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YANDRA ALZIRA PEREIRA DO NASCIMENTO

USO DE CISTEÍNO PROTEASES IMOBILIZADAS DO LÁTEX DE *Calotropis procera* PARA FABRICAÇÃO DE QUEIJOS

FORTALEZA
2022

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas

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RESUMO

Com o crescente aumento do consumo de queijos e o decaimento na produção de quimosina de origem animal, novas fontes de enzimas capazes de promover a coagulação do leite estão sendo estudadas. Dentre elas, as proteases cisteínicas originárias do látex de *Calotropis procera* apresentam aplicações promissoras na indústria biotecnológica de alimentos, incluindo a produção de queijo. O uso de enzimas livres possui algumas desvantagens, como a baixa estabilidade em diferentes temperaturas e pH, além da perda das enzimas no processo, aumentando o custo da produção. Uma possível alternativa para esses problemas é a imobilização enzimática, pois é uma técnica que auxilia na estabilidade da molécula, evita a contaminação do produto final pela enzima, assim como a enzima pode ser usada em vários ciclos do processo, o que se mostra de extrema relevância na indústria de alimentos. Desta forma, o presente trabalho prospectou a imobilização das proteases cisteínicas obtidas do látex de *C. procera* em suporte de glyoxyl-agarose e avaliou sua atividade na coagulação do leite e na fabricação de queijos. As enzimas foram imobilizadas, com sucesso, em suporte de glioxil-agarose (Glyoxyl-CpCPs). Quando imobilizadas, apresentaram atividades que variaram de 254% a 45%, de acordo com as cargas enzimáticas (5, 10, 20, 40 e 50 mg/g), quando comparadas às enzimas, em solução, em condições reacionais similares. A hidrólise da caseína por glioxil-CpCPs foi semelhante à sua forma solúvel. Além disso, glioxil-CpCPs exibiu melhor atividade específica de coagulação do leite do que sua forma solúvel e quimosina. As enzimas imobilizadas mantiveram atividade catalítica estável por pelo menos seis meses a 8 °C. As técnicas de microscopia de força atômica (AFM) e espalhamento dinâmico de luz (DLS) mostraram que o processo de agregação das micelas de caseína, após tratamento com glioxil-CpCPs foi muito semelhante àquele observando com o uso da quimosina solúvel. Por fim, o suporte glioxil-CpCPs, reutilizado em cinco ciclos de reação, de fabricação de queijos, manteve o mesmo rendimento. O estudo sugere que a imobilização das enzimas laticíferas potencializou sua utilização como um insumo, alternativo à quimosina, para a produção de queijo tipo coalho.

Palavras-chave: coagulação do leite; proteases vegetais; produção de queijo.

ABSTRACT

With the increasing consumption of cheese and the decline in the production of chymosin of animal origin, new sources of enzymes capable of promoting milk clotting are being studied. Among them, the cysteine proteases originating from the latex of Calotropis procera have promising applications in the biotechnological food industry, including cheese production. The use of free enzymes has some disadvantages, such as low stability at different temperatures and pH, in addition to the loss of enzymes in the process, increasing the cost of production. A possible alternative to these problems is enzymatic immobilization, as it is a technique that helps in the stability of the molecule, prevents contamination of the final product by the enzyme, as well as the enzyme can be used in several cycles of the process, which is extremely important. relevance in the food industry. Thus, the present work explored the immobilization of cysteine proteases obtained from C. procera latex on a glyoxyl-agarose support and evaluated its activity in milk coagulation and cheese production. The enzymes were successfully immobilized on glyoxyl-agarose support (Glyoxyl-CpCPs). When immobilized, they showed activities ranging from 254% to 45%, according to the enzymatic loads (5, 10, 20, 40 and 50 mg/g), when compared to the enzymes, in solution, under similar reaction conditions. The hydrolysis of casein by glyoxyl-CpCPs was similar to its soluble form. Furthermore, glyoxyl-CpCPs exhibited better specific milk clotting activity than their soluble form and chymosin. Immobilized enzymes maintained stable catalytic activity for at least six months at 8 °C. Atomic force microscopy (AFM) and dynamic light scattering (DLS) techniques showed that the aggregation process of casein micelles after treatment with glyoxyl-CpCPs was very similar to that observed with the use of soluble chymosin. Finally, the glyoxyl-CpCPs support, reused in five reaction cycles of cheese manufacturing, maintained the same yield. The study suggests that the immobilization of lacticiferous enzymes potentiated their use as an input, alternative to chymosin, for the production of coalho cheese.

Keywords: milk coagulation; plant proteases; cheesemaking.

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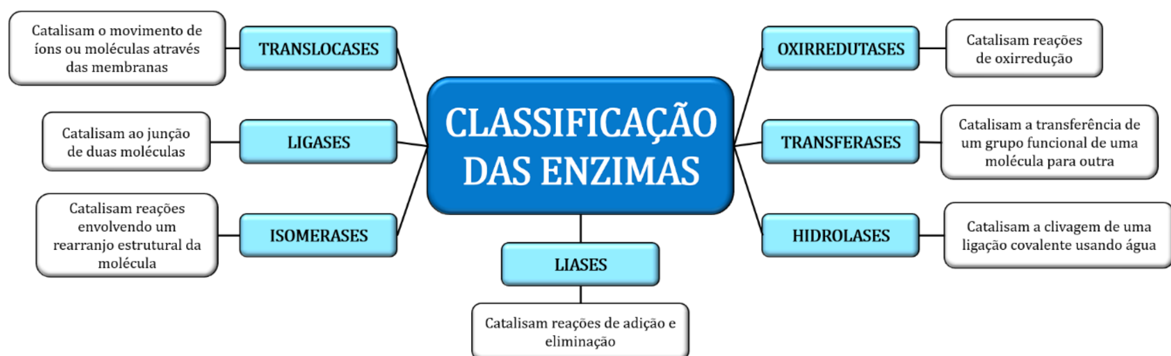
1. INTRODUÇÃO

Enzimas são catalisadores biológicos quase sempre de origem proteica, exceto por um pequeno grupo de moléculas de RNA catalíticas ("National Human Genome Research Institute", 2021). Esses catalizadores desempenham um papel essencial em diferentes processos biológicos, incluindo metabolismo, expressão gênica, divisão celular e reações importantes do sistema imunológico (PORTO DE SOUZA VANDENBERGHE et al., 2020) e são classificadas de acordo com a reação que catalisam, podendo ser alocadas em 7 classes: oxirredutases, transferases, hidrolases, liases, isomerases, ligases e translocases (Figura 1) (NELSON; COX, 2014). As hidrolases, especificamente, por serem um grupo muito abrangente de enzimas, apresentam subdivisões e dentre elas podem ser citadas as lipases, glicosidases e as proteases, que se destacam por sua extrema relevância na indústria alimentar (VERMELHO et al., 2016). As aplicações de enzimas na indústria alimentícia incluem panificação, laticínios, conversão de amido e processamento de bebidas (cerveja, vinho, frutas e sucos de vegetais) (HOMAEI et al., 2013).

Figura 1 – Classificação das enzimas de acordo com as reações que catalisam.



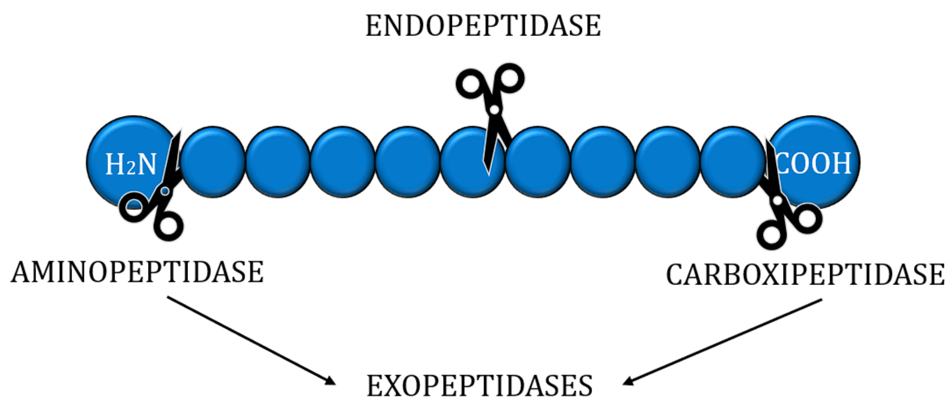
Fonte: (PORTO DE SOUZA VANDENBERGHE et al., 2020). Adaptado pelo autor.

1.1 Proteases

As proteases (peptidases ou enzimas proteolíticas) constituem um grande grupo de enzimas que catalisam a hidrólise de ligações peptídicas. A clivagem dessas ligações leva à degradação dos substratos proteicos em seus aminoácidos constituintes, ou pode ser mais específica, levando à clivagem seletiva da proteína para modificação e processamento pós-traducional (DE SOUZA et al., 2015). Elas fazem parte de uma subdivisão das hidrolases, sendo consideradas um dos grupos mais importantes de enzimas industriais e que atendem às necessidades de cerca de 60% do mercado mundial de enzimas (HUSAIN, 2018).

A classificação das proteases é baseada em seus mecanismos catalíticos, sendo divididas em dois grupos principais: as exopeptidases e as endopeptidases (AGARWAL, 1990). As exopeptidases executam sua atividade hidrolítica ou no C-terminal ou no N-terminal do polipeptídeo, podendo ser chamadas de carboxipeptidases e aminopeptidases, respectivamente (MÓTYÁN; TÓTH; TŐZSÉR, 2013). Já as endopeptidases realizam sua clivagem em aminoácidos que não estão nas extremidades do polipeptídeo (Figura 2) e podem ser classificadas de acordo com o aminoácido que é responsável pela atividade catalítica, sendo divididas em 6 grupos: i) cisteíno-proteases (EC 3.4.22); ii) serino-proteases (EC 3.4.21) ; iii) proteases de treonina (EC 3.4.25); (iv) proteases de ácido glutâmico (EC 3.4.23); (v) proteases de ácido aspártico (EC 3.4.23); e (vi) metaloproteases (EC 3.4.24) (GURUMALLESH et al., 2019).

Figura 2 – Sítio de clivagem das endopeptidases e exopeptidases



Fonte: Elaborada pelo autor

1.2 Proteases de origem vegetal e suas aplicações na indústria de alimentos

As proteases são encontradas em diversos organismos, seja procarioto ou eucarioto, animal ou vegetal (GURUMALLESH et al., 2019). Nas plantas, elas estão envolvidas na maioria das funções fisiológicas, como processamento de proteínas, crescimento, reprodução, defesa, apoptose e senescência (MAZORRA-MANZANO; RAMÍREZ-SUAREZ; YADA, 2018) e são, predominantemente, endopeptidases cisteínicas ou serínicas, sendo raramente observados outros tipos (GURUMALLESH et al., 2019). Além disso, as proteases de origem vegetal apresentam um alto valor agregado por continuarem ativas em amplas faixas de temperatura, pH, na presença de surfactantes, solventes orgânicos e desnaturantes, o que facilita o uso dessas enzimas em processos industriais (YADAV; PATEL; JAGANNADHAM, 2011).

Uma fonte importante de proteases de plantas é o látex e sua utilização na medicina tradicional e na indústria é bem conhecida (DOMSALLA; MELZIG, 2008). O látex é uma suspensão ou emulsão aquosa de vários tipos de partículas transportadas por células vivas, dentre elas encontram-se proteínas, alcaloides, amidos, açúcares, óleo, taninos, resinas e gomas (PRIYADARSHAN, 2017).

Enzimas como a papaína e a ficina, extraídas de *Carica papaya*, e *Ficus carica*, respectivamente, são as proteases de plantas mais conhecidas usadas no processamento de alimentos, produtos farmacêuticos e outros processos industriais, representando 5% das vendas globais de proteases (MAZORRA-MANZANO; RAMÍREZ-SUAREZ; YADA, 2018). O látex dessas plantas tem sido utilizado para a fabricação de queijos, nas indústrias cervejeira e farmacêutica, para amaciamento de carnes e na produção de peptídeos bioativos (SIAR et al., 2017).

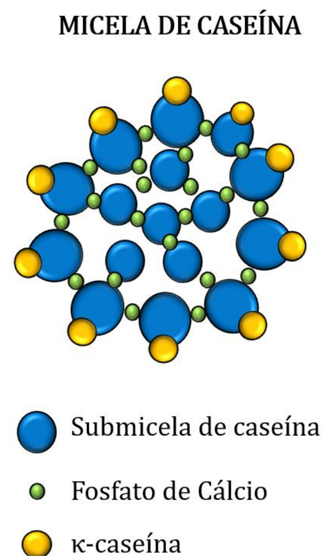
1.2.1 Uso de proteases vegetais na produção de queijo

O queijo é um dos produtos derivados do leite mais consumidos em todo o mundo. O processo de transformação do leite em queijo se inicia com a coagulação do leite (MAZORRA-MANZANO; MORENO-HERNÁNDEZ; RAMÍREZ-SUAREZ, 2018) e historicamente, a maioria das preparações enzimáticas usadas coagulação do leite são extratos de estômagos de ruminantes, especialmente do bezerro (FERNANDES, 2013). Esse extrato é chamado de coalho ou renina e ele contém quimosina (EC 3.4.23.4) como o principal

componente enzimático (HILL, 2000), responsável por hidrolisar as caseínas, principais proteínas do leite, especificamente a κ-caseína na ligação Phe_{105}–Met_{106}, resultando na formação de micelas (TUINIER; DE KRUIF, 2002). A coagulação do leite, por meio enzimático representa a modificação dessas micelas pela hidrólise limitada da caseína sob a ação das enzimas de coagulação do leite, seguida por uma rede de agregação de micelas induzida pela presença de íons cálcio (BENNETT; JOHNSTON, 2004)

As caseínas são as principais proteínas do leite, podendo ser obtidas pela precipitação em pH 4,6, sendo a fração sobrenadante "whey protein", outro componente importante na proteína do leite (KERN et al., 2020). Estruturalmente, as caseínas consistem em quatro subfrações anfifílicas, αS1-, αS2-, β- e κ-caseínas, que são caracterizadas como fosfoproteínas (KRUIF; HOLT, 2003). No leite, as caseínas estão organizadas em micelas, onde as subfrações ficam expostas ao meio aquoso e as micelas estão ligadas à íons de cálcio e realizando interações hidrofóbicas para permanecerem unidas (Figura 3) (WUSIGALE; LIANG; LUO, 2020).

Figura 3 – Modelo esquemático de uma micela de caseína.

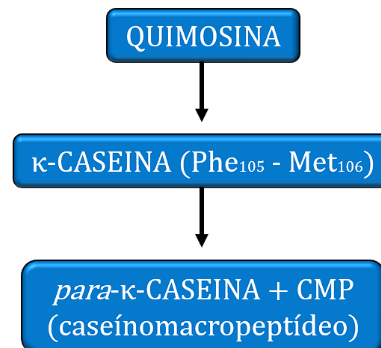


Fonte: Elaborada pelo autor

A coagulação do leite induzida pela quimosina pode ser descrita em três fases (NITU; GEICU-CRISTEA; MATEI, 2021):

FASE 1: Hidrólise enzimática da κ-caseína (Figura 4), que resulta na formação da *para*-κ-caseína, presente nas micelas (LUCIANA et al., 2017), e do caseínomacropeptídeo (CMP), que fica no soro e é uma molécula hidrofílica contendo 64 aminoácidos;

Figura 4 – Hidrólise enzimática da κ-caseína



Fonte: (NITU; GEICU-CRISTEA; MATEI, 2021) Adaptada pelo autor.

FASE 2: Formação do gel onde todos os componentes (proteínas, gordura, lactose e sais minerais) são aprisionados em uma estrutura tridimensional;

FASE 3: O soro é expelido da caseína por sinérese.

Com o crescente aumento do consumo de queijos, peptidases com atividade de coagulação de leites também têm sido obtidas de outras fontes como fungos, bactérias e plantas (FREITAS et al., 2016). Além disso, o alto preço do coalho, preocupações religiosas (como o islamismo e judaísmo), dieta (vegetarianismo) ou proibição do coalho de bezerro recombinante (na França, Alemanha e Holanda) estimularam ainda mais a busca por fontes alternativas de coagulação de leites (ROSEIRO et al., 2003), o que ainda promove maior aceitabilidade pelos vegetarianos e pode melhorar sua ingestão nutricional (DUARTE et al., 2009).

Nesse contexto, extratos brutos de plantas, como a *Calotropis procera,* vêm sendo usados como agentes coagulantes do leite (FREITAS et al., 2016). Estes extratos incluem enzimas como a papaína (*Carica papaya*), bromelaína (*Ananas comosus*) e a ficina (*Ficus carica*) (ABEBE; EMIRE, 2020). Além disso, já foi comprovado que frações proteolíticas e proteases purificadas do látex de *C. procera* são capazes de coagular o leite e produzir queijos com características necessárias para a produção industrial (FREITAS et al., 2016; SILVA et al., 2020).

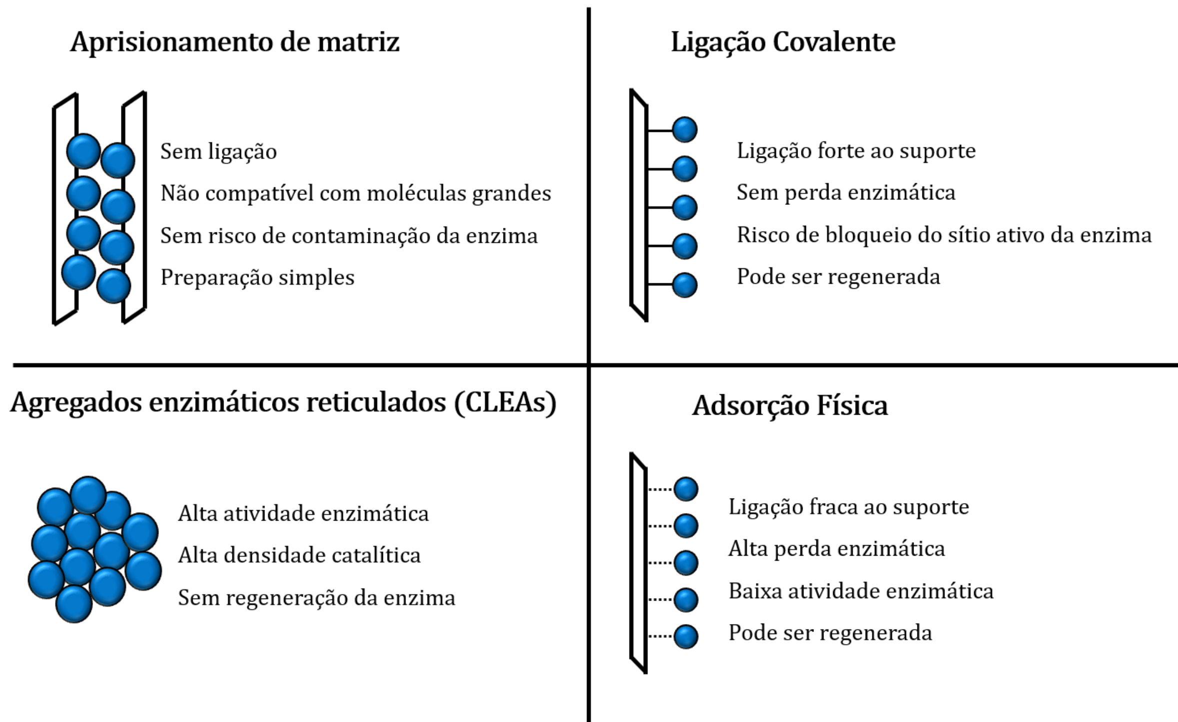
1.3 Imobilização enzimática

A obtenção de enzimas para o uso industrial, na maioria dos casos, é muito cara por conta dos custos com isolamento e purificação, além de serem moléculas altamente sensíveis a condições desnaturantes quando isoladas (HOMAEI et al., 2013). Um dos métodos mais bem sucedidos propostos para superar essas limitações é a utilização da técnica de imobilização enzimática (VAN DE VELDE et al., 2002).

A imobilização enzimática pode ser definida como o confinamento de moléculas em um suporte/matriz, fisicamente, quimicamente ou ambos, de forma que as enzimas mantenham sua atividade total ou a maior parte de sua atividade (AGGARWAL; PUNDIR, 2016). A imobilização de enzimas é um requisito para seu uso como biocatalisadores industriais na maioria do casos (RODRIGUES et al., 2013), tendo em vista que essa técnica permite a reutilização das enzimas (SPAHN; MINTEER, 2008), além de poder aprimorar a estabilidade da molécula, reduzir a inibição enzimática, aprimorar a especificidade da enzima e evitar a contaminação do produto pela enzima, o que tem extrema relevância na indústria de alimentos (GARCIA-GALAN et al., 2011). Em particular, a "reciclagem" enzimática por si só afeta drasticamente o impacto econômico de um processo em grande escala (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016).

De maneira geral, as tecnologias disponíveis para imobilizar enzimas podem ser divididas em três métodos tradicionais (Figura 5): (i) aprisionamento (dentro de um suporte inerte), (ii) ligação de portador (por adsorção física ou acoplamento covalente) e (iii) reticulação (formação de proteínas insolúveis) (BEZERRA et al., 2015). Independente do protocolo de imobilização a ser utilizado, alguns fatores precisam ser levados em conta para otimizar o processo, como as propriedades do suporte, o grupo ativo do suporte usado para imobilizar a enzima e o da molécula da enzima, bem como o próprio protocolo de imobilização (BOUDRANT; WOODLEY; FERNANDEZ-LAFUENTE, 2020).

Figura 5 – Métodos de imobilização enzimática e suas características.



Fonte: (KARAV et al., 2016) Adaptado pelo autor.

1.3.1 Suporte de glyoxyl agarose

O primeiro passo durante um processo de imobilização é a escolha de um suporte sólido que deve ser efetivo e econômico (SANTOS et al., 2015). Para isso, alguns requisitos devem ser cumpridos e dentre eles podem ser citados dois principais: a redução de custo (tanto do suporte quanto dos reagentes necessários) e a inércia química (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016).

Muitos fatores podem influenciar a seleção de um suporte em particular, e trabalhos de pesquisa mostraram que a hidrofilicidade é um dos fatores mais importantes para manter a atividade enzimática em um ambiente de suporte e, consequentemente, polímeros de polissacarídeos, que são muito hidrofílicos, são materiais de suporte populares para imobilização de enzimas (WANG; SHIYOU, 1997). Os resíduos de açúcar desses polímeros contêm grupos hidroxila, que são grupos funcionais ideais para ativação química para proporcionar a formação de ligações covalentes (SMITH et al., 2020).

Os suportes de glyoxyl possuem outras características especiais que determinam seu grande potencial (MATEO et al., 2007a). O braço espaçador curto, a ausência de

impedimentos estéricos para a reação entre os grupos químicos, presentes na enzima e o suporte e sua alta estabilidade são propriedades que favorecem uma intensa interação enzimática (MATEO et al., 2007b). Além disto, a baixa energia e reversibilidade da ligação imino são características que fazem os suportes de glyoxyl tão adequados para estabilizar enzimas, pois não introduzem fortes distorções da estrutura enzimática e direcionam a imobilização para a área onde existem grupos mais reativos na superfície da enzima (MATEO et al., 2007a). Isso significa que a enzima é imobilizada pela área onde é mais fácil obter uma ligação covalente muito intensa (MATEO et al., 2007b).

1.3.2 Imobilização enzimática por ligação covalente

O acoplamento covalente é um dos métodos mais utilizados para imobilização de enzimas porque as ligações formadas entre a enzima e o suporte são mais estáveis, impedindo a liberação da enzima no meio (BEZERRA et al., 2015). Este método de imobilização enzimático baseia-se na ligação de enzimas a carreadores insolúveis em água por meio de ligações covalentes (KARAV et al., 2016). Essa ligação é normalmente formada entre grupos funcionais presentes na superfície do suporte e grupos funcionais pertencentes a resíduos de aminoácidos na superfície da enzima (WANG; SHIYOU, 1997).

A imobilização enzimática por ligação covalente geralmente resulta em preparações muito estáveis e maior tempo de vida ativa quando comparadas com preparações de enzimas imobilizadas obtidas por outros métodos (NOROUZIAN, 2003).

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar a ação das proteases de cisteína de *C. procera*, imobilizadas em suporte de glyoxyl-agarose, na atividade de coagulação do leite e na fabricação de queijos.

2.2 Objetivos Específicos

- Obter as proteases purificadas do látex de Calotropis procera (CpCP1, CpCP2 e CpCP3)
- Realizar a imobilização das proteases purificadas (CpCPs)
- Analisar a atividade proteolítica das enzimas utilizando azocaseína como substrato.
- Comparar a atividade de coagulação do leite e a agregação micelar de caseínas utilizando as CpCPs e a quimosina comercial
- Produzir um queijo através da coagulação do leite

3 USE OF IMMOBILIZED CYSTEINE PROTEASES OF THE LATEX OF *CALOTROPIS PROCERA* FOR CHEESE MANUFACTURE

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Abstract

Cysteine proteases from *Calotropis procera* latex (CpCPs) have presented several biotechnological properties. Here, these enzymes were immobilized on glyoxyl-agarose support (Glyoxyl-CpCPs) and the recovered activities ranged from 254% to 45%, according to enzyme loadings (5, 10, 20, 40 and 50 mg/g). The casein hydrolysis by glyoxyl-CpCPs was similar to its soluble form. In addition, glyoxyl-CpCPs exhibited better specific milk-clotting activity than its soluble form and chymosin and even they were stable for at least 6 months stored at 8 °C. Atomic force microscopy (AFM) and dynamic light scattering (DLS) techniques showed that the process of casein micelle aggregation after treatment with glyoxyl-CpCPs was very similar to its soluble form and chymosin. Finally, glyoxyl-CpCPs was able to be used for five reaction cycles of cheese manufacture, maintaining the same yields. All results support the biotechnological potential of immobilized CpCPs as an alternative biocatalyst for cheesemaking.

1. Introduction

Cheese is one of the most consumed dairy products worldwide (Tilocca et al., 2020). Its manufacture usually involves the enzymatic coagulation by protease chymosin (EC 3.4.23.4), which is extracted from the fourth stomach of calves. Chymosin cleaves the bovine k-casein at Phe_{105}–Met_{106} bond, resulting in the disruption of the casein micelles and milk coagulation, and then producing the curd (Freitas et al., 2019). The high cost of chymosin, religious (Islam and Judaism) and dietary (vegetarianism) aspects as well as research by cheeses with new flavors and textures are some factors that have inspired studies to find alternative milk-clotting enzymes. Thus, new proteases with milk-clotting activity have been obtained from fungi, bacteria, and plants (Mazorra-Manzano, Moreno-Hernández, & Ramírez-Suarez, 2018).

The plant *Calotropis procera* has been studied as a suitable source of rennet, since its different parts have exhibited milk-clotting activity (Aworh & Muller, 1987). Another study reported that a mixture of cysteine proteases purified from *C. procera* latex was able to clot the milk and produce cheeses with good yields (Freitas et al., 2016). More recently, CpCP3 (a cysteine protease purified from *C. procera* latex) exhibited high milk clotting activity and the sensory analysis showed that the cheeses made with this enzyme had high acceptance (Silva et al., 2020). Moreover, standardized enzymatic preparation can be obtained from *C. procera* latex (Silveira et al., 2021). Therefore, all these results show the high potential of *C. procera* latex proteases as alternative enzymes for chymosin.

Enzyme immobilization can be a requirement for some industrial segments (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013). This technique allows the reuse of enzymes (Spahn, & Minteer, 2008), improve the stability of the molecule, reduce enzymatic inhibition, and avoid contamination of the product by the enzyme (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). All these

benefits are extremely relevant in the food industry. For example, during the cheesemaking, the chymosin is lost in the whey, representing a massive economic loss. Therefore, some studies have focused on immobilization of this enzyme, which has allowed continuous production of cheese curds (Liburdi, Spinelli, Benucci, Lombardelli, & Esti, 2018).

Recently, a study reported the immobilization, on different supports, of the cysteine proteases from *C. procera* latex. Among all supports tested, the proteases immobilized on glyoxyl-agarose showed broader pH stability, 60-fold more stable at 60 °C, and retained 70% of their initial activities after five reaction cycles (Oliveira et al., 2022). Therefore, the aim of this study was to evaluate the action of the *C. procera* cysteine proteases, immobilized on glyoxyl-agarose support, on milk-clotting activity and cheesemaking.

2. Material and Methods

2.1. Materials

CM-Sepharose fast-flow and agarose beads 6BCL were purchased from GE Healthcare (São Paulo, Brazil). Sodium borohydride, glycidol, sodium periodate, ethylenediamine, azocasein, BANA (Nα-Benzoyl-DL-arginine β-naphthylamide hydrochloride), DMACA (4-(Dimethylamino)cinnamaldehyde), k-casein, and L-cysteine were purchased from Sigma-Aldrich (São Paulo, Brazil). Cow's milk was purchased from local markets (Fortaleza, Ceará, Brazil). All other reagents were of analytical grade.

2.2. Purification of latex proteases and protein content

The latex of *C. procera* was collected in distilled water (1:1 ratio) and centrifuged (10,000 x *g*, 10 °C for 10 min) to eliminate the rubber. The supernatant was extensively dialyzed against distilled water at 8 °C using membranes with 10 kDa cutoff. Afterward, the material was centrifuged again, as previously described, and then the supernatant (latex

proteins) was lyophilized. The main latex proteases (CpCP1, CpCP2, and CpCP3) were purified by using two sequential ion-exchange chromatography steps (CM-Sepharose and Resource S) at pH 5.0 and 6.0, respectively (Ramos et al., 2013). Because the three cysteine proteases have very similar biochemical properties (Ramos et al., 2013), they were gathered as a fraction and are called here as *C. procera* cysteine proteases (CpCPs). The protein content was determined according to the method described by Bradford (1976), using bovine serum albumin as the protein standard.

2.3. Immobilization of CpCPs

CpCPs were immobilized on glyoxyl-agarose supports as described by Oliveira et al., 2022. Briefly, 1 g of glyoxyl-agarose was incubated with 4 mL of CpCPs solutions (in 200 mM bicarbonate buffer, pH 10.0), which were prepared at different concentrations in order to obtain enzyme loading of 5, 10, 20, 40, and 50 mg/g (enzyme:support). Immobilized CpCPs was termed Glyoxyl-CpCPs. The immobilizations also were performed in the presence of 20 mM L-cysteine (cysteine protease activator). That sample was named as Glyoxyl-CpCPs-(Cys). After 3 h under continuous stirring at 25 °C, the biocatalysts were reduced by adding $NaBH_4$ to a final concentration of 1 mg/mL and incubated for 30 min. Finally, the biocatalysts were rinsed with distilled water and vacuum filtered.

2.4. Proteolytic assays

The proteolytic assays were performed using azocasein or BANA as substrates, according to Freitas et al., (2007). For the assays using azocasein, 100 μL of soluble CpCPs (0.5 mg/mL) or 0.05 g of immobilized CpCPs was mixed with 200 μL of 1% azocasein and the final volume was adjusted to 500 μL with 50 mM sodium acetate buffer, pH 5.0. The reaction was performed for 40 min at 37 °C at 130 rpm. After the reaction, 300 μL of 12% trichloroacetic acid solution was added and then centrifuged at 10,000 x *g* for 5 min. Aliquots

of 400 µL of the supernatants were mixed with 400 µL of 2 M NaOH and the absorbance was measured at 420 nm. One unit of activity (U) was determined as the amount of soluble/immobilized CpCPs able to increase the absorbance by 0.01 unit per minute. For the assays using BANA, 100 µL of soluble CpCPs (0.5 mg/mL) or 0.05 g of immobilized CpCPs was mixed with 300 µL of 1 mM BANA and the final volume was adjusted to 500 µL with 50 mM sodium acetate buffer, pH 5.0. The reactions were performed at 130 rpm for 30 min at 37 °C. The reaction was stopped with 500 µL of 2% HCl solution in ethanol. Finally, 500 µL of each supernatant was mixed with 500 µL of 0.06% DMACA solution in ethanol. After 30 min, the absorbance was measured at 540 nm and one unit of activity (U) was defined as the amount of soluble/immobilized CpCPs able to increase the absorbance by 0.01 unit per minute.

2.5. Hydrolysis of the caseins

Casein purification from cow's milk was performed as described by Oliveira at al. (2019). Briefly, the skimmed milk was acidified up to pH 4.6 using 1 M HCl, and then centrifugated at 1,500 x *g* at 20 °C for 20 min. The precipitate (sodium caseinate) was washed three times with distilled water, followed by centrifugation as previously described. Afterward, the casein fraction was dialyzed against distilled water and lyophilized.

For casein hydrolysis, 5 mL of casein solution (10 mg/mL, in 200 mM sodium phosphate buffer, pH 7.0) was incubated with 0.125 g of immobilized CpCPs at different enzyme loading (5, 10, 20, 40, and 50 mg/g) or 100 µL of soluble enzymes at different concentrations (0.1, 0.2, 0.5, 1, and 2 mg/mL). The hydrolysis assays were performed at 37 °C, 130 rpm for 120 min. The casein hydrolysates were monitored spectrophotometrically by the increment in the absorbance at 550 nm caused by the aggregation of casein molecules (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020).

For two-steps casein hydrolysis, 5 mL of casein solution (10 mg/mL dissolved in 200 mM sodium phosphate buffer, pH 7.0) were incubated with 0.125 g of immobilized CpCPs at different enzyme loading (5, 10, 20, 40, and 50 mg/g). The hydrolysis assays were performed at 8 °C, under stirring at 130 rpm for 40 min. Subsequently, the solutions were vacuum filtered to collect the biocatalyst and the hydrolysates were allowed to coagulate at 37 °C. The coagulations were monitored spectrophotometrically by the increment in the absorbance at 550 nm (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020).

Casein hydrolysis by proteases was also evaluated by SDS-PAGE. For that, the hydrolysis reaction was performed as described above and, after each time points (0, 5, 10, 15, 20, 30, and 40 min), aliquots were withdraw and mixed with sample buffer (1:1, v:v) [0.0625 M Tris buffer (pH 6.8) containing 2% SDS and 1% 2-mercaptoethanol]. The runs were performed at 25 mA (25 °C for 2 h). The gels were stained with Coomassie Brilliant Blue R-350 in water:acetic acid:methanol (7:1:2, v:v:v). The de-coloration was performed with the same solution without the dye. In order to quantify the casein hydrolysis by proteases, the gels were scanned and the densitometries of each casein bands were plotted as a percentage of the unhydrolyzed caseins (time 0) using ImageJ software (https://imagej.nih.gov/ij/).

2.6. Milk-clotting activity

Initially, different amounts of the immobilized CpCPs (enzyme loading of 50 mg/g) were incubated with 10 mL of cow's milk at 8 °C and 130 rpm. Afterward, the milk was vacuum filtered to collect the biocatalysts and then, it was allowed to coagulate at 37 °C. The clotting time was recorded when discrete particles were visible in the milk. Soluble CpCPs and chymosin were used as controls. One specific milk-clotting activity (SMCA) was defined as the amount of enzyme (mg) that clotted 10 mL of milk within 40 min (Ahmed, Babiker, &

Mori, 2010), using the formula:

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

2.7. Casein micelle aggregation measured by Atomic Force Microscopy (AFM)

The AFM measurements were performed as described by Freitas et al. (2019), using an MFP3D-BIO microscope from Asylum Research (Oxford Instruments, Santa Barbara, California, USA). The micelle aggregation was imaged at different times after enzyme addition. For the reactions, 10 mL of skimmed milk were incubated with 0.125 g of glyoxyl-CpCPs (50 mg/g). The hydrolysis was performed at 8 °C, under stirring at 130 rpm for 40 min. After that, glyoxyl-CpCPs were removed and the hydrolysates were heated up to 37 °C to allow milk clotting. At each time point, aliquots were withdrawn and diluted (1:20, v/v) in 50 mM Tris-HCl buffer (pH 6.5), containing 10 mM $CaCl_2$, placed on glass surfaces (10 µl), spread, and dried at 25 °C. The size (diameter and height) of casein micelles was analyzed using the AFM native software from 5 acquired images of each sample (around 1000 micelles). The experiments were repeated twice with independent samples.

2.8. Size distribution and zeta potential of casein micelles

The size and zeta potential (ζ) of casein micelles after addition of soluble or immobilized CpCPs were calculated from the measured diffusion coefficient introduced in equations (for size and electrical mobility) derived from the Einstein-Smoluchowski relation (Silva, Bahri, Guyomarc'h, Beaucher, & Gaucheron, 2015). For the reactions using immobilized enzyme, 10 mL of skimmed milk were incubated with 0.125 g of glyoxyl-CpCPs (50 mg/g). The hydrolysis was performed at 8 °C, under stirring at 130 rpm for 40 min. After that, glyoxyl-CpCPs were removed and the hydrolysates were heated up to 37 °C to allow milk clotting. For the reactions using soluble enzyme, 10 mL of skimmed milk were incubated

with 100 μL of soluble CpCPs (2 mg/mL). The hydrolysis was performed at 37 °C for 40 min. A commercial chymosin (CoalhoPar®) was used as control according to fabricant specifications. At each time point, aliquots were withdrawn and diluted (1:20, v/v) in 50 mM Tris-HCl buffer (pH 6.5), containing 10 mM $CaCl_2$. The measurements were performed at 25 °C and using a scattering angle of 173°. The experiments were done in triplicates with independent samples. The diffusion coefficients were measured using dynamic light scattering (DLS), in a Zetasizer Nano ZS (Malvern Instruments, Swavesey, Cambridge, UK).

2.9. Cheese manufacture

The cheeses were manufactured as described for Freitas et al. (2016). Briefly, 10 g of immobilized CpCPs were incubated with 400 mL of cow's milk for 40 min at 8 °C and 130 rpm. Afterward, the milk was vacuum filtered, to collect the biocatalysts, and it was allowed to clotting at 37 °C for 40 min. Then, the coagulum formed was manually cut into cubes and heated at 85 °C. After constant stirring for 10 min, the whey was drained from the curd by pressurizing for 2 h at 25 °C using a handmade machine. The pressed materials represented the cheeses. Soluble CpCPs (10 mg of CpCPs/400 mL of milk) and a commercial chymosin (CoalhoPar®) (according to fabricant specifications) were used as controls for cheese manufacture. The reuse of the immobilized CpCPs was determined by using the same biocatalyst over five consecutives cycles of cheese manufacturing, as describe before.

3. Results and discussion

3.1. CpCPs immobilization on glyoxyl-agarose support

Agarose has been used as a versatile support for enzyme immobilization, since it allows different derivatization protocols, producing a wide range of carriers (Zucca, Fernandez-Lafuente, & Sanjust, 2016). Among them, glyoxyl-agarose emerges as a useful

support for high enzyme stabilization through multipoint covalent binding (Mateo et al., 2006). In addition, enzymes immobilized on glyoxyl-agarose has been used in different food technology processes, including the milk coagulation (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020). Recently, cysteine proteases purified from *C. procera* latex (CpCPs) were successfully immobilized on glyoxyl-agarose support by covalent binding, producing a biocatalyst with promising potential for applications in food technology (Oliveira et al., 2022). Here, the study of immobilized CpCPs was extended in order to better characterize this biocatalyst and evaluate its applicability for milk coagulation. For that, different CpCPs loading were evaluated since previous study showed the importance of enzyme loading in the milk clotting-activity of biocatalysts (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020). In all tested enzyme loading, the yield was around 90-95%. Using 5 mg/g, the glyoxyl-CpCPs exhibited the highest recovered activity (254%) (Supplementary Table 1).

Supplementary Table 1. Immobilization parameters of CpCPs on glioxyl-agarose at different enzyme loadings.

	Glyoxyl-CpCPs		**Glyoxyl-CpCPs(Cys)**	
Loadings (mg/g)	**Yield (%)**	**Recovered activity (%)**	**Yield (%)**	**Recovered activity (%)**
5	95.7 ± 0.2	254.1 ± 1.5	61.0 ± 2.0	116.1 ± 0.8
10	93.2 ± 0.7	186.9 ± 1.4	61.3 ± 1.7	86.9 ± 1.2
20	92.6 ± 0.1	144.4 ± 0.9	60.4 ± 1.8	60.0 ± 0.7
40	91.8 ± 0.5	89.5 ± 1.2	59.7 ± 1.4	35.3 ± 1.1
50	90.9 ± 0.2	45.9 ± 1.1	57.3 ± 2.1	26.5 ± 0.7

This result indicates that immobilization caused a hyperactivation of the proteases. Similarly, the activity of other immobilized milk-clotting enzymes was higher than its soluble forms (Esawy & Combet-Blanc, 2006). On the other hand, glyoxyl-CpCPs produced using the enzyme load of 50 mg/g exhibited the lowest recovered activity (46%). In general, greater the enzyme loading lower was recovered activity (Supplementary Table 1). This decreased activity can be due to diffusional limitation or steric hindrance (Boudrant, Woodley, &

Fernandez-Lafuente, 2020).

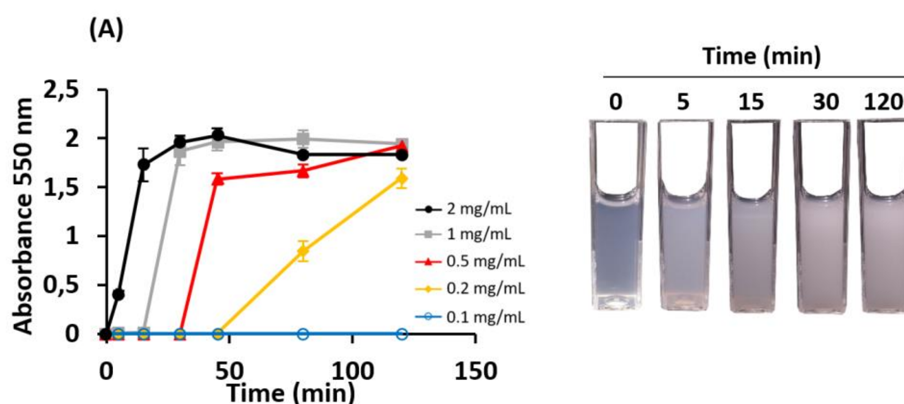
The immobilizations were also performed in the presence of L-cysteine, since CpCPs activity is highly dependent of cysteine protease activators (Freitas et al., 2007). Here, L-cysteine was chosen as additive during the immobilizations since it is a common nontoxic amino acid that can be used in food preparations. In addition, papain immobilization in the presence of L-cysteine enhanced the activity of the biocatalyst (Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010). Previously, when CpCPs immobilization was performed in the presence of L-cysteine, a drastic reduction of immobilization yields using an enzyme loading of 2 mg/g was obtained (Oliveira et al., 2022), but a better recovered activity was noticed against large substrate (azocasein). As shown in the Supplementary Table 1, CpCPs immobilized on glyoxyl support in the presence of L-cysteine [termed glyoxyl-CpCPs(Cys)], at enzyme loadings of 5-50 mg/g, exhibited immobilization yields and recovered activities considerably lower than those without L-cysteine (results obtained using a small substrate - BANA). The reduced immobilization yield is probably because L-cysteine present in immobilization medium is competing with the proteases for the glyoxyl reactive groups on the support. Although Oliveira et al. (2022) showed an interesting potential of glyoxyl-CpCPs(Cys), glyoxyl-CpCPs exhibited the best results regarding stability and reusability. Therefore, the biocatalyst obtained without L-cysteine was chosen to be used in all further assays.

3.2 Casein hydrolysis by immobilized CpCPs

The milk coagulation can occur in two phases: the primary (enzymatic) and the secondary (non-enzymatic) (Horne & Lucey, 2017). In the enzymatic phase, the hydrolysis of the casein micelles can be measured by the rate of their aggregation (clotting) along of the reaction time, which can be visually observed by the change in the turbidity of the casein

solution (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020). Thus, the casein hydrolysis by soluble and immobilized CpCPs was followed spectrophotometrically (Fig. 1). As observed, the clotting formation was highly dependent of CpCPs concentration (Fig 1A). Soluble CpCPs at 2 mg/ml started casein aggregation after 5 min and the maximum was reached at 30 min. Lower concentrations of CpCPs (1.0, 0.5, and 0.2 mg/mL) were also able to induce casein aggregation, but in a longer time. At 0.1 mg/mL, clotting was not observed. Similarly, ficin (a latex cysteine protease from *Ficus carica*) was also able to coagulate the caseins at low concentrations (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020).

Regarding immobilized CpCPs (Fig. 1B), the hydrolysis using the biocatalyst with high enzyme loading (50 and 40 mg/g) caused maximum casein aggregation after 30 min. However, this performance was observed only after 60 min when the enzyme loading was of 20 mg/g. Using 5 mg/g, the clotting was not observed. Ficin immobilized on glyoxyl-agarose using enzyme loading of 10, 30 and 85 mg/g also exhibited significant difference in the hydrolysis of caseins (Siar, Morellon-Sterling, Zidoune, & Fernandez-Lafuente, 2020).



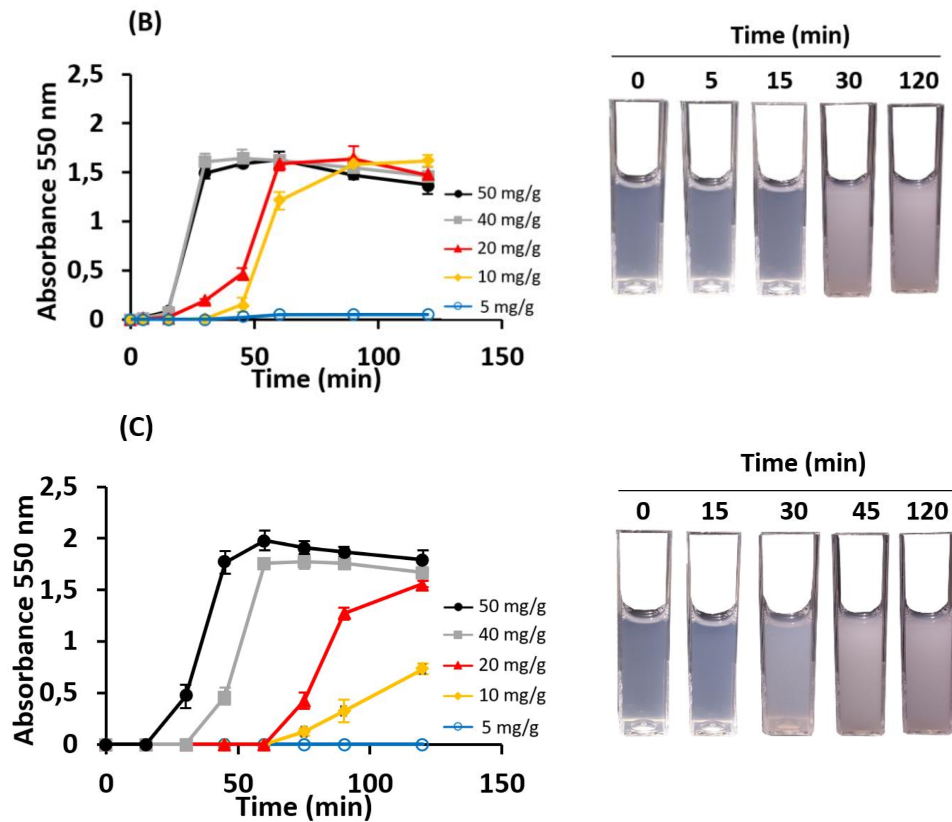


Fig. 1. **Effect of casein hydrolysis by soluble (A) and immobilized CpCPs (B and C)**. Soluble CpCPs were used in different concentration and immobilized CpCPs were used in different enzyme loadings (enzyme/support). The hydrolysis was performed at pH 6.5, 37 °C and 130 rpm and the clotting formation was measured by the increase of the absorbance at 550 nm. (C) For the double-step coagulation of caseins using immobilized CpCPs the reaction was performed pH 6.5, at 8 °C, 130 rpm, for 40 min. Then, immobilized enzymes were collected and then the milk was incubated at 37 ºC to allow the coagulation. The casein coagulation can also be visually demonstrated in the cuvettes throughout the time, using the higher concentration of soluble CpCPs (2 mg/mL) or the higher enzyme loading (50 mg/g) for immobilized CpCPs (B and C).

Although casein hydrolysis at 37 °C was rapid using immobilized CpCPs, milk coagulation under this condition is inadequate since immobilized enzyme would be trapped into casein coagulum, resulting in product contamination with the biocatalyst, besides hampering rennet reuse. Additionally, it is known that coagulum formation in the secondary phase is a temperature-dependent process and does not occur below 18 °C (Esteves, Lucey, Hyslop, & Pires, 2003). Therefore, when the hydrolysis reactions are performed at low temperatures, the immobilized enzymes can be easily recovered and the coagulation process is

allowed to continue at higher temperatures. Thus, new assays were performed to evaluate the viability of immobilized CpCPs in the hydrolysis of the caseins at low temperatures (Fig. 1C). For that, immobilized CpCPs were added to the casein solution at 8 °C for 40 min and then filtered in order to recover the biocatalyst. Afterward, the temperature of the hydrolysates was raised to 37 °C to follow casein coagulation, which was measured spectrophotometrically. Here, the hydrolysis time was fixed at 40 min since it is common time for hydrolysis by rennet for cheese manufacture (Mazorra-Manzano, Moreno-Hernández, & Ramírez-Suarez, 2018). As shown in Fig 1C, casein hydrolysis performed by glyoxyl-CpCPs with a load of 50, 40, and 20 mg/g was able to induced coagulum formation after 45, 60, and 120 min, respectively. This reduced enzymatic performance was caused by the influence of temperature in proteolytic activity of CpCPs, which was 10-fold lower at 8 °C when compared to 37 °C. As showed by Freitas et al. (2020), CpCP1, CpCP2, and CpCP3 have optimal activity at 35 °C.

The casein hydrolysis profile of each coagulant is shown in Fig 2. In addition, the degree of hydrolysis of each casein subunits (α-, β-, and κ-casein) was calculated using the intensity of the corresponding bands of the SDS-PAGE throughout the hydrolysis time (Fig. 2). Soluble and immobilized biocatalysts hydrolyzed the three casein components. However, the densitograms revealed that they were slightly more specific for the κ-casein hydrolysis, probably due to κ-casein external position in the micelle (Freitas et al., 2019), while α-casein band could still be observed in the SDS-PAGE after the hydrolytic reaction.

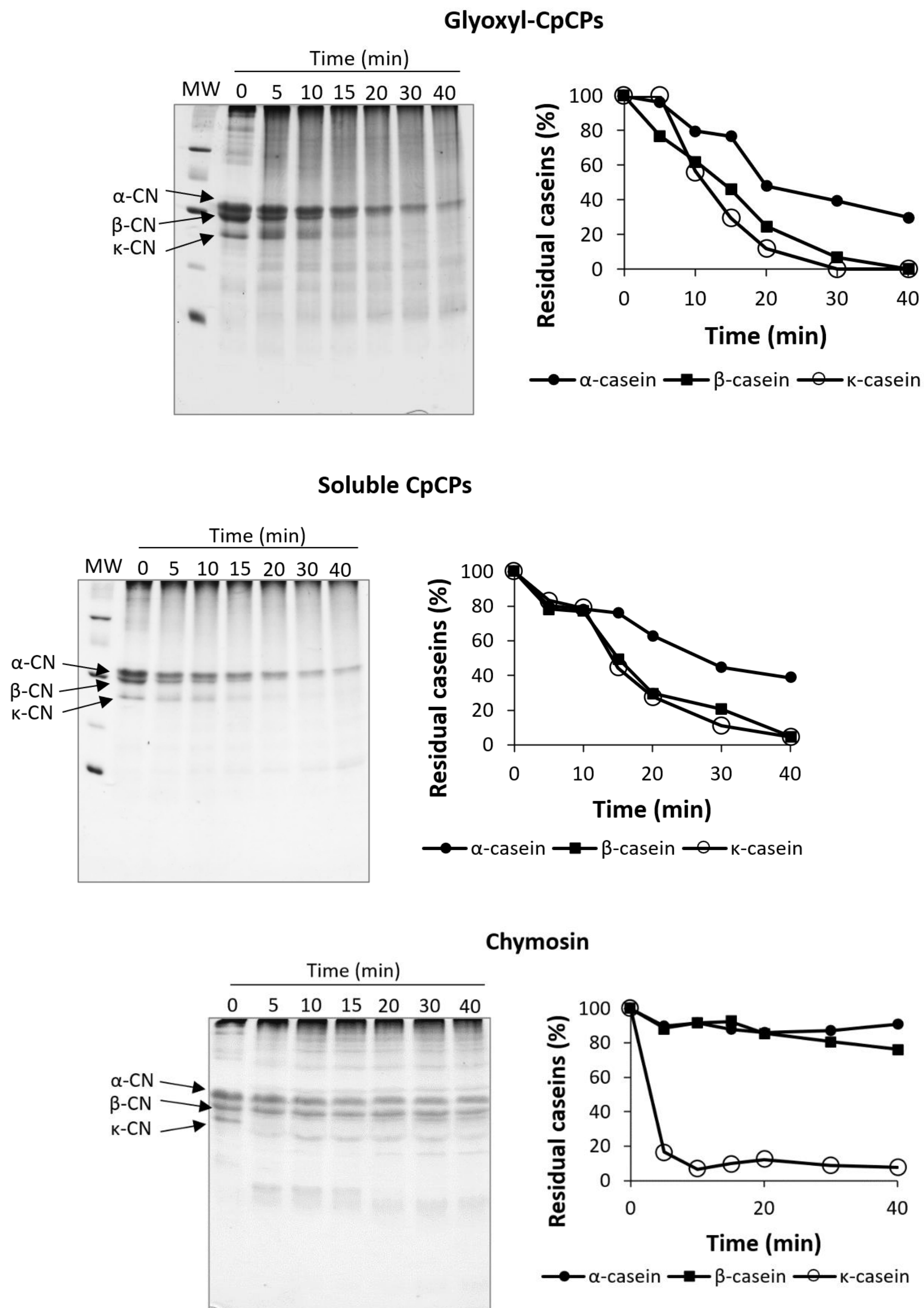


Fig. 2. Pattern of hydrolysis of the bovine caseins by immobilized and soluble CpCPs. Casein solution (10 mg/mL) was incubated with each biocatalyst for 40 min. For immobilized CpCPs, an amount of 0.125 g (10 mg/g) were incubated with 10 mL caseins solution at 8 °C and 130 rpm. Soluble CpCPs and chymosin (0.2 mg/mL) were incubated with caseins solution at 37 °C. The graphics show the degradation of caseins corresponding to densitograms from SDS-PAGE.

The hydrolysis specificity of immobilized CpCPs was κ-casein > β-casein > α-casein. Several vegetable rennet-like proteases have been described with different specificity for caseins hydrolysis (Mazorra-Manzano, Moreno-Hernández, & Ramírez-Suarez, 2018). Similarly, proteases from *Mucor miehei* covalently immobilized on aminated support (Pessela et al., 2004) and Alpine thistle aspartic protease immobilized on Immobead IB-150P (Esposito, Di Pierro, Dejonghe, Mariniello, & Porta, 2016) also exhibited higher specificity against κ-casein. As shown in Fig 2, immobilized CpCPs exhibited similar caseins cleavage pattern compared to its soluble form, which was able to produce cheeses with good taste and yields (Freitas et al., 2016; Silva et al., 2020).

As observed in Fig.2, chymosin hydrolyzed mainly the κ-casein molecule. It cleaves specifically between the Phe_{105} and Met_{106} bond of the k-casein, producing the para-k-casein and glycomacropeptide (Freitas et al., 2019). Previously, Freitas et al. (2016) reported that the proteases from *C. procera* latex exhibited similar κ-casein cleavage pattern compared to chymosin. The HPLC analysis showed the same peaks in the hydrolysates by *C. procera* proteases and chymosin, corresponding to para-k-casein and glycomacropeptide. In addition, the MS analysis of the protein band excised from SDS-PAGE exhibited molecular mass close to para-κ-casein. Thus, immobilized CpCPs may also exhibit similar cleavage site of κ-casein, resulting in analogous coagulation process.

3.3 Milk-clotting activity

In the search for new proteases as substitute for chymosin, some parameters must be evaluated. The milk-clotting activity of a protease is related to its ability to clot the milk in a suitable time, while the enzymatic activity is relevant to determine the extent of casein hydrolysis. Thus, the ratio of milk-clotting activity to proteolytic activity (SMCA/SPA) is an

important parameter to evaluate the use of a protease as rennet in cheesemaking (Freitas et al., 2016). A good coagulant should exhibit a high SMCA/SPA ratio, which is associated with high yields and good cheese characteristics (Mazorra-Manzano, Moreno-Hernández, & Ramírez-Suarez, 2018). As observed in Supplementary Table 2, the higher SMCA/SPA ratio was obtained for soluble CpCPs (1541.90), but immobilized enzyme exhibited similar value (1494.33). Mazorra-Manzano et al. (2018) reported that milk-clotting plant enzymes usually have shown lower SMCA/SPA ratio than chymosin. However, both soluble and immobilized CpCPs had very similar SMCA/SPA ratio compared to chymosin. Similar results were also observed for the proteolytic fraction of *C. procera* (Freitas et al., 2016). In contrast, purified CpCP3 exhibited lower SMCA/SPA ratio compared to chymosin (Silva et al., 2020).

Supplementary Table 2. Milk-clotting and proteolytic activities of the coagulants.

Enzymes	**SMCA** [a] (UA/mg)	**SPA** [b] (UA/mg)	**Ratio** (SMCA/SPA)
Glyoxyl-CpCPs	44.83 ± 6.64	0.03 ± 0.008	1494.33
Soluble CpCPs	477.99 ± 57.61	0.31 ± 0.11	1541.90
CoalhoPar®	326.18 ± 39.61	0.24 ± 0.04	1359.08

[a] SMCA: specific milk-coagulating activity.
[b] SPA: specific proteolytic activity.

3.4 Effect of temperature on clotting formation

A fixed mass of glyoxyl-CpCPs were screened in order to clot different volumes of milk. As shown in Supplementary Table 3, one gram of the biocatalyst was able to clot 80 mL of milk at 37 °C after 30 min. It is known that caseins aggregation is observed when approximately 70–80% of the k-caseins has been hydrolyzed (Mazorra-Manzano, Moreno-Hernández, & Ramírez-Suarez, 2018). Thus, using higher enzyme:substrate ratios, the hydrolysis degree of casein micelles may not be enough to start coagulation at 37 °C in a suitable time (up to 120 min). In comparison, 2.5 g of immobilized-agarose fungal protease

was adequate to coagulate 50 mL of milk at 30 °C (Pessela et al., 2004). When milk was hydrolyzed by glyoxyl-CpCPs and then heated at 40 °C, coagulation was observed even at a 1:120 ratio. As discussed before, the secondary phase is highly sensitive to the temperature. Thus, at high temperatures, the casein micelle aggregation may occur even if k-casein hydrolysis degree was low (Uniacke-Lowe & Fox, 2017). Moreover, at 45 °C, the coagulum formation was observed even at 1:160 ratio after 85 min.

Supplementary Table 3. Effect of temperature on clotting formation after hydrolysis by glyoxyl-CpCPs.

	Coagulation time (min)*		
Biocatalyst:milk ratio (g:mL)	**37 °C**	**40 °C**	**45 °C**
1:160	Nd	Nd	85 ± 10
1:120	Nd	88 ± 7.5	65 ± 8
1:80	30 ± 1.5	26 ± 3.0	21 ± 2.5
1:60	22 ± 2.0	15 ± 5.0	12 ± 2.0
1:40	18 ± 2.5	11 ± 3.0	6.5 ± 2.5

* Milk was hydrolyzed by glyoxyl-CpCPs during 40 min at 8 °C. Then, enzyme was recovered and the hydrolysates were heated up to allow clotting formation. Nd: not detected up to 120 min.

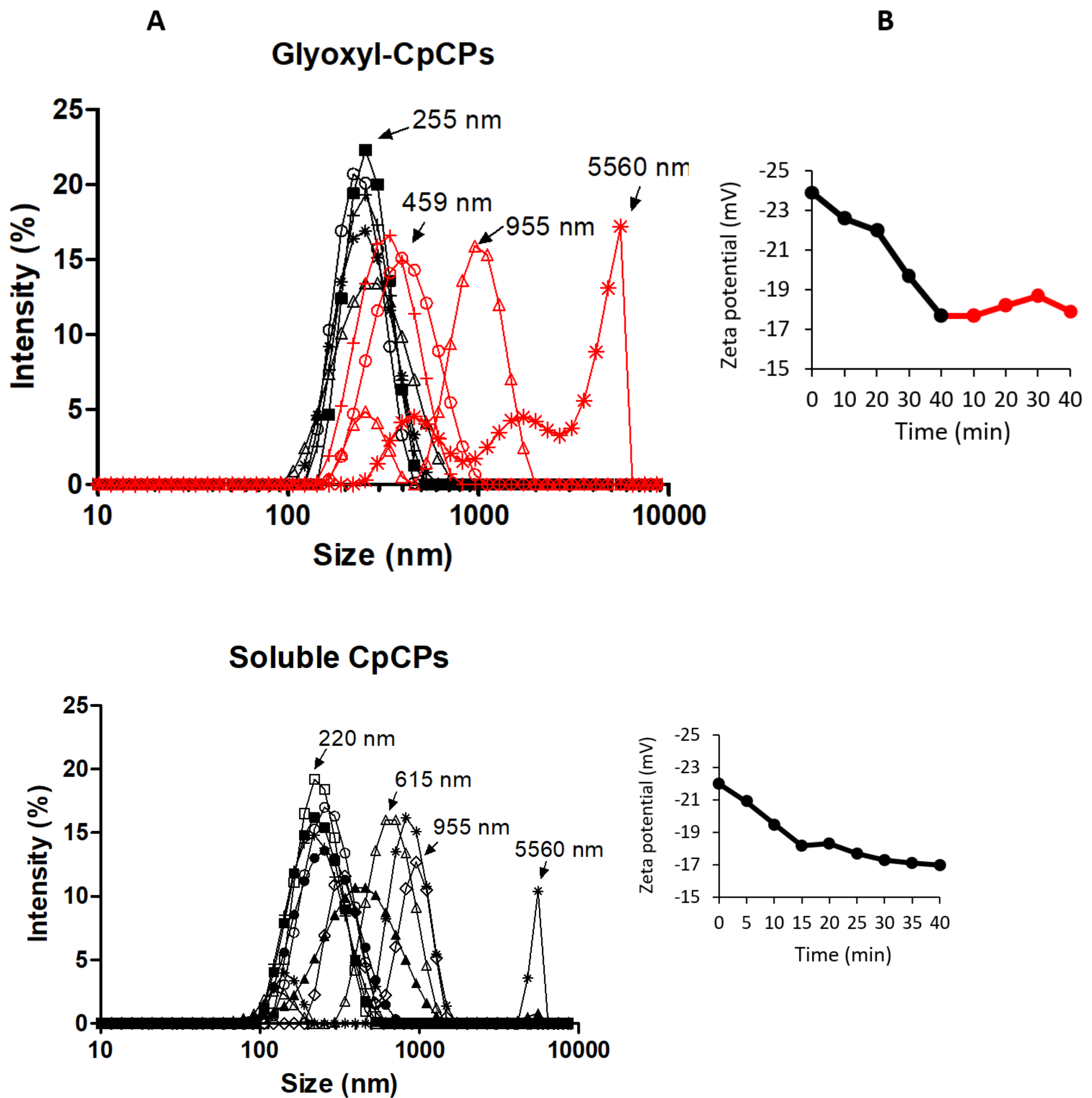
3.5 Action of coagulants on casein micelle aggregation

The analysis of particle size and zeta potential are important tools in the study of milk clotting (Freitas et al., 2019). It is known that size distribution of casein micelles can differ widely (80–550 nm), however their average diameters are around 200 nm (De Kruif & Holt, 2003). In addition, the external k-casein layer provides a high density of negative charges in the micelle structure, resulting in a zeta potential of approximately −23 mV. This surface charges are responsible for micelle stabilization in the milk by electrostatic repulsion (Green & Crutchfield, 1971). After renneting, the hydrolysis of the k-casein causes a decreasing in

the zeta potential and also result in micelle destabilization, and then aggregation (Freitas et al., 2019). The DLS analysis of the milk renneted with glyoxyl-CpCPs was evaluated during primary (enzymatic) and secondary (nonenzymatic) phase of milk coagulation. As observed in Fig 3, when Glyoxyl-CpCPs was added to the milk at low temperature (8 ºC), the zeta potential decreased approximately in a constant ratio. After 40 min, the zeta potential was −17.7 ± 3.63 mV, in comparison with −23.9 ± 4.12 mV for unhydrolyzed milk (0 min). Some studies reported that zeta potential can reduce up to 50% after rennet treatment (Horne & Lucey, 2017). Here, the reduction of zeta potential after enzymatic hydrolysis with glyoxyl-CpCPs corresponded to approximately 26%, which were sufficient to induce micelle aggregation. Similar results were reported by Freitas et al. (2019), using a commercial calf coagulant. The DLS assays also confirmed that size distributions of casein micelles were not altered and aggregation were not observed during all enzymatic phase. This result was confirmed by microscopy imaging using AFM (Fig. 4), which showed that most of the micelles were still isolated maintaining their shapes and sizes. This technique was recently used to characterize the coagulation process because avoid the use of additional dye, fluorophores or reagents, and its results are rapidly obtained and easily interpreted (Freitas et., 2019; Silva et al., 2020).

After the removal of the biocatalyst and incubation at 37 ºC, the secondary phase was characterized by micelle aggregation, which was observed by the increasing diameter of the micelle structures analyzed by DLS (Fig. 3) and AFM (Fig. 4). Following 10 min of the non-enzymatic phase, average size distribution of the particles was 342 nm. Meanwhile, AFM image indicate that casein micelles start to aggregate. After 30 min, the average diameter of the structures was 955 nm and finally, after 40 min, the size distribution of the particles was >5000 nm. At this time, macromolecular structures were observed in the milk, which start to acquire a solid aspect. No significant reduction of zeta potential was observed during the

secondary phase (Fig. 3). The casein micelles aggregation observed by AFM images were similar to those previously reported using chymosin (Freitas et al., 2019) and purified CpCP3 (Silva et al., 2020). Casein micelles gradually starts aggregation by hydrophobic interactions forming structures like bunches of grapes (Fig. 4, 10-20 min), that are grouped in larger clusters (Fig. 4, 30-40 min) and finally a three-dimensional gel is formed (not observed by AFM).



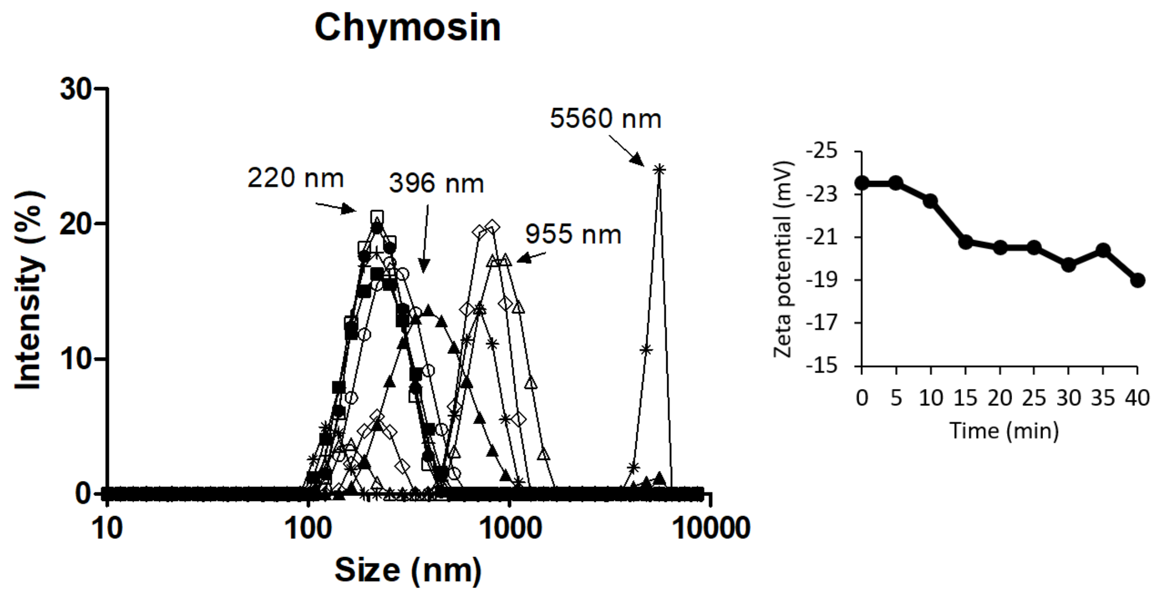


Fig. 3. (A) Particle size distribution and (B) zeta potential of milk casein micelles after addition of different coagulants measured by Dynamic Light Scattering (DLS). The dark lines in glyoxyl-CpCPs graphics correspond to the enzymatic phase and the red lines the non-enzymatic phase of milk coagulation. For immobilized CpCPs, an amount of 0.125g was incubated with 10 mL skimmed milk at 8 °C and 130 rpm. Soluble CpCPs and chymosin (0.2 mg/mL) were incubated with skimmed milk at 37 °C. After different time intervals, aliquots were withdrawn and diluted with 50 mM Tris-HCl buffer pH 6.5 (1:20, v/v). Legend: Milk without chymosin (■) and at intervals after adding coagulants of 5 min (□), 10 min (+), 15 min (●), 20 min (○), 25 min (▲), 30 min (Δ), 35 min (◊) and 40 min (*).

Using soluble CpCPs, the enzymatic stage was also characterized by a decreasing in zeta potential of the casein micelles. Following 25 min of CpCPs action, a sufficient number of k-casein molecules have been hydrolyzed and the aggregation started. At this point, the average diameter of the structures was 459 nm. Afterward, the aggregation phase overlapped the enzymatic phase, which means that hydrolysis of k-casein progressed (decreasing of zeta potential) while the micelles continued aggregating. The average size distribution was 615 nm and 825 nm after 30 and 35 min, respectively. After 40 min of reaction, the zeta potential was −17 ± 4,37 mV, corresponding to a reduction of approximately 22% by CpCPs treatment. At this point, particle size was > 5000 nm and macromolecular structures were observed in the milk.

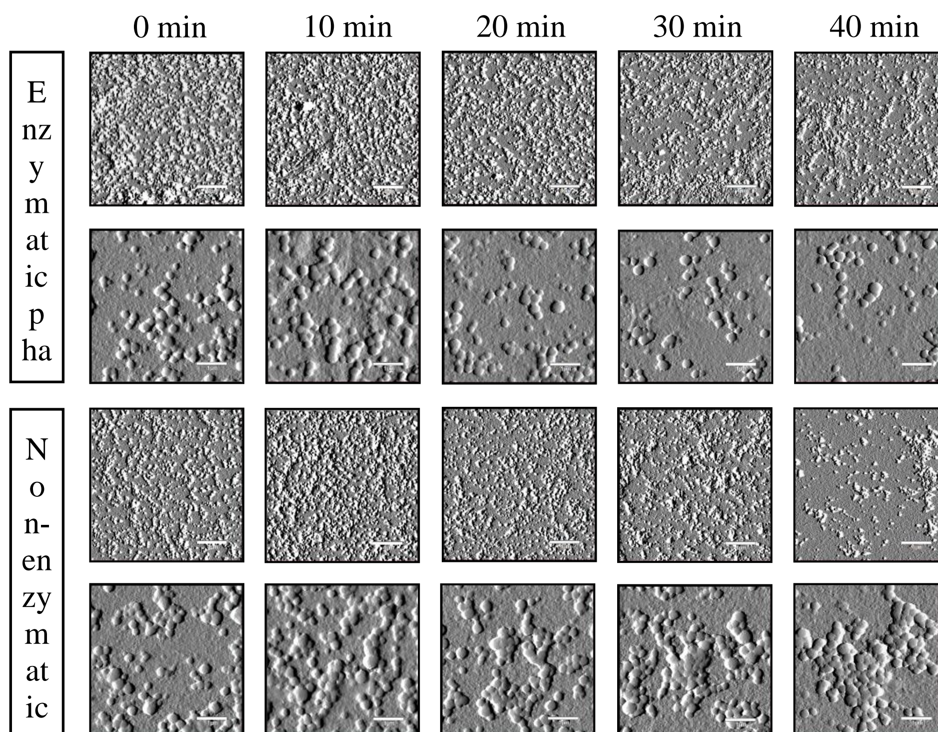


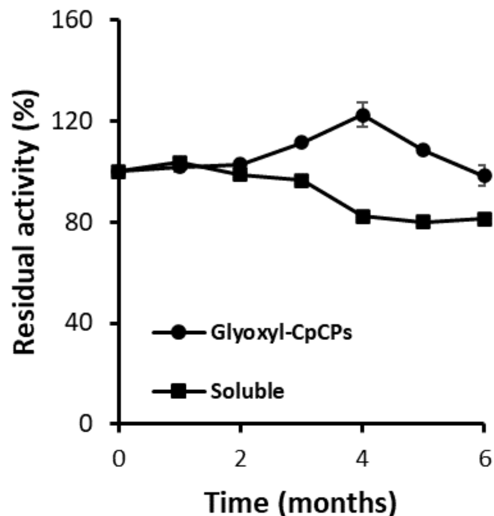
Fig. 4. Tapping mode AFM images of milk casein micelles after incubation with immobilized CpCPs at different time points (0, 10, 20, 30 and 40 min). For enzymatic phase, 10 mL of skimmed milk was incubated with 0.125 g of immobilized CpCPs at 8 °C and 130 rpm. For non-enzymatic phase, immobilized enzymes were collected from the milk and the hydrolisates were allowed to coagulate at 37 °C.

Milk treated with immobilized and soluble CpCPs exhibited similar results when compared to chymosin. The DLS analysis showed that after chymosin treatment, the reduction of zeta potential corresponded to about 20% of the initial values and the coagulation process was also characterized by the overlap of both enzymatic and aggregation phase. All these results were in accordance with previous report by Freitas et al. (2019). As observed, the reduction of zeta potential was slightly higher using glyoxyl-CpCPs than soluble CpCPs and chymosin. Dalgleish (1984) demonstrated that the zeta potential decreased with the extent of rennet action. Thus, these results suggest that immobilized biocatalyst exhibited higher hydrolysis of k-casein, likely by the fact that enzymatic phase is separated of aggregation phase, which allows higher hydrolytic action in the substrate without aggregation, since the enzymatic phase was performed at 8 °C.

3.6 Storage and operational stability

The storage stability of the immobilized CpCPs at load of 50 mg/g was evaluated over six months and the results are shown in Supplementary Fig 1. As observed, the enzymatic activity of glyoxyl-CpCPs slightly increased in the first four months. Probably, the reduction with sodium borohydride (final step of immobilization protocol) was unable to block all the reactive groups of the support and enzyme stabilization was observed during this period.

Over all, after six months, the residual activity of glyoxyl-CpCPs was close to initial (100%). El-Bendary, Moharam, & Ali (2009) reported that milk-clotting enzymes from *Bacillus sphaericus,* immobilized on silica gel by adsorption interaction, lost 10% of its activity in one month. The stability of soluble CpCPs was also evaluated (Supplementary Fig 1).



Supplementary Fig 1. Storage stability of immobilized CpCPs (50 mg/g). The biocatalysts were stored at 8 °C over six mouths and the proteolytic activity were measured using azocasein 1%. The proteolytic assay was performed using 0.062 g of immobilized CpCPs or 100 µL of soluble CpCPs (0.1 mg/mL).

As observed, the enzymes were stable during three months. After that, a slight loss in the enzymatic activity was observed and the residual activity was about 80% after six months. Similar results were reported by Silveira et al. (2021) using the whole protein fraction of *C.*

procera latex. The authors also showed that autolysis was negligible, which may explain the high stability of soluble CpCPs.

It is well known that one of the most important characteristics of the immobilized enzymes is their reutilization. Thus, the operational stability of glyoxyl-CpCPs was monitored over five cycles of milk renneting. Fig. 5 shows the schematic prosses of immobilized enzyme reuse during cheese manufacture.

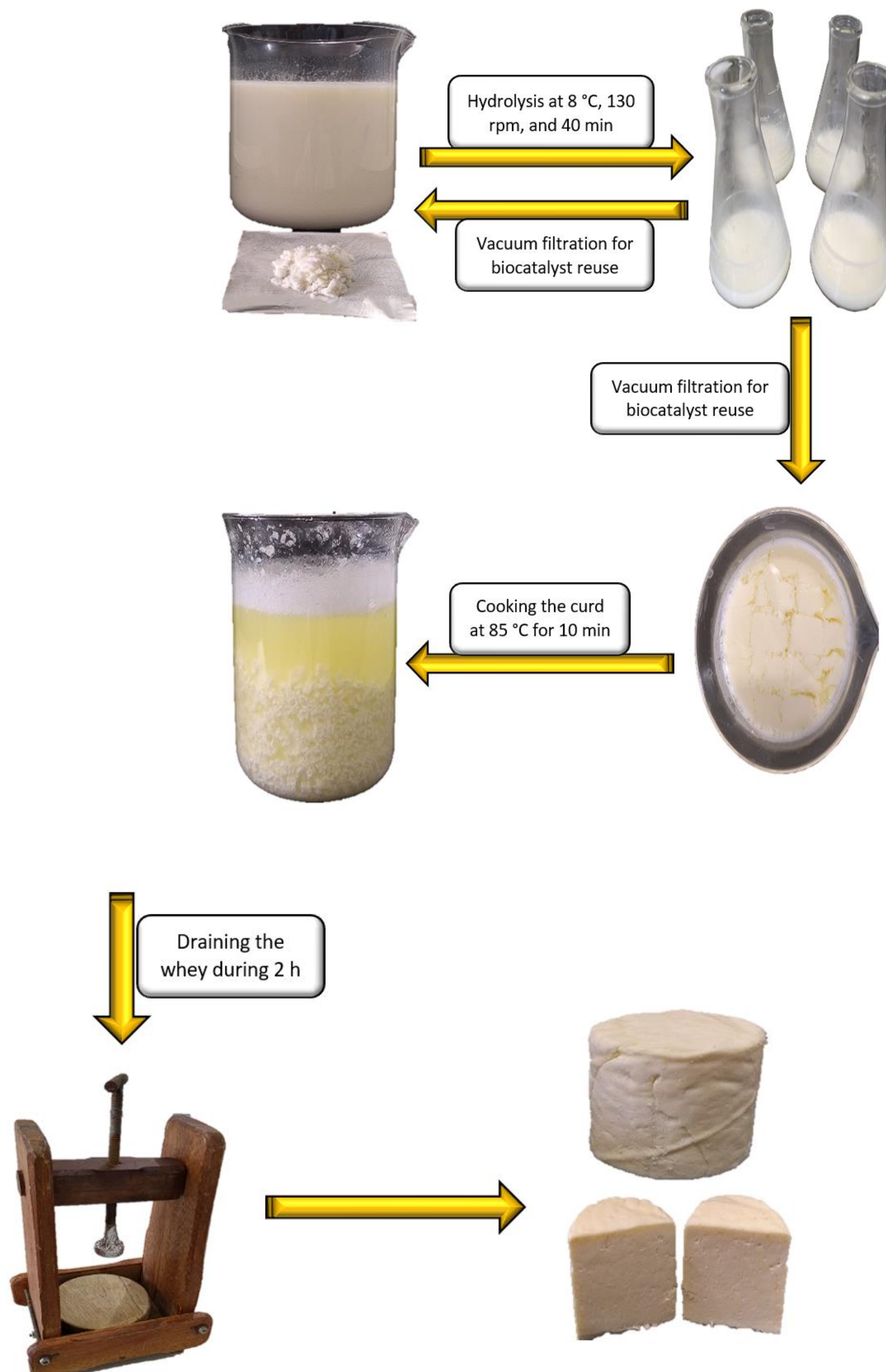


Fig. 5. Schematic showing the prosses of cheese manufacture using immobilized CpCPs.

As observed in Supplementary Table 4, similar yields were obtained in all the reaction cycles. In addition, the yields were comparable with cheese produced by soluble CpCPs and chymosin. Regarding enzymatic activity of glyoxyl-CpCPs, a slight reduction was observed and after five cycles, the residual activity was 78.5%. Thus, Glyoxyl-CpCPs was able to be used for at least five cycles of cheese making.

Supplementary Table 4. Some characteristics of the cheeses manufactured with Glyoxyl-CpCPs after five reuse cycles in comparison with soluble CpCPs and chymosin.

Glyoxyl-CpCPs (Reuse cycles)	**Yield (g/100 mL)**	**Residual activity (%)**
1st	9.01 ± 0.45	100
2nd	9.82 ± 0.80	95.1 ± 1.4
3rd	10.65 ± 0.21	89.7 ± 0.6
4th	9.69 ± 0.93	83.3 ± 1.3
5th	11.05 ± 1.33	78.5 ± 1.5
Other coagulants		
Soluble CpCPs	9.81 ± 0.15	-
CoalhoPar®	10.42 ± 1.12	-

Conclusion

The results show that immobilized CpCPs have biotechnological potential as an alternative enzyme for cheesemaking, since the immobilization technique produced biocatalysts with high yields and recovered activities. Immobilized enzymes were able to clot the milk in a suitable time and the hydrolysis pattern of the casein micelle was similar to soluble CpCPs, which produce cheese with high acceptance. Immobilized CpCPs also exhibited SMCA/SPA ratio higher than chymosin and was stable for six months of storage at 8 °C. In addition, immobilized CpCPs was able to be used for at least five reaction cycle, producing cheeses with constant yields in all the cycles. It is worth to note that the

coagulation process must be separated in two phases. The enzymatic hydrolysis must be performed at 8 °C to prevent micelle aggregation and allow the recovery of the biocatalyst. Then, hydrolysates must be heated up to 37 °C in order to form the curd.

References

Ahmed, I. A. M., Babiker, E. E., & Mori, N. (2010). pH stability and influence of salts on activity of a milk-clotting enzyme from Solanum dubium seeds and its enzymatic action on bovine caseins. LWT-Food Science and Technology, 43(5), 759-764.

Aworh, O. C., & Muller, H. G. (1987). Cheese-making properties of vegetable rennet from Sodom apple (Calotropis procera). Food chemistry, 26(1), 71-79.

Boudrant, J., Woodley, J. M., & Fernandez-Lafuente, R. (2020). Parameters necessary to define an immobilized enzyme preparation. Process Biochemistry, 90, 66-80. https://doi.org/10.1016/j.procbio.2019.11.026.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Dalgleish, D. G. (1984). Measurement of electrophoretic mobilities and zeta-potentials of particles from milk using laser Doppler electrophoresis. Journal of Dairy Research, 51(3), 425-438.

De Kruif, C. G., & Holt, C. (2003). Casein micelle structure, functions and interactions. In Advanced dairy chemistry—1 proteins (pp. 233-276). Springer, Boston, MA.

El-Bendary, M. A., Moharam, M. E., & Ali, T. H. (2009). Efficient immobilization of milk clotting enzyme produced by Bacillus sphaericus. Polish journal of food and nutrition sciences, 59(1).

Esawy, M. A., & Combet-Blanc, Y. (2006). Immobilization of Bacillus licheniformis 5A1

milk-clotting enzyme and characterization of its enzyme properties. World Journal of Microbiology and Biotechnology, 22(3), 197-200.

Esposito, M., Di Pierro, P., Dejonghe, W., Mariniello, L., & Porta, R. (2016). Enzymatic milk clotting activity in artichoke (Cynara scolymus) leaves and alpine thistle (Carduus defloratus) flowers. Immobilization of alpine thistle aspartic protease. Food chemistry, 204, 115-121.

Esteves, C. L., Lucey, J. A., Hyslop, D. B., & Pires, E. M. (2003). Effect of gelation temperature on the properties of skim milk gels made from plant coagulants and chymosin. International Dairy Journal, 13(11), 877-885.

Freitas, C. D., Leite, H. B., Oliveira, J. P. B., Amaral, J. L., Egito, A. S., Vairo-Cavalli, S., Lobo, M. D. P., Monteiro-Moreira, A. C. O., & Ramos, M. V. (2016). Insights into milk-clotting activity of latex peptidases from Calotropis procera and Cryptostegia grandiflora. Food Research International, 87, 50-59.

Freitas, C. D., Silva, M. Z., Oliveira, J. P., Silva, A. F., Ramos, M. V., & de Sousa, J. S. (2019). Study of milk coagulation induced by chymosin using atomic force microscopy. Food Bioscience, 29, 81-85.

Freitas, C. D., Silva, R. O., Ramos, M. V., Porfírio, C. T., Farias, D. F., Sousa, J. S., Oliveira, J. P. B., Souza, P. F. N., Dias, L. P., & Grangeiro, T. B. (2020). Identification, characterization, and antifungal activity of cysteine peptidases from Calotropis procera latex. Phytochemistry, 169, 112163.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. Advanced Synthesis & Catalysis, 353(16), 2885-2904.

Green, M. L., & Crutchfield, G. (1971). Density-gradient electrophoresis of native and of rennet-treated casein micelles. Journal of Dairy Research, 38(2), 151-164.

Homaei, A. A., Sajedi, R. H., Sariri, R., Seyfzadeh, S., & Stevanato, R. (2010). Cysteine

enhances activity and stability of immobilized papain. Amino acids, 38(3), 937-942. https://doi.org/10.1007/s00726-009-0302-3.

Horne, D. S., & Lucey, J. A. (2017). Rennet-induced coagulation of milk. Cheese, 115-143.

Liburdi, K., Spinelli, S. E., Benucci, I., Lombardelli, C., & Esti, M. (2018). A preliminary study of continuous milk coagulation using Cynara cardunculus flower extract and calf rennet immobilized on magnetic particles. Food chemistry, 239, 157-164.

Mateo, C., Palomo, J. M., Fuentes, M., Betancor, L., Grazu, V., López-Gallego, F., Pessela, B. C. C., Hidalgo, A., Fernández-Lorente, G., Fernández-Lafuente, R., & Guisán, J. M. (2006). Glyoxyl agarose: a fully inert and hydrophilic support for immobilization and high stabilization of proteins. Enzyme and Microbial Technology, 39(2), 274-280. https://doi.org/10.1016/j.enzmictec.2005.10.014.

Mazorra-Manzano, M. A., Moreno-Hernández, J. M., & Ramírez-Suarez, J. C. (2018). Milk-clotting plant proteases for cheesemaking. In Biotechnological applications of plant Proteolytic enzymes (pp. 21-41). Springer, Cham.

Oliveira, J. P. B., Candreva, A. M., Rizzo, G., Ramos, M. V., Oliveira, J. S., Oliveira, H. D., Ary, M. B., Docena, G., & Freitas, C. D. T. (2019). Allergenicity reduction of cow's milk proteins using latex peptidases. Food chemistry, 284, 245-253. https://doi.org/10.1016/j.foodchem.2019.01.123.

Pessela, B. C., Torres, R., Fuentes, M., Mateo, C., Fernandez-Lafuente, R., & Guisán, J. M. (2004). Immobilization of rennet from mucor m iehei via its sugar chain. Its use in milk coagulation. Biomacromolecules, 5(5), 2029-2033.

Ramos, M. V., Araújo, E. S., Jucá, T. L., Monteiro-Moreira, A. C. O., Vasconcelos, I. M., Moreira, R. A., Viana, C. A., Beltramini, L. M., Pereira, D. A., & Moreno, F. B. (2013). New insights into the complex mixture of latex cysteine peptidases in Calotropis procera. International journal of biological macromolecules, 58, 211-219.

https://doi.org/10.1016/j.ijbiomac.2013.04.001.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. Chemical Society Reviews, 42(15), 6290-6307.

Siar, E. H., Morellon-Sterling, R., Zidoune, M. N., & Fernandez-Lafuente, R. (2020). Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: Unexpected importance of the ficin loading on the biocatalysts. International journal of biological macromolecules, 144, 419-426.

Silva, M. Z., Oliveira, J. P. B., Ramos, M. V., Farias, D. F., Sá, C. A., Ribeiro, J. A. C., Silva, A. F. B., Sousa, J. S., Zambelli, R. A., Silva, A. C., Furtado, G. P. Grangeiro, T. B, Vasconcelos, M. S., Silveira, S. R., & Freitas, C. D. (2020). Biotechnological potential of a cysteine protease (CpCP3) from Calotropis procera latex for cheesemaking. Food chemistry, 307, 125574.

Silva, N. N., Bahri, A., Guyomarc'h, F., Beaucher, E., & Gaucheron, F. (2015). AFM study of casein micelles cross-linked by genipin: effects of acid pH and citrate. Dairy Science & Technology, 95(1), 75-86.

Silveira, S. R., Coelho, R. A., Sousa, B. F. E., Oliveira, J. S. D., Lopez, L. M. I., Lima-Filho, J. V. M., Júnior, P. A. V. R., Souza, D. P., Freitas, C. D. T., & Ramos, M. V. (2021). Standardized production of a homogeneous latex enzyme source overcoming seasonality and microenvironmental variables. Preparative Biochemistry & Biotechnology, 51(4), 375-385.

Spahn, C., & Minteer, S. D. (2008). Enzyme immobilization in biotechnology. Recent patents on engineering, 2(3), 195-200.

Tilocca, B., Costanzo, N., Morittu, V. M., Spina, A. A., Soggiu, A., Britti, D., Roncada, P., & Piras, C. (2020). Milk microbiota: Characterization methods and role in cheese production. Journal of Proteomics, 210, 103534.

Uniacke-Lowe, T., & Fox, P. F. (2017). Chymosin, pepsins and other aspartyl proteinases: Structures, functions, catalytic mechanism and milk-clotting properties. Cheese, 69-113.

Zucca, P., Fernandez-Lafuente, R., & Sanjust, E. (2016). Agarose and its derivatives as supports for enzyme immobilization. Molecules, 21(11), 1577. https://doi.org/10.3390/molecules21111577.

4 CONCLUSÃO

As proteases imobilizadas em suporte de glyoxyl-agarose de *C. procera* apresentaram um alto potencial biotecnológico como uma alternativa às enzimas do mercado, pois além de terem apresentado um alto rendimento na coagulação do leite, elas puderam ser recuperadas e reutilizadas em até cinco ciclos de reações, o que aumentaria a eficácia de uma produção de queijo à nível industrial. Além disso, a coagulação do leite deu-se de forma semelhante às proteases (CpCPs) livres, que já originam queijos de alta aceitação nos quesitos textura e sabor.

REFERÊNCIAS

ABEBE, B.; EMIRE, S. Manufacture of fresh cheese using east African Calotropis procera leaves extract crude enzyme as milk coagulant. **Food Science and Nutrition**, *[s.l.]* v. 8, n. 9, p. 4831–4842, 2020.

AGARWAL, S. K. Proteases cathepsins - A view. **Biochemical Education**, v. 18, n. 2, p. 67–72, 1990.

AGGARWAL, V.; PUNDIR, C. S. Rational Design of Nanoparticle Platforms for "cutting-the-Fat" Covalent Immobilization of Lipase, Glycerol Kinase, and Glycerol-3-Phosphate Oxidase on Metal Nanoparticles. 1. ed. *[s.l.]*, **Elsevier Inc**., 2016. v. 571.

BENNETT, R. J.; JOHNSTON, K. A. **General aspects of cheese technology**. [*s.l*: s.n.]. v. 2.

BEZERRA, C. S. et al. Enzyme immobilization onto renewable polymeric matrixes: Past, present, and future trends. **Journal of Applied Polymer Science**, *[s.l.]*, v. 132, n. 26, p. 1–15, 2015.

BOUDRANT, J.; WOODLEY, J. M.; FERNANDEZ-LAFUENTE, R. Parameters necessary to define an immobilized enzyme preparation. **Process Biochemistry**, *[s.l.]*, v. 90, n. November, p. 66–80, 2020.

DE SOUZA, P. M. et al. A biotechnology perspective of fungal proteases. **Brazilian Journal of Microbiology**, *[s.l.]*, v. 46, n. 2, p. 337–346, 2015.

DOMSALLA, A.; MELZIG, M. F. Occurrence and properties of proteases in plant latices. **Planta Medica**, *[s.l.]*, v. 74, n. 7, p. 699–711, 2008.

DUARTE, A. R. et al. Jacaratia corumbensis O. Kuntze a new vegetable source for milk-clotting enzymes. **Brazilian Archives of Biology and Technology**, *[s.l.]*, v. 52, n. 1, p. 1–9, 2009.

FERNANDES, J. Produção de queijo: origem dos coalho. **Publindústria**, *[s.l.]*, 2013.

FREITAS, C. D. T. et al. Insights into milk-clotting activity of latex peptidases from Calotropis procera and Cryptostegia grandiflora. **Food Research International**, *[s.l.]*, v. 87, p. 50–59, 2016.

GARCIA-GALAN, C. et al. Potential of different enzyme immobilization strategies to improve enzyme performance. **Advanced Synthesis and Catalysis**, *[s.l.]*, v. 353, n. 16, p. 2885–2904, 2011.

GURUMALLESH, P. et al. A systematic reconsideration on proteases. **International Journal of Biological Macromolecules**, *[s.l.]*, v. 128, p. 254–267, 2019.

HILL, A. **Technology of Cheesemaking**. [*s.l*: s.n.]. v. 33.

HOMAEI, A. A. et al. Enzyme immobilization: An update. **Journal of Chemical Biology**,

[s.l.], v. 6, n. 4, p. 185–205, 2013.

HUSAIN, Q. Nanocarriers immobilized proteases and their industrial applications: An overview. **Journal of Nanoscience and Nanotechnology**, *[s.l.]*, v. 18, n. 1, p. 486–499, 2018.

KARAV, S. et al. Recent Advances in Immobilization Strategies for Glycosidases. **Biotechnology Progress**, *[s.l.]*, p. 1–31, 2016.

KERN, L. et al. Chemical fractionation of caseins by differential precipitation: influence of pH, calcium addition, protein concentration and temperature on the depletion in α- and β-caseins. **International Journal of Food Science and Technology**, *[s.l.]*, v. 55, n. 2, p. 542–552, 2020.

KRUIF, C. .; HOLT, C. Casein micelle structure, functions and interactions. **Advanced Dairy Chemistry**, *[s.l.]*, v. 1, n. 3, 2003.

LUCIANA, M. et al. Detecção de caseinomacropeptídeo em leite cru. n. October 2019, p. 530–543, 2017.

MATEO, C. et al. Advances in the design of new epoxy supports for enzyme immobilization-stabilization. **Biochemical Society Transactions**, *[s.l.]*, v. 35, n. 6, p. 1593–1601, 2007a.

MATEO, C. et al. Improvement of enzyme activity, stability and selectivity via immobilization techniques. **Enzyme and Microbial Technology**, *[s.l.]*, v. 40, n. 6, p. 1451–1463, 2007b.

MAZORRA-MANZANO, M. A.; MORENO-HERNÁNDEZ, J. M.; RAMÍREZ-SUAREZ, J. C. **Milk-clotting plant proteases for cheesemaking**. [*s.l*: s.n.].

MAZORRA-MANZANO, M. A.; RAMÍREZ-SUAREZ, J. C.; YADA, R. Y. Plant proteases for bioactive peptides release: A review. **Critical Reviews in Food Science and Nutrition**, *[s.l.]*, v. 58, n. 13, p. 2147–2163, 2018.

MÓTYÁN, J.; TÓTH, F.; TŐZSÉR, J. Research Applications of Proteolytic Enzymes in Molecular Biology. **Biomolecules**, *[s.l.]*, v. 3, n. 4, p. 923–942, 2013.

National Human Genome Research Institute. Disponível em: https://www.genome.gov/genetics-glossary/Enzyme. Acesso em: 23 abr. 2021.

NELSON, D. L.; COX, M. M. **Princípios de Bioquímica de Lehninger**. 6a ed. Porto Alegre: Artmed, 2014.

NITU, S.; GEICU-CRISTEA, M.; MATEI, F. Milk-Clotting Enzymes Obtained From Plants in Cheesemaking-a Review. v. XXV, n. 1, 2021.

NOROUZIAN, D. Enzyme immobilization: the state of art in biotechnology. **Iranian Journal of Biotechnology**, *[s.l.]*, v. 1, n. 4, 2003.

PORTO DE SOUZA VANDENBERGHE, L. et al. Classification of enzymes and catalytic

properties. **Biomass, Biofuels, Biochemicals**, *[s.l.]*, n. August 2018, p. 11–30, 2020.

PRIYADARSHAN, P. M. **Biology of hevea rubber**. [*s.l*: s.n.].

RODRIGUES, R. C. et al. Modifying enzyme activity and selectivity by immobilization. **Chemical Society Reviews**, *[s.l.]*, v. 42, n. 15, p. 6290–6307, 2013.

ROSEIRO, L. B. et al. Cheesemaking with vegetable coagulants - The use of Cynara L. for the production of ovine milk cheeses. **International Journal of Dairy Technology**, *[s.l.]*, v. 56, n. 2, p. 76–85, 2003.

SANTOS, J. C. S. D. et al. Importance of the Support Properties for Immobilization or Purification of Enzymes. **ChemCatChem**, *[s.l.]*, v. 7, n. 16, p. 2413–2432, 2015.

SIAR, E. H. et al. Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins. **Process Biochemistry**, *[s.l.]*, v. 58, p. 98–104, 2017.

SILVA, M. Z. R. et al. Biotechnological potential of a cysteine protease (CpCP3) from Calotropis procera latex for cheesemaking. **Food Chemistry**, *[s.l.]*, v. 307, p. 125574, 2020.

SMITH, S. et al. A comprehensive review of the covalent immobilization of biomolecules onto electrospun nanofibers. **Nanomaterials**, *[s.l.]*, v. 10, n. 11, p. 1–39, 2020.

SPAHN, C.; MINTEER, S. Enzyme Immobilization in Biotechnology. **Recent Patents on Engineering**, *[s.l.]*, v. 2, n. 3, p. 195–200, 2008.

TUINIER, R.; DE KRUIF, C. G. Stability of casein micelles in milk. **Journal of Chemical Physics**, *[s.l.]*, v. 117, n. 3, p. 1290–1295, 2002.

VAN DE VELDE, F. et al. Carrageenan: A Food-Grade and Biocompatible Support for Immobilisation Techniques. **Advanced Synthesis and Catalysis**, *[s.l.]*, v. 344, n. 8, p. 815–835, 2002.

VERMELHO, A. B. et al. Application of Microbial Enzymes in the Food Industry. **Enzymes in Food Biotechnology: Production, Applications, and Future Prospects**. [*s.l*: s.n.]. p. 135–161.

WANG, H.; SHIYOU, L. Immobilization of enzymes and cells. **Chemistry Bulletin / Huaxue Tongbao**, *[s.l.]*, v. 1, n. 2, p. 22–27, 1997.

WUSIGALE; LIANG, L.; LUO, Y. Casein and pectin: Structures, interactions, and applications. **Trends in Food Science and Technology**, *[s.l.]*, v. 97, n. January, p. 391–403, 2020.

YADAV, R. P.; PATEL, A. K.; JAGANNADHAM, M. V. Purification and biochemical characterization of a chymotrypsin-like serine protease from Euphorbia neriifolia Linn. **Process Biochemistry**, *[s.l.]*, v. 46, n. 8, p. 1654–1662, 2011.

ZUCCA, P.; FERNANDEZ-LAFUENTE, R.; SANJUST, E. Agarose and its derivatives as

supports for enzyme immobilization. **Molecules**, *[s.l.]*, v. 21, n. 11, p. 1–25, 2016.