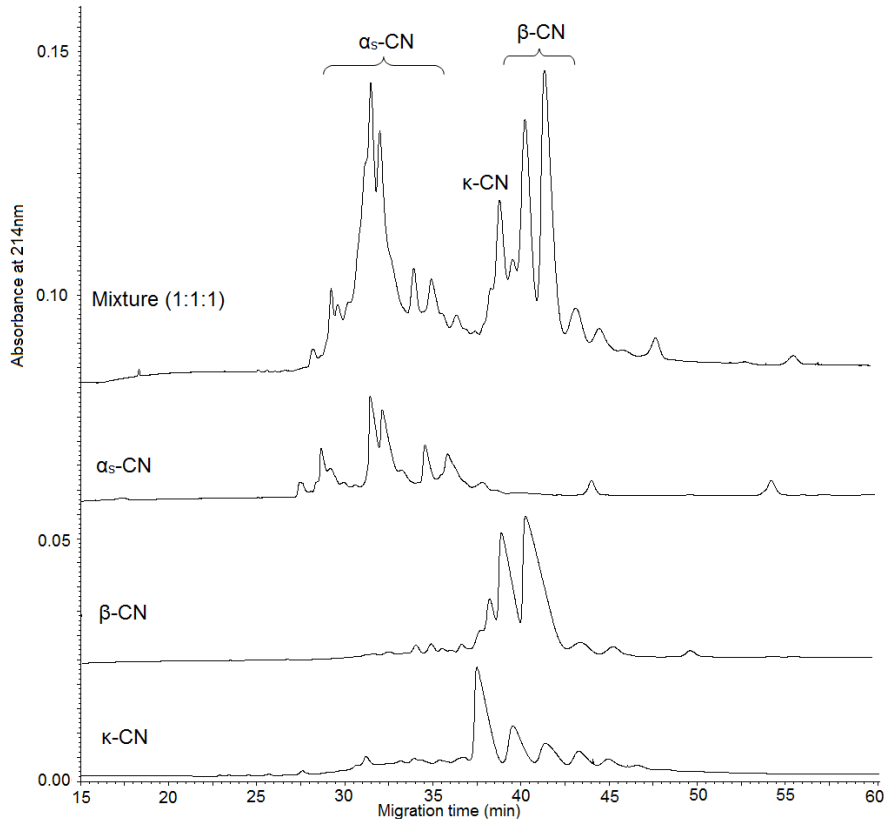

Figure 4.1 Capillary electropherogram separation of a 1:1:1 mixture of α_s-CN, β-CN and κ-CN and identification of major caseins by analysis of casein standards: α_s-casein (α_s-CN); β-casein (β-CN); κ-casein (κ-CN).

The comparison of casein gels obtained by using high pressure homogenized enzyme and non-processed one showed that the latter caused higher casein hydrolysis (especially on the α_s-CN fraction) with total hydrolysis of peaks 2, 3 and 4 after 6h of coagulation and formation of a new peak (11, not identified) after 18 h (this peak was not observed for gel produced by HPH enzyme after 24h). The lower proteolysis caused by HPH processed enzyme on α_s-CN fraction can be attributed to the reduction of the unspecific proteolytic profile of the calf rennet after HPH (Leite Junior, Tribst, & Cristianini, *in press*) On the contrary, the hydrolysis profiles of κ-CN were similar for non-processed and high pressure homogenized enzyme, which was expected since no changes on milk-clotting

activity were observed after HPH of this enzyme (Leite Junior, Tribst, & Cristianini, *in press*). Thus, the results evidenced that HPH is able to reduce the unspecific proteolytic behavior of calf rennet with no effects on the enzyme specificity.

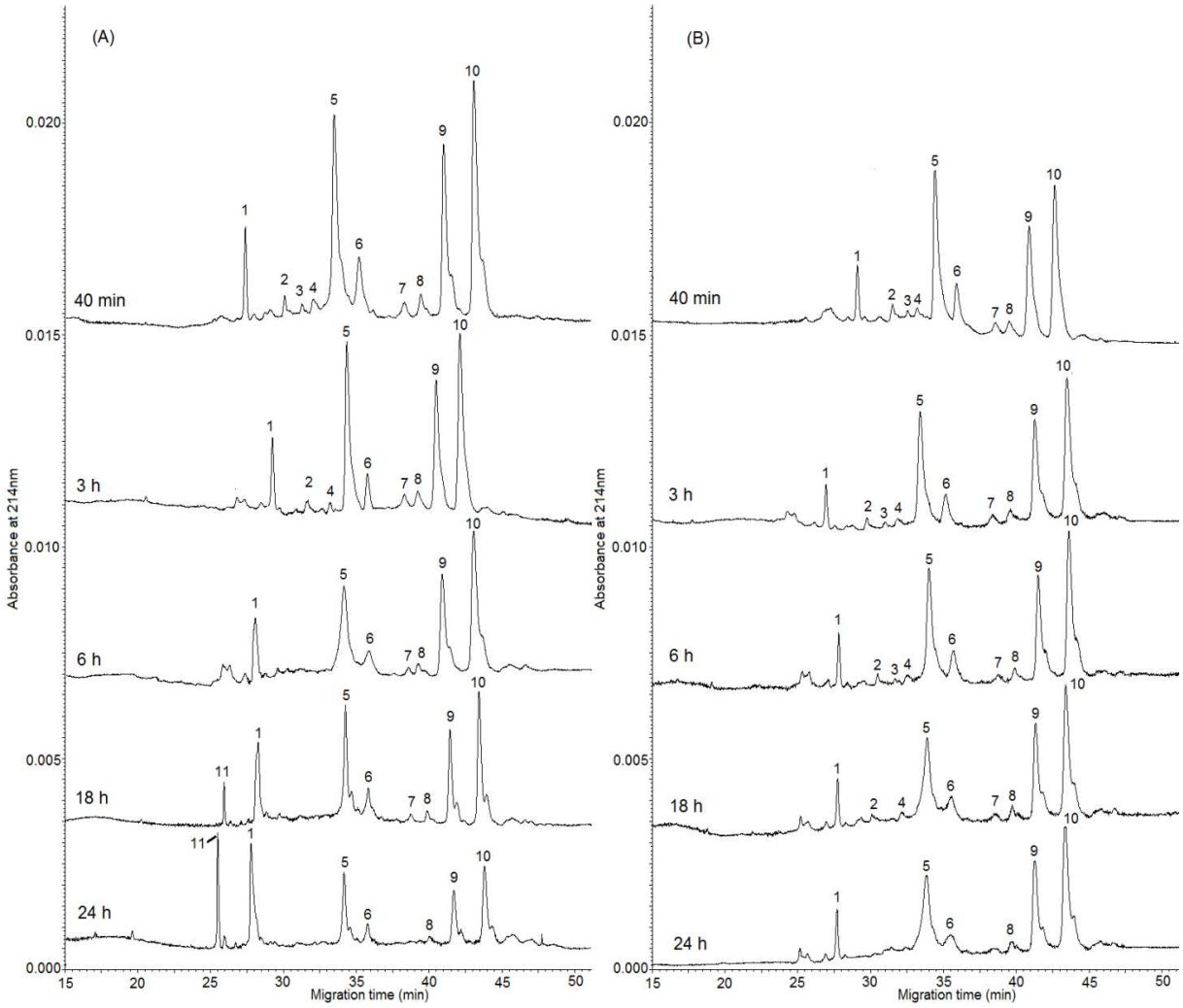

Figure 4.2 Capillary electropherogram of the rennet-induced gels using calf rennet non-processed (A) and processed by high pressure homogenization (B), throughout a 24 hours coagulation period at 35 ºC. Peak identification: 1: para-κ-CN; 2: α_{s2}-CN; 3: α_{s2}-CN; 4: α_{s2}-CN (three phosphorylation states of the α_{s2}-CN monomer were achieved); 5: α_{s1}-CN; 6: α_{s0}-CN (two phosphorylation states of α_{s1}-CN monomer were achieved); 7: κ-CN; 8: β-CN^{B}; 9: β-CN^{A1}; 10: β-CN^{A2} (two genetic variants of β-CN: A1 and A2); 11: unidentified.

From an industrial point of view, the effect of HPH on calf rennet is interesting since the observed reduction on proteolytic profile possibly limits the unspecific proteolysis during cheese storage. This is desirable once excessive proteolysis causes weakness of gel structure, protein loss in the whey and low yield of cheese dry mass. Additionally, the reduction on proteolytic activity prevents excessive protein hydrolysis during fresh cheese storage (being able to improve fresh cheese shelf life) as also during cheese ripening, ensuring in an adequate balance of intact proteins and peptides resulting in flavors, textures and functional characteristics typical of ripened cheese (Sousa, Ardo, & McSweeney, 2001).

4.3.2 Spontaneous syneresis and rheological assays of coagulation process and rennet-induced gels

Table 4.1 shows the results for whey separation during 24h at 35 ºC on the rennet-induced gels produced with non-processed and homogenized enzymes. Up to 3 hours of coagulation, the gels produced with the enzyme processed at 190 MPa did not differ from gels produced with the non-processed one ($p>0.05$). However, after 6 hours of coagulation, the gel produced with high pressure homogenized enzyme showed a significant higher syneresis than the produced with non-processed enzyme ($p<0.05$). The syneresis naturally happens in curd due to the formation of new bonds between para-casein micelles that results in compression of casein micelles package and expulsion of whey from the gel (Walstra & Jenness, 1984; Walstra, van Dijk, & Geurts, 1985; Walstra et al., 1999, Lodaite et al., 2000; Grundelius et al., 2000; Lucey et al., 2000; Lucey, 2001; Castillo et al., 2006).

Thus, the lower proteolytic activity of HPH enzyme possibly let the casein micelle more intact allowing to form more bounds between para-casein and leading to stronger contraction of casein matrix and higher syneresis.

Table 4.1 and *Figure 4.3* shows the results of rheological parameters of milk gels (during 24h) produced with using calf rennet non-processed and processed at

190 MPa. The G' values were higher for gels produced with HPH rennet ($p < 0.05$) and an increment on this value were observed up to 24h of evaluation. To the contrary, non-processed gels showed decay of the G' values after ~20h of coagulation.

These results demonstrate that gels obtained with HPH processed enzyme are initially more consistent and stronger and the contraction of casein micelles up to 24h increased even more the gel consistence. For the non-processed enzyme, the reduction on G' value after 20h can be attributed to the effect of unspecific proteolysis, which was reduced when enzyme was homogenized at 190 MPa. Therefore, it is possible to conclude that the HPH process of calf rennet results in more consistent and solid gels, with higher viscoelasticity at 24h favoring the formation of firm and compact cheese mass.

The G'' values reflects the water retention ability of the milk gels. The gel produced with HPH processed enzyme showed significant higher values of G'' than the gel obtained by milk coagulation with non-processed enzyme ($p<0.05$). It means that water diffusivity in casein matrix produced with enzyme processed at 190 MPa was lower. This phenomenon can be explained by the compact gels formed by homogenized enzymes, with strong linkages that difficult the whey diffusion.

Table 4.1. Rheological properties and whey separation of coagulation process and rennet-induced gels throughout a 24 hours coagulation period at 35 ºC using calf rennet processed by high pressure homogenization

Coagulation time (h)	Storage modulus – G' (Pa) *		Loss modulus – G''(Pa) *		Loss tangent (tan δ) *		Whey separation (%) **	
	Control	190 Mpa	Control	190 MPa	Control	190 MPa	Control	190 MPa
0.33 (20 min)	1.87 ± 0.1^{b}	2.47 ± 0.1^{a}	0.7± 0.0^{b}	0.9 ± 0.0^{a}	0.359 ± 0.003^{a}	0.360 ± 0.002^{a}	ND	ND
0.67 (40 min)	10.4 ± 0.6^{b}	11.5 ± 0.2^{a}	3.7 ± 0.2^{b}	4.1 ± 0.0^{a}	0.358 ± 0.003^{a}	0.357 ± 0.004^{a}	2.52 ± 0.37^{a}	2.80 ± 0.49^{a}
3	38.8 ± 1.0^{b}	40.2 ± 1.1^{a}	14.0 ± 0.4^{b}	14.6 ± 0.3^{a}	0.362 ± 0.002^{a}	0.365 ± 0.007^{a}	4.62 ± 0.74^{a}	4.68 ± 0.68^{a}
6	51.3 ± 0.9^{a}	52.2 ± 2.0^{a}	19.0 ± 0.3^{b}	19.6 ± 0.3^{a}	0.370 ± 0.001^{a}	0.374 ± 0.010^{a}	9.66 ± 1.01^{b}	11.89 ± 1.54^{a}
18	59.0 ± 3.7^{b}	67.4 ± 4.1^{a}	23.1 ± 0.7^{b}	27.3 ± 0.7^{a}	0.392 ± 0.013^{a}	0.406 ± 0.014^{a}	17.97 ± 1.55^{b}	19.67 ± 1.74^{a}
24	56.6 ± 5.3^{b}	69.9 ± 4.6^{a}	22.0 ± 1.4^{b}	28.6 ± 0.7^{a}	0.390 ± 0.012^{b}	0.412 ± 0.016^{a}	19.96 ± 1,97^{b}	21.64 ± 1.20^{a}

* Values are means from replicates (n = 6).

** Values are means from replicates (n = 9).

^{a,b} Means ± standard deviation in the row, with different superscripts, are different ($p<0.05$).

ND: Not Determined.

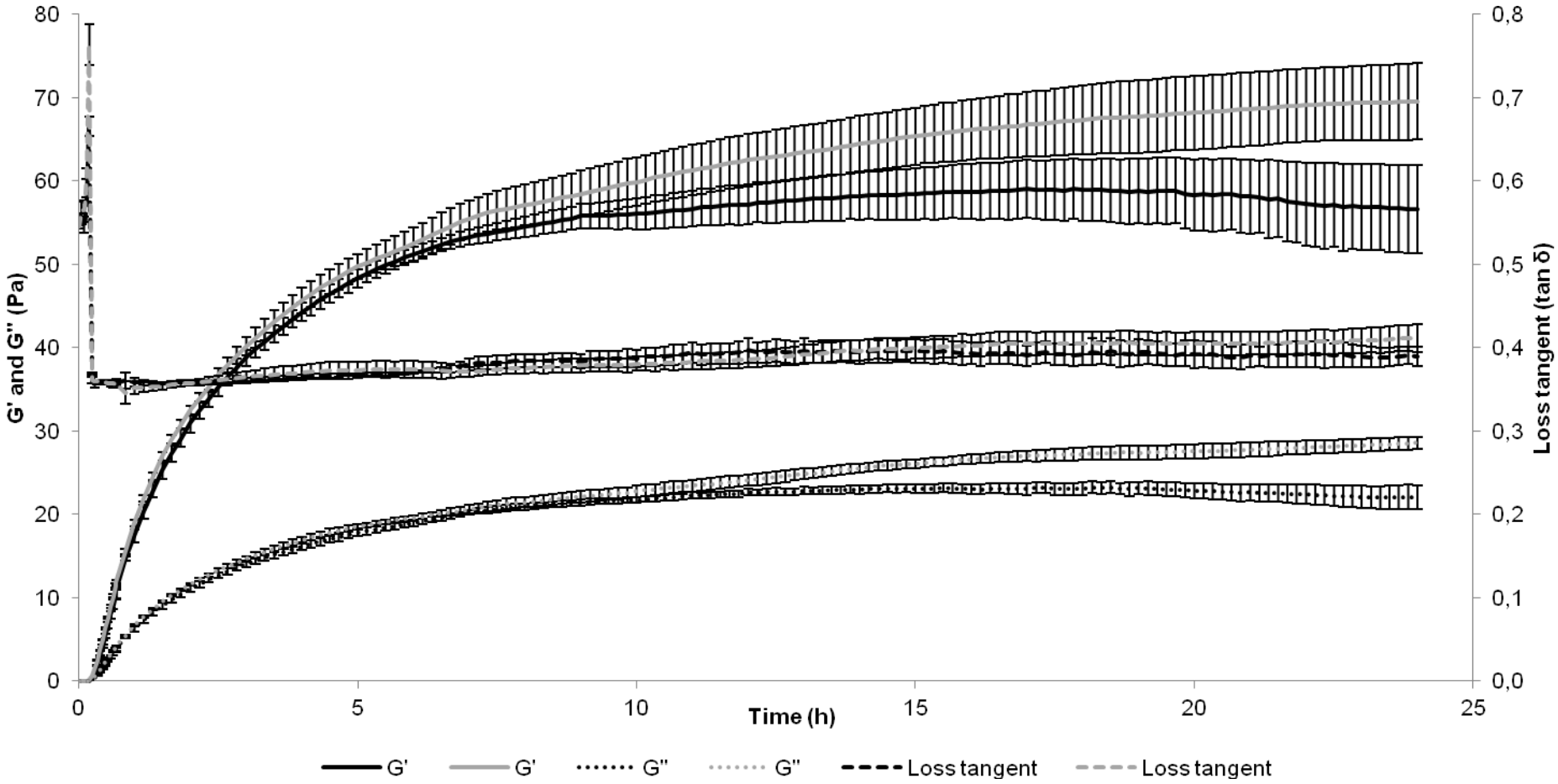

*Figure 4.3 Evaluation of milk coagulation using calf rennet enzyme subjected to high pressure homogenization (processed at 190 MPa - gray lines) and non-processed (control - black lines), immediately after the process throughout a 24 hours coagulation period at 35 ºC. ** Values are means from replicates (n = 6).*

In rennet-induced gels, the susceptibility to syneresis has been associated with a high value for the loss tangent at long time scales (van Vliet et al., 1991), being related to the tendency of the network to rearrange after gel formation (Lucey, 2001). Many studies of the relations between syneresis of casein gels and their rheological properties have been presented, however reports of the effects of the storage modulus (G') on the syneresis behavior are not conclusive (Lelievre, 1977; Zoon, van Vliet, & Walstra, 1988). The analysis of van Vliet et al. (1991) concludes that syneresis is not dependent on G', but increases with increasing tan δ (=G''/G').

The data of tan δ (loss tangent) were also presented on *Table 4.1* and *Figure 4.3*. A tendency of loss tangent to be higher for gels prepared with high pressure homogenized enzyme was observed. This difference became statistically significant after 24h of coagulation ($p < 0.05$) and endorses the hypothesis of the increasing of whey expulsion during gel formation due to the higher contraction of protein matrix, forming a dense network.

Therefore, the results of syneresis and gel rheology highlight that protein network formed was stronger since the beginning of coagulation to the end of rennet-induced gel development. The reduction of the hydrolysis of casein fractions allowed the formation of a more compact, cohesive, firm and dense protein network, increasing whey loss accompanied by a reduction in the water diffusivity. These effects observed in rennet-induced gels can be interesting for cheese production, since it may allow higher cheese yield (due to the increment on water binding capacity of protein) and increased dry matter (due to the reduction of small peptides loss in the whey).

4.3.3 Microstructure of coagulation process and rennet-induced gels by confocal scanning laser microscopy (CSLM)

Figure 4.4 shows the images obtained by CSLM during formation of calf rennet-induced gel. The porosity of the gel, total number of pores and the average pore area are showed in *Figure 4.5* and *Table 4.2*.

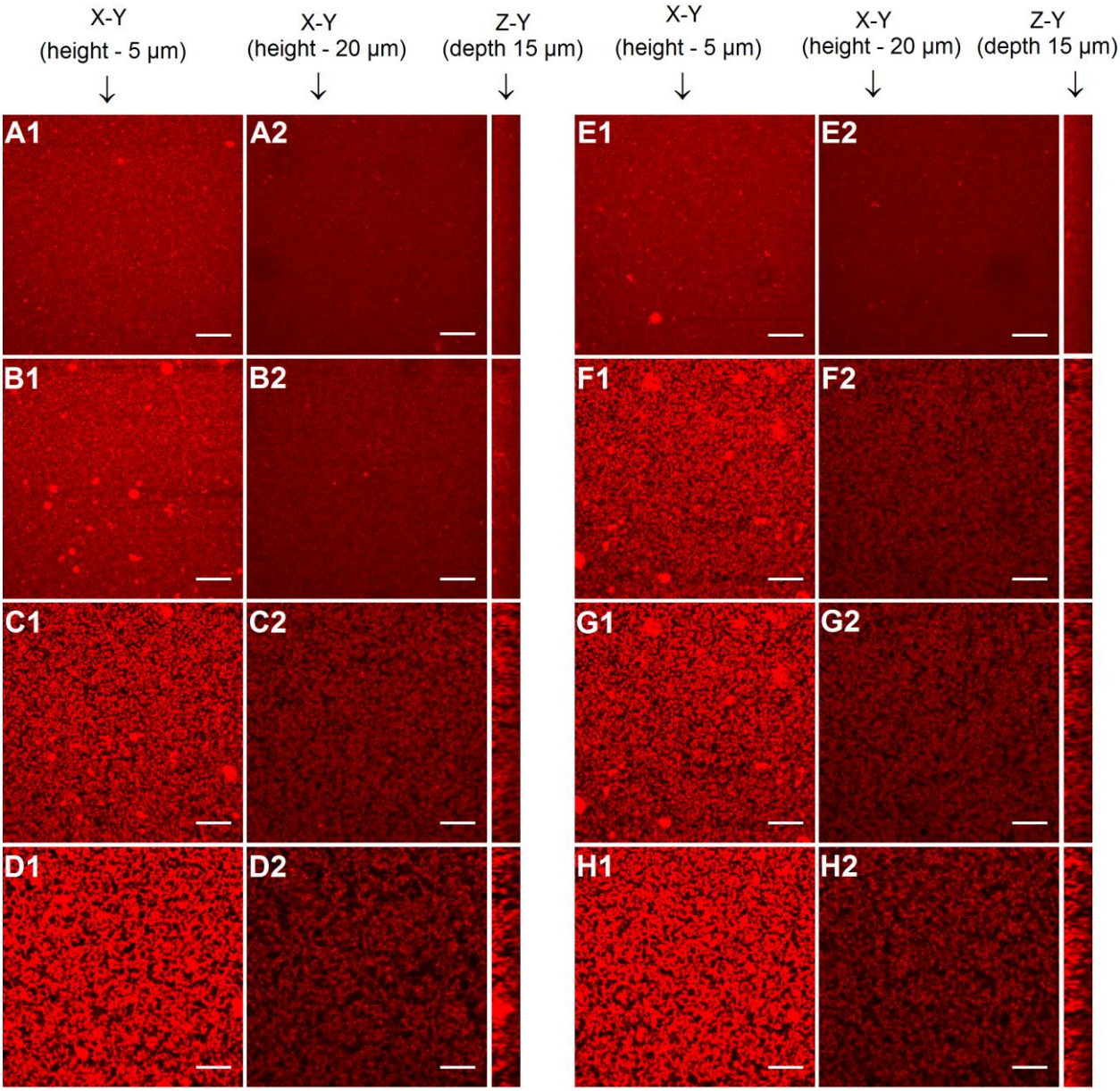

Figure 4.4 CLSM micrographs of rennet-induced gels using calf rennet processed by high pressure homogenization and calf rennet non-processed (A1-D1: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; A2-D2: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm; E1-H1: 190 MPa

at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; E2-H2: 190 MPa at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm) throughout a 24 hours period at 35 ºC. The fast-green FCF stained protein appears red and the serum phase appears black in these images. Each set of images is presented in two views: the X–Y (a height of 5 and 20 µm from the bottom – the surface of the coverglass) and the Z–Y (right) projections. For each sample, 20 adjacent planes were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. The scale bars are 20 µm in length.

The images elucidate the effect of HPH on the calf rennet behavior during the rennet-induced gel formation and development. The images clearly shows that, after 20 minutes of enzyme addition, the protein aggregation is faster for gels obtained by high pressure homogenized enzyme. Nevertheless, at the end of coagulation step (40 minutes), no visual differences were observed on the gels images obtained using non-processed and homogenized enzyme at 190 MPa. After 24 h, more compact protein network was observed for gel obtained by using HPH enzyme, being compatible with the results obtained of rheology and syneresis analysis.

The gel porosity at 5 µm from the sample bottom was reduced due to the aggregation and sedimentation of the protein network. On the contrary, the porosity at 20 µm increased due to the excessive whey expulsion from the protein matrix. Comparing the results obtained for each enzyme it was observed that, after 20 minutes of coagulation, the gel produced with high pressure homogenized enzyme, the porosity was lower at 5 µm and higher at 20 µm than the gel produced with non processed enzyme ($p<0.05$). Similarly, after 24h, it was found significant differences between gels obtained with non-processed and homogenized enzyme.

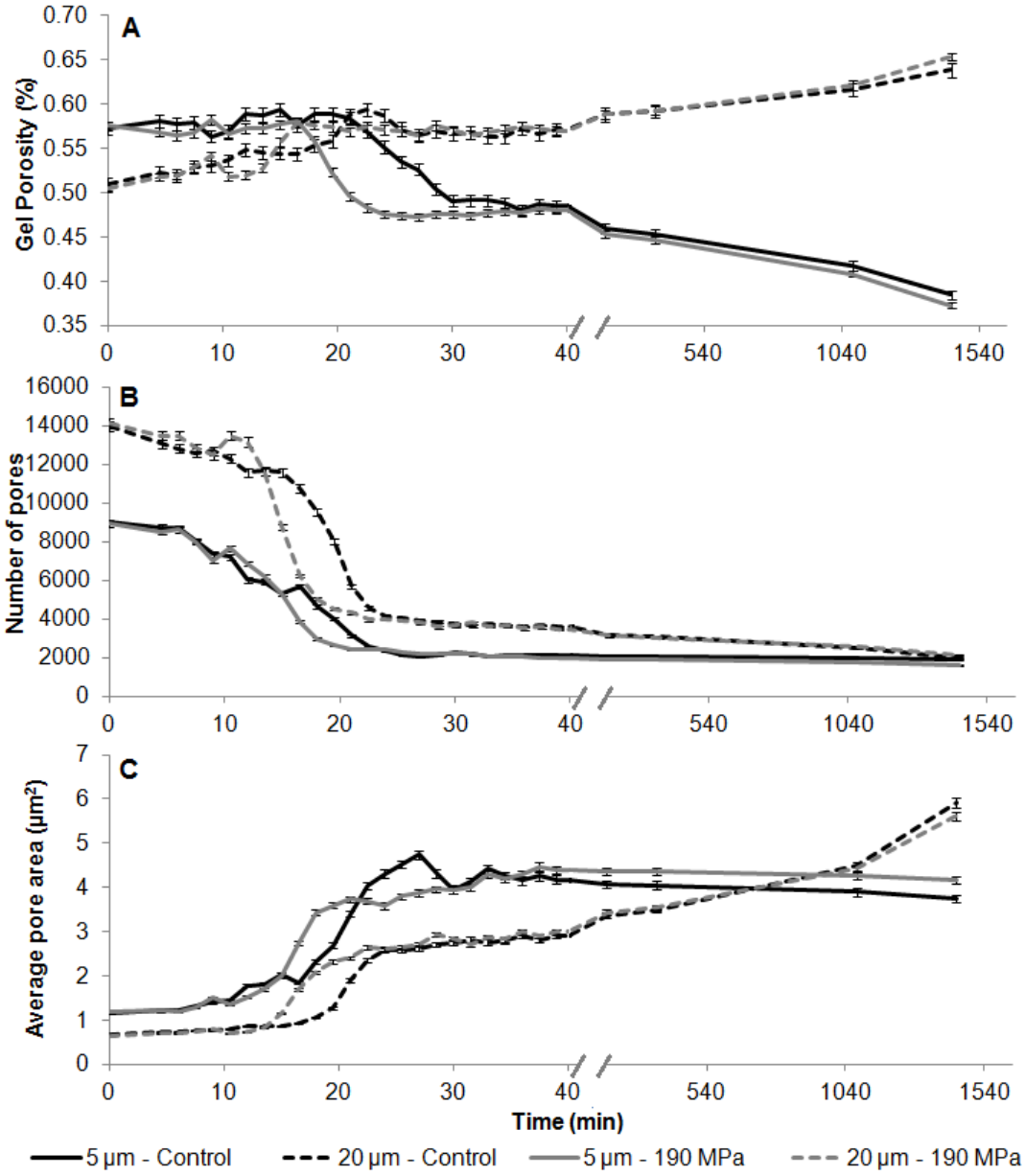

Figure 4.5 Gel porosity (A), number of pores (B) and average pore area (C) of rennet-induced gels using calf rennet processed by high pressure homogenization and calf rennet non-processed. Results are expressed as the mean ± the standard deviation (n=6).

Results of total number of pores and the average pore area at 5 µm showed a reduction of total number of pores with increase of the average pore area along the time, which is directly linked to the protein aggregation. The rennet-induced gel

obtained with HPH enzyme showed a faster reduction of total pores, presenting a significant difference (from 20 to 40 minutes of coagulation) when compared with gel produced using non-processed enzyme. From 40 minutes to 24h, it was observed a reduction in the number of pores and an increase in the average pore area, due to the compaction of protein network. Again, the milk gels produced with HPH enzyme were significantly different of gel obtained with non-processed enzyme ($p<0.05$).

Imagens recorded at a distance of 20 μm from the surface of coverglass, results also showed a reduction of total number of pores and an increase in the average pore area along the coagulation time (40 minutes). Again, from 40 minutes to 24h, it was observed a reduction in the number of pores and an increase in the average pore area. However, comparing the results of gels obtained with non-processed and HPH processed enzyme, it was observed a high number of pores and lower average pore area for gel produced with processed enzyme. This phenomena can possible be attributed to the reduction of proteolytic activity of the enzyme after HPH, with consequent maintenance of the protein network structure, which was previously evidenced by G' and G'' values.

Therefore, the gel produced by using calf rennet processed at 190 MPa is overall less porous, when compared to a similar gel manufactured by using non-processed calf rennet. This effect occurred due to changes in the activity and specificity of the enzyme by HPH processing, which directly affects the aggregation of the protein network. In the present work, the speed up of k-CN cleavage promotes a greater number of connections between fractions of caseins in the presence of calcium ions. Considering the highest rate of milk-clotting for calf rennet processed by HPH (Leite Junior, Tribst, & Cristianini, in press), it is suggested that the integrity of protein matrix of milk gel formed by HPH enzyme was the main reason for the higher gel contraction observed. The direct consequence of this enzyme action was the formation of a compact, dense and consistent protein network.

Table 4.2 Gel porosity, average pore area and number of pores of rennet-induced gels using calf rennet processed by high pressure homogenization

		Sample height 5 µm			
	Sample	0 min	20 min	40 min	24 h
Gel Porosity (%)	Control	0.5894 ± 0.0073 [a]	0.5885 ± 0.0075 [a]	0.4850 ± 0.0062 [a]	0.3843 ± 0.0049 [a]
	190 MPa	0.5757 ± 0.0052 [a]	0.5232 ± 0.0047 [b]	0.4801 ± 0.0043 [a]	0.3723 ± 0.0034 [b]
Number of pores	Control	8989 ± 123 [a]	3986 ± 76 [a]	2115 ± 40 [a]	1875 ± 36 [a]
	190 MPa	8925 ± 152 [a]	2655 ± 45 [b]	1984 ± 34 [b]	1624 ± 28 [b]
Average pore area (μm^2)	Control	1.16 ± 0.03 [a]	2.69 ± 0.06 [a]	4.18 ± 0.09 [a]	3.73 ± 0.08 [a]
	190 MPa	1.17 ± 0.02 [a]	3.59 ± 0.07 [b]	4.41 ± 0.08 [b]	4.17 ± 0.08 [b]
		Sample height 20 µm			
	Sample	0 min	20 min	40 min	24 h
Gel Porosity (%)	Control	0.5092 ± 0.0071 [a]	0.5588 ± 0.0072 [a]	0.5714 ± 0.0073 [a]	0.6389 ± 0.0082 [a]
	190 MPa	0.5055 ± 0.0046 [a]	0.5747 ± 0.0052 [b]	0.5704 ± 0.0052 [a]	0.6537 ± 0.0044 [b]
Number of pores	Control	13983 ± 253 [a]	7989 ± 152 [a]	3588 ± 68 [a]	1968 ± 38 [a]
	190 MPa	14128 ± 241 [a]	4508 ± 76 [b]	3479 ± 59 [a]	2110 ± 41 [b]
Average pore area (μm^2)	Control	0.66 ± 0.02 [a]	1.27 ± 0.03 [a]	2.90 ± 0.06 [a]	5.91 ± 0.13 [a]
	190 MPa	0.65 ± 0.01 [a]	2.32 ± 0.04 [b]	2.99 ± 0.06 [a]	5.64 ± 0.09 [b]

Results are expressed as the mean ± the standard deviation (n = 6). [a,b] Means ± standard deviation in the row, with different superscripts, are significantly different ($p<0.05$).

4.4 Conclusion

The results evidenced that HPH was able to reduce the unspecific proteolytic activity of calf rennet (random proteolysis) and increase the rate of milk clotting. The milk gel obtained using calf rennet processed at 190MPa was more compact, firm, dense and cohesive than one obtained by using non-processed enzyme. Additionally, the gels produced with the HPH processed enzyme showed higher whey expulsion from protein matrix. After 24h of coagulation, the gel produced using HPH enzyme kept its consistence due to the lowered proteolytic activity after the HPH process. Therefore, the results highlights the HPH is a process able to improve the hydrolytic characteristics of calf rennet, allowing to obtaining stronger, compact and more consistent milk gels which may result in gels with higher yield (due to the increment on water binding capacity of protein) and increased dry matter (due to the reduction of small peptides loss in the whey). Moreover, the expected of low proteolysis in the gel can be interesting for the extension of shelf-life of fresh cheese.

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Capítulo 5. Effect of high pressure homogenization on commercial bovine rennet and characterization of the coagulation process and gel development

Resumo

Esse trabalho avaliou o efeito da HAP (até 190 MPa) nas atividades proteolítica e de coagulação do leite do coalho de bovino adulto e a consequência do uso da enzima processada na formação e no desenvolvimento do gel quanto ao perfil reológico, proteólise, sinérese e microestrutura. A HAP causou uma redução na atividade proteolítica (AP) (redução máxima de ~7 % na enzima processada a 190 MPa). A atividade de coagulação de leite (ACL) aumentou após a enzima ser processada pela HAP a 150 MPa (7 % de aumento). A enzima processada a 150 MPa apresentou maior taxa de coagulação quando comparada com a enzima não processada e o gel produzido mostrou maior consistência. Adicionalmente, os géis produzidos com a enzima processada a 150 MPa apresentaram maior sinérese (formando uma rede proteica mais compacta), menor porosidade (evidenciada pela microscopia confocal) e maior aprisionamento de água (maiores valores de G''). Estes efeitos no gel de leite podem ser atribuídos ao aumento da ACL e redução da AP causada pela HAP no coalho de bovino. Portanto, os resultados destacam que a HAP é um processo capaz de melhorar as características proteolíticas do coalho bovino adulto permitindo novos mercados para este produto na indústria de fabricação de queijos.

Palavras-Chave: *Alta pressão dinâmica • Coalho bovino adulto • Coagulação enzimática do leite • Atividade proteolítica • Atividade de coagulação do leite • Ensaio reológico*

Abstract

This work evaluated the effect of HPH (up to 190 MPa) on adult bovine rennet proteolytic and milk-clotting activities and the consequences of using this processed enzyme in the milk gel formation and its characteristics (rheological profile, proteolysis, syneresis and microstructure). The HPH caused a reduction in proteolytic activity (PA) (maximum reduction of ~7% in the enzyme processed at 190 MPa). The milk-clotting activity (MCA) increased after enzyme processed by HPH at 150 MPa (increased of 7%). The enzyme processed at 150 MPa showed higher rate of milk-clotting activity when compared with non-processed one and the gel produced showed greater consistency. Additionally, the gels produced with the enzyme processed at 150 MPa showed higher syneresis (forming a more compact protein network), lower porosity (evidenced by confocal microscopy) and higher trapping of water (higher G'' values). These effects on milk gel can be linked to the increment of MCA and reduction of PA caused by HPH in the bovine rennet. Therefore, the results highlight that HPH is a process able to improve the proteolytic characteristics of bovine rennet, enabling new markets for this product in the cheese making industry.

Key-words: *Dynamic high pressure • Bovine rennet • Enzymatic coagulation of milk • Proteolytic activity • Milk-clotting activity • Rheological assay*

5.1 Introduction

Proteinases found in the abomasum of ruminants are chymosin and pepsin (Rolet-Répécaud et al., 2013). The chymosin is characterized by a highly specific enzyme activity (Phe_{105}-Met_{106} of κ-caseína) during the coagulation phase (Hyslop, 2003; Crabbe, 2004; Møller et al., 2012). However, the pepsin has lower specificity hydrolyzing connections that have aminoacids such as Phe, Tyr, Leu or Val (Agudelo et al., 2004; Papoff et al., 2004), thus leading to the release of medium-chain peptides with bitterness flavour, despite having hydrolytic activity higher than the chymosin (Fox et al., 2004). The concentration of chymosin and pepsin varies depending on the age of the animal, being it found in the proportion of 80% chymosin and 20% pepsin in calves and in at a inverse proportion to adult animals (Fox et al., 2004; Jacob, Jaros, & Rohm, 2011).

Over the last decades, a large number of coagulants from animal, microbial and plant origin were considered as potential substitutes for calf rennet (Jacob, Jaros, & Rohm, 2011; Yegin, Goksungur, & Fernandez-Lahore, 2012), however, the application of these enzymes in the coagulation of milk could result in problems such as reduction of yield at manufacturing and the appearance of defects in flavour and texture (Møller et al., 2012).

High pressure homogenization (HPH) is an emerging technology developed for food preservation with minimum sensory and nutritional damage (Tribst et al., 2011; Franchi, Tribst, & Cristianini, 2012). Recently, HPH was also proposed as a physical method to change proteins, being able to improve (Liu et al., 2009 a,b; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012b,c), reduce (Velázquez-Estrada et al., 2012; Tribst, Augusto, & Cristianini, 2012b; Leite Júnior, Tribst, & Cristianini, *in press*) or not alter (Tribst & Cristianini, 2012a) the activity and stability of enzymes. The effects of HPH were dependent on the level of pressure homogenization applied, the temperature of the enzyme during the process, the nature of enzyme studied and pH of homogenization (Tribst & Cristianini, 2012a,b,c; Tribst, Augusto, & Cristianini, 2012, a,b).

Leite Júnior, Tribst, & Cristianini, (*in press*) found that the high pressure homogenization (HPH) on calf rennet at 190 MPa caused reduction in proteolytic activity (52% loss of activity), did not alter the milk-clotting activity and improved the G' values obtained during milk coagulation using this enzyme. These changes in activity are usually attributed to changes caused by HPH in quaternary, tertiary and secondary structures of enzymes (Lacroix, Fliss, & Makhlouf, 2005; Liu et al., 2009b; Liu et al., 2010).

Thus, HPH may be an interesting tool in the process of modifying enzymes, once, well as promoting increased activity and improved specificity, can also result in increased enzymatic stability (Leite Júnior, Tribst, & Cristianini, *in press*, Tribst, Augusto, & Cristianini, 2012; Tribst & Cristianini, 2012b; Tribst & Cristianini, 2012c), turning adult bovine rennet into a potential substitute to calf rennet. Therefore, this study aimed to evaluate the influence of high pressure homogenization on the proteolytic and milk-clotting activities and stability of commercial adult bovine rennet used in manufacturing cheese and evaluate the formation of the gels by 24 hours at 35 ºC.

5.2 Material and Methods

5.2.1 Bovine rennet protease and high pressure homogenization

A commercial adult bovine rennet (Bela Vista Produtos Enzimáticos Indústria e Comércio Ltda, Santa Catarina, Brazil) was used in the experiments.

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the assays. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

5.2.2 Effect of high pressure homogenization in commercial bovine rennet protease

5.2.2.1. High pressure homogenization processing

A volume of 2 L of the bovine rennet solution was prepared at 3 % (w/v) in 0.2 M sodium acetate buffer (pH 5.6) and homogenized under pressures of 0, 50, 100, 150 and 190 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately in an ice bath after exiting the homogenizing valve. A non-processed sample of porcine pepsin was evaluated as the control sample.

5.2.2.2. Relative proteolytic activity (RPA) determination

The proteolytic activity of bovine rennet was measured according to Merheb-Dini et al. (2010): the enzyme solution (3 % w/v) was prepared in a 0.2 M acetate buffer (pH 5.6). 600 µL of the enzymatic solution was added to 400 µL of a 0.5 % (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at 35 ºC/40 min in a shaken water bath (62 rpm) and 1 mL of 10 % (w/v) trichloroacetic acid (TCA) then added to stop the hydrolysis. The samples were centrifuged at 2300 *g*/5 min/10 ºC and the absorbance was measured at 280 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution, and the ΔAbs_{280nm} was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to *Equation 5.1*.

$U/mL = (\Delta Abs_{280nm} \times 10 \times \text{dilution factor} / 0.6 \times 40)$ (*Equation 5.1*)

RPA assays were carried out immediately after processing (time 0 h) and after 1, 7 and 14 days. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed sample was used for a comparative evaluation. The relative proteolytic activity (RPA) was calculated considering the

activity of the HPH processed and non-processed samples, according to *Equation 5.2*:

RPA = (enzyme activity$_{after_HPH_and/or_storage}$/ enzyme activity $_{non\text{-}processed\_sample\_at\_0h}$)*100 (*Equation 5.2*)

5.2.2.3. Relative milk-clotting activity (RMCA) determination

The milk-clotting activity was determined according to Merheb-Dini et al. (2010). A volume of 5 mL of skimmed milk powder, reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) was added to a 0.01 M $CaCl_2$ solution and pre-incubated at 35 °C/10 min. An aliquot of 0.5 mL of the enzyme solution (0.005 %, w/v, prepared in 0.1 M sodium acetate buffer, pH 5.6) was then added and the time count started. Clot formation was determined by manual tube rotation and the time taken for the first particles to form measured. One milk-clotting activity unit (MCA) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 ºC. The MCA was calculated using *Equation 5.3*:

Unit of milk-clotting activity (MCA) = 2400/*t* x *S*/*E* (*Equation 5.3*)

Where *t* is the time (seconds) necessary for clot formation, *S* is the milk volume and *E* is the enzyme volume.

MCA assays were carried out immediately after processing (time 0 h) and after 1, 7 and 14 days. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed enzyme was also used for a comparative evaluation. The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to *Equation 5.4*:

RMCA = (MCA$_{after_HPH_and/or_storage}$/ MCA $_{non\text{-}processed\_sample\_at\_0h}$)*100 (*Equation 5.4*)

5.2.2.4. Rheological assays

The milk coagulation was evaluated using a time sweep low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments,

USA). These assays were carried out with the processed and non-processed bovine rennets.

The experiments were carried out according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skimmed milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) with the addition of a 0.01 M $CaCl_2$ solution. This mixture was pre-incubated at 35 °C/10 min, and subsequently 0.8 mL of enzyme solution (0.5 %, w/v) prepared in 0.1 M acetate buffer (pH 5.6) was added. The mixture was immediately transferred to the rheometer cup (30 mm diameter and 80 mm height), which had a *vaned quarter* geometry (with 28mm of diameter and 42 mm of length) and a 4 mm *gap*. The stress was set at 0.1 Pa and frequency at 0.1 Hz. The parameter G' (storage modulus) was measured at 3 min intervals for 90 min of the clotting process at 35 °C. The temperature was controlled by a *Peltier* system. The rheological assays were carried out immediately after processing (time 0 h) and after 60 days. The samples were stored under refrigeration (4 ºC) throughout this period.

The rate of milk-clotting was calculated as the variation of G' with variation in time as the log (dG'/dt) at three min intervals, expressed in $Pa.min^{-1}$. The storage modulus (G') describes the elastic (solid) behavior of the product, and consequently the energy stored and released in each oscillatory cycle.

5.2.3 Characterization of the coagulation process and the gel formed using commercial bovine rennet processed by high pressure homogenization

5.2.3.1. High pressure homogenization processing

A volume of 1 L of the commercial bovine rennet solution 3 % (w/v) prepared in sodium acetate buffer 0.2M (pH 5.6) was homogenized under pressure of 150 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately after exiting the homogenizing valve in an ice bath. A non-

processed sample of commercial bovine rennet was evaluated as the control sample.

5.2.3.2. Capillary zone electrophoresis method of bovine rennet-induced gels

The capillary electrophoresis analysis was determined according to the conditions described by Ortega et al. (2003) and Otte et al. (1997). Firstly, the samples preparation were carried out: an aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added by 0.01 M $CaCl_2$ and by 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 800 µL of enzyme solution (0.5 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added and time started. After 40 min, 3h, 6h, 18h and 24h of coagulation at 35 ºC, 20 mg of samples were collected and dissolved in 1 mL of 10 mM sodium phosphate buffer solution containing 8 M urea (Merck, Darmstadt, Germany) and 10 mM of dithiothreitol (DTT, Sigma Chemical Co, St. Louis, USA) at pH 8, and left for 1 h at temperature of 23 ºC before filtration (0.22 µm Millex-GV_{13}, Millipore, Molsheim, France).

Then, Capilary zone electrophoresis (CZE) analysis was carried out using a Beckman P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) controlled by 32 Karat software (Beckman Coulter). The separations were performed using a fused-silica capillary (eCap™, Beckman Instruments Inc., San Ramon, CA, USA) of 57 cm (50-cm effective length to detector) x 75 µm I.D. The running buffer was prepared with 10 mM sodium phosphate containing 6 *M* urea and 0.05% hydroxypropyl methylcellulose (HPMC, Sigma Chemical Co, St. Louis, USA); the pH was adjusted to 3.0 with 1 M HCl. Buffer solutions were filtered through 0.22 µm filters (Millipore, Molsheim, France) before use.

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 5 s at 0.5 psi. During sample analysis, a constant voltage (18.5 kV ~35 µA) was applied and the temperature of the separation was kept at 23 ºC circulating coolant

surrounding the capillary. For all experiments, electrophoreses was carried for 70 min and detection was at 214 nm (data collection rate 5 Hz). In CZE, the capillaries were conditioned by washing with 0.5 M NaOH for 5 min, then with pure water for 5 min, and finally with running buffer for 5 min after each electrophoretic runs.

The first electropherogram in a series was always discarded. The repeatability of peak areas was assessed by replicate injections (n = 3). Standard curves were made with a mixture of α_s-casein (C-6780), β-casein (C-6905) and κ-casein (C-0406) at 5 mg.mL^{-1} obtained from Sigma Chemical Co (St. Louis, MO, USA) in the ratio of 1:1:1. Assignment of peaks was based on previous reports by Ortega et al. (2003) and Albillos et al. (2006).

5.2.3.3. Determination of spontaneous syneresis of bovine rennet-induced gels by the siphon method

The level of spontaneous whey separation in undisturbed bovine rennet-induced gels was determined using a siphon method in according Amatayakul et al. (2006). An aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 800 µL of enzyme solution (0.5 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added and time started. After 40 min, 3h, 6h, 18h and 24h coagulation at 35 ºC samples were cooled to 5 ºC and inclined at an angle of 45 º and maintained at rest for a period of 1 hour. Then, the serum exceeded was collected from the surface of the samples (using a syringe) and weighed. The syneresis was expressed as the percent weight of the whey divided the initial weight of the gels sample, according to *Equation 5.5*.

Whey separation (%) = $(S_F/P_T - P_E) \times 100$ (*Equation 5.5)*

Where, S_F is the weight of the separated whey, P_T is the weight of sample plus packaging and P_E is the weight of the empty package.

5.2.3.4. Rheological assays of coagulation process and bovine rennet-induced gels

The milk coagulation and development of formed gel were evaluated by monitoring the milk coagulation process through time sweep using low deformation oscillatory test in rheometer with controlled stress (AR2000ex, TA Instruments, USA).

The experiments were carried out according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany). This mixture was pre-incubated at 35 °C/10 minutes. Subsequently, 800 µL of enzyme solution (0.5 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added. Immediately after enzyme addition, the sample was transferred to the rheometer cup (30 mm diameter and 80 mm height). It was used a *Vaned Quarter* geometry (with 28mm of diameter and 42 mm of length) and a *gap* of 4 mm. The stress was set at 0.1 Pa, frequency 0.1 Hz. The parameters G' (storage modulus) and G'' (loss modulus) were measured at 3 minute intervals (at first's 40 minutes) and then at 10 minute intervals (up to 24 hours) of clotting process at 35 ºC. The tan δ (loss tangent) was calculated by G''/G' ratio. The temperature was controlled by a *Peltier* system.

5.2.3.5. Three dimensional (3D) microstructure of coagulation process and bovine rennet-induced gels by confocal scanning laser microscopy (CSLM)

An aliquot of 1 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 13,5 µL of enzyme solution (0.5 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) and 25 µL of fast-green FCF (0.1% w/v, in distilled water, Sigma–Aldrich, Ireland) (used to observe the protein matrix in a confocal

microscope) were added and time started. Immediately after addition, 200 µL of the solution was transferred to coverglass (a cavity dish of 10 mm in depth), covered with a glass coverslip (0.17 mm thick) with 8 chambers (Lab-Tek® II Chambered Coverglass, USA) and the confocal imaging was performed using a Zeiss Upright LSM780-NLO microscope (Carl Zeiss AG, Germany) with temperature control set to 35 ºC.

CLSM is able to penetrate deeply but noninvasively through the sample to obtain a large number of sequential, thin optical sections that could then be assembled by image-analysis software to produce 3D reconstructions and projections. For each sample, 20 adjacent planes (2D layers 512×512 pixels in resolution from a 134.95 µm × 134.95 µm sample area) were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. Images were recorded at a distance of 5 µm and 20 µm from the surface of the coverglass (bottom). Images of representative areas of each sample were taken every 90 seconds for 40 minutes and after 3 h, 6 h, 18 h and 24 h using an oil immersion 63x objective lens (numerical aperture = 1.40) at excitation wavelengths of 633 nm to fast-green (He/Ne laser), in which the fast-green FCF stained protein appears red, in contrast, the serum phase appears black in these images. The images were acquired in RGB color (8 bits), of 512 x 512 pixels in size to give final resolutions of 0.26 µm/pixel (Ong et al., 2010).

5.2.3.6. Image analysis

Image analysis of CLSM micrographs was performed using image J software (Research Service Branch, National Institute of Health, Maryland, USA) equipped with “Pore Analysis” and “ComputeStats” plug-ins. The images were enhanced and flattened using a bandpass filter. The enhanced image was then transformed to a binary image using an Otsu threshold algorithm binarisation with all structural features contributing black pixels and all backgrounds features contributing white pixels (Impoco et al., 2006).

The porosity was calculated as an average area of pores and as the fraction of pore area with respect to the total sample area (0.018 mm^2). The porosity calculated here is only equal to a two-dimensional pore area and fraction and not the absolute porosity of the sample (Ong et al., 2011). The average pore area is also calculated as total pore area with respect to total sample area divided by total numbers of pores and Image J analysis was used to quantify the porosity and total numbers of pores from CLSM micrographs (Hussain, Grandison, & Bell, 2012).

5.2.4 Statistical analysis

The processes and analyses were carried out with three repetitions and each experimental unit was carried out in triplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and the Tukey test to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software– (StatiSoft, Inc., Tulsa, Okla., U.S.A.) and the results were presented as the mean ± standard deviation.

5.3 Results and Discussion

5.3.1 Effect of high pressure homogenization in commercial bovine rennet protease

5.3.1.1. Proteolytic activity

Figure 5.1 shows the results of relative proteolytic activity during 14 days of storage after enzyme process at different high pressure homogenization. The greater the applied pressure higher the reduction in relative proteolytic activity of the bovine rennet, being observed a maximum reduction of ~7% in the enzyme processed at 190 MPa evaluated after processing ($p<0.05$). After one day the

storage of the enzyme solution it was observed a reduction in all samples, reaching a minimum of 83% in RPA after processing at 190 MPa ($p<0.05$). Until the 14th day it was observed maintenance of activity in all samples. Samples processed at 100 MPa, 150 MPa and 190 MPa showed lower activity compared to the non-processed enzyme (13%, 13% and 16% lower, respectively) ($p<0.05$). Thus HPH process reduces the proteolytic activity bovine rennet according to the increased pressure. Leite Júnior, Tribst, & Cristianini, (*in press*) found reduction of 52% in the relative proteolytic activity of the calf rennet after processing at 190 MPa ($p<0.05$) and no significant differences in activity during 5 days of storage.

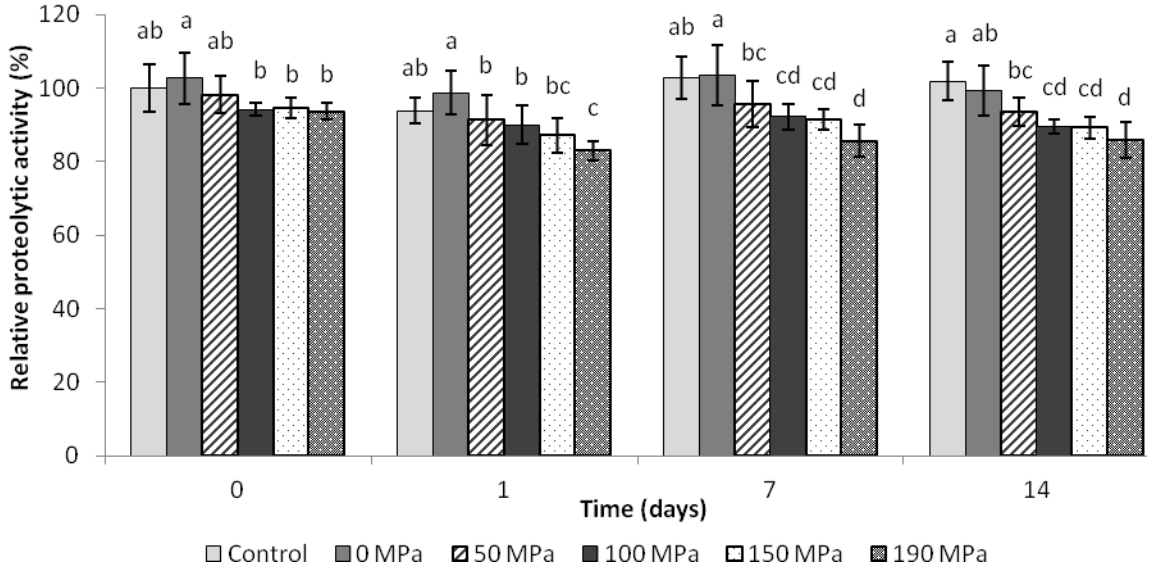

Figure 5.1 Relative proteolytic activity of the bovine rennet subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed bovine rennet samples evaluated at each time.

5.3.1.2. Milk-clotting activity

Milk-clotting activity, i.e. the capability for specific κ-casein hydrolysis, is the most important property of enzymes used in cheese production (Jacob, Jaros, & Rohm, 2011). This cleavage destabilizes the casein micelles, which coagulates in

the presence of Ca^{2+} (Andrén, 2011). *Figure 5.2* shows the results for relative milk-clotting activity of the non-processed and processed bovine rennet samples immediately after HPH processing up to 14 days of enzyme processing.

The results obtained immediately after HPH showed an increase in milk-clotting activity in which the enzyme processed at 150 MPa showed RMCA 7% higher compared to the control sample ($p<0.05$). The similar behavior was observed during the storage of enzymes solution. After 14 days of storage all samples showed slight reduction on RMCA, where the enzyme processed at 150 MPa kept the difference in milk clotting activity compared to the control sample (~ 8% higher) ($p<0.05$). Leite Júnior, Tribst, & Cristianini (*in press*) showed that HPH processing did not alter the milk-clotting activity of calf rennet, with no significant differences between the non-processed and processed samples up to 190 MPa ($p>0.05$).

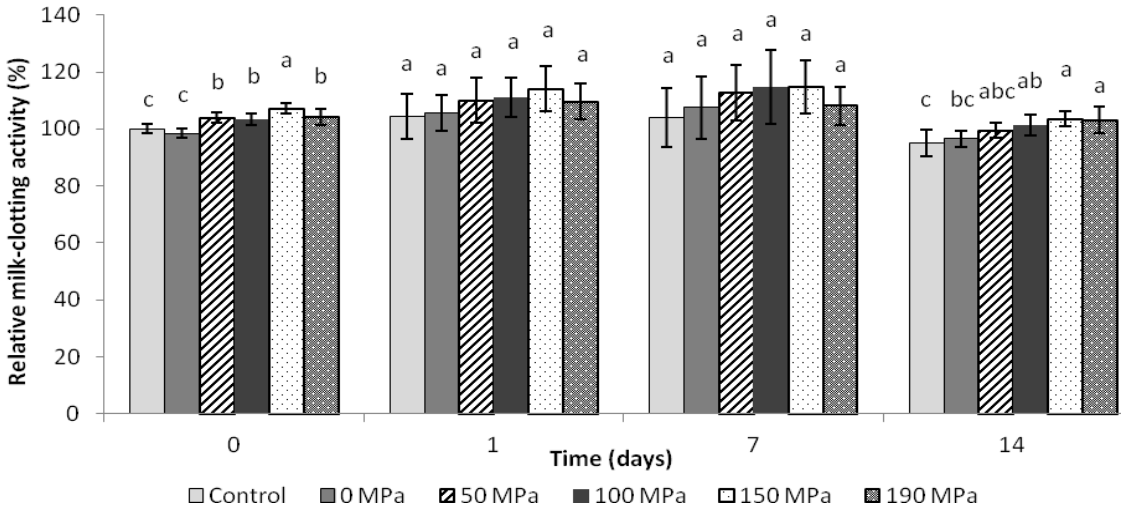

Figure 5.2 Relative milk-clotting activity of the bovine rennet subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed bovine rennet samples evaluated at each time.

Therefore, considering the results for proteolytic (section 5.3.1.1) and milk-clotting activities, it was concluded that HPH processing caused a reduction in

enzyme activity (proteolytic performance) and increase in its specific proteolysis (responsible for milk coagulation). Thus, HPH process in bovine rennet showed a potential improvement in the proteolytic profile of this enzyme, due to the reduction of unspecific activity and improvements on milk-clotting activity.

5.3.1.3. Rheological evaluation

An effective way to monitor the behavior of milk coagulation is dynamic rheometry, by applying a strain (or stress) to the gelling sample; this input must be small enough to ensure undisturbed network formation (Sandra, & Dalgleish 2007; Jacob, Jaros, & Rohm, 2011), and results in a response stress (or strain). Stress normalized to unit strain is denoted as modulus which, in this case, as storage modulus G' (Pa), refers to the measure of gel stiffness in a visco-elastic system. Therefore, the storage modulus (G') describes the elastic (solid) behavior of the product, and starts to increase at the beginning of the coagulation process, characterized by micelle aggregation.

Thus, the rheological data are important to evaluate the behavior of milk coagulation using the HPH processed and non-processed enzymes, and gels consistency at the end of the coagulation process (*Figure 5.3*). In addition, the beginning of the aggregation can be better visualized using a log scale (*Figure 5.3, a2 and b2*). Immediately after the process (*Figure 5.3 a1),* the results showed that the aggregation process started at approximately 12 min for the sample that used the enzyme processed at 50 MPa, 100 MPa, 150 MPa and 190 MPa and the non-processed enzyme. For samples processed at 0 MPa aggregation started at 14 min. After 90 min of coagulation, bovine rennet homogenized at 150 MPa produced the gel with G’ values 4 % higher than non-processed one.

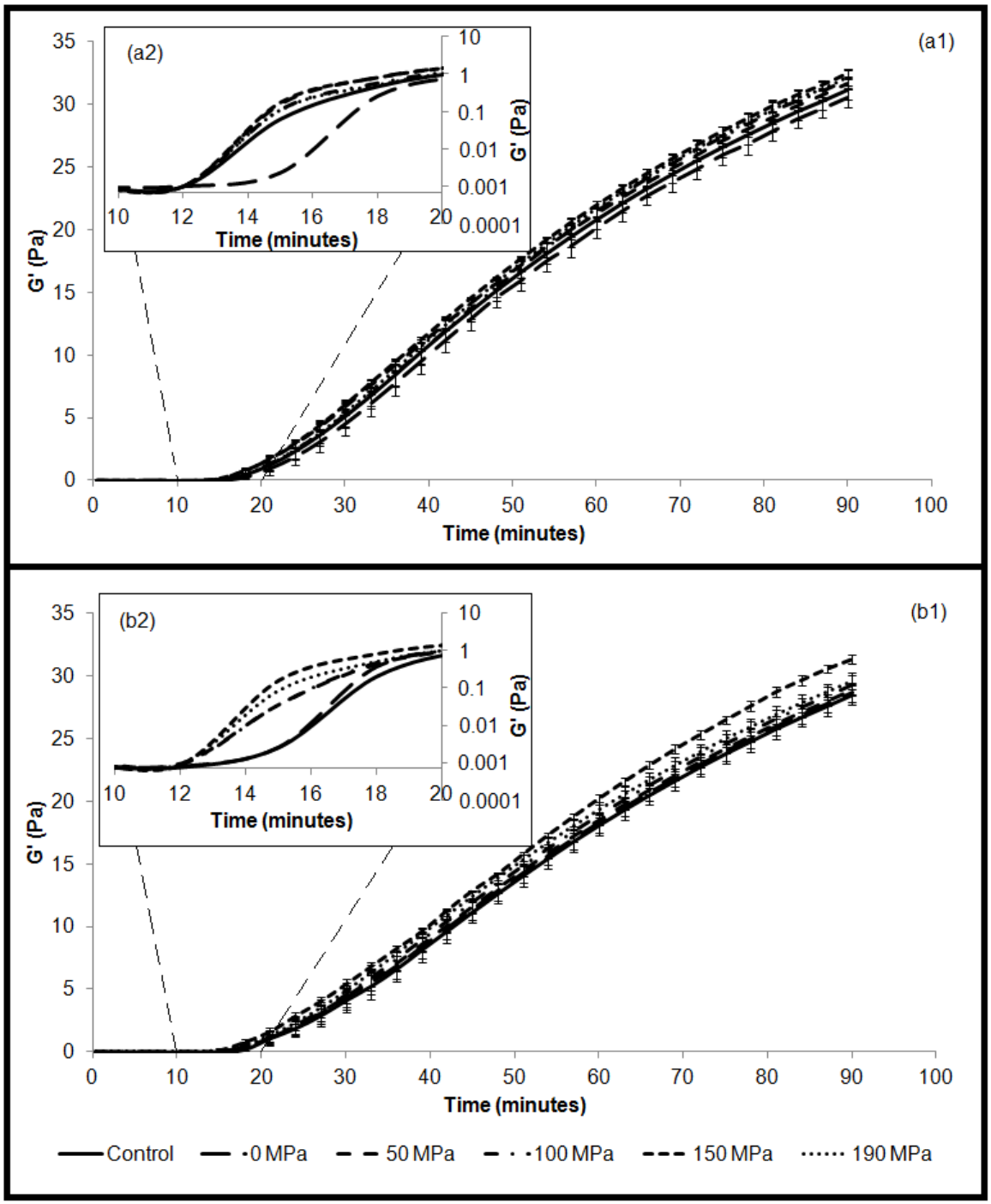

Figure 5.3 Evaluation of milk coagulation (a1) immediately after the process (0h) (a2) log scale, and (b1) after 14 days stored at 4 °C (b2) log scale.

Another rheological evaluation was carried out after 14 days of storage of the bovine rennet solutions at 4 °C, to determine if storage in solution affected the coagulation profile of the enzymes (*Figure 5.3 b1*). The storage period caused a loss of milk-clotting activity for non-processed enzymes, which was evidenced by the increase in the time taken to start the aggregation process (beginning at 14 min). On the contrary, the samples homogenized 50 MPa to 190 MPa maintained the initial time for aggregation (beginning at 12 min). Therefore coagulation using the enzyme processed at 150 MPa occurred 15 % faster than using the non-processed one and the G' value after 90 minutes of coagulation was 10% higher for enzyme processed at 150 MPa than for non-processed one, which is possibly linked to improvements in enzyme stability during storage in aqueous solution after the HPH process, being compatible with the results of RMCA.

Figure 5.4 shows the rate of milk-clotting, calculated as the variation in G' at the every three minutes. The graph shows the moment at which aggregation started, represented by the higher values for log(dG'/dt) reached by each curve (higher speed). It can be observed that the enzyme processed at 150 MPa achieved a greater initial velocity immediately after processing and, after 14 days of storage, presented a faster start and achieved a greater initial velocity compared with non-processed one. Consequently, the protein aggregation was faster and the gel produced was firmer when enzyme processed at 150 MPa was used, decreasing the milk coagulation time and improving the consistency of the final product.

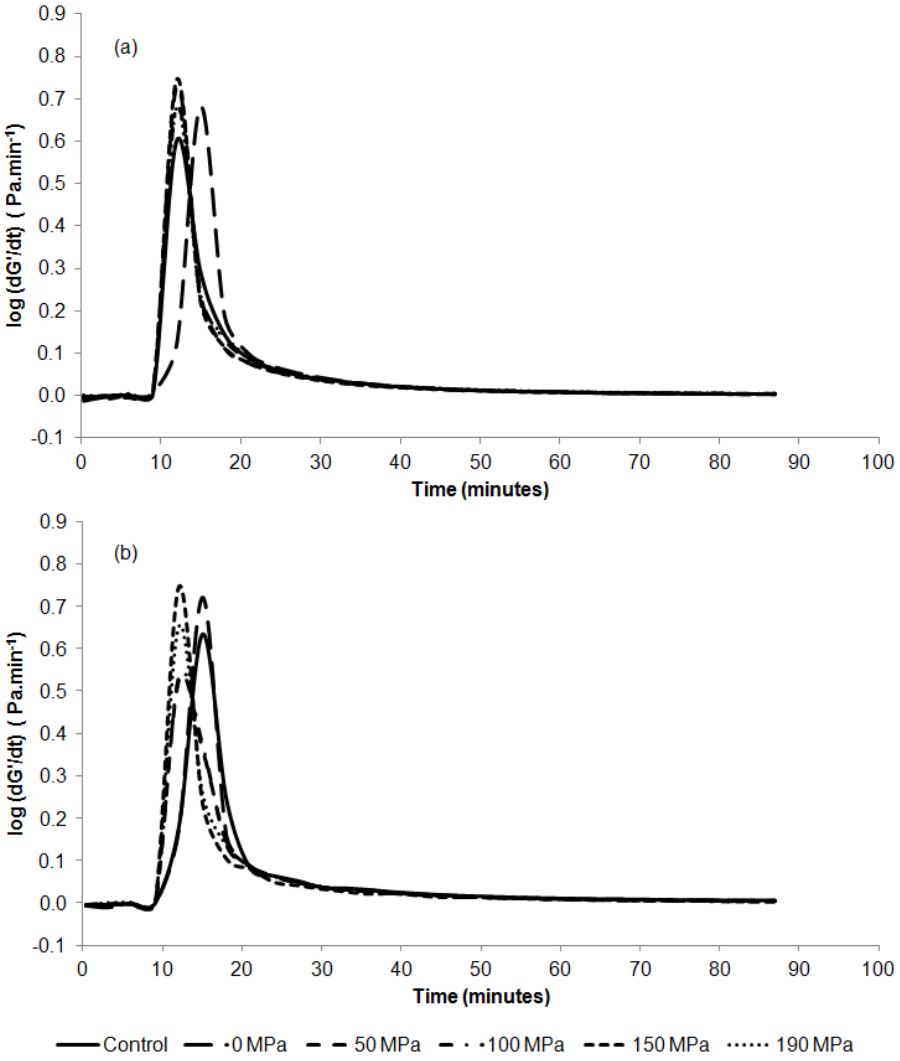

Figure 5.4 Rate of milk-clotting by the action of the enzyme subjected to high pressure homogenization, (a) immediately after the process (0h) and (b) after 14 days storage at 4 °C.

5.3.2 Characterization of the coagulation process and the gel formed using commercial bovine rennet processed by high pressure homogenization

5.3.2.1. Proteolysis of bovine rennet-induced gels by capillary electrophoresis

Electropherograms for the casein standards and bovine rennet-induced gels during 24 hours at 35 ºC are shown in *Figure 5.5* and *Figure 5.6*, respectively. The peaks were indicated on the electropherograms with serial numbers and the identification of peaks is detailed in the figure caption.

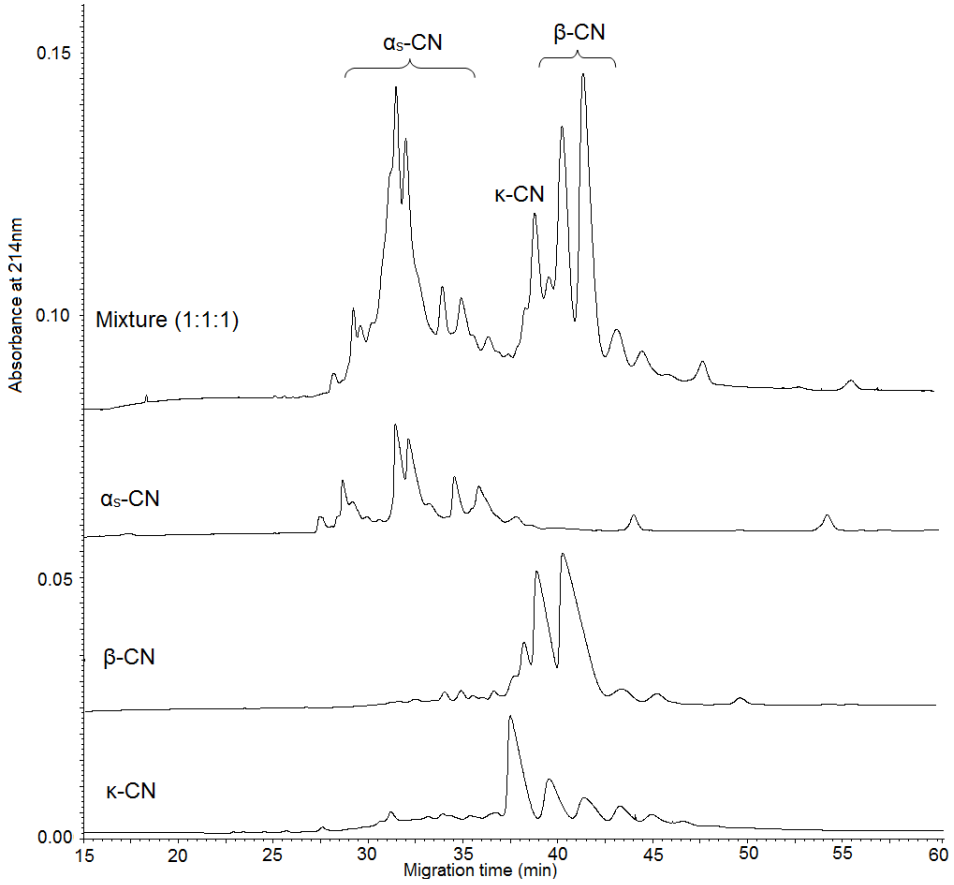

Figure 5.5 Capillary electropherogram separation of a 1:1:1 mixture of α_s-CN, β-CN and κ-CN and identification of major caseins by analysis of casein standards: α_s-casein (α_s-CN); β-casein (β-CN); κ-casein (κ-CN).

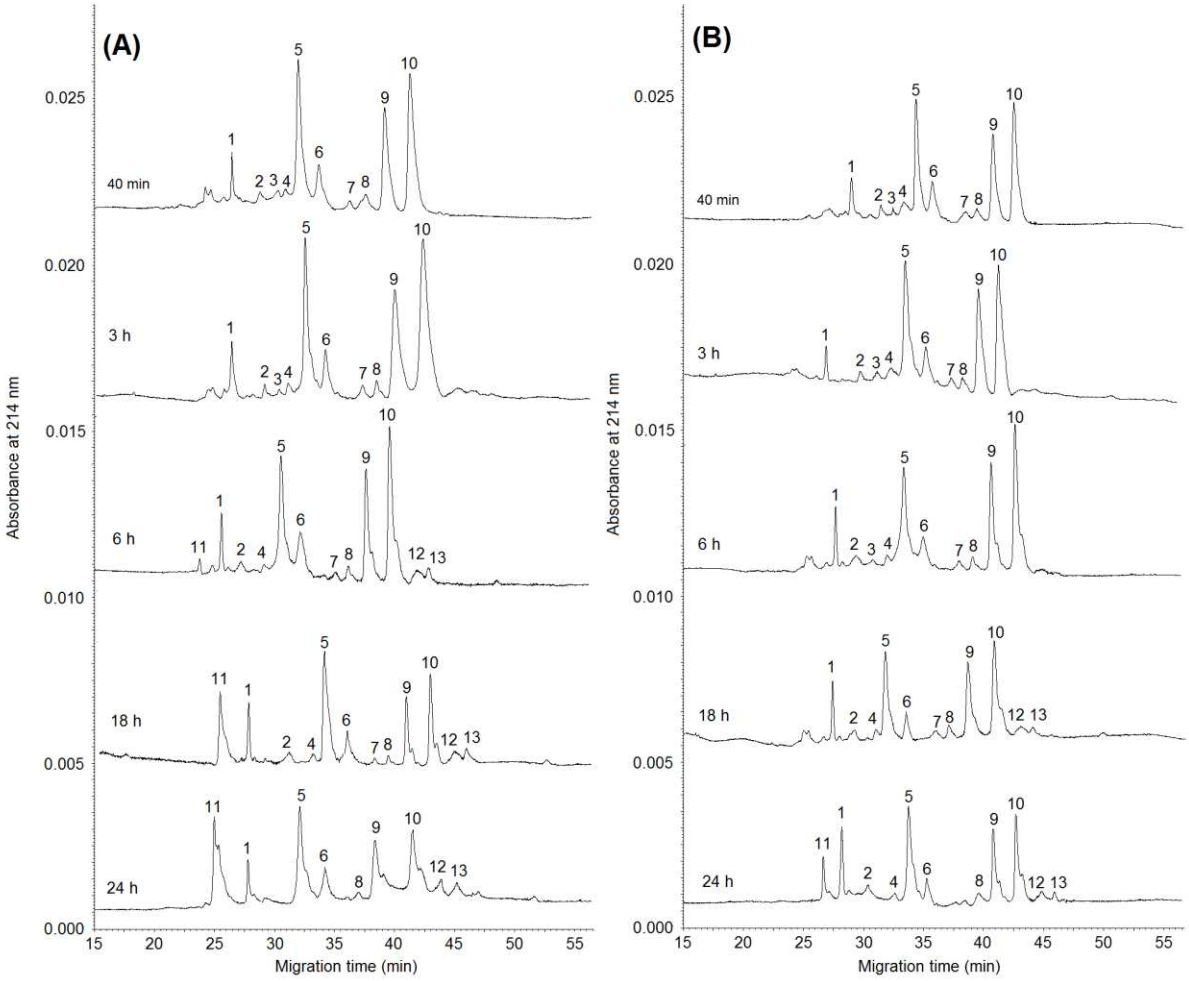

Figure 5.6 Capillary electropherogram of the bovine rennet-induced gels using (A) non-processed (A) and (B) processed by high pressure homogenization at 150 MPa, throughout a 24 hours coagulation period at 35 ºC. Peak identification: 1: para-κ-CN; 2: α_{s2}-CN; 3: α_{s2}-CN; 4: α_{s2}-CN (three phosphorylation states of the α_{s2}-CN monomer were achieved); 5: α_{s1}-CN; 6: α_{s0}-CN (two phosphorylation states of α_{s1}-CN monomer were achieved); 7: κ-CN; 8: β-CN^{B}; 9: β-CN^{A1}; 10: β-CN^{A2} (two genetic variants of β-CN: A1 and A2); 11, 12 and 13: unidentified.

The comparison of casein gels obtained by using high pressure homogenized enzyme and non-processed one showed that, overall, hydrolysis profiles were similar throughout time. The non-processed sample caused a slightly higher casein hydrolysis (especially on the β-CN fraction) with formation of the new

peaks 11, 12 and 13 (not identified, after 6 h). Those peaks were also observed for gel produced by HPH enzyme but only after 18 h (peaks 12 and 13) and 24 h (peak 11). Furthermore, a higher hydrolysis after 24 hours for the non-processed enzyme was observed, where peaks 2, 3 and 4 (α_{s2}-casein fraction) were completely gone. On the other hand, the gel produced with the enzyme processed at 150 MPa after 24 h the peaks 2 and 4 were still present indicating the absence or a slowly hydrolysis up to 24 h at 35 ºC. This can be attributed to the reduction of the proteolytic activity of the bovine rennet after HPH (as observed in section 5.3.1.1, in *Figure 5.1*). The hydrolysis profiles of κ-CN after 40 minutes and during storage of gel were similar to the non-processed and HPH processed enzyme. Thus, the results evidenced that HPH was able to reduce the unspecific proteolytic behavior of bovine rennet after the gel formation with no effects on the enzyme specificity.

Reduction on proteolytic profile might limits the high unspecific proteolysis during cheese storage. This is desirable once excessive proteolysis, mainly in fractions of β-casein – specific for pepsin activity (larger fraction in adult bovine rennet), promotes the release of hydrophobic peptides that confer bitterness flavour (Hassan et al., 2013). Furthermore, the intense proteolysis could cause weakness of gel structure, protein loss in the whey and low yield of dry matter. Additionally, the reduction on proteolytic activity prevents excessive protein hydrolysis during fresh cheese storage (being able to improve fresh cheese shelf life) (Sousa, Ardo, & McSweeney, 2001).

5.3.2.2. Spontaneous syneresis and rheological assays of coagulation process and development bovine rennet-induced gels

Table 5.1 shows the results for whey separation during 24h at 35 ºC on the bovine rennet-induced gels produced with non-processed and homogenized enzymes.

Table 5.1 Rheological properties and whey separation of milk gels throughout a 24 hours coagulation period at 35 ºC using bovine rennet processed by high pressure homogenization

Coagulation time (h)	Storage modulus – G' (Pa) *		Loss modulus – G'' (Pa) *		Loss tangent (tan δ) *		Whey separation (%) **	
	Control	150 MPa	Control	150 MPa	Control	150 MPa	Control	150 MPa
0.33 (20 min)	0.8 ± 0.1^{b}	1.3 ± 0.3^{a}	0.2 ± 0.0^{a}	0.4 ± 0.3^{a}	0.306 ± 0.02^{b}	0.336 ± 0.01^{a}	ND	ND
0.67 (40 min)	4.4 ± 0.1^{b}	5.4 ± 0.8^{a}	1.5 ± 0.1^{a}	1.9 ± 0.5^{a}	0.337±0.001^{b}	0.340±0.001^{a}	2.07 ± 0.19^{b}	3.59 ± 0.47^{a}
3	33.3 ± 0.9^{a}	35.3 ± 1.8^{a}	11.5 ± 0.3^{a}	12.3 ± 0.6^{a}	0.345±0.001^{b}	0.349±0.003^{a}	5.29 ± 0.83^{a}	6.31 ± 1.33^{a}
6	45.9 ± 1.2^{a}	48.9 ± 2.3^{a}	16.1 ± 0.3^{b}	17.3 ± 0.8^{a}	0.352±0.002^{a}	0.354±0.005^{a}	12.56 ± 2.29^{a}	14.35 ± 1.58^{a}
18	63.0 ± 1.8^{b}	68.1 ± 2.3^{a}	22.7 ± 0.5^{b}	24.5 ± 0.9^{a}	0.359±0.004^{a}	0.360±0.005^{a}	21.68 ± 2.51^{b}	28.39 ± 3.94^{a}
24	66.1 ± 2.4^{b}	72.6 ± 2.8^{a}	23.9 ± 1.1^{b}	26.3 ± 1.0^{a}	0.362±0.002^{a}	0.363±0.005^{a}	28.50 ± 4.34^{b}	32.40 ± 2.20^{a}

* Values are LS-means from replicates (n=6).

** Values are LS-means from replicates (n=9).

Means ± standard deviation in the column, with different superscripts, are different ($p<0.05$).

ND: Not Determined.

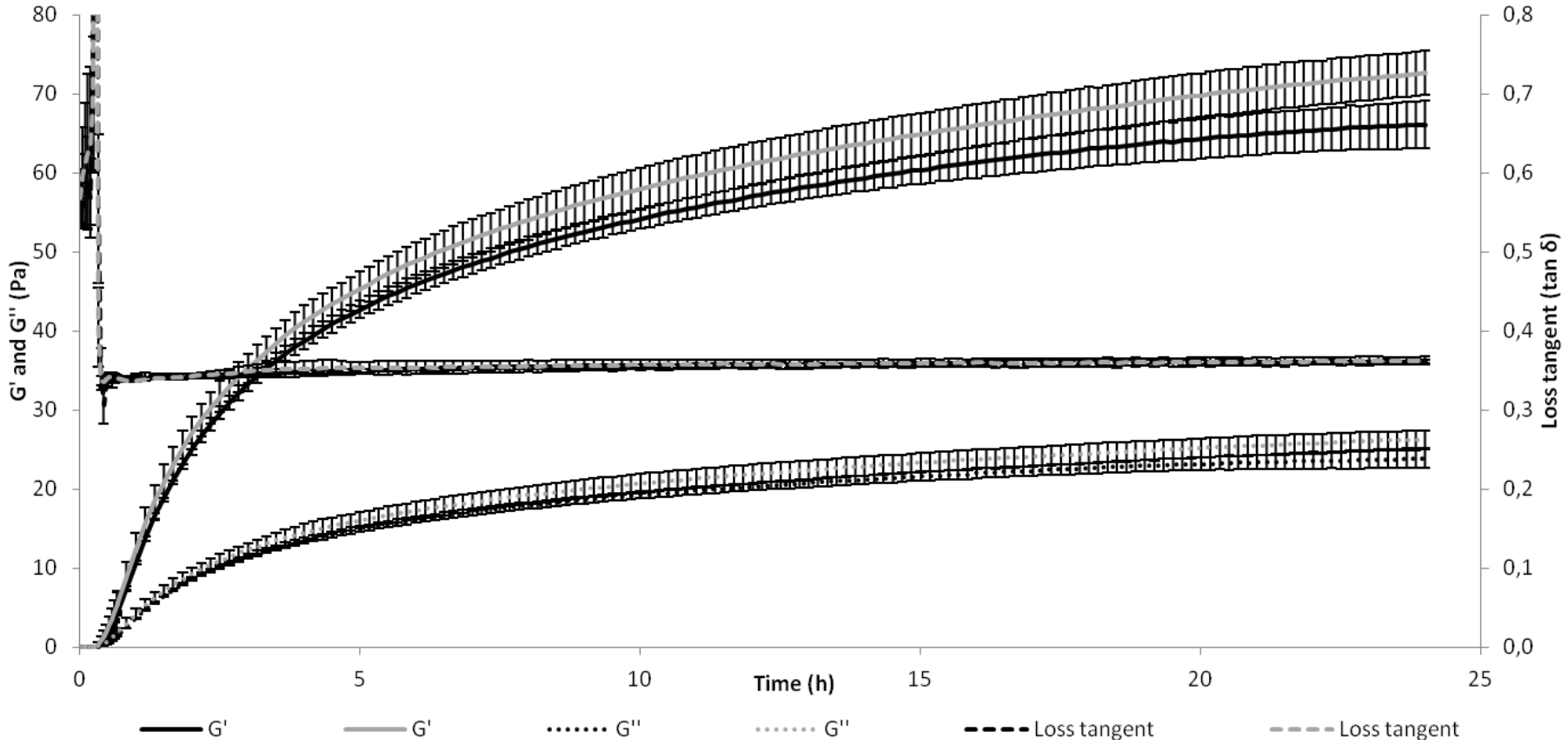

*Figure 5.7 Evaluation of milk coagulation using bovine rennet enzyme subjected to high pressure homogenization (processed at 150 MPa - gray lines) and non-processed (control - black lines), immediately after the process throughout a 24 hours coagulation period at 35 ºC. ** Values are means from replicates (n = 6).*

After 40 minutes of coagulation, the gels produced with the enzyme processed at 150 MPa showed a significant higher syneresis than the produced with non-processed enzyme ($p<0.05$), from 3 to 6 hours of clotting. The gel produced with high pressure homogenized enzyme did not differ from gels produced with the non-processed enzyme ($p>0.05$), after 18 h and 24 hours was observed syneresis higher for the gel coagulated with the enzyme processed ($p<0.05$).

The syneresis naturally happens in curd due to the formation of new bonds between para-casein micelles that results in compression of casein micelles package and expulsion of whey from the gel (Lodaite et al., 2000; Lucey et al., 2000; Lucey, 2001; Castillo et al., 2006). Thus, the higher milk-clotting activity of HPH processed enzyme enables the formation of more linkages in the para-κ-casein in the presence of calcium ions during the coagulation phase (first 40 minutes) promoting a greater compression network with higher expulsion of whey. In addition, the lower proteolytic activity of HPH enzyme possibly let the casein micelle more intact allowing the formation of more bounds between para-casein and leading to stronger contraction of casein matrix and higher syneresis during the 24 hours coagulation.

Table 5.1 and *Figure 5.7* shows the results of rheological parameters of milk gels (during 24h) produced with using bovine rennet non-processed and processed at 150 MPa. The G' values were higher for gels produced with HPH bovine rennet during the first 40 minutes and after 18 hours of coagulation ($p< 0.05$) and an increment on this value were observed up to 24h of evaluation for both enzymes. These results demonstrate that gels obtained with HPH processed enzyme are initially more consistent and stronger and the contraction of casein micelles up to 24h increased more the gel consistence. The non-processed sample showed a similar behavior, however, due to the less contraction of the network (less linkages para-k-casein micelles and higher proteolysis), lower G' values were observed during the 24 hours of observation. Therefore, it is possible to conclude that the HPH process of bovine rennet results in more consistent and solid gels, with higher

viscoelasticity after 24h of coagulation, favoring the formation of firm and compact cheese mass.

The G'' values can be related the water retention properties of the milk gels. The gel produced with HPH processed enzyme showed significant higher values of G'' than the gel obtained by milk coagulation with non-processed enzyme after 6 hours of coagulation to 24 hours ($p<0.05$). That suggests that water diffusivity in casein matrix produced with enzyme processed at 150 MPa is lower. This phenomenon can be explained by the compact gels formed by homogenized enzymes, with more linkages and strong linkages which difficult the whey diffusion.

In gels obtained by enzymatic coagulation, the susceptibility to syneresis has been associated with a high value for the loss tangent at long time scales (van Vliet et al., 1991), being related to the propensity of the network to rearrange after gel formation (Lucey, 2001). Many studies of the relations between syneresis of casein gels and their rheological properties have been presented, however, reports of the effects of the storage modulus (G') on the syneresis behavior are not conclusive (Lelievre, 1977; Zoon, van Vliet, & Walstra, 1988). The analysis of van Vliet et al. (1991) concludes that syneresis is not dependent on G', but increases with increasing tan δ (=G''/G').

The data of tan δ (loss tangent) were also presented on *Table 5.1* and *Figure 5.7*. A tendency of higher loss tangent value for gels prepared with high pressure homogenized enzyme was observed during the 24 hours coagulation. This difference became statistically significant during the first 3 hours ($p< 0.05$), which ratifies the hypothesis of whey expulsion increasing after gel formation due to the higher contraction of protein matrix, leading to formation of a dense network.

Therefore, the results of syneresis and gel rheology highlight that protein network formed was stronger since the beginning of coagulation to the end of bovine rennet-induced gel development. The reduction in proteolytic activity and increase in milk-clotting activity of HPH enzyme allows the formation of more para-κ-casein linkages in the presence of calcium ions and reduced the hydrolysis of casein fractions, leading the formation of a more compact, cohesive, firm and

dense protein network, increasing whey loss accompanied by a reduction in the water diffusivity. These effects observed in gels can be interesting for cheese production, resulting in higher cheese yield (due to the increment on water binding capacity of protein) and higher dry matter (due to the reduction of small peptides loss in the whey).

5.3.2.3. Microstructure of milk coagulation process by bovine rennet

Figure 5.8 shows the images obtained by confocal scanning laser microscopy CSLM during the milk enzymatic coagulation and of the gel formed by using non-processed and homogenized enzyme at 150 MPa. The porosity of the gel, total number of pores and the average pore area are showed in *Figure 5.9* and *Table 5.2*.

These images elucidate the effect of HPH on the bovine rennet behavior during the gel formation and development. The images clearly shows that, after 20 minutes of enzyme addition, the protein aggregation is faster for gels obtained by high pressure homogenized enzyme. Consequently, after 40 minutes, it was observed that the gel obtained with the processed enzyme shows a greater protein aggregation with less pores larger sizes. After 24 h, more compact protein network was observed for gel obtained by using HPH enzyme, being compatible with the results obtained of rheology and syneresis analysis.

The gel porosity at 5 μm from the sample bottom was reduced due to the aggregation and sedimentation of the protein network. On the contrary, the porosity at 20 μm increased because the excessive whey expulsion from the protein matrix. Comparing the results obtained for each enzyme it was observed that during coagulation, the porosity at 5 μm was lower for gel produced with high pressure homogenized enzyme presenting a significant difference after 24 hours of coagulation ($p<0.05$). To the contrary, at 20 μm, the gel porosity were statistically different just after 40 minutes of coagulation, being more porous the gel produced with processed enzyme ($p<0.05$).

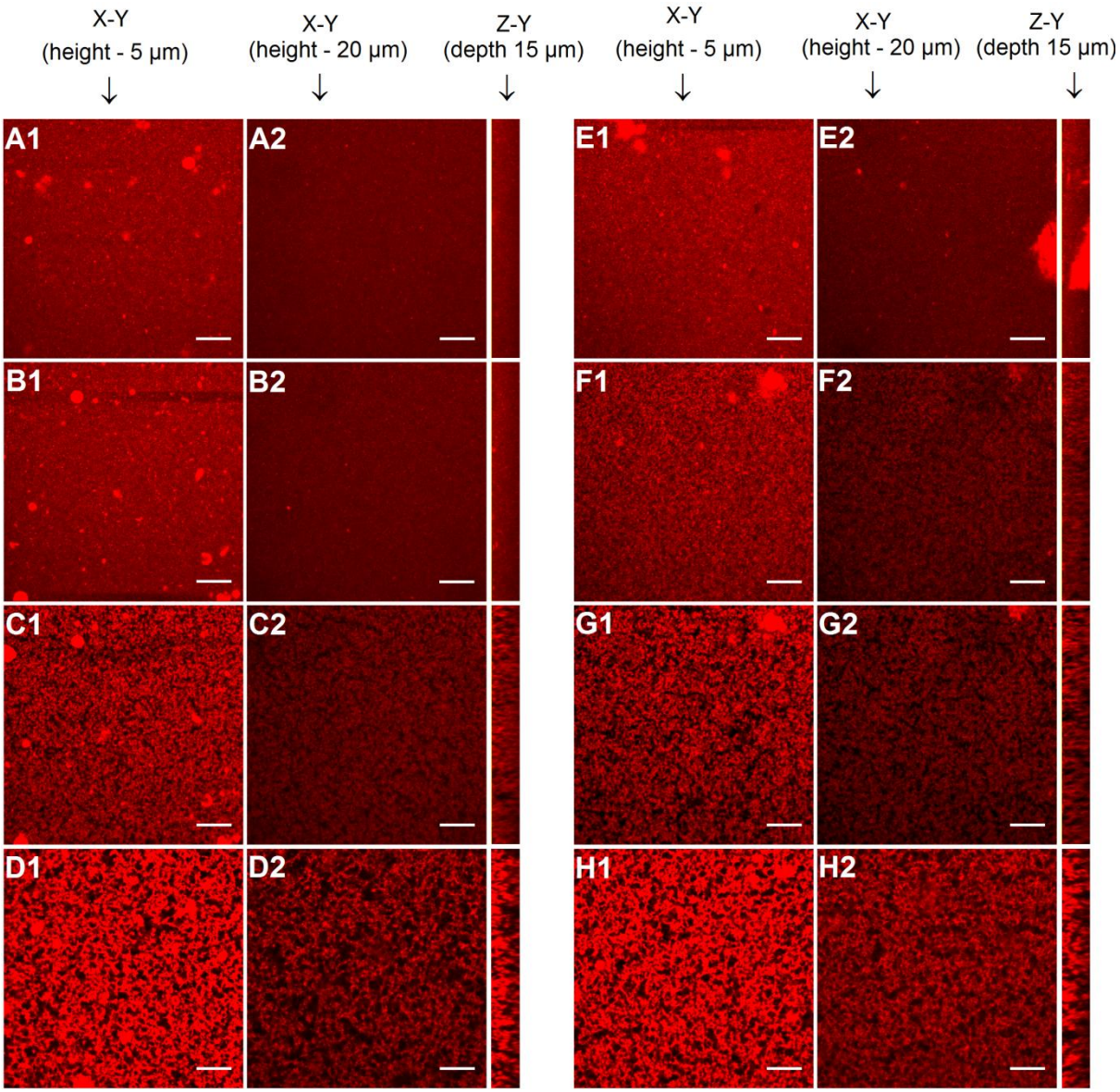

Figure 5.8 CLSM micrographs of rennet-induced gels using bovine rennet processed by high pressure homogenization and bovine rennet non-processed (A1-D1: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; A2-D2: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm; E1-H1: 190 MPa at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; E2-H2: 190 MPa at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm) throughout a 24 hours period at 35 ºC. The fast-green FCF stained protein appears red and the serum phase appears black in these images. Each set of

images is presented in two views: the X–Y (a height of 5 and 20 µm from the bottom – the surface of the coverglass) and the Z–Y (right) projections. For each sample, 20 adjacent planes were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. The scale bars are 20 µm in length.

Additionally, at 5 µm, reduction of total number of pores and increase of the average pore area were observed along the time, which is directly linked to the protein aggregation. The gel obtained with HPH enzyme showed a faster reduction of total pores with increasing pore area, presenting a significant difference (until 40 minutes of coagulation) when compared with gel produced using non-processed enzyme ($p<0.05$). From 40 minutes to 24 hours, it was observed an inversion on the behavior of gels produced with processed and non-processed enzyme, with pores in larger number and smaller areas for gel produced using HPH enzyme.

Images recorded at a distance of 20 µm from the surface of the coverglass, results also showed a reduction of total number of pores and an increase in the average pore area along the coagulation time. Again the gel obtained with HPH processed enzyme showed a faster reduction of total pores with increasing pore area during the first 40 minutes ($p<0.05$). From 40 minutes to 24 hours, it was observed similar behavior with a higher number of pores and lower average pore area for gel produced with processed enzyme ($p<0.05$). This phenomena can possible be attributed to the higher number of linkages para-κ-casein due to increase on milk-clotting activity, resulting in higher compaction and sedimentation of protein network and maintenance of links between casein micelles due to reduction in the proteolytic activity of the HPH processed enzyme.

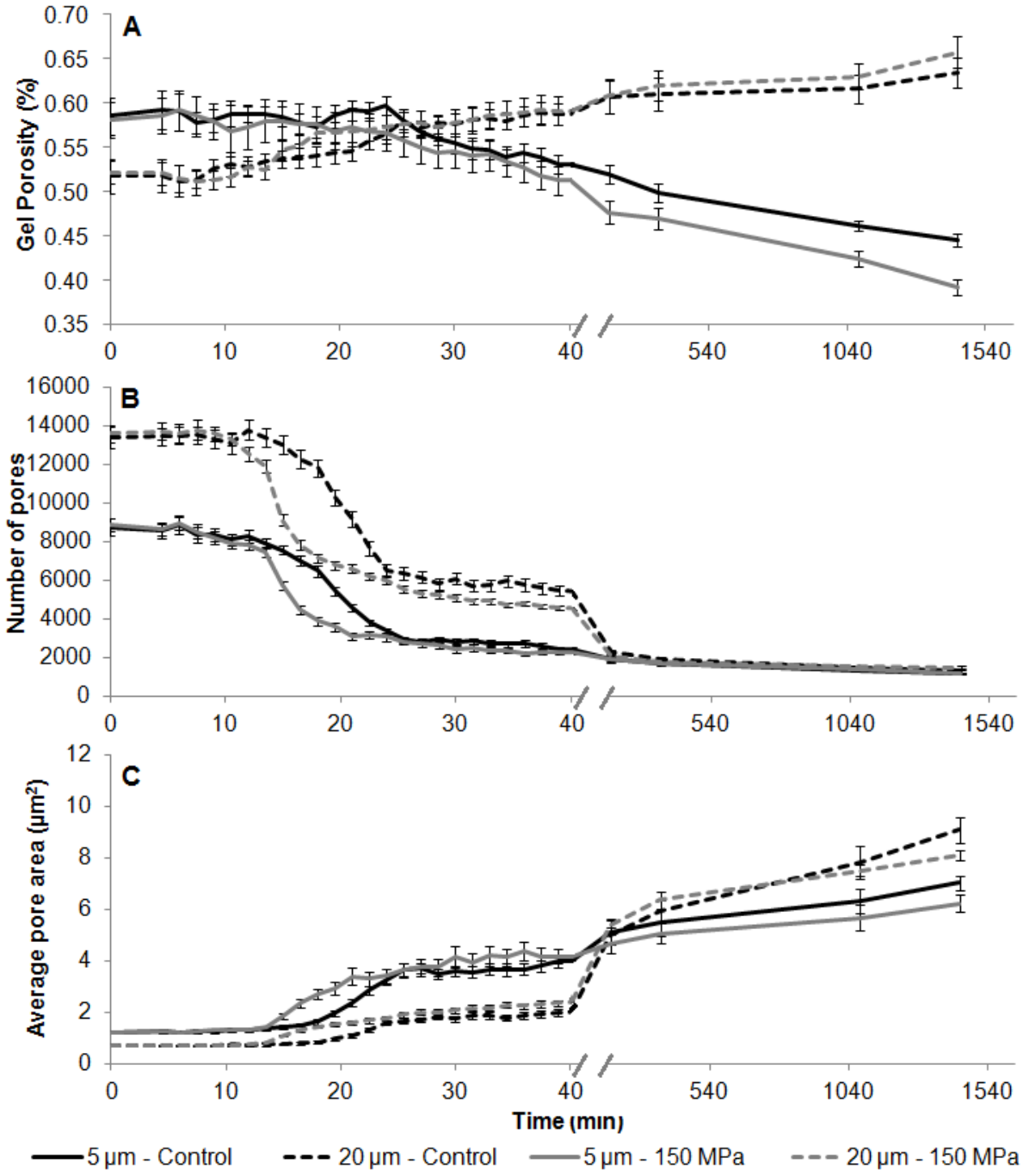

Figure 5.9 Gel porosity (A), number of pores (B) and average pore area (C) of rennet-induced gels using bovine rennet processed by high pressure homogenization and bovine rennet non-processed. Results are expressed as the mean ± the standard deviation (n = 6).

Table 5.2 Gel porosity, average pore area and number of pores of rennet-induced gels using bovine rennet processed by high pressure homogenization and non-processed enzyme

		Sample height 5 µm			
	Sample	0 min (Blank)	20 min	40 min	24 h
Gel Porosity (%)	Control	0.5854 ± 0.0213 [a]	0.5850 ± 0.0135 [a]	0.5313 ± 0.0108 [a]	0.4455 ± 0.0074 [a]
	190 MPa	0.5807 ± 0.0198 [a]	0.5687 ± 0.0210 [a]	0.5126 ± 0.0168 [a]	0.3916 ± 0.0082 [b]
Number of pores	Control	8730 ± 430 [a]	5436 ± 236 [a]	2443 ± 118 [a]	1160 ± 60 [a]
	190 MPa	8832 ± 331 [a]	3564 ± 224 [b]	2242 ± 93 [b]	1168 ± 44 [a]
Average pore area (μm^2)	Control	1.22 ± 0.07 [a]	1.96 ± 0.12 [a]	3.97 ± 0.24 [a]	7.00 ± 0.26 [a]
	190 MPa	1.20 ± 0.05 [a]	2.92 ± 0.24 [b]	4.17 ± 0.27 [a]	6.22 ± 0.35 [b]
		Sample height 20 µm			
	Sample	0 min (Blank)	20 min	40 min	24 h
Gel Porosity (%)	Control	0.5171 ± 0.0188 [a]	0.5435 ± 0.0126 [a]	0.5872 ± 0.0119 [a]	0.6344 ± 0.0168 [a]
	190 MPa	0.5214 ± 0.0130 [a]	0.5657 ± 0.0121 [b]	0.5913 ± 0.0168 [a]	0.6577 ± 0.0182 [a]
Number of pores	Control	13376 ± 529 [a]	10249 ± 416 [a]	5448 ± 289 [a]	1278 ± 95 [a]
	190 MPa	13584 ± 442 [a]	6771 ± 226 [b]	4598 ± 117 [b]	1482 ± 66 [b]
Average pore area (μm^2)	Control	0.70 ± 0.03 [a]	0.97 ± 0.06 [a]	1.97 ± 0.14 [a]	9.07 ± 0.49 [a]
	190 MPa	0.70 ± 0.03 [a]	1.52 ± 0.08 [b]	2.34 ± 0.12 [b]	8.09 ± 0.20 [b]

Results are expressed as the mean ± the standard deviation (n = 6). [a,b] Means ± standard deviation in the row, with different superscripts, are significantly different ($p<0.05$).

Therefore, the gel produced by using bovine rennet processed at 150 MPa is overall less porous, when compared to a similar gel manufactured by using non-processed bovine rennet. This effect occurred due to changes in the activity and specificity of the enzyme by HPH processing, which directly affects the aggregation of the protein network. In the present work, the reduction in proteolytic activity and increase in milk-clotting activity promotes a greater number of connections between fractions of caseins in the presence of calcium ions.

5.4 Conclusion

The high pressure homogenization process is capable to reduce the proteolytic activity of bovine rennet and increase its milk-clotting activity, especially for enzyme processed at 150 MPa. In addition, the gel produced with enzyme processed at 150 MPa had higher rate of milk-clotting resulting in gels more consistent than the one obtained with non-processed enzyme. In addition, after 14 days of storage the coagulation using the enzyme processed at 150 MPa occured 15 % faster and the G' value after 90 minutes of coagulation was 10% higher than the non-processed one, which is possibly linked to improvements in enzyme stability after the HPH process. This highlights that HPH processing can be applied as tool for bovine rennet performance improvement. Moreover, the milk gel produced with bovine rennet processed at 150 MPa was more compact, firm, and cohesive and cause higher whey expulsion from protein matrix, when compared with gel obtained using non-processed enzyme. After 24h of coagulation, the gel produced using high pressure homogenized enzyme kept its consistence due to the lowered proteolytic profile. Therefore, the results highlight the HPH is a process able to improve the hydrolytic characteristics of bovine rennet and can open new markets for this enzyme in cheese making industry.

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Capítulo 6. Influence of high pressure homogenization on commercial protease from *Rhizomucor miehei*: effects on proteolytic and milk-clotting activities

Resumo

Este trabalho estudou a influência da homogeneização a pressão alta (HAP) sobre a atividade de uma protease fúngica comercial. A solução enzimática (2%) foi processada até 190 MPa e atividade proteolítica (AP) e atividade de coagulação do leite (ACL) foram avaliadas durante 30 dias, mas não foram observados diferenças na AP e ACL entre as amostras em quase todos os tempos avaliados, possivelmente indicando que a energia promovida pelo processo não foi capaz de modificar a estrutura da enzima. Os efeitos de múltiplas passagens (três ciclos) e uma alta concentração de solução de enzima (20%) também foram avaliados a 25 MPa e 190 MPa. As múltiplas passagens a 190 MPa aumentaram a AP da enzima (aumento de ~ 6 %), mas não alteraram a ACL. Aumento na AP (~ 3 %) e ACL (~ 10 %) foram observados para enzima processada (190 MPa) em solução com alta concentração e consequentemente melhorou a fase de coagulação (rápida coagulação e maior consistência do gel). Portanto, HAP pode ser usada como uma operação unitária para melhorar a competitividade dessa protease fúngica como coagulante do leite.

Palavras-Chave: *Alta pressão dinâmica • Protease fúngica • Atividade proteolítica • Atividade de coagulação do leite*

Abstract

This work studied the influence of high pressure homogenization (HPH) on the activity of a commercial fungal protease. The enzyme solution (2%) was processed up to 190 MPa, and the proteolytic activity (PA) and milk-clotting activity (MCA) were evaluated during 30 days, but no differences in PA and MCA were observed between the samples, possibly indicating that the energy provided by the process not modify the structures of this enzyme. The effects of multi-pass (three cycles) HPH and a high concentration of enzyme (20%) were also evaluated at 25 MPa and 190 MPa. The multi-pass at 190 MPa increased PA (~6%) but did not alter MCA. Increases in PA (~3%) and MCA (~10%) were observed for the enzymes processed (190 MPa) at high concentration and consequently improved the coagulation phase (faster clotting and higher consistency of the milk gel). Therefore, HPH can be used as a unitary operation to improve the competitiveness of this fungal protease as milk coagulant.

Key-words: *Dynamic high pressure • Fungal protease • Proteolytic activity • Milk-clotting activity*

6.1 Introduction

Traditionally, cheese makers extensively use calf rennet, a clotting preparation extracted from calf abomasums that contains two enzymes: chymosin and pepsin (Rolet-Répécaud et al., 2013). However, the supply of calf rennet is decreasing considerably while the world cheese production is increasing at around 4% annually (Food Agriculture Organization [FAO], 2010; Euromonitor International, 2012). Hence currently, only 20-30% of the cheeses produced worldwide are produced using calf rennet (Jacob, Jaros, & ROHM, 2011) and there is therefore a growing interest in alternative enzymes to replace calf rennet (Walstra et al., 2006; Møller et al., 2012).

Potential substitutes should emulate the specific properties, with high specificity and good proteolytic activity at the pH value and temperature of cheese manufacturing (Fox & Kelly, 2004; Kumar et al., 2010). Furthermore, it must comply with the rules and regulations and with the technological and economic constraints, as well as the demands of the target market (kosher certification, organic or vegetarian approval) (Rolet-Répécaud et al., 2013).

The most used rennet substitutes from microbial sources are the *Miehei* coagulant (*R. miehei* proteinase), *Pusillus* coagulant (*R. pusillus* proteinase) and the *Parasitica* coagulant (*C. parasitica* proteinase) (Andrén, 2011). The *Miehei* coagulant dominates the microbial coagulant market (35% of the coagulants used in France) and is produced in two different heat-labile forms, which are less proteolytic than the native one (Syndicat Professionnel des Producteurs d'Auxiliaires Pour l'Industrie Laitière [SPPAIL], 2010; Andrén, 2011).

High pressure homogenization (HPH) has been proposed as a physical process capable of modifying the structure of enzymes, with consequent improvement of their activity, specificity and stability (Tribst, Augusto, & Cristianini, 2012b; Tribst, Augusto, & Cristianini, 2013). However, other authors did not observe such changes in protein conformation after the HPH processing of specific enzymes (Bouaouina et al., 2006; Tribst, Augusto, & Cristianini, 2012a).

These changes are usually attributed to modifications caused by HPH in the quaternary, tertiary and secondary structures of the enzymes (Lacroix, Fliss, & Makhlouf, 2005; Liu et al., 2009b; Liu et al., 2010). Chemically, HPH causes an increase in the exposure of sulfhydryl groups and a reduction in the number of SH groups available, indicating that the process causes molecular denaturation and unfolding at the same time, which favors the formation of disulfide bridges (Liu et al., 2009b, Liu et al., 2010). In addition, HPH processing increases the exposure of tyrosine and tryptophan residues (Liu et al., 2009b, Liu et al., 2010), modifies molecule hydrophobicity (Liu et al., 2009b, Liu et al., 2010), alters exposure of the enzyme active sites (Vannini et al., 2004; Lanciotti et al., 2007; Iucci et al., 2007), reduces inter and intra molecular hydrogen bonding (Liu et al., 2010) and changes the composition (α-helix and β-sheet) of the enzyme secondary structure (Liu et al., 2009b).

Moreover, multi-pass HPH may improve the activity of some enzymes (Liu et al., 2009a; Liu et al., 2009b; Tribst, Augusto, & Cristianini, 2013). The main advantage of the multi-pass process is the possibility of applying relatively low pressures, with a consequent reduction in processing costs (equipment and operation).

Thus, this study evaluated the influence of high pressure homogenization on the proteolytic and milk-clotting activities and stability of a commercial *Rhizomucor miehei* protease used in cheese manufacture and the effect of multi-pass HPH and high enzyme concentrations on HPH processing.

6.2 Material and Methods

6.2.1 *Rhizomucor miehei* protease and high pressure homogenization processing

A commercial *Rhizomucor miehei* protease was used in the experiments (Marzyme 150 MG Powder Microbial Rennet, Danisco, Vinay, France).

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the trials. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

A volume of 2 L of a 2.0% (w/v) *Rhizomucor miehei* protease solution prepared in 0.2M sodium acetate buffer (pH 5.6), was processed at pressures of 0, 50, 100, 150 and 190 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled in an ice bath to 23 ºC. A non-processed sample of *Rhizomucor miehei* protease was evaluated as the control sample. The experiments were carried out on different days using different enzyme solutions.

6.2.2 Relative proteolytic activity (RPA) determination

The proteolytic activity of the *Rhizomucor miehei* protease was measured according to Merheb-Dini et al. (2010): the enzyme solution (2.0% w/v) was prepared in a 0.2 M acetate buffer (pH 5.6) and 600 µL of the enzymatic solution added to 400 µL of a 0.5% (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at 35ºC/40 min in a shaken water bath (62 rpm) and 1 mL of 10% (w/v) trichloroacetic acid (TCA) then added to stop the hydrolysis. The samples were centrifuged at 2300 *g*/5 min/10 ºC and the absorbance measured at 280 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution and the ΔAbs_{280nm} was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to *Equation 6.1*.

$U/mL = (\Delta Abs_{280nm} \times 10 \times \text{dilution factor} / 0.6 \times 40)$ (*Equation 6.1*)

RPA assays were carried out immediately after processing (time 0h) and after 1, 2, 3, 4, 5 and 30 days. The samples were stored under refrigeration (4ºC) throughout the period, and a non-processed sample used for comparative

purposes. The relative proteolytic activity (RPA) was calculated considering the activity of the HPH and non-processed samples according to *Equation 6.2*:

RPA = (enzyme activity$_{after_HPH_and/or_storage}$/ enzyme activity $_{non\text{-}processed\_sample\_at\_0h}$)*100 (*Equation 6.2*)

6.2.3 Relative milk-clotting activity (RMCA) determination

The milk-clotting activity was determined according to Merheb-Dini et al. (2010). A volume of 5 mL of skimmed milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids, Tagará Foods, Brazil) was added to a 0.01 M $CaCl_2$ solution and pre-incubated at 35 °C/10 min. A 0.5 ml aliquot of the enzyme solution (0.002%, w/v, prepared in 0.1 M sodium acetate buffer, pH 5.6) was then added and the time count started. Clot formation was determined by manual tube rotation and the time taken for the first particles to form measured. One milk-clotting activity unit (MCA) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 ºC. The MCA was calculated using *Equation 6.3*:

Units of milk-clotting activity (MCA) = 2400/*t* x S/E (*Equation 6.3*)

Where *t* is the time (seconds) necessary for clot formation, S is the milk volume and E the enzyme volume.

MCA assays were carried out immediately after processing (time 0h) and after 1, 2, 3, 4, 5 and 30 days. The samples were stored under refrigeration (4ºC) throughout the period and a non-processed sample used for comparative purposes. The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to *Equation 6.4*:

RMCA = (MCA$_{after_HPH_and/or_storage}$/ MCA $_{non\text{-}processed\_sample\_at\_0h}$)*100 (*Equation 6.4*)

6.2.4 Rheological assays

The milk coagulation was evaluated by monitoring the milk coagulation process by way of a time sweep using a low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments, USA). These assays were carried out with the processed and non-processed *Rhizomucor miehei* protease samples.

The experiments were carried out according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skimmed milk powder reconstituted at 10% (w/v) (pH 6.65, 3.2% protein, 9.2% non-fat solids, Tagará Foods, Brazil) with the addition of a 0.01 M $CaCl_2$ solution. This mixture was pre-incubated at 35 °C/10 min, and 0.8 mL of enzyme solution (0.02%, w/v) prepared in 0.1M acetate buffer (pH 5.6) subsequently added. The mixture was immediately transferred to the rheometer cup (30 mm diameter and 80 mm height), which had a *vaned quarter* geometry (with 28mm of diameter and 42 mm of length) and a 4 mm *gap*. The stress was set at 0.1 Pa and frequency at 0.1 Hz. The parameter G' (storage modulus) was measured at 3 min intervals for 90 min of the clotting process at 35 °C. The temperature was controlled by a *Peltier* system. The rheological assays were carried out immediately after processing (time 0h) and after 30 days. The samples were stored under refrigeration (4ºC) throughout the period.

Furthermore, the rate of milk-clotting was calculated as the variation in G' with the variation in time as the log (dG'/dt) at three min intervals, and expressed in $Pa.min^{-1}$. The storage modulus (G') describes the elastic (solid) behavior of the product, and consequently the energy stored and released in each oscillatory cycle.

6.2.5 High pressure homogenization processing with multi-passes

A volume of 3 L of enzyme solution 2.0% (w/v) was prepared using 0.2 M sodium acetate buffer (pH 5.6). The samples were then homogenized using

pressures of 25 and 190 MPa and an inlet temperature of 23 ºC. The samples were homogenized using 3 consecutive passes. After each pass, the samples were immediately cooled to 23 °C using a shell and tube heat exchanger, aiming to guarantee that the final temperature after each homogenization step was the same. The residence time at the temperature reached after the HPH valve (<10 s) was estimated considering the equipment flow and the distance between the homogenization valve and the heat exchange inlet. The time spent between the consecutive processes was lower than 5 min and the final process time was around 15 min.

After each pass (one, two and three), a total of 100 mL sample was collected and the non-processed enzyme was evaluated as the control (zero passes). The temperatures were measured using a digital T type thermocouple (Multithermometer ® (Brazil)). The RPA and RMCA were determined and the rheological assays carried out immediately after the end of the process. The maximum pressure levels were chosen considering the operational capacity of the equipment.

6.2.6 Effect of HPH processing on high enzyme concentrations

A volume of 2 L enzyme solution at concentrations of 20% (w/v) was prepared using 0.2 M of sodium acetate buffer (pH 5.6) and homogenized under pressure of 25 and 190 MPa using an inlet temperature of 23 ºC. Then, 200 mL sample were collected and cooled in an ice bath to 23 ºC. Non-processed enzyme was evaluated as the control (zero passes, 20% (w/v)).

Analyses of RPA, RMCA and rheological assays were measured immediately after the end of the HPH process. The maximum pressure levels were chosen considering the operational capacity of the equipment. The experiments were carried out on different days using different enzymes solutions.

6.2.7 Statistical analysis

The processes and analyses were carried out with three repetitions and each experimental unit was carried out in quadruplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and the Tukey test to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software– (StatiSoft, Inc., Tulsa, Okla., U.S.A.) and the results were presented as the mean ± standard deviation.

6.3 Results and Discussion

6.3.1 Effect of high pressure homogenization on the relative proteolytic activity

Figure 6.1 shows the results for relative proteolytic activity after the enzyme process at different pressures and during 30 days of storage. For the activity measured immediately after HPH, the RPA results showed a slight increase at 150 MPa (~105%) and 190 MPa (~104%), although not statistically significant ($p>0.05$). During storage, no difference was observed between the processed and non-processed samples ($p>0.05$). In addition, after 30 days of storage, the RPA of the control sample and the samples processed at 150 and 190 MPa were 95, 95 and 92%. These results confirm that this enzyme is highly stable when stored in solution at pH 5.6 and refrigerated temperatures.

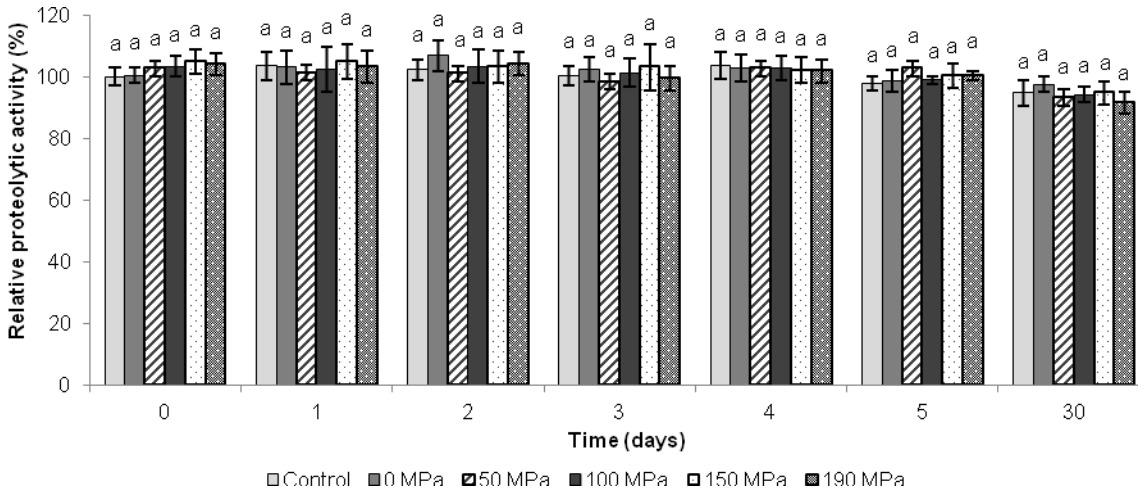

Figure 6.1 Evaluation of the proteolytic activity of the Rhizomucor miehei protease subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed Rhizomucor miehei protease samples evaluated at each time point

Other researchers also evaluated the effect of HPH on enzymes and observed that the process was able to improve (Liu et al., 2009 a,b; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012b,c), reduce (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009; Velázquez-Estrada et al., 2012; Tribst, Augusto, & Cristianini, 2012b) or not alter (Tribst & Cristianini, 2012a) the activity of the enzymes. The effects of HPH were dependent on the pressure level of homogenization applied, the temperature of the enzyme during the process, the nature of the enzyme studied, the pH of homogenization and the presence/absence of substrate during homogenization (Liu et al., 2009 a,b; Tribst & Cristianini, 2012a,b,c; Tribst, Augusto, & Cristianini, 2012 a,b). In general, enzymes with no quaternary structure and showing good thermal resistance were also resistant to HPH processing (Tribst, Augusto, & Cristianini, 2012 a).

6.3.2 Effect of high pressure homogenization on the relative milk-clotting activity

Figure 6.2 shows the results for milk-clotting activity after enzyme processing at different pressures and during 30 days of storage. Similar to the results obtained for RPA, no differences were observed between the processed and non-processed samples immediately after treatment ($p>0.05$). After 3 days of storage, the samples processed at 0 MPa, 50 MPa and 100 MPa showed slightly higher milk-clotting activity (103.68%, 103.12%, 104.61%, respectively) than the enzyme processed at 190 MPa (98.39%) ($p<0.05$). However, from the 5th to the 30th day no difference was observed amongst the samples ($p>0.05$). The RMCA of all the samples (non-processed and HPH processed) only showed a small reduction during the storage period, suggesting that the configurations of the active sites of the enzyme were preserved even when the enzyme was processed at 190 MPa.

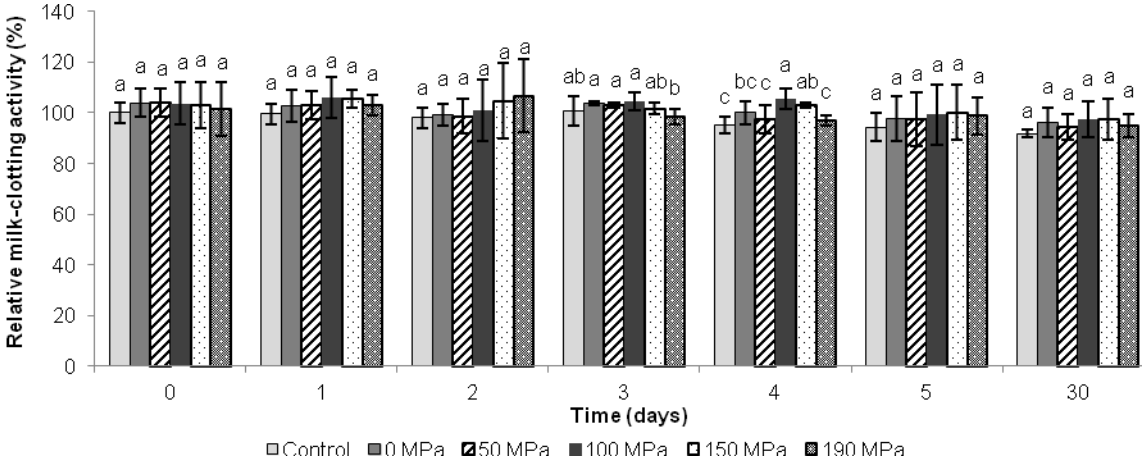

Figure 6.2 Evaluation of the milk-clotting activity of the Rhizomucor miehei protease subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed Rhizomucor miehei protease samples evaluated at each time point

The aspartic protease produced by *Rhizomucor miehei* consists of a single polypeptide chain with great similarity to chymosin in its three-dimensional structure (Chitpinityol & Crabbe 1998). As with chymosin, the proteinase from *Rhizomucor miehei* preferably hydrolyzes the Phe_{105}-Met_{106} bonds of κ-casein, although it can also cleave other bonds of the κ-casein (Chitpinityol & Crabbe, 1998; Fox et al., 2000).

Most of the enzymatic activity of the coagulant added to the milk during cheese making is lost in the whey, and only 0–15% of the activity remains in the curd (Sousa, Ardo, & McSweeney, 2001). However, the coagulant retained in the curd, which also exhibits proteolytic action, is one of the main agents responsible for the breakdown of caseins during cheese ripening, a process known as primary proteolysis, especially during the first 24 h after milk-clotting (Silva & Malcata, 2004).

Considering the results obtained for RPA and RMCA it was concluded that HPH did not alter the enzyme studied. Therefore no differences were expected between the milk coagulation profile and the characteristics of the cheeses obtained using the non-processed and HPH processed enzymes.

6.3.3 Rheological evaluation

Figure 6.3 shows the storage modulus (G'), which describes the elastic (solid) behavior of the product and can represent the phenomenon of milk coagulation. The beginning of aggregation can be better visualized using a log scale (*Figure 6.3 a2 and b2*). It was observed that the aggregation process started after approximately 24 min for the sample processed at 190 MPa, after 27 min for samples processed at 50 MPa, 100 MPa and 150 MPa and for the control sample, and after 30 min for the sample processed at 0 MPa. Ninety min after coagulation, it was found that the sample treated at 190 MPa and the non-processed sample had the highest values for G', being more consistent that the samples processed at 0 MPa, 50 MPa, 100 MPa and 150 MPa.

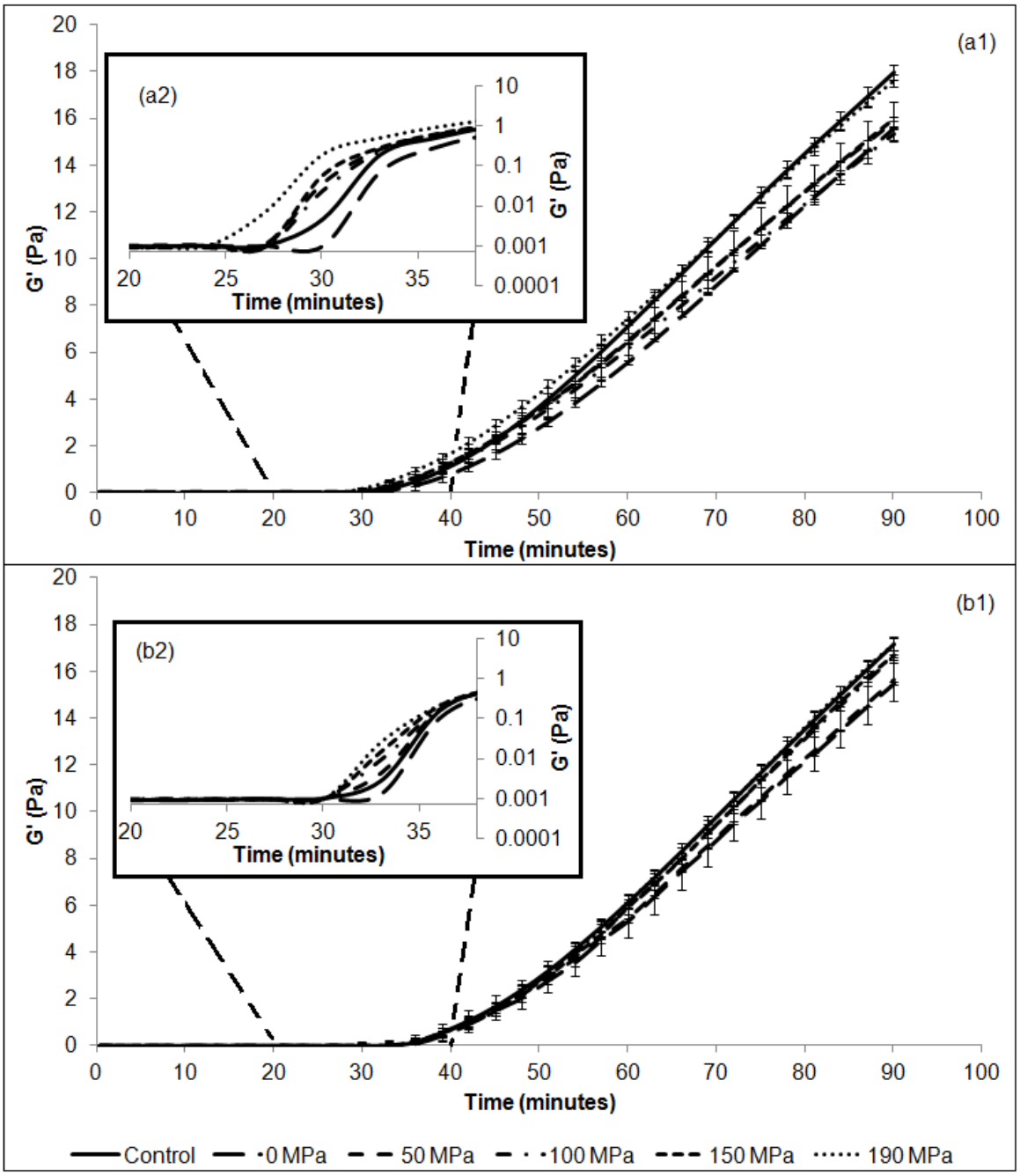

Figure 6.3 Evaluation of milk coagulation (a1) immediately after the process (0h) (a2) log scale, and (b1) after 30 days stored at 4 °C (b2) log scale

After 30 days of storage at 4 °C the rheological assays showed a decrease in the milk-clotting activity after storage, evidenced by the increase in time to start

the aggregation process. An increase in the pressure applied improved the stability of the enzyme during storage in solution, which was verified by the more rapid start of aggregation and the higher values for G' at the end of coagulation.

The rate of milk-clotting is calculated from the variation in G' with time and the highest speeds occurred at the moment aggregation began. *Figure 6.4* shows the milk-clotting rate obtained using the non-processed and HPH processed enzymes.

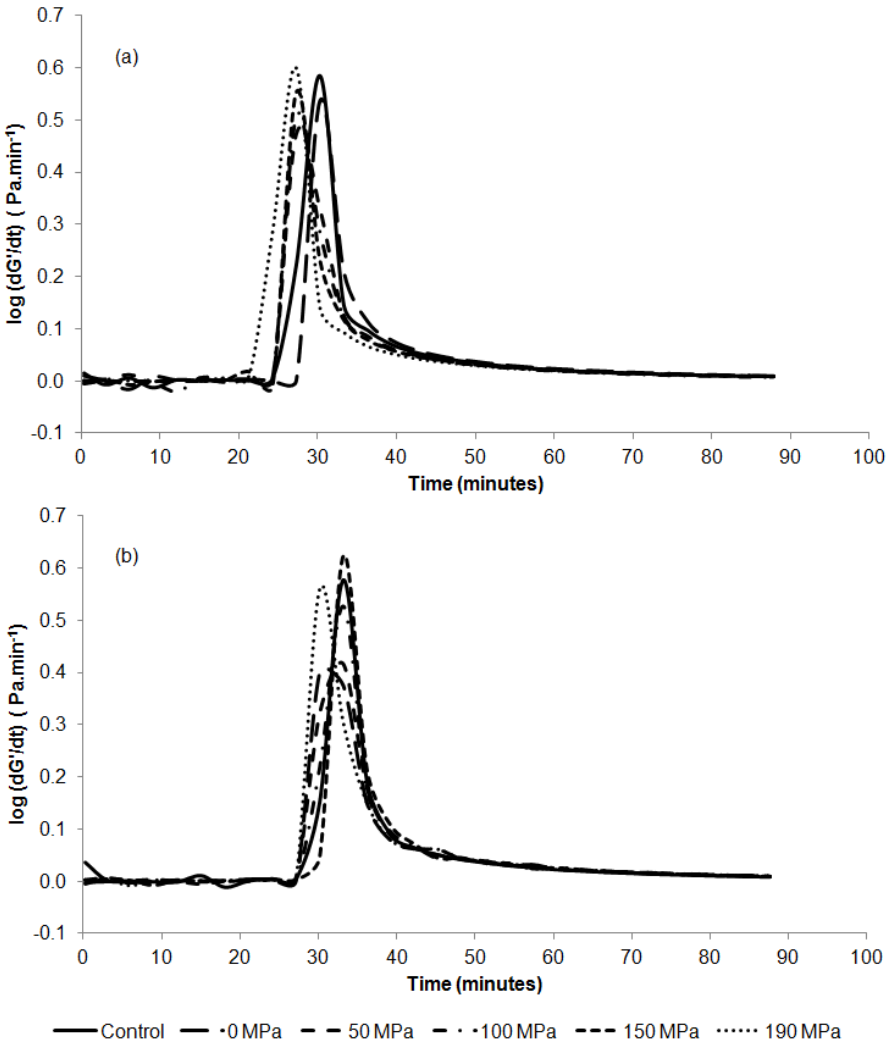

Figure 6.4 Rate of milk-clotting by the action of the enzyme subjected to high pressure homogenization (a) immediately after the process (0h) and (b) after 30 days storage at 4 °C

The results showed that the sample processed at 190 MPa presented a faster start and a greater initial velocity. Consequently, protein aggregation in cheese produced with an enzyme processed above 190 MPa was faster, and the curd obtained was more rigid, improving the cheese manufacturing time and the consistency of the product. Therefore, the results obtained suggested that the HPH process positively improved the milk-clotting kinetic parameters.

6.3.4 Multi-passes of high pressure homogenization processing

Figure 6.5 shows the results obtained for RPA and RMCA for the enzyme processed with up to three passes at 25 MPa and 190 MPa. The results showed that a significant increment in RPA (105.99%) was only observed after 3 passes at 190 MPa. Although statistically significant, this was only a small increment with no effects on the cheese manufacture. For RMCA, only the results for the sample processed at 25 MPa showed a significant change; with a reduction in activity to 92.5% (two passes) and 90.7% (three passes). Thus the results corroborate the idea that this enzyme is highly stable to the HPH process, requiring 3 consecutive passes to obtain slight changes in the RPA and RMCA values.

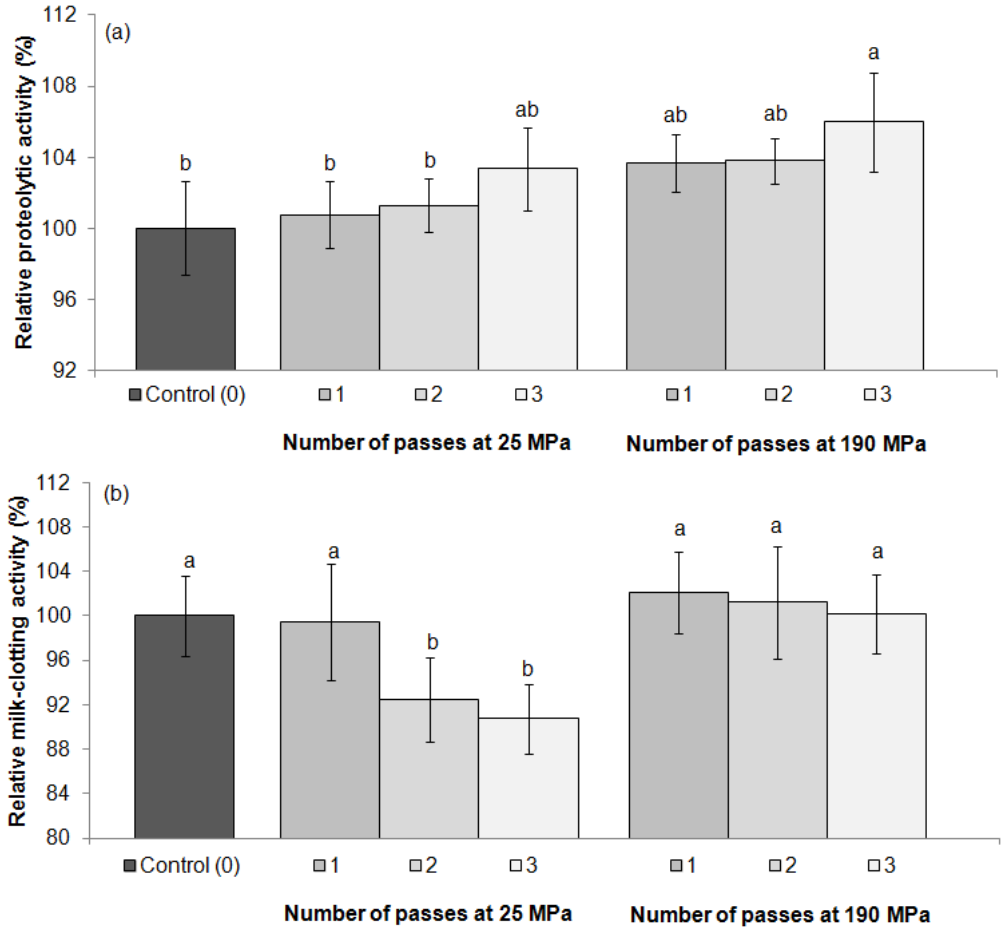

Figure 6.5 Effects of the number of passes on (a) the relative proteolytic activity and (b) the relative milk-clotting activity of Rhizomucor miehei protease evaluated immediately after processing at 25 and 190 MPa

Tribst, Augusto, & Cristianini (2013) found that the process at 150 and 200 MPa with up to three passes caused a 60% reduction in the activity of a neutral protease at 60ºC. To the contrary, a 20% increase in the protease activity was observed at 20 ºC after two passes at 200 MPa. Other studies demonstrated that the activity of pectin methylesterase was not affected by five HPH passes at 100 MPa (Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009) or at 170 MPa (Lacroix, Fliss, & Makhlouf, 2005), while the polyphenol oxidases from mushrooms and from pears showed a significant improvement in activity after three HPH

passes at 150 and 160 MPa, respectively (Liu et al., 2009a, Liu et al., 2009b). Therefore, it is not possible to establish a standard behavior for enzymes processed with multi-passes by HPH.

Figure 6.6 shows the milk storage modulus (G') as a function of time during milk coagulation using the non-processed enzyme and those processed at 25 and 190 MPa for one, two and three passes. The results showed that coagulation started after 27 min for samples processed at 190 MPa (independent of the number of passes), after 28 min for the non-processed sample and after 29/30 min for samples processed at 25 MPa. After 90 min, it was observed that the process at 190 MPa was able to increase the G' value (17.36, 17.13 and 16.89 for 1, 2 and 3 passes) when compared with the G' value of the non-processed sample (G' = 16.20). On the contrary, the process at 25 MPa reduced the G' values (15.17, 13.72 and 12.28 for 1, 2 and 3 passes). Thus these results indicated that the application of multi-passes at low pressure slightly reduced the curd formation ability of the enzyme and the consistency of the curd. However, multi-passes at high pressure (190 MPa) caused the opposite effect, incrementing the ability of the protease to form milk curds and increasing the consistency of these curds. Although the differences were small, this may indicate that different levels of pressure might affect the structure of the protease in different ways.

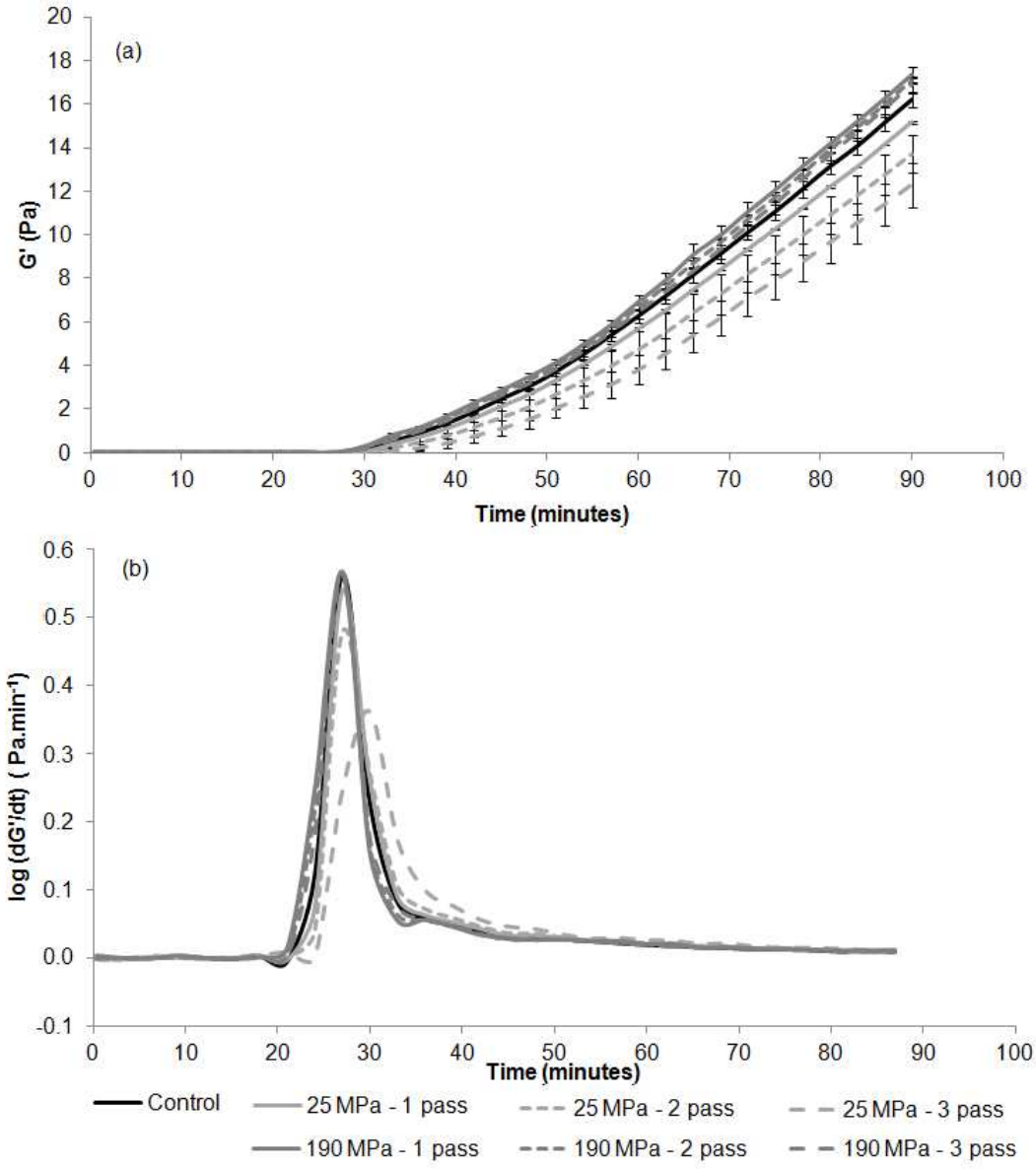

Figure 6.6 Effects of the number of passes on (a) the milk coagulation and (b) milk-clotting rate

6.3.5 Effect of HPH processing on high enzyme concentration

The results shown in *Figure 6.7* represent the values obtained for the RPA and RMCA of the protease processed at a concentration of 20%. At this

concentration, a slight increase in proteolytic activity (102.65%) was observed as well as in the milk-clotting activity (109.97%), ($p<0.05$) after processing at 190 MPa.

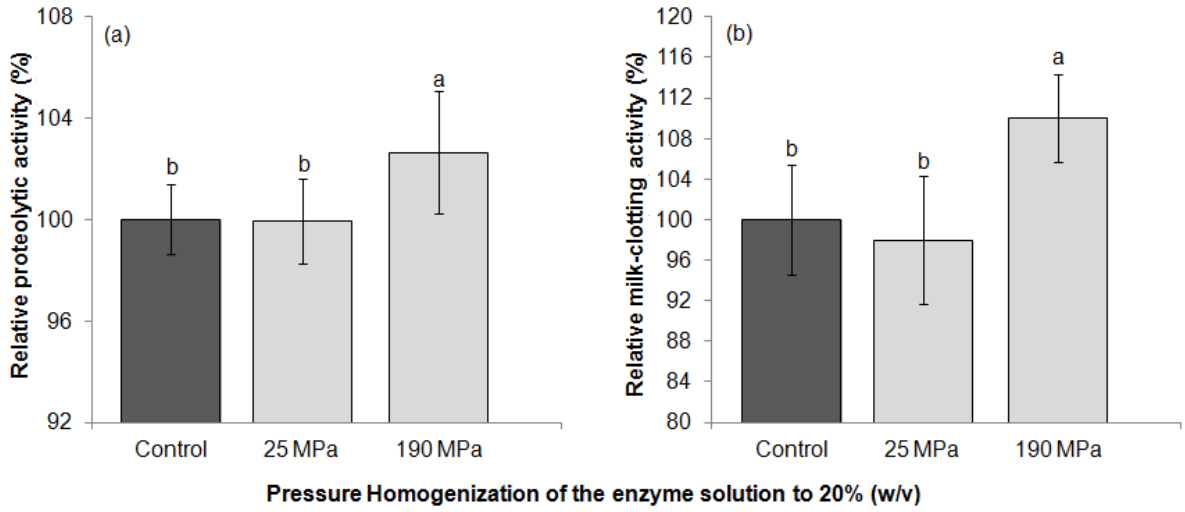

Figure 6.7 The relative proteolytic activity (a) and the relative milk-clotting activity (b) of a high enzyme concentration (20%) of Rhizomucor miehei subjected to HPH processing

The coagulation curves (*Figure 6.8*) show that the gel formed using the enzyme processed at 190 MPa starts aggregating after 27 min, whilst that processed at 25 MPa and the control started aggregating after 30 min. Ninety min after coagulation, it was found that the G' values of the gels were 14.69, 14.54 and 16.32 Pa, using the non-processed enzyme and those processed at 25 and 190 MPa, respectively. Thus the sample processed at 190 MPa presented slightly faster gel formation and a more consistent gel.

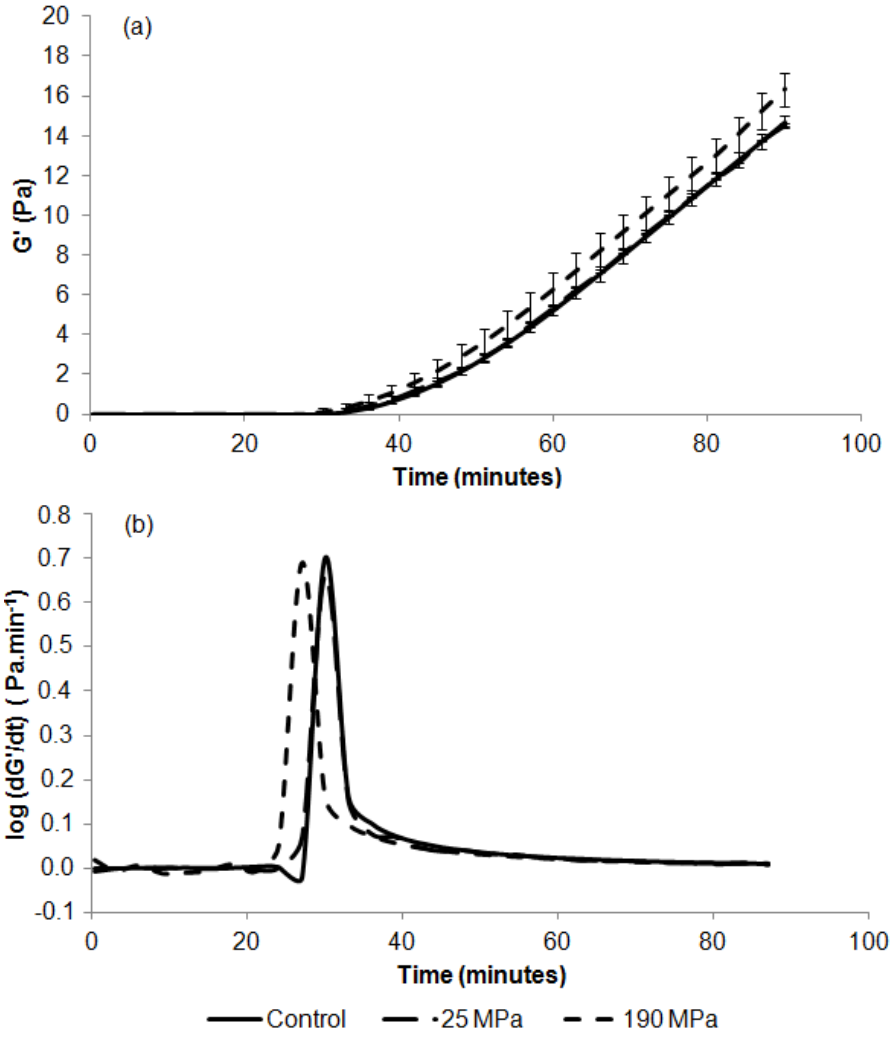

Figure 6.8 High enzyme concentration (20%) subjected to HPH processing: Effects on (a) the milk coagulation and (b) the milk-clotting rate

6.4 Conclusions

The fungal protease from *Rhizomucor miehei* is highly resistant to the high pressure homogenization process, with no changes in the proteolytic and milk-clotting activities when processed at pressures of up to 190 MPa. However, the sample processed at 190 MPa had a higher coagulation rate and formed more consistent gels. For enzyme high pressure homogenized at high concentration

(20%), the process increased milk-clotting activity, improving the coagulation phase and the quality of the product (faster clotting and higher consistency of the milk gel). Therefore, the HPH can be used as a unitary operation to improve the competitiveness of this fungal protease as milk coagulant.

Acknowledgements

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Capítulo 7. Porcine pepsin protease modified by high pressure homogenization: characterization of the coagulation process and gel development

Resumo

Esse trabalho estudou o efeito da HAP (até 190 MPa) na pepsina suína (atividades proteolíticas e de coagulação do leite) e a consequência do uso desta enzima processada na formação e desenvolvimento do gel (perfil reológico, proteólise, sinérese e microestrutura). A atividade proteolítica (AP) reduziu durante a estocagem da enzima; amostras homogeneizadas a 50, 100 e 150 MPa apresentaram uma menor AP (~5%) comparado com as amostras processada 190 e não processada. A atividade de coagulação de leite (ACL) aumentou após a enzima ser homogeneizada a 150 MPa, sendo 15 % maior que a amostra não processada após 60 dias de estocagem. A enzima processada a 150 MPa produziu uma coagulação mais rápida e um gel mais consistente (G' 92% maior aos 90 minutos) quando comparado com a não processada. Além disso, o gel produzido com a enzima processada a 150 MPa apresentou maior sinérese (formando uma rede proteica mais compacta), menor porosidade (evidenciado pela análise de microscopia de confocal) e maior aprisionamento de água (maiores valores de G''). Estes efeitos no gel podem ser ligados ao aumento da ACL e a redução da AP causada pela HAP na pepsina. Portanto, os resultados destacam a HAP como um processo capaz de melhorar as características hidrolíticas de pepsina suína permitindo a inserção desta enzima na fabricação de queijos.

Palavras-chave: *Alta pressão dinâmica* • *Pepsina suína* • *Coagulação enzimática do leite*

Abstract

This work studied the effect of HPH (up to 190 MPa) on porcine pepsin (proteolytic and milk-clotting activities) and the consequences of using this processed enzyme in the milk gel formation and its characteristics (rheological profile, proteolysis, syneresis and microstructure). The proteolytic activity (PA) reduced during the enzyme storage; samples homogenized at 50, 100 and 150 MPa showed lower PA (~5%) than control and processed sample at 190 MPa. The milk-clotting activity (MCA) increased after enzyme homogenized at 150 MPa, being 15% higher than non-processed sample after 60 days of storage. The enzyme processed at 150 MPa produced a faster and consistent milk gel (G' value 92% higher at 90 minutes) when compared with non-processed one. Additionally, the gels produced with the enzyme processed at 150 MPa showed higher syneresis (forming a more compact protein network), lower porosity (evidenced by confocal microscopy) and higher trapping of water (higher G'' values). These effects on milk gel can be linked to the increment of MCA and reduction of PA caused by HPH in the pepsin. Therefore, the results highlight the HPH as a process able to improve the proteolytic characteristics of pepsin porcine, supporting the insertion of this enzyme for cheesemaking.

Key-words: *Dynamic high pressure • Porcine pepsin • Enzymatic coagulation of milk*

7.1 Introduction

The search for enzymes to replace calf rennet is increasing due to the decrease in supply of this rennet (Walstra et al., 2006), since there is a tendency to reduce early slaughter of steers due to the low achievers in terms of meat production (Food Agriculture Organization [FAO], 2010) and the growth of global production of cheese (about 4% per year) (Euromonitor International, 2012).

Potential replacements should emulate their specific properties, with high specificity and good proteolytic activity at pH and temperature of cheesemaking (Fox & Kelly, 2004; Kumar et al., 2010). However, the application of these enzymes in the coagulation of milk could result in problems such as reduction of yield at manufacturing (due to the intense activity of these proteolytic enzymes) and the appearance of defects in flavour and texture (especially the bitterness flavour and brittle texture) (Møller et al., 2012).

Porcine pepsin, enzymatically classified as a pepsin B (EC 3.4.23.2), is a protease found in porcine stomachs. This enzyme has milk-clotting activity (Nielsen & Foltmann, 1995; Harboe, Broe, & Qvist, 2010), however, its proteolytic activity show low specificity in the hydrolysis of bounds with Phe, Tyr, Leu or Val residues (Agudelo et al., 2004; Papoff et al., 2004). Therefore, the application of this enzyme as a calf rennet substitute is rare (Fox et al., 2004).

The high pressure homogenization (HPH), also known as dynamic high pressure (DHP) is a non-conventional process applied for pump able food (Pinho et al., 2011). Recently, some researchers have been dedicated to evaluate the effect of the process on enzymes, in which HPH was able to improve (Liu et al., 2009 a,b; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012b,c), reduce (Velázquez-Estrada et al., 2012; Tribst, Augusto, & Cristianini, 2012b, Leite Júnior, Tribst, & Cristianini, *in press*) or not alter (Tribst & Cristianini, 2012a) the activity and stability of enzymes. The effects of HPH were dependent on the level of pressure homogenization applied, the temperature of the enzyme during the process, the nature of enzyme studied and pH of homogenization (Liu et

al., 2009 a,b; Tribst & Cristianini, 2012a,b,c; Tribst, Augusto, & Cristianini, 2012, a,b). Specifically for milk coagulants, the HPH was able to reduce the general proteolytic activity of calf rennet, increases its specificity in the milk-clotting formation and improve the enzyme stability over time (Leite Júnior, Tribst, & Cristianini, in press).

Therefore, HPH may be an interesting tool in the process of modifying enzymes, once it can increase or decrease activity as well as improved specificity and promote enzymatic stability. So, reduced activity and/or improvement in milk clotting activity in porcine pepsin could open new markets for this enzyme in the cheese making industry. Thus, this study aimed to evaluate the influence of high pressure homogenization on the proteolytic and milk-clotting activities and stability of commercial porcine pepsin protease used in manufacturing cheese and evaluate the formation of the gels by 24 hours at 35 ºC using porcine pepsin subjected to high pressure homogenization (HPH).

7.2 Material and methods

7.2.1 Porcine pepsin protease and high pressure homogenization

A commercial porcine pepsin protease was used in the experiments (freeze dried powder Porcine Pepsin) PEPSINA SUINA TS (Bela Vista, Santa Catarina, Brazil).

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the assays. This equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa and operates at a flow rate of 9 $L.h^{-1}$.

7.2.2 Effect of high pressure homogenization in commercial porcine pepsin protease

7.2.2.1. High pressure homogenization processing

A volume of 2 L of the porcine pepsin solution was prepared at 0.1 % (w/v) in 0.2 M sodium acetate buffer (pH 5.6) and homogenized under pressures of 0, 50, 100, 150 and 190 MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately in an ice bath after exiting the homogenizing valve. A non-processed sample of porcine pepsin was evaluated as the control sample.

7.2.2.2. Relative proteolytic activity (RPA) determination

The proteolytic activity of porcine pepsin protease was measured according to Merheb-Dini et al. (2010): the enzyme solution (0.1 % w/v) was prepared in a 0.2 M acetate buffer (pH 5.6). 600 µL of the enzymatic solution was added to 400 µL of a 0.5 % (w/v) sodium caseinate solution (Sigma Aldrich®, USA) prepared in the same buffer. The reaction was carried out at 35 ºC/40 min in a shaken water bath (62 rpm) and 1 mL of 10 % (w/v) trichloroacetic acid (TCA) then added to stop the hydrolysis. The samples were centrifuged at 2300 *g*/5 min/10 ºC and the absorbance was measured at 280 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount required to increase the absorbance at 280 nm by 0.1 under the assay conditions. The blank samples were prepared by adding the TCA to the tubes before the addition of the enzymatic solution, and the ΔAbs_{280nm} was determined from the difference in absorbance between the sample and the blank. The enzymatic activity was calculated according to *Equation 7.1*.

$U/mL = (\Delta Abs_{280nm} \times 10 \times \text{dilution factor} / 0.6 \times 40)$ (*Equation 7.1*)

RPA assays were carried out immediately after processing (time 0 h) and after 7, 14, 30 and 60 days. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed sample was used for a comparative evaluation. The relative proteolytic activity (RPA) was calculated considering the

activity of the HPH processed and non-processed samples, according to *Equation 7.2*:

RPA = (enzyme $activity_{after\_HPH\_and/or\_storage}$/ enzyme $activity_{non-processed\_sample\_at\_0h}$)*100 (*Equation 7.2*)

7.2.2.3. Relative milk-clotting activity (RMCA) determination

The milk-clotting activity was determined according to Merheb-Dini et al. (2010). A volume of 5 mL of skimmed milk powder, reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) was added to a 0.01 M $CaCl_2$ solution and pre-incubated at 35 °C/10 min. An aliquot of 0.5 mL of the enzyme solution (0.0015 %, w/v, prepared in 0.1 M sodium acetate buffer, pH 5.6) was then added and the time count started. Clot formation was determined by manual tube rotation and the time taken for the first particles to form measured. One milk-clotting activity unit (MCA) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 ºC. The MCA was calculated using *Equation 7.3*:

Unit of milk-clotting activity (MCA) = 2400/*t* x *S*/*E* (*Equation 7.3*)

Where *t* is the time (seconds) necessary for clot formation, *S* is the milk volume and *E* is the enzyme volume.

MCA assays were carried out immediately after processing (time 0 h) and after 1, 7, 14, 30 and 60 days. The samples were stored under refrigeration (4 ºC) throughout this period. A non-processed sample was also used for a comparative evaluation. The relative MCA (RMCA) was calculated considering the MCA of the HPH processed and non-processed samples, according to *Equation 7.4*:

RMCA = ($MCA_{after\_HPH\_and/or\_storage}$/ $MCA_{non-processed\_sample\_at\_0h}$)*100 (*Equation 7.4*)

7.2.2.4. Rheological assays

The milk coagulation was evaluated by using a time sweep low deformation oscillatory test in a rheometer with controlled stress (AR2000ex, TA Instruments, USA). These assays were carried out with the processed and non-processed porcine pepsin proteases.

The experiments were carried out according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skimmed milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) with the addition of a 0.01 M $CaCl_2$ solution. This mixture was pre-incubated at 35 °C/10 min, and subsequently 0.8 mL of enzyme solution (0.015 %, w/v) prepared in 0.1 M acetate buffer (pH 5.6) was added. The mixture was immediately transferred to the rheometer cup (30 mm diameter and 80 mm height), which had a *vaned quarter* geometry (with 28mm of diameter and 42 mm of length) and a 4 mm *gap*. The stress was set at 0.1 Pa and frequency at 0.1 Hz. The parameter G' (storage modulus) was measured at 3 min intervals for 90 min of the clotting process at 35 °C. The temperature was controlled by a *Peltier* system. The rheological assays were carried out immediately after processing (time 0 h) and after 60 days. The samples were stored under refrigeration (4 ºC) throughout this period.

Furthermore, the rate of milk-clotting was calculated as the variation of G' with variation in time as the log (dG'/dt) at three min intervals, and expressed in $Pa.min^{-1}$. The storage modulus (G') describes the elastic (solid) behavior of the product, and consequently the energy stored and released in each oscillatory cycle.

7.2.3 Characterization of the coagulation process and the gel formed using commercial porcine pepsin processed by high pressure homogenization

7.2.3.1. High pressure homogenization processing

A volume of 1 L of the commercial porcine pepsin solution 0.1 % (w/v) prepared in sodium acetate buffer 0.2M (pH 5.6) was homogenized under 150

MPa, using an inlet temperature of 23 ºC. Samples (200 mL) were collected and cooled immediately after exiting the homogenizing valve in an ice bath. A non-processed sample of commercial porcine pepsin was evaluated as the control sample.

7.2.3.2. Capillary zone electrophoresis method of porcine pepsin-induced gels

The capillary electrophoresis analysis was determined according to the conditions described by Ortega et al. (2003) and Otte et al. (1997). Firstly, the samples preparation were carried out: an aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added by 0.01 M $CaCl_2$ and by 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 360 µL of enzyme solution (0.1 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added and time started. After 40 min, 3 h, 6 h, 18 h and 24 h of coagulation at 35 ºC, 20 mg of samples were collected and dissolved in 1 mL of 10 mM sodium phosphate buffer solution containing 8 M urea (Merck, Darmstadt, Germany) and 10 mM of dithiothreitol (DTT, Sigma Chemical Co, St. Louis, USA) at pH 8, and left for 1 h at temperature of 23 ºC before filtration (0.22 µm Millex-GV_{13}, Millipore, Molsheim, France).

Then, Capilary zone electrophoresis (CZE) analysis was carried out using a Beckman P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) controlled by 32 Karat software (Beckman Coulter). The separations were performed using a fused-silica capillary (eCap™, Beckman Instruments Inc., San Ramon, CA, USA) of 57 cm (50-cm effective length to detector) x 75 µm I.D. The running buffer was prepared with 10 mM sodium phosphate containing 6 *M* urea and 0.05% hydroxypropyl methylcellulose (HPMC, Sigma Chemical Co, St. Louis, USA); the pH was adjusted to 3.0 with 1 M HCl. Buffer solutions were filtered through 0.22 µm filters (Millipore, Molsheim, France) before use.

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 5 s at 0.5 psi. During sample analysis, a constant voltage (18.5 kV ~35 µA) was applied and the temperature of the separation was kept at 23 ºC circulating coolant surrounding the capillary. For all experiments, electrophoreses was carried for 70 min and detection was at 214 nm (data collection rate 5 Hz). In CZE, the capillaries were conditioned by washing with 0.5 M NaOH for 5 min, then with pure water for 5 min, and finally with running buffer for 5 min after each electrophoretic runs.

The first electropherogram in a series was always discarded. The repeatability of peak areas was assessed by replicate injections (n = 3). Standard curves were made with a mixture of α_s-casein (C-6780), β-casein (C-6905) and κ-casein (C-0406) at 5 $mg.mL^{-1}$ obtained from Sigma Chemical Co (St. Louis, MO, USA) in the ratio of 1:1:1. Assignment of peaks was based on previous reports by Ortega et al. (2003) and Albillos et al. (2006).

7.2.3.3. Determination of spontaneous syneresis of porcine pepsin-induced gels by the siphon method

The level of spontaneous whey separation in undisturbed porcine pepsin-induced gels was determined using a siphon method in according Amatayakul et al. (2006). An aliquot of 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10 minutes. Subsequently, 360 µL of enzyme solution (0.1 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added and time started. After 40 min, 3h, 6h, 18h and 24h coagulation at 35 ºC samples were cooled to 5 ºC and inclined at an angle of 45 º and maintained at rest for a period of 1 hour. Then, the serum exceeded was collected from the surface of the samples (using a syringe) and weighed. The syneresis was expressed as the percent weight of the whey divided the initial weight of the gels sample, according to *Equation 7.5*.

Whey separation (%) = $(S_F/P_T - P_E) \times 100$ (*Equation 7.5*)

Where, S_F is the weight of the separated whey, P_T is the weight of sample plus packaging and P_E is the weight of the empty package.

7.2.3.4. Rheological assays of coagulation process and porcine pepsin-induced gels

The milk coagulation and development of formed gel were evaluated by monitoring the milk coagulation process through time sweep using low deformation oscillatory test in rheometer with controlled stress (AR2000ex, TA Instruments, USA).

The experiments were determined according to Leite Júnior, Tribst, & Cristianini (*in press*) using 60 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany). This mixture was pre-incubated at 35 °C/10 minutes. Subsequently, 360 µL of enzyme solution (0.1 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) was added. Immediately after enzyme addition, the sample was transferred to the rheometer cup (30 mm diameter and 80 mm height). It was used a *Vaned Quarter* geometry (with 28mm of diameter and 42 mm of length) and a *gap* of 4 mm. The stress was set at 0.1 Pa, frequency 0.1 Hz. The parameters G' (storage modulus) and G'' (loss modulus) were measured at 3 minute intervals (at first's 40 minutes) and then at 10 minute intervals (up to 24 hours) of clotting process at 35 ºC. The tan δ (loss tangent) was calculated by G''/G' ratio. The temperature was controlled by a *Peltier* system.

7.2.3.5. Three dimensional (3D) microstructure of coagulation process and porcine pepsin-induced gels by confocal scanning laser microscopy (CSLM)

An aliquot of 1 mL of skim milk powder reconstituted at 10 % (w/v) (pH 6.65, 3.2 % protein, 9.2 % non-fat solids) added of 0.01 M $CaCl_2$ and 0.05 % (w/v) of sodium azide (Merck, Darmstadt, Germany) was pre-incubated at 35 °C/10

minutes. Subsequently, 6 µL of enzyme solution (0.1 %, w/v) prepared in acetate buffer 0.1M (pH 5.6) and 25 µL of fast-green FCF (0.1% w/v, in distilled water, Sigma–Aldrich, Ireland) (used to observe the protein matrix in a confocal microscope) were added and time started. Immediately after addition, 200 µL of the solution was transferred to a coverglass (a cavity dish of 10 mm in depth), covered with a glass coverslip (0.17 mm thick) with 8 chambers (Lab-Tek® II Chambered Coverglass, USA) and the confocal imaging was performed using a Zeiss Upright LSM780-NLO microscope (Carl Zeiss AG, Germany) with temperature control set to 35 ºC.

CLSM is able to penetrate deeply but noninvasively through the sample to obtain a large number of sequential, thin optical sections that may then be assembled by image-analysis software to produce 3D reconstructions and projections. For each sample, 20 adjacent planes (2D layers 512×512 pixels in resolution from a 134.95 µm × 134.95 µm sample area) were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. Images were recorded at a distance of 5 µm and 20 µm from the surface of the coverglass (bottom). Images of representative areas of each sample were taken every 90 seconds for 40 minutes and after 3 h, 6 h, 18 h and 24 h using an oil immersion 63x objective lens (numerical aperture = 1.40) at excitation wavelengths of 633 nm to fast-green (He/Ne laser), in which the fast-green FCF stained protein appears red, in contrast, the serum phase appears black in these images. The images were acquired in RGB color (8 bits), of 512 x 512 pixels in size to give final resolutions of 0.26 µm/pixel (Ong et al., 2010).

7.2.3.6. Image analysis

Image analysis of CLSM micrographs was performed using image J software (Research Service Branch, National Institute of Health, Maryland, USA) equipped with “Pore Analysis” and “ComputeStats” plug-ins. The images were enhanced and flattened using a bandpass filter. The enhanced image was then

transformed to a binary image using an Otsu threshold algorithm binarisation with all structural features contributing black pixels and all backgrounds features contributing white pixels (Impoco et al., 2006).

The porosity was calculated as an average area of pores and as the fraction of pore area with respect to the total sample area (0.018 mm^2). The porosity calculated here is only equal to a two-dimensional pore area and fraction and not the absolute porosity of the sample (Ong et al., 2011). The average pore area is also calculated as total pore area with respect to total sample area divided by total numbers of pores and Image J analysis was used to quantify the porosity and total numbers of pores from CLSM micrographs (Hussain, Grandison, & Bell, 2012).

7.2.4 Statistical analysis

The processes and analyses were carried out with three repetitions and each experimental unit was carried out in triplicate. The analysis of variance (ANOVA) was used to compare the effects of the different treatments and the Tukey test to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 7.0 software– (StatiSoft, Inc., Tulsa, Okla., U.S.A.) and the results were presented as the mean ± standard deviation.

7.3 Results and Discussion

7.3.1 Effect of high pressure homogenization in commercial porcine pepsin protease

7.3.1.1. Proteolytic activity

Figure 7.1 shows the results of relative proteolytic activity during 60 days of storage after enzyme process at different high pressure homogenization. Until the

14th day storage, it was not observed any change in proteolytic activity among the non-processed (control) and processed samples ($p>0.05$), and a continuous proteolytic activity reduction was observed for all samples, reaching ~85% after 14 days. After 30 days storage, samples processed at 50 MPa, 100 MPa and 150 MPa showed lower activity (5 to 8% less) than the sample processed at 190 MPa and non-processed ($p<0.05$).

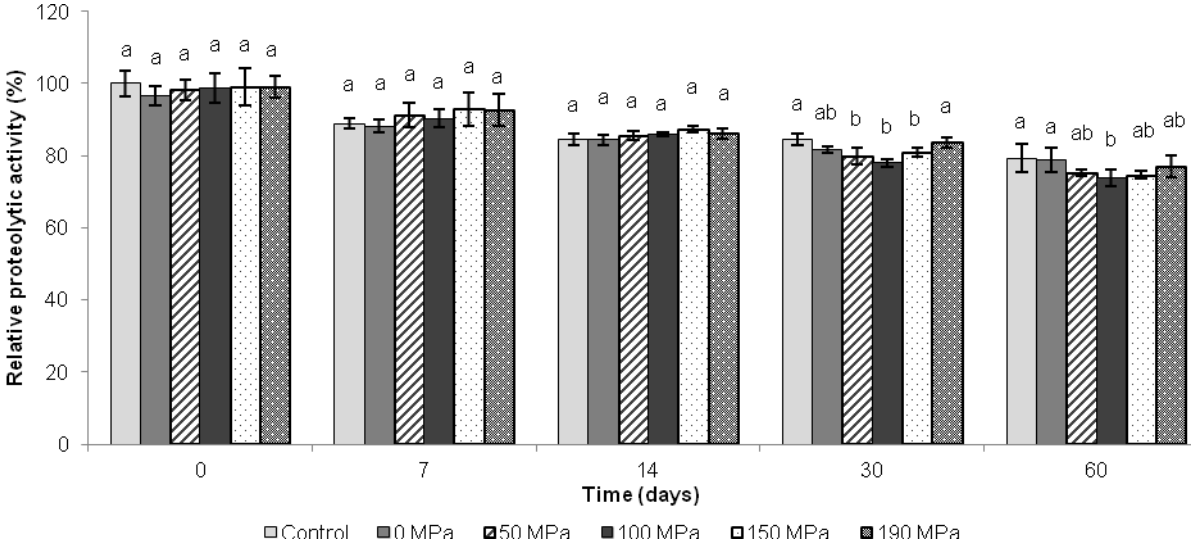

Figure 7.1 Relative proteolytic activity of the porcine pepsin subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed porcine pepsin samples evaluated at each time.

Similar behavior was observed after 60 days of storage, in which the sample processed at 100 MPa showed lower activity (~5% less) than the control sample and those processed at 0 MPa ($p<0.05$). Thus, the HPH process can reduce the proteolytic activity of porcine pepsin after 60 days in solution. From the cheese making point of view, the reduction of excessive proteolytic activity may be interesting for improving cheese yield, flavor and texture.

7.3.1.2. Milk-clotting activity

The milk-clotting activity is an indirect way of evaluating the specificity of milk coagulants in the cleavage the site Phe_{105}-Met_{106} of κ-casein. This cleavage destabilizes the casein micelles, which coagulates in the presence of Ca^{2+} (Andrén, 2011). *Figure 7.2* shows the results for milk-clotting of the non-processed and processed porcine pepsin samples immediately after HPH processing and up to 60 days of enzyme processing.

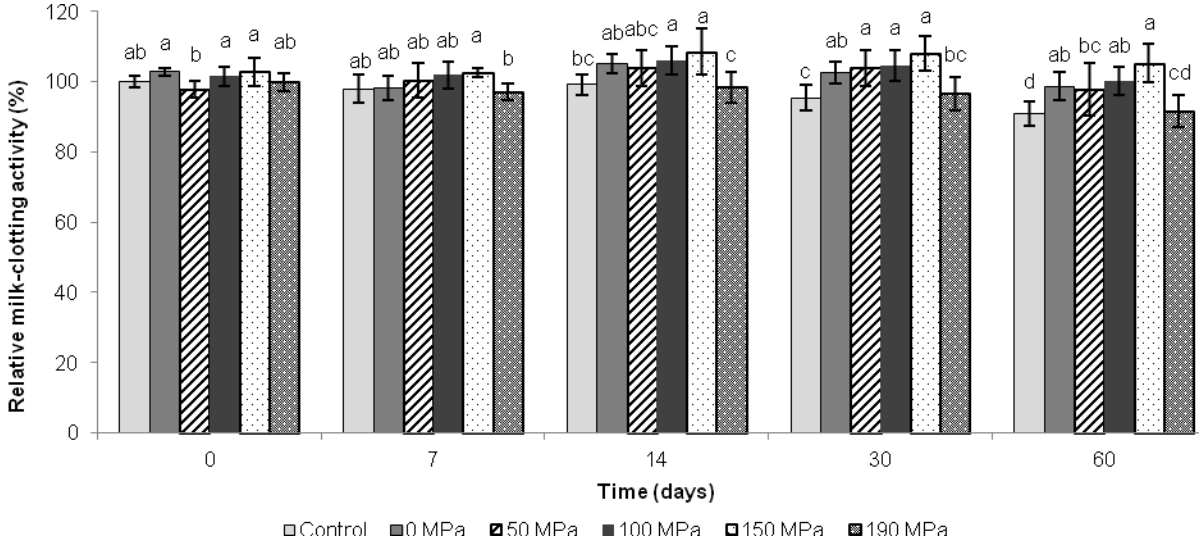

Figure 7.2 Relative milk-clotting activity of the porcine pepsin subjected to the high pressure homogenization process. Different letters mean significant difference ($p<0.05$) between the non-processed and processed porcine pepsin samples evaluated at each time.

Immediately after processing, no samples showed significant differences of RMCA when compared with the control sample, although some of them had shown differences between other HPH processed enzyme. From the 14th to 60th day of storage, the samples processed at 150 MPa and 100 MPa showed higher RMCA than non-processed enzyme and processed at 190 MPa ($p<0.05$). A maximum difference on MCA was obtained between sample processed at 150 MPa and control after 60 days of storage, being the activity of HPH sample 15% higher.

HPH process at 150 MPa was able to promote greater enzymatic stability to milk-clotting activity, while process at 190 MPa just reduced the RMCA. Therefore, the effect of HPH on the RMCA is dependent on the applied pressure.

The evaluation of proteolytic and milk-clotting activities of porcine pepsin after HPH indicated that processes at 150 MPa and 100 MPa showed the most satisfactory and interesting results from the cheese production point of view. Although no differences were found immediately after homogenization, the enzyme processed at 150 MPa showed a potential improvement of its proteolytic profile after 60 days of storage, due to the reduction of proteolytic activity and improvements on milk-clotting activity.

7.3.1.3. Rheological evaluation

The storage modulus (G') describes the product elastic (solid) behavior and can represent the phenomenon of milk coagulation (*Figure 7.3*). The beginning of the aggregation can be better visualized using a log scale (*Figure 7.3, a2* and *b2*). It was observed that the aggregation process started 36 minutes when gel was obtained using porcine pepsin homogenized at 150 MPa. On the contrary, the aggregation starts just after 45 minutes for control and homogenized samples at 50 and 190 MPa. Therefore coagulation using the enzyme processed at 150 MPa occured 20% faster than using the non-processed one. This possibly happened due to the fast cleavage of the specific hydrophilic glycomacropeptide, preferably at the site Phe_{105}-Met_{106} of the κ-CN in the first phase of coagulation. After 90 minutes of coagulation, porcine pepsin homogenized at 150 MPa produced the gel with G' values 92% higher than non-processed one ($p<0.05$). The differences between the G' values of the samples highlighted that each pressure increment possible cause slights changes in the enzyme conformation with consequent minimal alterations in the coagulation performance.

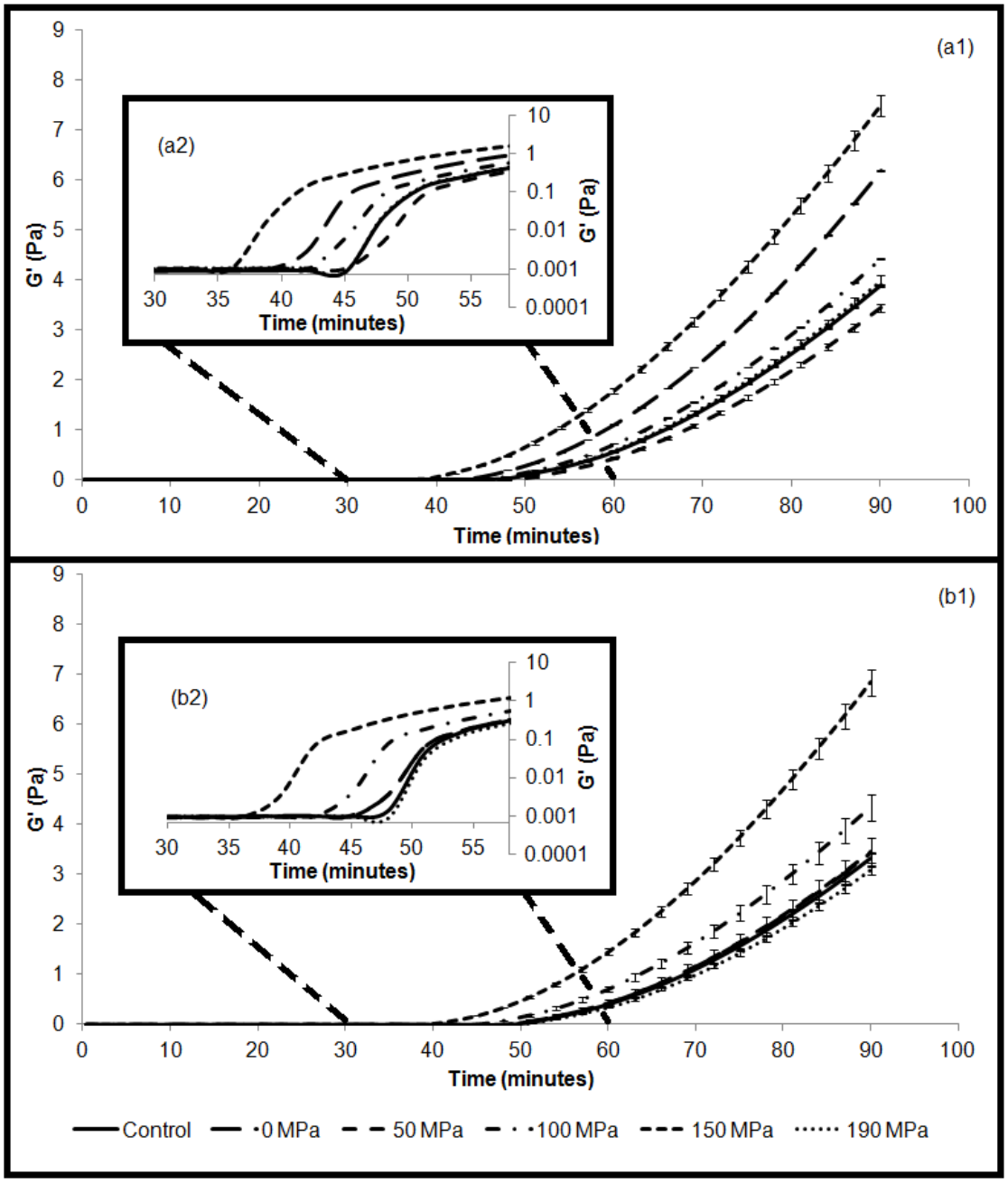

Figure 7.3 Evaluation of milk coagulation (a1) immediately after the process (0h) (a2) log scale, and (b1) after 60 days stored at 4 °C (b2) log scale.

The differences between the results of the rheological analysis and RMCA can be attributed to the sensibility of the methods, since RMCA is a visual evaluation while rheological is an instrumental method with high sensibility. By the

rheological data, it was observed that 150 MPa positively changed the porcine pepsin, reducing the time of coagulation and improving the gel consistence.

Another rheological evaluation was carried out after 60 days of porcine pepsin storage in solution at 4 °C, aiming to determine if storage in solution affected the coagulation profile of the enzymes. The storage period caused a loss of milk-clotting activity for both processed and non-processed enzymes, which was evidenced by the increase in the time taken to start the aggregation process and being compatible with the results of RMCA. Comparing the results of 0 and 60 days of enzyme storage, similar G' values profiles were observed. Again, coagulation using the enzyme processed at 150 MPa occurred rapidly than using the non-processed one (time spent to aggregation beginning was 25% less for 150 MPa processed-enzyme). The G' value after 90 minutes of coagulation was 106% higher for enzyme processed at 150 MPa than for non-processed ones. These results corroborate to the increase of enzyme stability in solution after the HPH process.

The rate of milk-clotting (*Figure 7.4*) was calculated as the variation of G' every 3 minutes. This graph shows the moment at which aggregation started (higher speed), it was observed that the gel produced with enzyme processed at 190 MPa resulted in higher speed of milk protein aggregation; however, the gel produced with enzyme processed at 150 MPa resulted in faster start of milk protein aggregation. Consequently, the gels obtained using enzyme processed at 150 MPa was firmer. Comparing the results of 0 and 60 days of enzyme storage, similar rates of milk-clotting profiles were observed for the majority of samples. Therefore, the rheological results obtained highlights the HPH process is able to improve the milk-clotting parameters, which is dependent on the level of pressure applied.

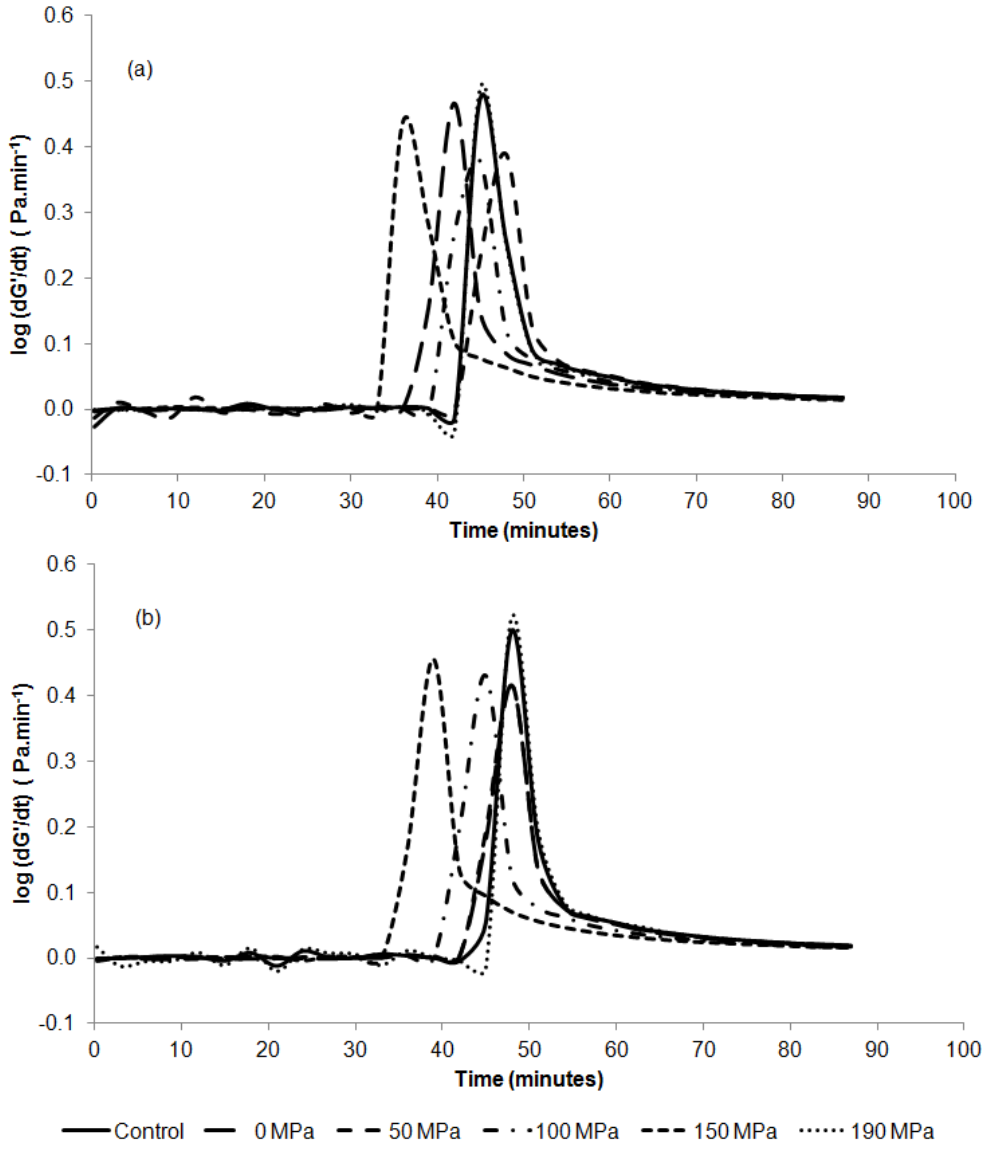

Figure 7.4 Rate of milk-clotting by the action of the enzyme subjected to high pressure homogenization (a) immediately after the process (0h) and (b) after 60 days storage at 4 °C.

7.3.2 Characterization of the coagulation process and the gel formed using commercial porcine pepsin processed by high pressure homogenization

7.3.2.1. Proteolysis of porcine pepsin-induced gels by capillary electrophoresis

Electropherograms of casein standards are shown in *Figure 7.5* and *Figure 7.6* shows the electropherograms of the porcine pepsin-induced gels during 24 hours at 35 ºC. The peaks were indicated on the electropherograms with serial numbers and the identification of peaks is detailed in the figure caption.

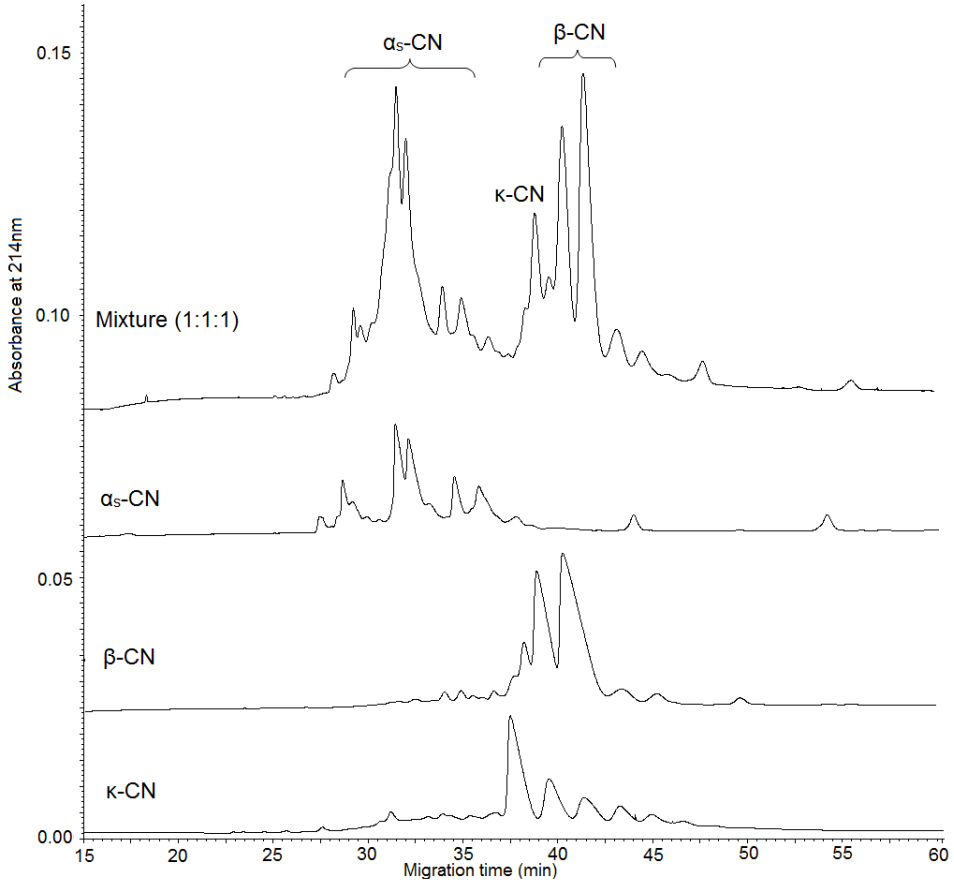

Figure 7.5 Capillary electropherogram separation of a 1:1:1 mixture of α_s-CN, β-CN and κ-CN and identification of major caseins by analysis of casein standards: α_s-casein (α_s-CN); β-casein (β-CN); κ-casein (κ-CN).

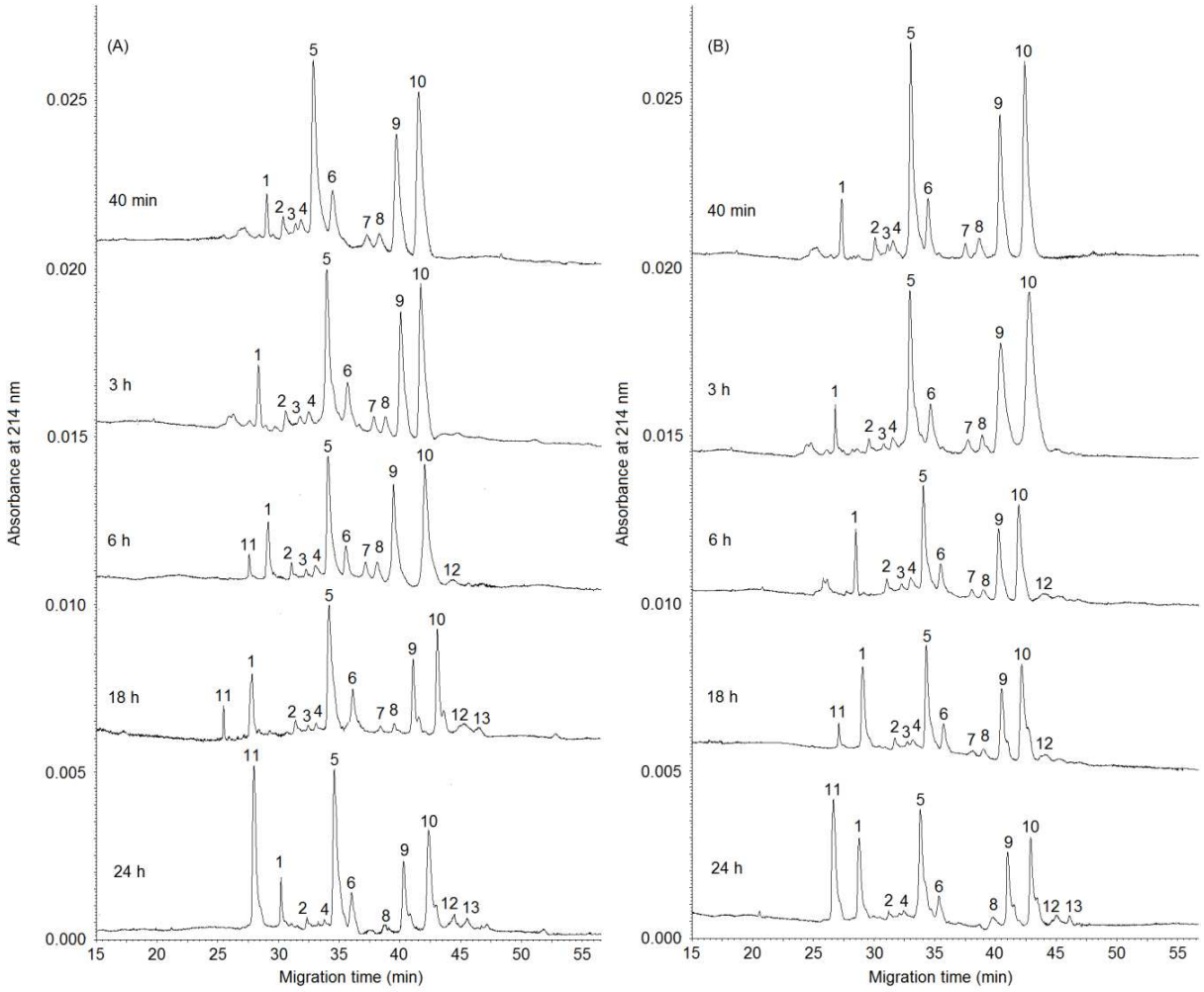

Figure 7.6 Capillary electropherogram of the porcine pepsin-induced gels using (A) non-processed and (B) processed by high pressure homogenization at 150 MPa, throughout a 24 hours coagulation period at 35 ºC. Peak identification: 1: para-κ-CN; 2: α_{s2}-CN; 3: α_{s2}-CN; 4: α_{s2}-CN (three phosphorylation states of the α_{s2}-CN monomer were achieved); 5: α_{s1}-CN; 6: α_{s0}-CN (two phosphorylation states of α_{s1}-CN monomer were achieved); 7: κ-CN; 8: β-CN^{B}; 9: β-CN^{A1}; 10: β-CN^{A2} (two genetic variants of β-CN: A1 and A2); 11, 12 and 13: unidentified.

The comparison of casein gels obtained using high pressure homogenized enzyme and non-processed one showed that, overall, hydrolysis profiles were similar throughout time, whereas the non-processed sample caused a slightly

higher casein hydrolysis (especially on the β-CN fraction) with formation of new peaks (#11, after 6 h and #13, after 18h). These peaks were observed for gel produced by HPH enzyme only after 18 h and 24 h, respectively. The lower proteolysis caused by HPH processed enzyme can be attributed to the reduction of the proteolytic activity of the HPH processed porcine pepsin during storage (as observed in section 7.3.1.1, in *Figure 7.1*). The hydrolysis profiles of κ-CN after 40 minutes and during storage of gel were similar to the non-processed and processed enzyme. Thus, the results evidenced that HPH was able to reduce the unspecific proteolytic behavior of porcine pepsin after the gel formation with no effects on the enzyme specificity.

From an industrial point of view, the effect of HPH on porcine pepsin is interesting since the observed reduction on proteolytic profile might limit the high unspecific proteolysis during cheese storage. This is desirable once excessive proteolysis, mainly in fractions of β-casein – specific for pepsin activity, promotes the release of hydrophobic peptides that confer bitterness flavour (Hassan et al., 2013). Furthermore, the intense proteolysis could cause weakness of gel structure, protein loss in the whey and low yield of dry matter. Additionally, the reduction on proteolytic activity prevents excessive protein hydrolysis during fresh cheese storage (being able to improve fresh cheese shelf life) as well as during cheese ripening, ensuring in an adequate balance of intact proteins and peptides resulting in flavors, textures and functional characteristics typical of ripened cheese (Sousa, Ardo, & McSweeney, 2001).

7.3.2.2. Spontaneous syneresis and rheological assays of coagulation process and development porcine pepsin-induced gels

Table 7.1 shows the results for whey separation during 24h at 35 ºC of the porcine pepsin-induced gels produced with non-processed and homogenized enzymes.

After 40 minutes of coagulation, the gels produced with the enzyme processed at 150 MPa showed a significant higher syneresis than the produced with non-processed enzyme ($p<0.05$). After 3 hours of coagulation, the gel produced with high pressure homogenized enzyme did not differ from gels produced with the non-processed enzyme ($p>0.05$) up to 24 hours.

The syneresis naturally happens in curd due to the formation of new bonds between para-casein micelles that results in compression of casein micelles package and expulsion of whey from the gel (Lodaite et al., 2000; Lucey et al., 2000; Lucey, 2001; Castillo et al., 2006). Thus, the higher milk-clotting activity of HPH enzyme enables the formation of more para-κ-casein linkages in the presence of calcium ions during the coagulation phase (first 40 minutes) promoting a greater compression network with higher expulsion of whey.

Table 1 and *Figure 7* shows the results of rheological parameters of milk gels (during 24h) produced using porcine pepsin non-processed and processed at 150 MPa. The G' values were higher for gels produced with HPH pepsin during the first 40 minutes and after 18 hours of coagulation ($p< 0.05$) and an increment on this value were observed up to 24h of evaluation for both enzymes. These results demonstrate that gels obtained with HPH processed enzyme are initially more consistent and stronger and the contraction of casein micelles up to 24h increased the gel consistence. The non-processed sample showed a similar behavior, however, due to the less contraction of the network (less linkages para-k-casein micelles), lower G' values were observed during the 24 hours period. Therefore, it is possible to conclude that the HPH process of porcine pepsin results in more consistent and solid gels, with higher viscoelasticity after 24h of coagulation, favoring the formation of firm and compact cheese mass.

Table 7.1 Rheological properties and whey separation of milk gels throughout a 24 hours coagulation period at 35 ºC using porcine pepsin processed by high pressure homogenization

Coagulation time (h)	Storage modulus - G' (Pa) *		Loss modulus - G'' (Pa) *		Loss tangent (tan δ) *		Whey separation (%) **	
	Control	150 MPa	Control	150 MPa	Control	150 MPa	Control	150 MPa
0.33 (20 min)	2.4 ± 0.3^{b}	3.2 ± 0.5 ^{a}	0.8 ± 0.1^{b}	1.1 ± 0.2^{a}	0.338±0.003^{b}	0.350±0.011^{a}	ND	ND
0.67 (40 min)	9.2 ± 0.4^{b}	10.3 ± 0.7^{a}	3.1 ± 0.1^{b}	3.6 ± 0.3^{a}	0.340±0.002^{b}	0.346±0.003^{a}	1.68 ± 0.17^{b}	2.33 ± 0.47^{a}
3	31.5 ± 0.7^{a}	32.8 ± 1.4^{a}	11.0 ± 0.3^{a}	11.5 ± 0.5^{a}	0.349±0.002^{a}	0.350±0.002^{a}	5.02 ± 0.84^{a}	5.38 ± 0.50^{a}
6	42.3 ± 0.8^{a}	43.7 ± 1.6^{a}	15.0 ± 0.3^{a}	15.5 ± 0.5^{a}	0.355±0.002^{a}	0.355±0.003^{a}	6.18 ± 0.79^{a}	6.84 ± 0.81^{a}
18	57.8 ± 0.7^{b}	60.0 ± 1.4^{a}	20.6 ± 0.2^{b}	21.5 ± 0.5^{a}	0.356±0.003^{a}	0.359±0.002^{a}	11.05 ± 0.80^{a}	12.14 ± 1.33^{a}
24	61.3 ± 0.7^{b}	64.2 ± 1.7^{a}	22.0 ± 0.2^{b}	23.2 ± 0.5^{a}	0.359±0.003^{a}	0.361±0.002^{a}	14.82 ± 1.10^{a}	15.87 ± 1.96^{a}

* Values are LS-means from replicates (n=6).

** Values are LS-means from replicates (n=9).

Means ± standard deviation in the column, with different superscripts, are different ($p<0.05$).

ND: Not Determined.

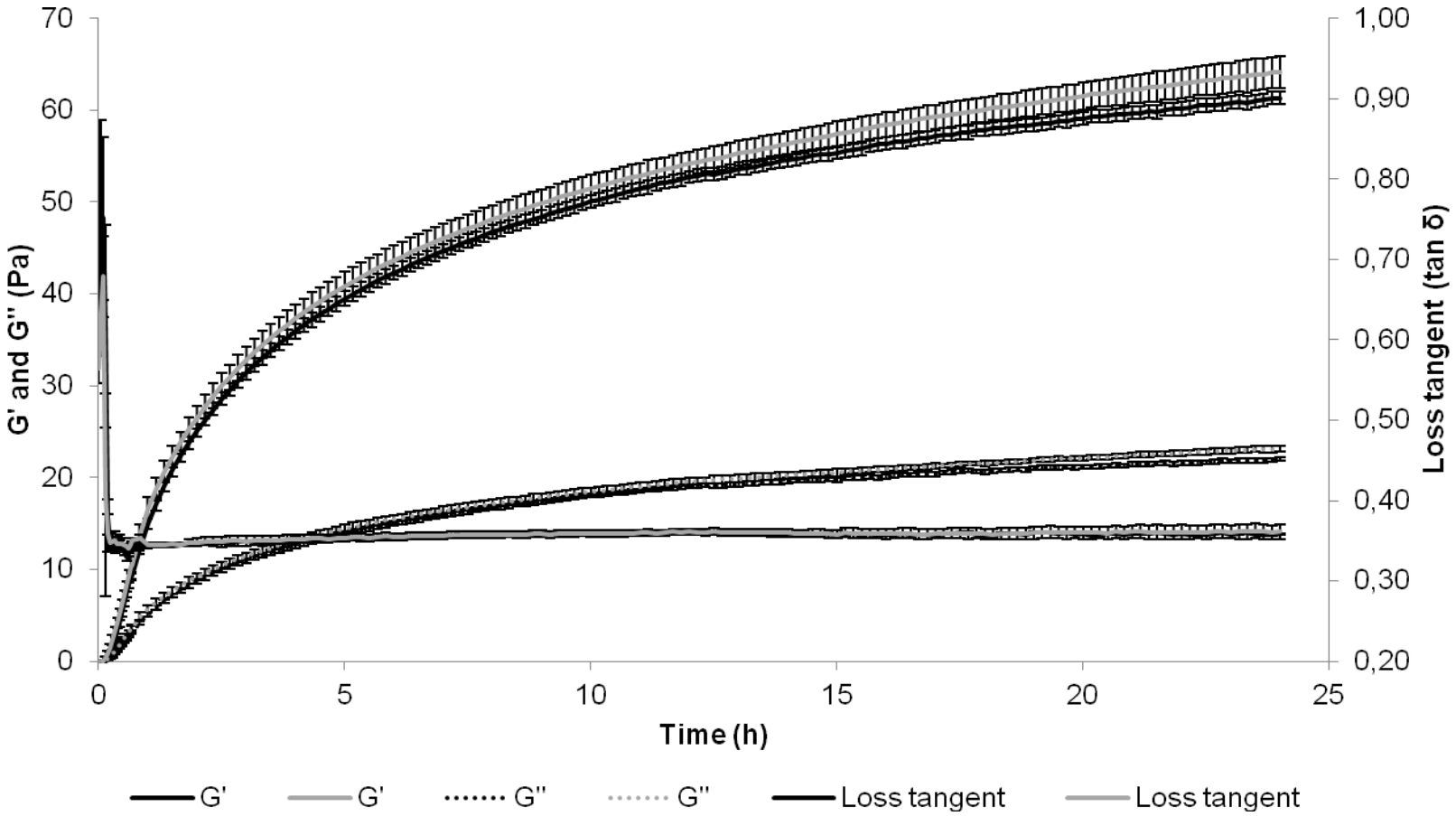

*Figure 7.7 Evaluation of milk coagulation using porcine pepsin enzyme subjected to high pressure homogenization (processed at 150 MPa - gray lines) and non-processed (control - black lines) immediately after the process throughout a 24 hours period at 35 ºC. ** Values are means from replicates (n = 6).*

The G'' values can be related to the water retention ability of the milk gels. The gel produced with HPH processed enzyme showed significant higher values of G'' than the gel obtained by milk coagulation with non-processed enzyme ($p<0.05$) during the first 40 minutes and after 18 hours of coagulation. It means that water diffusivity in casein matrix produced with enzyme processed at 150 MPa was lower. This phenomenon can be explained by the compact gels formed by homogenized enzymes, with more and strong linkages which difficult the whey diffusion.

In gels obtained by enzymatic coagulation, the susceptibility to syneresis has been associated with a high value for the loss tangent at long time scales (van Vliet et al., 1991), being related to the propensity of the network to rearrange after gel formation (Lucey, 2001). Many studies of the relations between syneresis of casein gels and their rheological properties have been presented, however. Reports of the effects of the storage modulus (G') on the syneresis behavior are not conclusive (Lelievre, 1977; Zoon, van Vliet, Walstra, 1988). The analysis of van Vliet et al. (1991) indicating that syneresis is not dependent on G', but increases with the increasing tan δ (=G''/G').

The data of tan δ (loss tangent) were also presented on *Table 7.1* and *Figure 7.7*. A tendency of higher loss tangent for gels prepared with high pressure homogenized enzyme was observed. This difference became statistically significant after 3 hours of coagulation ($p< 0.05$), which ratifies the hypothesis of whey expulsion increasing after gel formation due to the higher contraction of protein matrix, leading to formation of a dense network.

Therefore, the results of syneresis and gel rheology highlight that protein network formed was stronger from the beginning of coagulation to the end of porcine pepsin-induced gel development. The increase in milk-clotting activity of HPH enzyme allows the formation of more para-κ-casein linkages in the presence of calcium ions and reduced the hydrolysis of casein fractions, leading the formation of a more compact, cohesive, firm and dense protein network, increasing whey loss accompanied by a reduction in the water diffusivity. These effects

observed in gels can be interesting for cheese production, resulting in higher cheese yield (due to the increment on water binding capacity of protein) and higher dry matter (due to the reduction of small peptides loss in the whey).

7.3.2.3. Microstructure of milk coagulation process by porcine pepsin

Figure 8 shows the images obtained by confocal scanning laser microscopy (CSLM) during the milk enzymatic coagulation and of the gel formed by using non-processed and homogenized enzyme at 150 MPa. The porosity of the gel, total number of pores and the average pore area are showed in *Figure 9* and *Table 2*.

These images elucidate the effect of HPH on the porcine pepsin behavior during the gel formation and development. The images clearly show that, after 20 minutes of enzyme addition, the protein aggregation is faster for gels obtained by high pressure homogenized enzymes (150MPa). Nevertheless, at the end of coagulation step (40 minutes), similar micrographs were observed in the gels. After 24 h, more compact protein network was observed for gel obtained by using HPH processed enzyme, being compatible with the results obtained for rheology and syneresis analysis.

The gel porosity at 5 μm from the sample bottom was reduced due to the aggregation and sedimentation of the protein network. On the contrary, the porosity at 20 μm increased due the excessive whey expulsion from the protein matrix. Comparing the results obtained for each enzyme it was observed that, after 20 minutes and during the 24 hours of coagulation, the porosity at 5 μm was lower for gel produced with high pressure homogenized enzyme ($p<0.05$). To the contrary, at 20 μm, the gel porosity were different just after 24 hours of coagulation, being more porous the gel produced with processed enzyme ($p<0.05$).

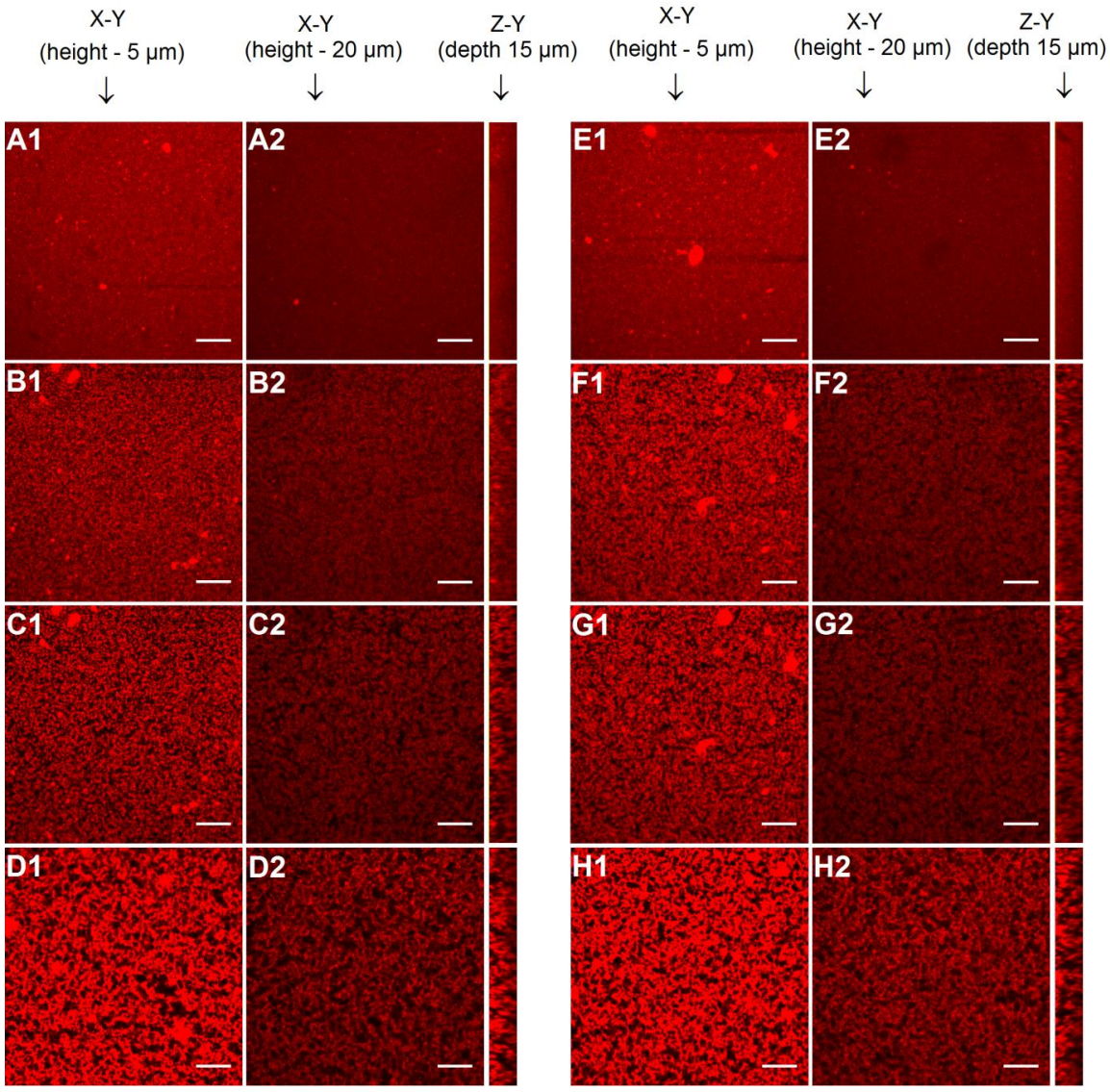

Figure 7.8 CLSM micrographs of porcine pepsin-induced gels processed by high pressure homogenization and porcine pepsin non-processed (A1-D1: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; A2-D2: control at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm; E1-H1: 150 MPa at 0 min, 20 min, 40 min and 24 h, respectively at a height of 5 µm; E2-H2: 150 MPa at 0 min, 20 min, 40 min and 24 h, respectively at a height of 20 µm) throughout a 24 hours period at 35 ºC. The fast-green FCF stained protein appears red and the serum phase appears black in these images. Each set of images is presented in two views: the X–Y (a height of 5 and 20 µm from the bottom – the

surface of the coverglass) and the Z–Y (right) projections. For each sample, 20 adjacent planes were acquired with the separation between the planes kept constant at 0.75 µm, giving a total observation depth of 15 µm. The scale bars are 20 µm in length.

Additionally, at 5 µm, reduction of total number of pores and increase of the average pore area were observed along the time, which is directly linked to the protein aggregation. The gel obtained with HPH enzyme showed a faster reduction of total pores with increasing pore area, presenting a significant difference (until 40 minutes of coagulation) when compared with gel produced using non-processed enzyme ($p<0.05$). From 40 minutes to 24 hours, it was observed an inversion on the behavior of gels produced with processed and non-processed enzyme, with pores in larger number and smaller areas for gel produced using HPH enzyme.

Imagens recorded at a distance of 20 µm from the surface of coverglass, results also showed a reduction of total number of pores and an increase in the average pore area along the coagulation time. Again the gel obtained with HPH enzyme showed a faster reduction of total pores with increasing pore area during the first 40 minutes. From 40 minutes to 24 hours, it was observed similar behavior with a higher number of pores and lower average pore area for gel produced with processed enzyme. This phenomena can possibly be attributed to the higher number of para-κ-casein linkages due to increase on milk-clotting activity, resulting in higher compaction and sedimentation of protein network and maintenance of links between casein micelles due to reduction in the proteolytic activity of the HPH processed enzyme.

Table 7.2 Gel porosity, average pore area and number of pores of porcine pepsin-induced gels processed by high pressure homogenization and non-processed enzyme.

		Sample height 5 µm			
	Sample	0 min (Blank)	20 min	40 min	24 h
Gel Porosity (%)	Control	0,5834 ± 0,0104 [a]	0,5686 ± 0,0191 [a]	0,5606 ± 0,0188 [a]	0,4910 ± 0,0186 [a]
	150 MPa	0,5814 ± 0,0095 [a]	0,5380 ± 0,0037 [b]	0,5269 ± 0,0054 [b]	0,4457 ± 0,0079 [b]
Number of pores	Control	8254 ± 271 [a]	2567 ± 101 [a]	2139 ± 76 [a]	925 ± 71 [a]
	150 MPa	8300 ± 273 [a]	1852 ± 139 [b]	1601 ± 123 [b]	1009 ± 85 [a]
Average pore area (μm^2)	Control	1,28 ± 0,03 [a]	4,04 ± 0,23 [a]	4,78 ± 0,23 [a]	9,71 ± 0,89 [a]
	150 MPa	1,28 ± 0,02 [a]	5,31 ± 0,43 [b]	6,02 ± 0,51 [b]	8,09 ± 0,78 [b]
		Sample height 20 µm			
	Sample	0 min (Blank)	20 min	40 min	24 h
Gel Porosity (%)	Control	0,5315 ± 0,0057 [a]	0,5739 ± 0,0098 [a]	0,5804 ± 0,0088 [a]	0,6546 ± 0,0067 [a]
	150 MPa	0,5323 ± 0,0095 [a]	0,5731 ± 0,0052 [a]	0,5864 ± 0,0094 [a]	0,6698 ± 0,0099 [b]
Number of pores	Control	13094 ± 291 [a]	5086 ± 122 [a]	4432 ± 198 [a]	1044 ± 77 [a]
	150 MPa	13180 ± 359 [a]	4107 ± 160 [b]	3447 ± 185 [b]	1162 ± 66 [a]
Average pore area (μm^2)	Control	0,74 ± 0,03 [a]	2,06 ± 0,05 [a]	2,39 ± 0,09 [a]	11,46 ± 0,77 [a]
	150 MPa	0,73 ± 0,01 [a]	2,54 ± 0,10 [b]	3,10 ± 0,17 [b]	10,53 ± 0,69 [a]

Results are expressed as the mean ± the standard deviation (n = 6). [a,b] Means ± standard deviation in the row, with different superscripts, are significantly different ($p<0.05$).

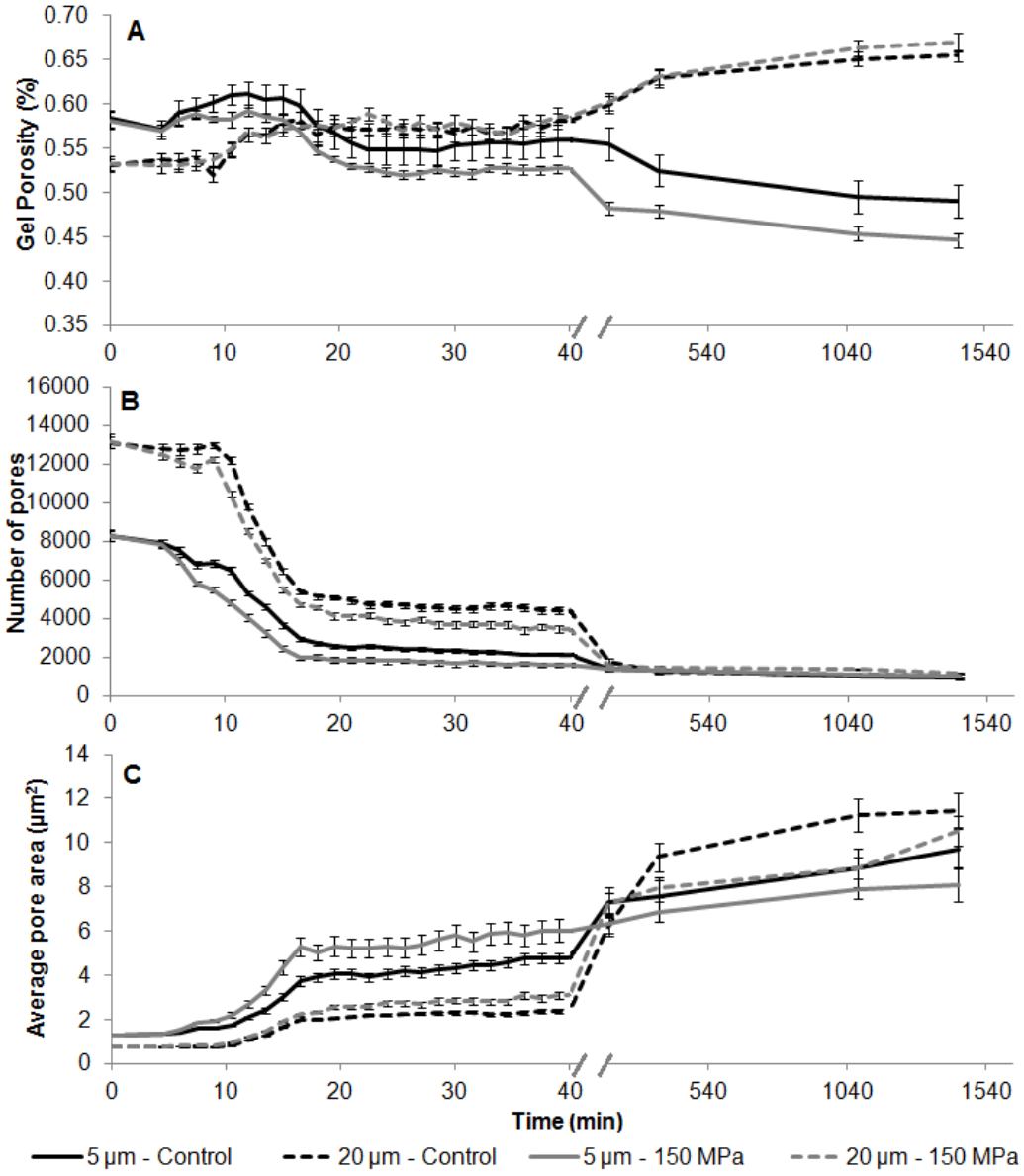

Figure 7.9 Gel porosity (A), number of pores (B) and average pore area (C) of porcine pepsin-induced gels processed by high pressure homogenization and porcine pepsin non-processed. Results are expressed as the mean ± the standard deviation (n = 6).

Therefore, the gel produced by using porcine pepsin processed at 150 MPa is overall less porous, when compared to a similar gel manufactured by using non-

processed porcine pepsin. This effect occurred due to changes in the activity and specificity of the enzyme by HPH processing, which directly affects the aggregation of the protein network. In the present work, the increase on milk-clotting activity promotes a greater number of connections between fractions of caseins in the presence of calcium ions, which was previously evidenced by G' and G'' values.

7.4 Conclusions

The high pressure homogenization process did not affect proteolytic and milk clotting activities of porcine pepsin immediately after the processing, however, during storage of processed and non-processed enzymes was found a reduction in the proteolytic activity and increase milk-clotting activity, especially for enzyme processed at 150 MPa. In addition, the gel produced with processed enzyme at 150 MPa was formed faster (20%) and was more consistent (G' value 92% higher at 90 minutes immediately after processing) than the gel obtained by non-processed enzyme. This highlights that HPH processing can be applied as tool for porcine pepsin performance improvement. Moreover, the milk gel produced with porcine pepsin processed at 150 MPa was more compact, firm, and less porous and promoted higher whey expulsion from protein matrix, when compared with gel obtained using non-processed enzyme. After 24h of coagulation, the gel produced using high pressure homogenized enzyme kept its consistence due to the lowered proteolytic profile and higher milk-clotting activity. Therefore, the results highlight the HPH as a process able to improve the hydrolytic characteristics of pepsin porcine allowing the insertion of this enzyme for cheesemaking.

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Conclusões Gerais

A partir dos resultados obtidos do efeito do processo de homogeneização à alta pressão sobre as enzimas coagulantes do leite algumas conclusões foram obtidas individualmente para cada enzima.

Para o coalho de vitelo o processo de HAP reduziu a atividade proteolítica (com máximo de redução de 52% após processamento a 190 MPa) e aumentou a taxa de coagulação do leite. Os géis obtidos pela ação da enzima processada a 190 MPa apresentaram maior consistência com menor proteólise resultando numa rede proteica mais compacta, firme e menos porosa com maior expulsão de soro do que o gel obtido pelo uso da enzima não processado. Assim, destaca-se que o processamento a HAP pode ser aplicado como uma tecnologia para melhorar as características hidrolíticas do coalho de vitelo permitindo a obtenção de géis com maior rendimento (devido a maior dificuldade de difusidade da água nestes géis) e aumento de matéria seca (devido à redução da proteólise). Além disso, a menor proteólise no gel pode resultar numa extensão da vida de prateleira de queijos frescos.

O processamento a HAP no coalho de bovino adulto foi capaz de reduzir a atividade proteolítica e aumentar a atividade e estabilidade de coagulação do leite após o processamento da enzima a 150 MPa. Além disso, igualmente ao que foi verificado para coalho de vitelo, o processo foi capaz de aumentar a taxa de coagulação do leite pela enzima processada 150 MPa, resultando num gel com maior consistência. O acompanhamento do gel por 24 h mostrou que o gel obtido com a enzima processada foi mais compacto, firme com maior expulsão do soro da matriz proteica quando comparado com o gel obtido pela enzima não processada. Portanto, destaque-se que, novamente, o processo de HAP foi capaz de melhorar as características hidrolíticas do coalho de bovino adulto, o que pode promover a abertura de novos mercados para esta enzima na fabricação de queijos.

A protease fúngica do *Rhizomucor miehei* foi altamente resistente ao processo de HAP, não sofrendo alterações na atividade proteolítica e de coagulação do leite quando processada uma vez até 190 MPa. As múltiplas

passagens a 190 MPa aumentaram a atividade proteolítica da enzima (aumento de ~ 6 %), mas não alteraram a atividade de coagulação do leite. Aumento na atividade proteolítica (~ 3 %) e na atividade de coagulação do leite (~ 10 %) foram observados para enzima processada a 190 MPa em solução com alta concentração o que, consequentemente, melhorou a fase de coagulação (rápida coagulação e maior consistência do gel).

Para pepsina suína, as alterações na atividade proteolítica e de coagulação do leite só foram observadas durante a estocagem refrigerada das enzimas, sendo que a enzima processada a 150 MPa apresentou uma redução na atividade proteolítica e um aumento na atividade de coagulação do leite. No entanto, na avaliação reológica realizada imediatamente após o processamento, o leite produzido com a enzima processada a 150 MPa apresentou uma coagulação mais rápida resultando num gel mais consistente quando comparada ao gel obtido pela mesma enzima sem processamento. Além disso, o gel produzido com a enzima processada a 150 MPa foi mais compacto, firme e menos poroso com maior liberação de soro da matriz proteica.

De uma forma geral, foi possível concluir que as enzimas processadas nas maiores pressões (coalho de vitelo, 190 MPa; coalho de bovino adulto, 150 MPa; pepsina suína, 150 MPa) apresentaram uma redução na atividade proteolítica e um aumento na atividade de coagulação de leite, apresentando um melhor desempenho durante o processo de coagulação. Esses ganhos resultaram em géis com menores níveis de proteólises, favorecendo a manutenção da rede proteica rígida, firme e coesa (conforme observado pelos resultados de sinérese, pelos valores de G' e G'' obtidos nas análises reológicas e pelos dados de análises microestruturais).

Desta forma, a avaliação global dos resultados permite concluir que a HAP é um processo promissor para modificação de enzimas coagulantes do leite, especialmente quando se deseja diminuir atividade proteolítica e aumentar sua atividade de coagulação do leite. Entretanto, foi observado que não é possível fazer uma generalização dos efeitos em enzimas coagulantes do leite, sendo

necessária a avaliação individual para cada enzima. Alguns resultados obtidos no presente trabalho apresentam melhorias para características hidrolíticas das enzimas (coalho de vitelo, coalho de bovino adulto e pepsina suína) dependendo do nível de pressão aplicada. A protease do micro-organismo *Rhizomucor miehei* foi, entre as enzimas avaliadas, a que apresentou maior resistência ao processo, o que possivelmente significa que o nível de energia fornecido para as moléculas pelo processo não resultou em uma grande modificação de sua estrutura.

Assim, a utilização de enzimas coagulantes do leite processadas a HAP permite obter géis mais consistentes com menor proteólise (podendo promover um aumento de rendimento e aumento de matéria seca em queijos). Além disso, a redução da proteólise verificada nestes géis pode desacelerar as alterações no sabor e odor de queijos frescos, possivelmente, resultando em aumento da extensão de vida de prateleira destes produtos.

Sugestões para trabalhos futuros

Os resultados obtidos nesta tese aumentam substancialmente as informações disponíveis sobre o efeito da HAP em enzimas, especialmente em enzimas comerciais utilizadas na fabricação de queijos. Sugere-se para trabalhos futuros avaliar isoladamente o efeito do processo HAP sobre quimosina e pepsina bovina para elucidar de fato qual o efeito do processo para cada enzima.

Além disso, sugere-se que novos trabalhos sejam realizados com enzimas com alto grau de pureza a partir de técnicas bioquímicas de separação possibilitando uma análise biofísica da molécula após a HAP para determinar os efeitos do processo nas estruturas da enzima e, consequentemente, estabelecer o papel de cada estrutura e da conformação final das enzimas na atividade, especificidade e estabilidade das mesmas.

Por fim, sugere-se a continuidade do trabalho na produção de diversos tipos de queijos utilizando as diferentes enzimas processadas na condição ótima para avaliação e certificação de como essas alterações ocorridas na enzima processada interferem nas características finais dos queijos produzidos.

Apêndice I

Tabela 8 Effect of high pressure homogenization (HPH) on the catalytic properties of milk-clotting enzymes and characterization of gels obtained by enzymes processed at HPH

Enzyme	Catalytic properties					Gel during 24 hours					
	Proteolytic activity	**Milk-clotting activity**	**Rate Milk-clotting**	**Start of aggregation (Beginning G' values)**	**G' Values at the final of coagulation**	**Protelysis**	**Synerisis**	**Porosity**	**G' values**	**G'' values**	**Loss tangent**
Calf rennet	Reduced with increase of pressure; maximum of 52% at 190 MPa	Not affected	Higher to 190 MPa processed enzyme	The samples produced with the enzyme processed at 150 MPa, 190 MPa and the non-processed enzyme were the first with no difference between them	Higher G' for sample produced with enzyme processed at 190 MPa (7% higher than non-processed one)	Reduction of non-specific proteolysis using the enzyme processed at 190 MPa	Increased using the 190 MPa processe d enzyme	Reduced porosity of the gel using the enzyme processed at 190 MPa	Higher values of G' using the enzyme processe d at 190 MPa	Higher values of G' using the enzyme processed at 190 MPa	Higher values of Loss tangent using the enzyme processed at 190 MPa
Adult bovine rennet	Reduced with increase of pressure; maximum of 7% at 190 MPa	Increased with increase of pressure; maximum of 7% at 150 MPa	150 MPa achieved a greater initial velocity after processing and after 14 days of storage	No difference after processing and after 14 days of storage the start aggregation occured 15 % quicker by enzyme processed at 150 MPa than using the non-processed one	Higher G' for sample produced with enzyme processed at 150 MPa (4% higher than non-processed one after processing and after 14 days of storage 10% higher)	Reduction of non-specific proteolysis using the enzyme processed at 150 MPa	Increased using the 150 MPa processe d enzyme	Reduced porosity of the gel using the enzyme processed at 150 MPa	Higher values of G' using the enzyme processe d at 150 MPa	Higher values of G' using the enzyme processed at 150 MPa	Higher values of Loss tangent using the enzyme processed at 150 MPa
***Rhizomucor miehei* protease**	Not affected	Not affected	Sample processed at 190 MPa achieved greater initial velocity	Sample processed at 190 MPa presents a faster start	Sample processed at 190 MPa and non-processed sample have the highest values of G'	ND	ND	ND	ND	ND	ND
Porcine pepsin	No difference after processing and after 60 days of storage the enzymes processed at 50, 100 and 150 MPa showed lower PA (~5%) than control and processed sample at 190 MPa	Not affected immediately after processing and after 60 days storage the enzyme processed at 150 MPa was 15% higher compared to the control	Sample processed at 190 MPa achieved greater initial velocity	The coagulation using the enzyme processed at 150 MPa occured 20% quicker than using the non-processed one after processing and after 60 days of storage occured 25% quicker	The porcine pepsin homogenized at 150 MPa produced the gel with G' values 92% higher than non-processed one after processing and after 60 days of storage obtained G' values 106% higher	Reduction of non-specific proteolysis using the enzyme processed at 150 MPa	Increased using the 150 MPa processe d enzyme	Reduced porosity of the gel using the enzyme processed at 150 MPa	Higher values of G' using the enzyme processe d at 150 MPa	Higher values of G' using the enzyme processed at 150 MPa	Higher values of Loss tangent using the enzyme processed at 150 MPa

ND: Not determined