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JOÃO PEDRO BRASIL DE OLIVEIRA

IMOBILIZAÇÃO DE PEPTIDASES LATICÍFERAS EM DIFERENTES SUPORTES PARA APLICAÇÃO NA HIDRÓLISE DE ALÉRGENOS DO LEITE BOVINO

FORTALEZA

2022

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica, do Departamento de Bioquímica e Biologia Molecular, da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Bioquímica. Área de concentração: Bioquímica vegetal

Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas

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BANCA EXAMINADORA

_____

Prof. Dr. Cleverson Diniz Teixeira de Freitas (Orientador)
Universidade Federal do Ceará (UFC)

_____

Prof. Dra. Luciana Rocha Barros Gonçalves
Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Rafael Audino Zambelli
Universidade Federal do Ceará (UFC)

_____

Prof. Dra. Nathalia Saraiva Rios
Universidade Federal do Rio Grande do Norte (UFRN)

_____

Prof. Dr. Pedro Filho Noronha de Souza
Universidade Federal do Ceará (UFC)

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RESUMO

A alergia ao leite é um problema de saúde pública que afeta principalmente os lactentes. Para estes, as fórmulas hidrolisadas tornam-se a principal fonte de nutrição. Nesse contexto, o objetivo deste estudo foi desenvolver biocatalisadores a partir da imobilização das peptidases do látex de *C. procera* (CpCPs), visando a hidrólise das proteínas alergênicas do leite. CpCPs foram rapidamente imobilizadas em sulfopropil-agarose (suporte com carga negativa), porém com baixa atividade recuperada (4%). Ao imobilizar CpCPs em MANAE-agarose e DEAE-agarose (suportes com carga positiva), foram observados valores de rendimento de 33% e 54% e atividades recuperadas de 23% e 20%, respectivamente. A imobilização covalente foi realizada em glioxil-agarose e MANAE-agarose (ativado com glutaraldeído, MANAE-GLU). Usando glioxil (glioxil-CpCPs), o rendimento de imobilização foi aproximadamente 100%, com atividade recuperada de 11%. Contudo, ao utilizar L-cisteína na imobilização [glioxil-CpCPs(Cys)], foi obtido atividade recuperada de 60%. A imobilização em MANAE-GLU gerou diferentes biocatalisadores com elevado rendimento (> 90%) e atividade recuperada (> 200%), este último foi obtido utilizando BANA como substrato. Em relação à caracterização dos biocatalisadores, CpCPs imobilizadas em glioxil foi estável em valores de pH ácidos; enquanto que CpCPs imobilizadas em MANAE (adsorção e covalente) foi observado uma mudança no pH ótimo da atividade enzimática para valores de pH mais básicos, comparado à CpCPs solúvel. Em relação à estabilidade térmica, CpCPs imobilizadas em glioxil foi 60 vezes mais estável a 60 °C e pH 10.0, quando comparado a CpCPs solúvel. Após cinco ciclos de reação, a atividade residual foi de 90%, 70%, 60% e 30% para MANAE-GLU-CpCPs, glioxil-CpCPs, glioxil-CpCPs(Cys) e MANAE-CpCPs, respectivamente. Os parâmetros cinéticos de CpCPs imobilizadas em glioxil foram avaliados. Foi observado que glioxil-CpCPs apresentou valores de K_m e V_{max} próximos a CpCPs solúvel; enquanto que glioxil-CpCPs(Cys) apresentou baixos valores de K_m e V_{max}. Análises de FTIR mostraram mudanças nas bandas correspondentes a amida I e II, o que pode sugerir mudanças na estrutura secundária de CpCPs após a imobilização. Em relação à hidrólise das proteínas do leite, CpCPs imobilizadas em glioxil foi capaz de hidrolisar extensivamente as caseínas (alergenicidade residual - 3%) e parcialmente as proteínas do soro (alergenicidade residual - 35%), apresentando maior eficiência quando comparados a uma fórmula comercial parcialmente hidrolisada (Enfamil Gentlease). Além disso, CpCPs imobilizado em glioxil foi eficiente na hidrólise das proteínas do leite por pelo menos 5 ciclos de reação. Com isso, pode-se concluir que a imobilização de

CpCPs resultou em biocatalisadores com potencial promissor para aplicação no processamento de proteínas do leite bovino para produção de fórmulas hipoalergênicas.

Palavras-chave: adsorção; alergia; proteases; imobilização covalente.

ABSTRACT

Milk allergy is a public health problem that affect mainly the infants. For these, hydrolyzed formulas become the main source of nutrients. In this context, the aim of this study was to develop biocatalysts by the immobilization of peptidases from the latex of *Calotropis procera* (CpCPs) on different supports, aiming the hydrolysis of allergenic proteins of bovine milk. CpCPs were rapidly immobilized on sulfopropyl-agarose (support with negative charge), but with low recovered activity (4%). When immobilizations of CpCPs were performed on MANAE-agarose and DEAE-agarose (supports with positive charge), yield of 33% and 54% and recovered activities of 23% and 20% were observed, respectively. Covalent immobilizations were performed on glyoxyl-agarose and MANAE-agarose (activated with glutaraldehyde, MANAE-GLU). Using glyoxyl (glyoxyl-CpCPs), the immobilization yield was approximately 100%, with recovered activity of 11%. However, when L-cysteine was used in the immobilization [glyoxyl-CpCPs(Cys)], recovered activity of 60% was obtained. Immobilization on MANAE-GLU generated different biocatalysts with high yield (> 90%) and recovered activity (> 200%), that latter was obtained using BANA as substrate. Regarding the characterization of the biocatalysts, CpCPs immobilized in glyoxyl was stable at acidic pH values; while CpCPs immobilized in MANAE (by adsorption or covalently) caused a change of the optimal pH for enzymatic activity to more basic pH values, compared to soluble CpCPs. Regarding thermal stability, CpCPs immobilized in glyoxyl were 60-fold more stable at 60 °C and pH 10.0, when compared to soluble CpCPs. After five reaction cycles, the residual activity was 90%, 70%, 60% and 30% for MANAE-GLU-CpCPs, glyoxyl-CpCPs, glyoxyl-CpCPs(Cys) and MANAE-CpCPs, respectively. The kinetic parameters of glyoxyl-immobilized CpCPs were evaluated. It was observed that glyoxyl-CpCPs presented Km and Vmax values close to soluble CpCPs; while glyoxyl-CpCPs(Cys) showed low values of Km and Vmax. FTIR analyzes exhibited changes in the bands corresponding to amide I and II, which may suggest alteration of CpCPs secondary structure after immobilization. Regarding the hydrolysis of milk proteins, CpCPs immobilized on glyoxil was able to extensively hydrolyze the caseins (residual allergenicity - 3%) and partially hydrolyze the whey proteins (residual allergenicity - 35%), showing greater efficiency when compared to a commercial partially hydrolyzed formula (Enfamil Gentlease). Furthermore, CpCPs immobilized on glyoxyl was efficient in the hydrolysis of milk proteins for at least 5 reaction cycles. Thus, it can be concluded that the immobilization of CpCPs resulted in biocatalysts with promising

potential for application in the hydrolysis of proteins from bovine milk for the production of hypoallergenic formulas.

Keywords: adsorption; allergy; protease; covalent immobilization.

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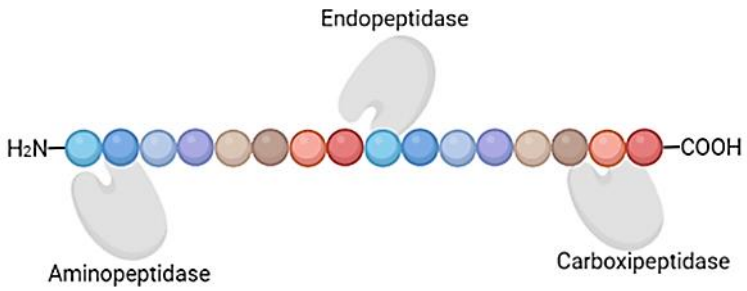
1 INTRODUÇÃO

1.1 Enzimas

As enzimas são, em geral, proteínas que atuam como catalisadores biológicos. Essas moléculas reduzem a energia de ativação, acelerando as reações químicas sem interferir no seu equilíbrio. A hidrólise enzimática pode ser realizada sob condições brandas de temperatura, pressão e pH; diferindo dos catalisadores sintéticos que exigem meios reacionais mais extremos (TAVANO, 2013). Além disso, a hidrólise enzimática é mais econômica, gera menos resíduos e é mais eficiente energeticamente (TAVANO et al., 2018). Assim, os biocatalisadores têm emergido como uma importante tecnologia para atender à crescente demanda da indústria.

Dentre os diferentes tipos de enzimas, pode-se destacar as peptidases. Estas moléculas, também conhecidas como proteases, proteinases ou enzimas proteolíticas, são enzimas responsáveis por clivar ligações peptídicas em um polipeptídeo. O Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (NC-IUBMB, sigla em inglês), classifica as peptidases como hidrolases (EC 3) e entre as hidrolases, as que clivam ligações peptídicas (EC 3.4). As peptidases são classificadas de acordo com a posição da ligação peptídica clivada (Figura 1). As exopeptidases clivam ligações na extremidade do polipeptídeo e subdividem-se em aminopeptidases (ligações do lado N-terminal) e carboxipeptidases (ligações do lado C-terminal); enquanto que as endopeptidases clivam ligações no interior do polipeptídeo (NAVEED et al., 2021).

Figura 1 – Sítios de clivagem das endopeptidases e exopeptidases.



Fonte: Elaborada pelo autor.

De acordo com o mecanismo de ação, as endopeptidases podem ser classificadas como do tipo serina, cisteína, aspártica, glutâmica, asparagina ou treonina, com base no aminoácido presente no seu sítio ativo, ou metalopeptidase, quando é necessário um metal para a sua ativação (GURUMALLESH et al., 2019). Peptidases cisteínicas (o foco deste estudo) são designadas como EC 3.4.22 e possuem a papaína (EC 3.4.22.2) e bromelaína (EC 3.4.22.32) como representantes mais conhecidos.

Em relação às aplicações, as peptidases são as enzimas mais utilizadas na indústria, correspondendo a 60% do mercado global, com destaque para a indústria alimentícia e de detergentes (NAVEED et al., 2021). Atualmente, a maioria das peptidases com aplicações biotecnológicas são de origem microbiana (SINGH et al., 2016; NAVEED et al., 2021). Contudo, novas peptidases de origem vegetal estão sendo descobertas com propriedades atraentes para a indústria (FEIJOO-SIOTA; VILLA, 2011; NAVEED et al., 2021).

1.2 Peptidases vegetais

As peptidases de plantas destacam-se por serem capazes de hidrolisar uma ampla variedade de proteínas, atuando em uma extensa faixa de pH e temperatura (DUBEY et al., 2007). Essas moléculas já foram isoladas de diferentes tecidos ou órgãos, sendo a maioria pertencente à classe das peptidases serínicas e cisteínicas (SOMAVARAPU; VEMULA; REDDY, 2019). O látex vegetal é uma rica fonte de peptidases. Estudos conduzido por Domsalla e Melzig (2008) mostraram que, dentre as plantas laticíferas, mais de 110 famílias apresentam pelo menos uma enzima proteolítica no seu látex. Além disso, essas biomoléculas correspondem a cerca de 90% do conteúdo proteico em algumas espécies (ZARE et al., 2013).

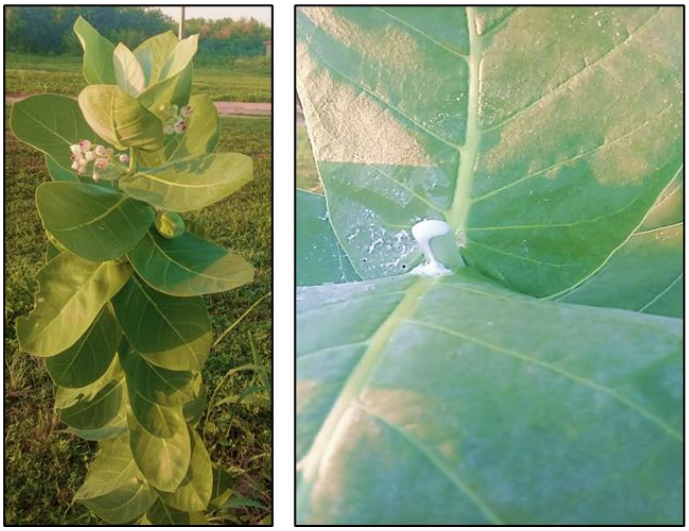
Algumas plantas laticíferas destacam-se devido às propriedades e aplicabilidade de suas peptidases. Papaína, por exemplo, é a peptidase de origem vegetal mais conhecida e estudada (AMRI; MAMBOYA, 2012). Esta enzima é purificada do látex de *Carica papaya* e é empregada no amaciamento de carnes, clarificação de bebidas, na coagulação do leite, produção de hidrolisados proteicos e no desenvolvimento de medicamentos (GUPTA et al., 2016; GURUMALLESH et al., 2019). Além da papaína, outras peptidases cisteínicas são encontradas no látex de *C. papaya*, sendo estas: quimopapaína (A e B) e papaína proteinases (A e B) (MEZHLUMYAN; KASYMOVA; YULDASHEV, 2003). Outra peptidase laticífera de destaque é a ficina, que é purificada do látex de plantas do gênero *Ficus* e pode ser utilizada para a produção de hidrolisados proteicos (MESHRAM et al., 2019). Essa diversidade de

aplicações evidenciam o potencial das peptidases laticíferas e estimula o estudo dessas enzimas no látex de outras espécies.

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

Calotropis procera, também conhecida como ciúme ou hortência, é uma planta laticífera da família Apocynaceae (Figura 2). Uma característica marcante desta espécie é a capacidade de produzir látex em abundancia, presente em quase todas as partes da planta, com exceção das sementes e raízes (RAMOS et al., 2007).

Figura 2 – Aspecto geral da planta *C. procera*, evidenciando seu látex sendo exsudado após uma injúria mecânica.



Fonte: Elaborada pelo autor.

Em estudo conduzido por Kwon e colaboradores (2015), os genes de 20 peptidases cisteínicas de *C. procera* foram sequenciados. A presença de múltiplas peptidases aumenta o potencial biotecnológico desta espécie. Até o momento, cinco destas peptidases (Proceraína, Proceraína B, CpCP1, CpCP2 e CpCP3) foram purificadas e caracterizadas, mostrando atividade em uma ampla faixa de pH e temperatura (DUBEY; JAGANNADHAM, 2003; SINGH et al. 2010; RAMOS et al. 2013).

A planta *C. procera* tem recebido atenção especial devido ao seu uso na medicina popular na Índia. Diversas atividades biológicas foram relatadas para a fração proteica do látex

dessa espécie, como por exemplo, propriedades anti-inflamatória (ALENCAR et al., 2004), coagulante (RAMOS et al., 2012) e antitumoral (OLIVEIRA et al., 2007). Essas proteínas também são usadas no tratamento de lepra, úlceras, hemorroidas, doenças do baço, fígado e abdómen (KUMAR; ARYA, 2006). Além das propriedades farmacológicas, é destacado a atividade inseticida contra o mosquito *Aedes aegypti* e diferentes pragas agrícolas (RAMOS et al., 2006, 2007), assim como atividade contra diferentes fungos fitopatogênicos (SOUZA et al., 2011).

As aplicações biotecnológicas das peptidases do látex de *C. procera* também se estendem para a área da tecnologia de alimentos. Freitas e colaboradores (2016) mostraram que as peptidases laticíferas foram capazes de coagular o leite de forma semelhante ao coalho animal (quimosina). Além disso, essas peptidases foram capazes de hidrolisar os principais alérgenos do leite bovino, demonstrando que são enzimas promissoras para o desenvolvimento de novas fórmulas hipoalergênicas (OLIVEIRA et al., 2019).

1.3 Alergia ao leite bovino

Alergia é um termo utilizado para descrever uma reação anormal do corpo (reação de hipersensibilidade) desencadeada pelo sistema imunológico após o contato com um alérgeno, que pode ocorrer pela interação com a pele, inalação, ingestão ou injeção. Em geral, podem ser observados sintomas cutâneos, respiratórios, gastrointestinais, cardiovasculares e neurológicos (FIOCCHI et al., 2010). No caso da alergia ao leite bovino, este quadro clinico é desencadeado pela exposição às proteínas do leite, que são inofensivas à maioria das pessoas, e pode ser mediada por qualquer um dos 4 tipos de reação de hipersensibilidade, com destaque para a reação de hipersensibilidade tipo I (FIOCCHI et al., 2010). Resumidamente, o sistema imunológico reconhece algumas das proteínas do leite como danosas (alérgenos) e inicia a produção de imunoglobulinas E (IgE) para neutralizá-las, etapa conhecida como sensibilização. Após a sensibilização, em exposições futuras aos alérgenos, os anticorpos IgE sinalizarão para a liberação de histaminas, causando as respostas inflamatórias e os sintomas da alergia (DOCENA et al., 2016).

O leite é um fluido biológico complexo constituído de água (87,3%), proteínas (3,2%), carboidratos (4,6%), gordura (3,9%) e minerais (0,7%) (LÉONIL et al., 2000). Em relação as proteínas do leite, estas podem ser divididas em duas frações: caseínas e proteínas do soro/lactosoro. As caseínas representam aproximadamente 80% das proteínas do leite

bovino e são os principais alérgenos deste alimento (DOCENA et al., 1996). O termo caseínas refere-se a um grupo de quatro proteínas fosforiladas (α_{s1}-, α_{s2}-, β- e κ-caseína), as quais diferem em suas sequências aminoacídicas, massa molecular e modificações pós-traducionais (WANG et al., 2013). No leite, as caseínas se associam naturalmente e, junto com o fosfato de cálcio inorgânico, formam estruturas coloidais denominadas micelas. As micelas de caseínas podem variar de 80-400 nm, possuindo diâmetro médio de 200 nm (DALGLEISH, 2011). Todas as caseínas contêm quantidades significativas de aminoácidos hidrofóbicos. Além disso, a proteína κ-caseína possui uma porção hidrofílica glicosilada. Assim, as micelas de caseínas possuem um interior hidrofóbico formado por α_{s1}-, α_{s2}- e β-caseína e uma superfície formada, principalmente, por *k*-caseína, em que a parte hidrofílica desta proteína se projeta da superfície da micela, formando uma "camada peluda" responsável pela estabilização dessas estruturas no leite (DALGLEISH, 2011).

As proteínas do soro correspondem a 20% do conteúdo proteico total do leite e são comumente utilizadas na suplementação alimentar. A composição proteica da fração lactosoro varia entre as espécies de mamíferos, estando presente no leite bovino as seguintes proteínas: α-lactoalbumina (α-LA), β-lactoglobulina (β-LG), albumina sérica bovina, lactoferrina e imunoglobulinas. Trabalhos na literatura relatam o potencial alergênico de todas as proteínas da fração lactossoro do leite bovino, com destaque para α-lactoalbumina e β-lactoglobulina (HOCHWALLNER et al., 2014). A α-lactoalbumina é uma proteína globular com elevado teor de aminoácidos essenciais, que participa da via de biossíntese da lactose. Esta proteína é encontrada em todos os leites já estudados (FIOCCHI et al., 2010) e, além disso, sua composição aminoacídica se assemelha entre bovinos e humanos (LAJNAF et al., 2022). Em relação à β-Lactoglobulina, esta é uma proteína globular responsável pelo transporte de ácidos graxos, retinol e vitaminas (LAJNAF et al., 2022). Esta proteína é a mais abundante da fração lactosoro e é um dos principais alérgenos do leite bovino (QI et al., 2021). A alergenicidade da β-LG pode estar relacionada ao fato de esta proteína não ser sintetizada em humanos (LUCENA et al., 2006). Entretanto, ainda pode ser encontrada no leite materno, pois é absorvida pela mucosa do intestino e depositada nas glândulas mamárias (WAL, 2004). A β-LG possui estrutura estável e elevada resistência a hidrolise enzimática. Assim, vários trabalhos na literatura buscam estratégias para aumentar o grau de hidrolise desta proteína (LIANG et al., 2021).

Após o diagnóstico da alergia às proteínas do leite, a forma mais eficaz de evitar os sintomas é interromper o consumo deste alimento. No entanto, essa pratica pode desencadear

problemas no desenvolvimento da criança, pois o leite é um alimento com elevado valor nutricional, sendo fonte de calorias, íons, alguns aminoácidos essenciais, entre outros. Assim, o processamento das proteínas do leite visando modificar ou destruir a estrutura dos alérgenos é uma solução para reduzir a alergenicidade desse alimento. Diversas técnicas podem ser utilizadas no processamento das proteínas do leite. Bu e colaboradores (2013) destacam a utilização de altas pressões, desnaturação térmica, reações de glicação, fermentação e hidrolise enzimática. Dessas estratégias, a hidrólise enzimática se destaca, pois é a forma mais eficiente para eliminar os epítopos alergênicos das proteínas e prevenir as interações com os anticorpos, principalmente IgE.

Alguns dos alérgenos presentes no leite bovino são resistentes à proteólise por enzimas comerciais, como a papaína e tripsina (CHEISON et al., 2011; QUINTIERI et al., 2017). As peptidases de *C. procera*, por exemplo, hidrolisaram extensivamente as caseínas e parcialmente as proteínas do soro (OLIVEIRA et al., 2019). A hidrólise extensiva dessas proteínas demanda grandes quantidades de enzimas, assim como uma diversidade dessas moléculas. Contudo, em concentrações elevadas, enzimas solúveis podem agregar e/ou precipitar. Além disso, as enzimas estão sujeitas a processos de autólise, resultando na diminuição da atividade enzimática e, consequentemente, na perda da eficiência do biocatalisador. Nesse contexto, as técnicas de imobilização destacam-se como uma ferramenta para evitar os problemas acima citados (GARCIA-GALAN et al., 2011).

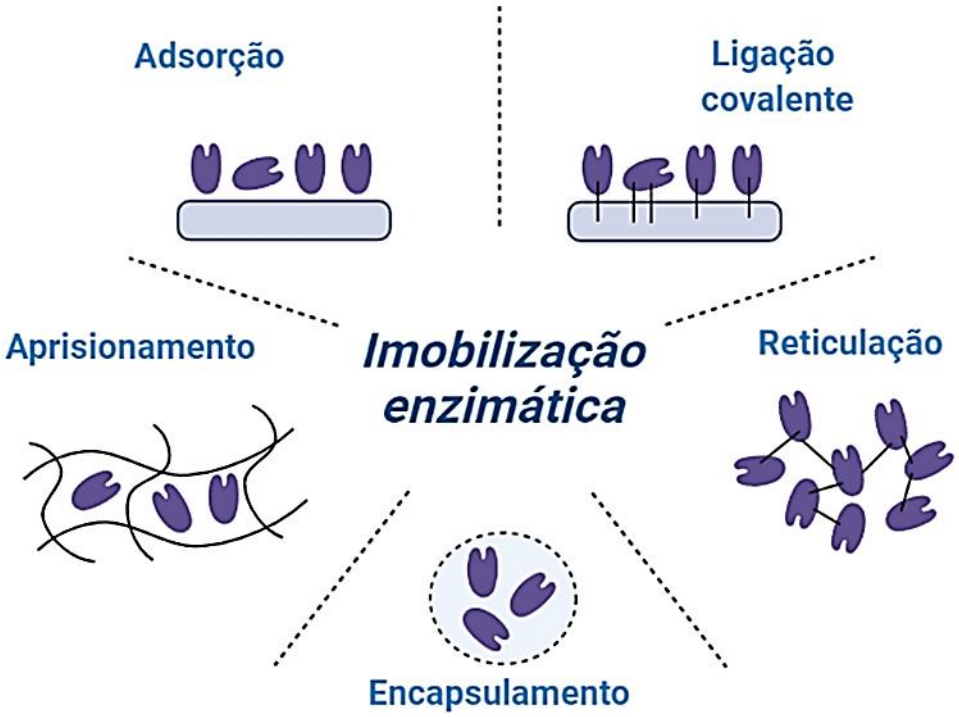
1.4 Imobilização enzimática

O termo “imobilização enzimática” refere-se ao ato de confinamento de uma enzima em uma matriz ou suporte, mantendo total ou parcialmente sua atividade (AGGARWAL; PUNDIR, 2016). Algumas das aplicações de enzimas na indústria alimentícia requerem que essas moléculas sejam previamente imobilizadas, facilitando a manipulação e aumentando a resistência à desnaturação. Além disso, a imobilização viabiliza a reutilização do biocatalisador e diminui a contaminação do produto final pela enzima utilizada (BILAL et al., 2019). Assim, de acordo com Garcia-Galan e colaboradores (2011), os biocatalisadores mais adequados para o uso industrial devem apresentar resistência mecânica, simplicidade no protocolo de imobilização, elevada estabilidade enzimática e resistência a inibição.

Os métodos de imobilização enzimática são divididos em aprisionamento/encapsulamento, reticulação e ligação a um suporte (Figura 3). A imobilização

por aprisionamento/encapsulamento consiste em inserir a enzima em uma membrana, polímero ou microcápsula que aprisionam a enzima, permitindo apenas a difusão dos substratos e produtos (BEZERRA et al., 2015). Nesta técnica, o biocatalisador pode ser obtido com a polimerização do suporte na presença da enzima (SHELDON; VAN PELT, 2013). Com isso, a enzima não está diretamente ligada ao suporte, evitando assim efeitos negativos em sua estrutura terciária (HOMAEI et al., 2013). Outra estratégia de imobilização ocorre através da reticulação de enzimas agregadas (CLEAs) ou cristais enzimáticos (CLECs). Nessa estratégia, os biocatalisadores são produzidos utilizando reagentes bifuncionais, como glutaraldeído, para promover ligações entre as enzimas gerando um produto com elevada concentração enzimática e baixo custo por não utilizar suporte (SHELDON, 2019; BEZERRA et al., 2015).

Figura 3 – Principais estratégias para imobilização enzimática.



Fonte: Elaborada pelo autor.

A imobilização por ligação ao suporte pode ocorrer por adsorção ou ligação covalente. Na adsorção, as interações físicas são geralmente mediadas por ligações de hidrogênio, forças de Van der Waals ou atrações hidrofóbicas, enquanto que as iônicas são mediadas por forças eletrostáticas (SHELDON; VAN PELT, 2013). Na adsorção iônica, os grupos funcionais ionizáveis do suporte atraem os resíduos de aminoácidos de carga oposta que

estão presentes na superfície da enzima. Contudo, a interação enzima-suporte é considerada fraca podendo ocorrer o desacoplamento da enzima no meio reacional. Assim, as condições ótimas de imobilização devem ser controladas através do pH e da força iônica do tampão de imobilização (BRAHAM et al., 2019). A adsorção por força iônica possui a vantagem de ser um método simples, menos prejudicial para as enzimas, o custo é relativamente baixo e, uma vez que é possível desacoplar a enzima do suporte, este pode ser reutilizado para outras imobilizações (AGYEI; SHANBHAG; HE, 2015). A imobilização por ligação covalente é uma estratégia que envolve o acoplamento irreversível da enzima ao suporte. Esta técnica é uma das formas mais estáveis de imobilização e, portanto, requer protocolos mais complexos comparados à imobilização por adsorção. Além disso, a formação da ligação covalente pode requerer tempos de incubação mais longos (NGUYEN; KIM, 2017). Diversas estratégias podem ser utilizadas e, na maioria dos casos, o suporte precisa ser previamente modificado com o objetivo de inserir grupos funcionais reativos, processo chamado de ativação. Alguns reagentes são amplamente utilizados na ativação do suporte, sendo eles: glutaraldeido, glicidol e divinilssulfona (BARBOSA, et al., 2014; SANTOS et al., 2015; SANTOS et al., 2020). Após a ativação, a imobilização por ligação covalente ocorre através da reação com outros grupos funcionais presentes na superfície da enzima, como por exemplo: grupo amino, carboxílico, fenólico, sulfidrila, tiol, imidazol, indol e hidroxila (NGUYEN; KIM, 2017).

Uma vez que cada protocolo de imobilização apresenta suas vantagens e desvantagens, a escolha do método mais adequado depende da enzima, dos suportes, das condições de reação e do reator utilizado. Assim, uma imobilização adequada pode melhorar as propriedades da enzima nativa, como atividade, estabilidade, especificidade e seletividade (FERNANDEZ-LOPEZ et al., 2017), além de aumentar os ciclos de reuso do biocatalisador (GARCIA-GALAN et al., 2011).

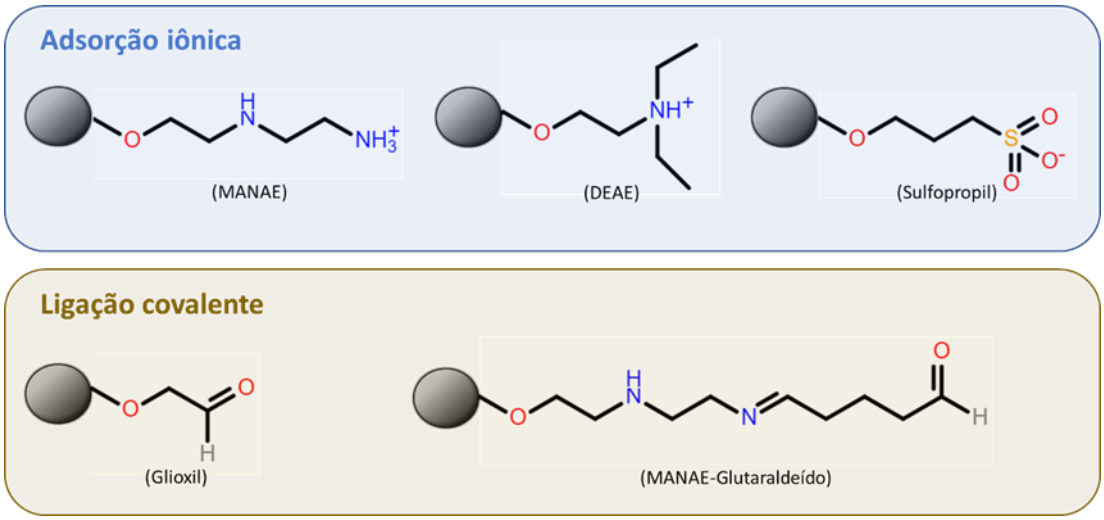
1.4.1 Imobilização em agarose

A escolha do suporte para imobilização enzimática é uma etapa fundamental. Este deve apresentar elevada estabilidade, ser resistente ao ataque microbiano, apresentar durabilidade e capacidade de reutilização, elevada área superficial, compatibilidade com a enzima e com o substrato, tamanho e forma adequados, baixo custo e facilidade de manuseio (GARCIA-GALAN et al., 2011). Nesse contexto, a agarose destaca-se por apresentar várias das propriedades acima citadas (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016).

A agarose é um heteropolissacarídeo obtido a partir do ágar extraído de algumas espécies de algas marinhas dos gêneros *Gelidium*, *Gelidiella*, *Gracilaria*, *Pterocladia* e *Ahnfeltia* (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016). Essa molécula pode ser preparada na forma de microesferas com tamanho e porosidade variados, sendo muito utilizada para a imobilização enzimática. Por apresentar um alto grau de polimerização, a agarose pode formar estruturas com ampla área superficial para a interação enzima-suporte, permitindo produzir biocatalisadores com elevada carga de enzima. Além disso, os grupos funcionais hidroxilas presentes na agarose podem ser modificados gerando outras funções químicas no polímero, como por exemplo amina, carboxila, sulfonato, entre outros (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016). Essa característica demonstra a versatilidade da agarose que pode ser aplicada em diferentes protocolos de imobilização para a obtenção de biocatalisadores com características distintas.

A Figura 4 apresenta modelos de agarose modificada com alguns grupos para imobilização enzimática. Como observado, sulfopropil agarose é negativamente carregado, enquanto monoaminoetil-N-etil agarose (MANAE) e dietilaminoetanol agarose (DEAE) são positivamente carregados, sendo utilizados na imobilização por adsorção iônica, através da interação com resíduos de aminoácidos de carga oposta presentes na superfície da enzima. Por outro lado, grupos aldeídos podem ser úteis para imobilização por ligação covalente. Glioxil-agarose, por exemplo, é um suporte bastante utilizado para imobilização/estabilização de diversas enzimas (MATEO et al., 2006). Nesse suporte, os grupos glioxil reagem com os grupos aminos dos resíduos de lisina presentes na superfície da enzima. Esta reação deve ocorre em pH 10.0 devido à ionização da lisina (pKa 10,5). Além disso, são necessários pelo menos dois resíduos de lisina para a imobilização utilizando glioxil.

Figura 4 – Exemplos de suportes obtidos a partir da agarose.



Fonte: Elaborada pelo autor.

O suporte MANAE agarose pode ser “ativado” utilizando o reagente bifuncional glutaraldeído. O grupo aldeído do glutaraldeido é mais reativo que o aldeído do glioxil, podendo ser utilizado em uma faixa de pH 7.0-8.5 (RODRIGUES et al., 2008) e são capazes de reagir com os resíduos de aminoácidos lisina, asparagina, glutamina e arginina da superfície da enzima e o amino N-terminal quando exposto (SANTOS et al., 2020). Além disso, a formação de apenas uma ligação enzima-suporte é suficiente para a imobilização (RODRIGUES et al., 2008).

1.4.2 Enzimas imobilizadas na indústria de alimentos

O interesse por enzimas aplicadas na indústria cresceu consideravelmente nos últimos anos. Estima-se que em 2024 o mercado global de enzimas corresponda a 10,5 bilhões de dólares (CAVALCANTE et al., 2021), com grande destaque para a área de alimentos. Em relação as enzimas imobilizadas, alguns produtos são atualmente comercializados para utilização em larga escala (OLIVEIRA; FREITAS, 2021). Dentre esses, a enzima glicose isomerase destaca-se como um dos principais exemplos, sendo utilizada para adoçar alimentos e bebidas (DICOSIMO et al., 2013). Além do uso como adoçante, diversos trabalhos na literatura destacam que os biocatalisadores imobilizados também podem ser empregados no empacotamento de alimentos, clarificação de sucos, produção de leite sem lactose, maturação de queijo, processamento de vinhos, produção de biosensores, entre outros exemplos (BASHIR; SOOD; BANDRAL, 2020). Essa diversidade de aplicações demonstra que o interesse por

enzimas imobilizadas na tecnologia de alimentos cresceu consideravelmente nos últimos anos com aumento no número de publicações científicas e patentes desenvolvidas na área (OLIVEIRA; FREITAS, 2021).

Em relação à hidrolise das proteínas do leite, Abd El-Salam e El-Shibiny (2021) destacam o uso de biocatalisadores imobilizados como uma forma eficiente para a obtenção de hidrolisados proteicos. Nesse contexto, vários estudos foram conduzidos utilizando diferentes enzimas, suportes e protocolos de imobilização. Por exemplo, tripsina e papaína imobilizadas em nanopartículas magnéticas foram eficientes na hidrólise das proteínas do leite (ATACAN; ÇAKIROĞLU; ÖZACAR, 2016; FENG et al., 2020). Alcalase imobilizada em glioxil-agarose foi utilizada para a obtenção de hidrolisados da fração lactosoro (PESSATO et a., 2016). Santos e colaboradores (2019) compararam a hidrólise das caseínas bovina utilizando biocatalisadores produzidos através da imobilização da pepsina por interação iônica e ligação covalente em biocarvão obtido da palmeira pupunheira. Além disso, enzimas bacterianas imobilizadas por reticulação (CLEA) foram utilizadas na hidrólise das caseínas (STRESSLER et al., 2015). Embora alguns estudos mostrem a eficiência do uso de enzimas imobilizadas na hidrólise das proteínas do leite bovino, poucos trabalhos tiveram como objetivo determinar a alergenicidade residual dos hidrolisados obtidos, visando a produção de um leite hipoalergênico.

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver biocatalisadores a partir da imobilização das peptidases do látex de *C. procera*, em diferentes suportes, visando a hidrólise das proteínas alergênicas do leite bovino.

2.2 Objetivos específicos

- Obter a fração proteica e purificar as peptidases do látex de *C. procera*;

- Imobilizar as peptidases laticíferas em diferentes suportes;

- Comparar a atividade enzimática, a estabilidade e determinar os parâmetros cinéticos das enzimas solúveis e imobilizadas;

- Purificar as proteínas do leite bovino;

- Hidrolisar as proteínas do leite bovino com as enzimas solúveis e imobilizadas;

- Caracterizar os hidrolisados quanto ao grau de hidrólise e antigenicidade residual;

3 IMMOBILIZATION AND CHARACTERIZATION OF LATEX CYSTEINE PEPTIDASES ON DIFFERENT SUPPORTS AND APPLICATION FOR COW'S MILK PROTEIN HYDROLYSIS

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João P.B. Oliveira[a], Luciana R.B. Gonçalves[b], Kímberle P.S. Amorim[b], Bruna B. Pinheiro[b], Márcio V. Ramos[a], Pedro F.N. Souza[a], Jefferson S. Oliveira[c], Deborah C. Freitas[a], Cleverson D. T. Freitas[a*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, CEP 60.440-554, Fortaleza, Ceará, Brazil.

[b] Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, CEP 60.440-554, Fortaleza, Ceará, Brazil.

[c] Laboratório de Bioquímica de Plantas Laticíferas (LABPL), Universidade Federal do Delta do Parnaíba - UFDPar, CEP 64.202-020, Parnaíba, Piauí, Brazil

*Corresponding author: Cleverson D.T. Freitas (cleversondiniz@ufc.br). Phone number: +55 85 3366 9816.

Author E-mails

João P.B. Oliveira (joaopedrobol@hotmail.com); Luciana R.B. Gonçalves (lrg@ufc.br), Kímberle P.S. Amorim (kimberleps@gmail.com), Bruna B. Pinheiro (bruna.b.pinheiro@hotmail.com), Márcio V. Ramos (r_marcio@hotmail.com); Pedro F.N. Souza (pedrofilhobio@gmail.com), Jefferson S. Oliveira (jefferson.oliveira@gmail.com), Deborah C. Freitas (deborahcarfre@yahoo.com.br), Cleverson D. T. Freitas (cleversondiniz@ufc.br).

Abstract

Calotropis procera cysteine peptidases (CpCPs) have been used for reducing cow's milk allergenicity or as rennet in cheesemaking. Due to their residual presence in food products, the present study evaluated, for the first time, different protocols for their immobilization on different supports. Although the yield of immobilization on sulfopropyl-agarose (99%, at pH 7.0) was better than when using DEAE- and MANAE-agarose (40% and 15%, respectively), the derivatives had low recovered activity (4%). On the other hand, MANAE-agarose at pH 10.0 (200 mM buffer) exhibited the highest recovered enzymatic activity (~23%). Regarding the covalent immobilization, the peptidases immobilized on glyoxyl-agarose (glyoxyl-CpCPs) showed broader pH stability (pH 3.0-10.0), 60-fold more stable at 60 °C, and retained 70% of their initial activities after five reaction cycles, even though the immobilization has induced some structural changes analyzed by Fourier-Transform Infrared (FTIR) spectroscopy as well as altered some of enzyme kinetic parameters (V_{max}, K_m, K_{cat}, and catalytic efficiency). In addition, this biocatalyst (glyoxyl-CpCPs) hydrolyzed the major cow's milk allergens (whey proteins) to a greater extent (65%) than the soluble enzymes (8%) and a commercial hypoallergenic formula (50%).

Keywords: *Calotropis procera*; Laticifer; Milk allergy; Protease.

1. Introduction

Peptidases catalyze several reactions of great interest to different industrial segments, such as textiles, foods, detergents, pharmaceuticals, and leather making [1]. Therefore, the growing global enzyme market has stimulated studies to find new peptidases from different organisms. In this respect, plant latex peptidases have been highlighted because of their diverse industrial applications [2].

The plant *Calotropis procera* (Aiton) Dryand (Apocynaceae) is well known for producing a huge amount of latex, which has several cysteine peptidase isoforms. Five cysteine peptidases, named Procerain, Procerain B, CpCP1, CpCP2, and CpCP3, have been purified and characterized from its latex [3–5]. Regarding their biotechnological potential, these latex cysteine peptidases have been used to dehair hides to make leather [6], to hydrolyze cow's milk proteins and reduce their allergenicity [7], and to serve as rennet in cheesemaking [8].

Despite this great potential, there are some drawbacks related to usage of soluble enzymes, such as low stability at different pH and temperatures [1]. Therefore, the immobilization has been widely used to overcome some of these problems, since it allows to reuse the enzyme several times, increases the enzymatic stability, and enables the removal of the protein to avoid possible toxic or allergenic effects [9]. For instance, a previous study showed that the immobilization of Procerain B, a cysteine peptidase from *C. procera* latex, on glutaraldehyde-activated chitosan matrix, enhanced the enzyme stability at higher temperature and pH ranges. In addition, this technique enabled the reuse of the biocatalyst for ten reaction cycles, with residual activity near 50% [10]. Positive results of enzyme stabilization were also reported after the immobilization of ficin (another latex cysteine peptidase) on glyoxyl-agarose support. The biocatalyst was 40-fold more stable than the free enzyme at pH 5.0 and had three-fold more activity at 80 °C [11].

For enzyme immobilization, agarose is one of the most used supports in both laboratory and industrial settings due to its stability, reproducibility, and chemical and physical resistance [12]. In addition, agarose can be functionalized with different reactive groups and used as support for enzyme immobilization, either by adsorption or covalent binding [13]. Ionic adsorption is one of the simplest immobilization methods, consisting of reversible interactions between the enzyme and support. This process occurs through electrostatic interactions, so it is dependent on the protein net charge and the surface distribution of its positively and negatively charged amino acids [14]. On the other hand, covalent attachment allows higher enzyme

stabilization under harsh operational conditions and prevents enzyme leaching from the carrier [15]. In this respect, glyoxyl supports have been successfully used for multipoint covalent immobilization, causing low impact on the activity of the immobilized enzymes [15].

Accordingly, we hypothesized that the immobilization would stabilize the three cysteine peptidases from *C. procera* latex (CpCP1, CpCP2, and CpCP3), which have shown interesting biotechnological applications in the food industry, and enhance their performance as biocatalysts. In this respect, the three peptidases were purified and immobilized on different supports to further analyze their stability and suitability to digest the main cow's milk allergens (caseins and whey proteins). There are no reports about the immobilization of CpCP1, CpCP2, and CpCP3, and a few studies have focused on the use of immobilized peptidases for the production of cow's milk hypoallergenic hydrolysates [16].

2. Material and Methods

2.1. Materials

Sulfopropyl- and DEAE (diethylaminoethyl)-agarose supports and the columns CM (carboxymethyl)-Sepharose fast-flow and Resource S (1 mL) were purchased from GE Healthcare (São Paulo, Brazil). Glutaraldehyde, sodium borohydride, sodium periodate, ethylenediamine, N-benzoyl-dl-arginine β-naphthylamide hydrochloride (BANA), Nα-benzoyl-DL-arginine p-nitroanilide hydrochloride (BApNA), 4-(dimethylamino)-cinnamaldehyde (DMACA), azocasein, and sodium tetrathionate were purchased from Sigma-Aldrich (São Paulo, Brazil). Skimmed cow's milk and Enfamil Gentlease (partially hydrolyzed milk formula, PHM) were purchased from local markets. All other reagents were of analytical grade.

2.2. Purification of latex peptidases (CpCP1, CpCP2, and CpCP3)

The *C. procera* latex, obtained by cutting the end branches of the plant, 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 dialyzed against distilled water for two days at 8 °C using membranes with 10 kDa cutoff. Afterward, the material was centrifuged again as previously described, and then the supernatant was lyophilized. The peptidases CpCP1, CpCP2, and CpCP3 were purified according to Ramos et al. [5], by using two sequential ion-exchange

chromatography steps (CM-Sepharose and Resource S) at pH 5.0 and 6.0, respectively. The purity of each protein was determined by 12.5% SDS-PAGE. Because the three cysteine peptidases have very similar biochemical properties [5], they were gathered as a fraction and are called here *C. procera* cysteine peptidases (CpCPs). The CpCPs fraction was used in all further assays.

2.3 Molecular modeling

The three-dimensional models of CpCP1, CpCP2, and CpCP3 were obtained using the SWISS-MODEL server (http://swissmodel.expasy.org) to identify the amino acids present on their surfaces, which could be available for interaction with the supports used during the ionic and covalent immobilization assays. The complete amino acid sequences of the three peptidases were obtained from Freitas et al. [17], and the crystal structure of papain (PDB: 1PPO) was used to construct the models. The structural and stereochemical quality of all models was checked using the programs PROCHECK version 3.5 (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) and Molprobity version 4.4 (http://molprobity.biochem.duke.edu/) [18]. The 3D structures were visualized using the PyMOL software (https://pymol.org/2/).

2.4 Immobilization of CpCPs

CpCPs fraction was immobilized by adsorption or covalently using the following supports: sulfopropyl-, DEAE-, and MANAE-agarose (adsorption) and glyoxyl-agarose (covalently). MANAE-agarose, after activation using 5% glutaraldehyde (named MANAE-GLU-agarose), was also used as a support for covalent binding. Sulfopropyl- and DEAE-agarose were purchased, while MANAE- and glyoxyl-agarose were prepared according to Guisán [19] and Fernandez-Lafuente et al. [20], respectively. The agarose beads (50-150 µm) used for the immobilizations had high porosity and surface area of 25 m^2/mL [19].

For the immobilization by adsorption, 0.25 g (dry) of each support (sulfopropyl-, DEAE- or MANAE-agarose) was mixed with 1 mL of CpCPs solutions (0.5 mg/mL), dissolved in different buffers at 5 mM or 200 mM: sodium phosphate (pH 7.0) and bicarbonate (pH 10.0). Thus, the protein load was 2 mg/g (enzyme/support). The suspensions were gently shaken at 25 °C and samples were periodically withdrawn at different intervals (0.5, 3, 8, 16, and 24 h) to

monitor the immobilization process. The samples were centrifuged (10,000 x *g*, 10 °C for 10 min) and the supernatants were used to determine the immobilization yield and recovered activity. Both were calculated according to Boudrant et al. [21], considering the protein content (measured using the Bradford reagent) and proteolytic activity (section 2.5) of the supernatants compared with the initial CpCPs solution. The immobilization yield relates the initially offered protein content and the final protein content observed in the supernatant, while the recovered activity relates the immobilized enzyme activity and the theoretical activity, which is calculated using the offered activity and immobilization yield.

$$\text{Yields (\%)} = [(\text{Protein content}_{(\text{Initial})} - \text{Protein content}_{(\text{Final})}) \times 100] / \text{Protein content}_{(\text{Initial})}]$$

$$\text{Theoretical activity} = \text{Yield} \times \text{Activity}_{(\text{Offered})};$$

$$\text{Recovered activity (\%)} = (\text{Derivative activity} / \text{Theoretical activity}) \times 100.$$

For the covalent immobilization, MANAE-agarose was activated using 5% glutaraldehyde solution [1 g support / 10 mL of glutaraldehyde solution] at 25 °C, pH 7.0, for 1 h, under stirring. After that, 0.25 g (dry) of pre-activated MANAE-agarose (named MANAE-GLU-agarose) or glyoxyl-agarose was suspended in 1 mL of CpCPs solution (0.5 mg/mL), dissolved in different buffers at 5 mM or 200 mM: sodium phosphate (pH 7.0) and bicarbonate (pH 10.0). Thus, the protein load was 2 mg/g (enzyme/support). The suspensions were gently shaken for 3 h at 25 °C. The samples were centrifuged (10,000 x *g*, 10 °C for 10 min) and the supernatants were used to determine the immobilization yield and recovered activity, as described before. Afterward, the supports were mixed with sodium borohydride (1 mg/mL) for 30 min at 25 °C, and then were rinsed abundantly with distilled water.

Additionally, to achieve better recovered activity of immobilized CpCPs, some tests were performed using glyoxyl-agarose in 200 mM bicarbonate buffer (pH 10.0), considering that this was the best combination of support and pH level among all those analyzed. Soluble CpCPs were pre-incubated with L-cysteine (1, 5, 10, 20, and 50 mM), 30 µM sodium tetrathionate (a reversible cysteine peptidase inhibitor) or urea (2 and 4 M), and then immobilized for different times on the support. Different protein loads (0.5 and 1 mg/g: enzyme/support) were also used in the immobilizations.

After the attempts to establish the best immobilization protocol, the type of interaction (adsorption or covalent binding) between the enzymes and supports was confirmed

by SDS-PAGE. The immobilized CpCPs on different supports were mixed with SDS-PAGE sample buffer (containing 2% SDS and 1% 2-mercaptoethanol), heated at 100 °C for 10 min, centrifuged (10,000 x *g*, 10 °C for 10 min), and then the supernatants were applied in 12.5% SDS-PAGE. The presence of protein bands was detected by using Coomassie brilliant blue dye [5].

2.5 Proteolytic assays

The proteolytic assays were performed using 1% azocasein or 1 mM BANA (as unspecific and specific substrate for cysteine peptidases, respectively) [22]. Before the assays, all samples were incubated with 1 mM L-cysteine (a reducing agent) at 25 °C for activation of the cysteine peptidases.

For the assays using azocasein, 100 µL of soluble CpCPs (0.5 mg/mL) or 0.25 g of supports containing immobilized CpCPs was mixed with 200 µL of different buffers (pH 3.0-10.0). Then, 200 µL of 1% azocasein was added and the reaction was performed at 120 rpm for 40 min at 37 °C. 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 defined as the amount of soluble/immobilized CpCPs able to increase the absorbance by 0.01 unit per minute [22].

For the assays using BANA, 200 µL of soluble CpCPs (0.5 mg/mL) or 0.25 g of supports containing immobilized CpCPs was mixed with 200 µL of different buffers (pH 7.0 and pH 10.0). Then, 300 µL of 1 mM BANA was added and the reaction was performed at 120 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 [22].

The kinetic parameters of soluble and immobilized CpCPs were determined using Nα-benzoyl-DL-arginine p-nitroanilide hydrochloride (BApNA) as substrate, according to Freitas et al. [17]. The hydrolysis of BApNA at the bond between arginine and p-nitroaniline releases the chromophore p-nitroaniline, whose amount can be measured by spectrophotometry

using $\varepsilon_{410\ nm} = 8800\ M^{-1}\ cm^{-1}$ [17]. For the reactions, 300 µL of soluble CpCPs (10 mg/mL) or 0.125 g of supports containing immobilized CpCPs was mixed with 1 mL of BApNA (0-9 mM). The reactions were stopped after 30 min by adding 500 µL of 50% acetic acid and the absorbances were measured at 410 nm. One unit of activity (nkat) was defined as the amount of enzyme able to hydrolyze 1 nmoL of substrate per second. K_m (Michaelis-Menten constant) and V_{max} (maximal velocity) were determined using different concentrations of BApNA (at 37 °C and pH 5.0) from a nonlinear curve fit of the untransformed data using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA) [17]. K_{cat} (turnover number; $K_{cat} = V_{max}/[E]$) and catalytic efficiency (K_{cat}/K_m) were also calculated. All assays were performed in triplicate. The results used to define the kinetic parameters were best-fit estimate ± SD.

2.6 Effect of pH and temperature on the enzyme stability and operational stability

The effect of pH on the activity of the enzymes after immobilization was evaluated using the following buffers at 50 mM: glycine-HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris-HCl (pH 8.0), and ammonium bicarbonate (pH 9.0 and 10.0). The thermal stability of the immobilized enzymes was studied by incubating the biocatalysts at 60 °C in the following buffers at 5 mM: sodium acetate (pH 5.0), sodium phosphate (pH 7.0) or ammonium bicarbonate (pH 10.0). Periodically, samples were withdrawn to determine the thermal stability. The half-life values were calculated according to model proposed by Henley and Sadana [23], using the Origin software. Enzyme stabilization was calculated comparing the half-life of the immobilized CpCPs with the half-life of soluble CpCPs. The operational stability was determined by measuring the residual activity of the immobilized enzymes after five reaction cycles, considering the first cycle as having 100% activity. All tests were performed using azocasein as substrate according to section 2.5. Comparative tests with soluble enzymes were performed similarly.

2.7 Determination of enzyme structural changes by FTIR

Fourier-transform infrared (FTIR) spectra were obtained using a Bruker infrared spectrophotometer (model Vertex 70), containing the accessory Golden Gate Single Reflection Diamond ATR System. The soluble and immobilized CpCPs were lyophilized and the infrared

spectra were obtained in the range from 4000 to 500 cm^{-1} in order to understand the possible enzyme structural modifications caused by different immobilization protocols.

2.8 Hydrolysis of milk proteins

Immobilized CpCPs were tested for the ability to hydrolyze cow's milk proteins (caseins and whey proteins), according to Oliveira et al. [7]. Briefly, equivalent amounts of activity units (U) of soluble and immobilized CpCPs were mixed with 1 mL of cow's milk proteins (10 mg/mL in 50 mM Tris-HCl buffer, pH 6.5). The reaction was performed under 150 rpm stirring at 37 °C for 2 h and then the volumes were centrifuged (5,000 x *g*, 25 °C for 5 min) to remove the immobilized CpCPs. Similarly, the hydrolysis was also evaluated using the milk protein solution previously heated at 85 °C for 30 min. In the reactions with soluble CpCPs, after each time point, the aliquots were withdrawn and boiled for 5 min to stop the reaction. The operational stability (reuse) was determined using native and heated-milk proteins as substrates, with reaction cycles of 2 h at 37 °C, pH 6.5, and 150 rpm stirring. The degree of hydrolysis was evaluated by SDS-PAGE and ELISA [7]. For the SDS-PAGE (15%), aliquots of the hydrolysates were mixed with sample buffer (1:1, v:v) [0.0625 M Tris buffer (pH 6.8) containing 2% SDS and 1% 2-mercaptoethanol] and 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 decoloration was performed with the same solution without the dye. The residual antigenicity of the hydrolysates was assessed by ELISA using anti-casein and anti-whey protein polyclonal antibodies produced in rabbits, as described by Oliveira et al. [7].

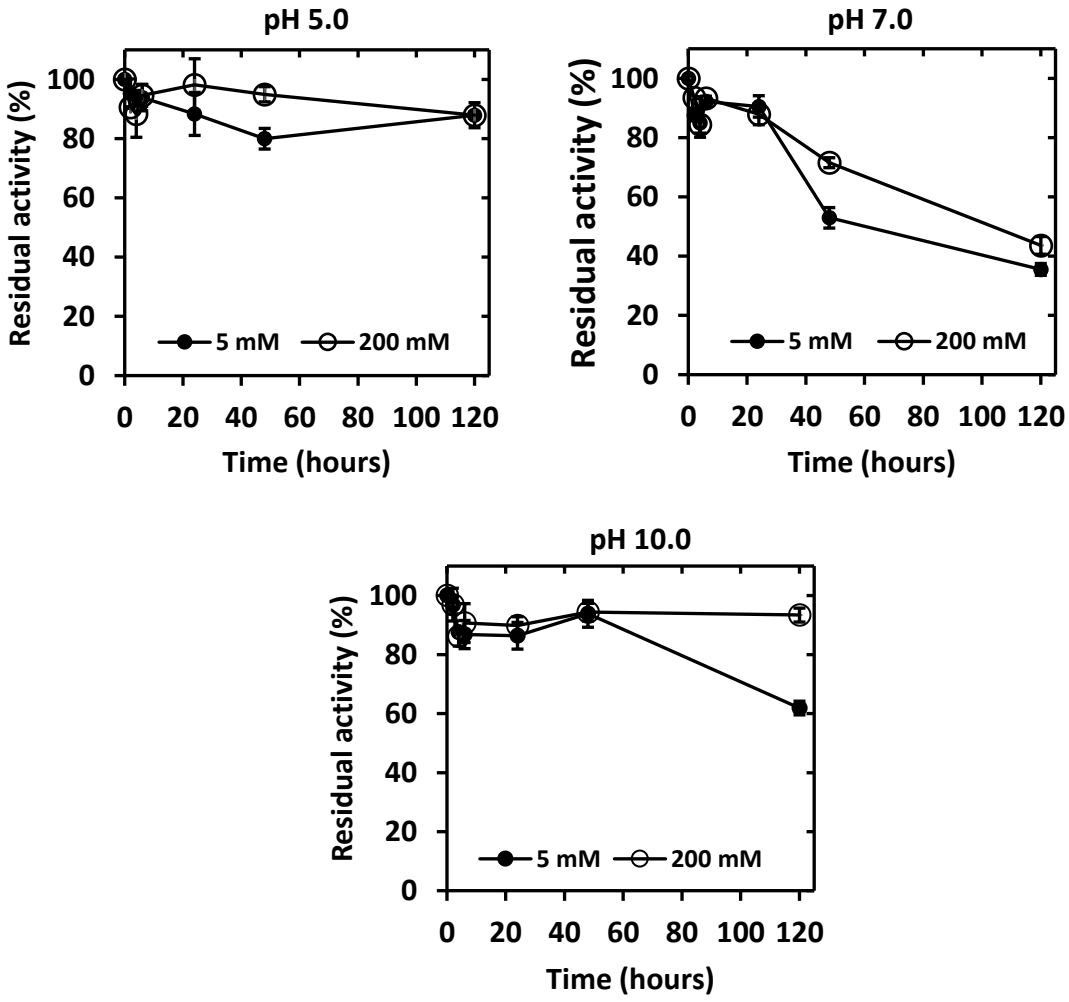
3. Results and Discussion

3.1 CpCPs stability

One of the most limiting factors for protein immobilization is that some protocols submit the proteins to severe pH levels and ionic strengths, which can result in their inactivation. Enzymes are particularly implicated in these constraints. Therefore, the stability of soluble CpCPs was evaluated at different pH levels and ionic strengths (buffer concentration, 5 mM or 200 mM). As shown in Figure 5, in general, CpCPs were most stable at pH 5.0, followed by 10.0 and 7.0. CpCPs were quite stable after 24 h at pH 5.0 (98%) and 10.0 (90%)

in 200 mM buffer. Substantial losses in proteolytic activity were detected only after 48 h of incubation at pH 7.0 (~50%) in 5 mM buffer. The residual activity of soluble CpCPs after 120 h was about 90%, at pH 5.0 or 10.0, and 46% at pH 7.0, using 200 mM buffers. In comparison, the activity of papain (a latex cysteine peptidase from *Carica papaya*) decreased at basic pH values [24]. High stability at pH 10.0 is necessary for immobilization by multipoint covalent attachment due to the ionization state of lysine residues (pKa = 10.0).

Figure 5 – Proteolytic activity of soluble *Calotropis procera* cysteine peptidases (CpCPs) at 25 °C under different conditions of pH and ionic strength (buffer concentration, 5 mM or 200 mM). The enzyme activity was measured using 1% azocasein as substrate. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.



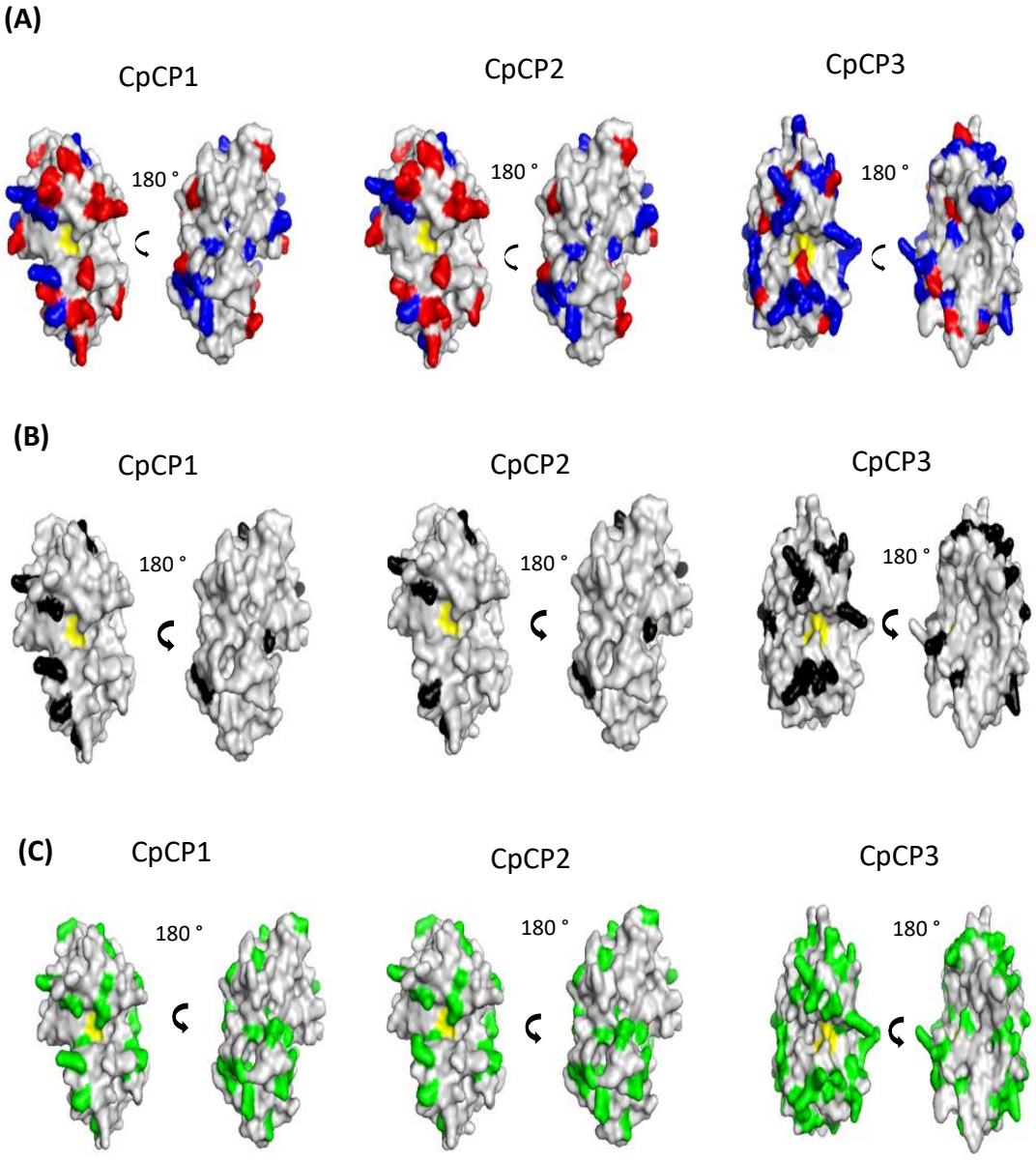
Source: elaborated by the author.

The ionic strength also had a significant influence on enzyme stability. In all pH, soluble CpCPs were more stable in buffer with higher ionic strength (200 mM) (Figure 5), likely because the 'salting in' effect can prevent protein aggregation and maintain enzyme activity. Similar behavior was observed by *Ficus racemosa* latex peptidases, which were more stable in buffers with higher ionic strengths [25]. In addition, a previous study showed that 1 M NaCl had no inhibitory effect on proteolytic activity of CpCPs [8].

3.2 Ionic immobilization using different supports

The superficial charge distribution is mainly responsible for protein orientation and anchoring on ionic supports. As shown in a study with four isoforms of ficin (a latex cysteine peptidase from *Ficus carica*), the ionizable residue distribution on its surface explained its low immobilization on some ionic supports [13]. In this respect, CpCP1, CpCP2, and CpCP3 were modeled to show the ionizable residues on their surfaces. As shown in Figure 6, abundant cationic and anionic residues were found in all three peptidases. CpCP1 and CpCP2 are very similar. Both have 23 anionic amino acid residues, located mainly close to active site. In addition, the cationic residues of CpCP1 (13) and CpCP2 (12) are distributed over all proteins. In contrast, CpCP3 has more cationic (27) than anionic (15) amino acid residues and both are distributed over the entire protein (Figure 6).

Figure 6 – Three-dimensional models of *Calotropis procera* cysteine peptidases (CpCP1, CpCP2, and CpCP3) showing their active sites (yellow), as well as **(A)** their positive (blue) and negative (red) amino acid residues, and **(B)** glyoxyl (black) and **(C)** glutaraldehyde (green) biding sites.



Source: elaborated by the author.

Initially, different ionic supports were tested and the immobilization results are shown in Figure 7 and Table 1. In this study, one cation exchanger (sulfopropyl) and two anion exchangers (DEAE and MANAE) were used as supports. Using sulfopropyl-agarose, the CpCPs were rapidly immobilized at pH 7.0 using 5 mM buffer. The immobilization was higher at pH 7.0 than pH 10.0, likely due to ionization of amine groups of CpCPs. In addition, buffers with lower ionic strength produced better immobilization yields (Figure 7 and Table 1). This can be explained because the adsorption rate can decrease when the buffer's ionic strength increases, since buffer ions can hinder protein-support interaction. Despite the high immobilization using low ionic strength buffers (5 mM), the recovered activities of all sulfopropyl-CpCPs were negligible (~ 4%) (Table 1). This might have been caused by blockage of the substrate to the active site of the enzymes, since all three peptidases have positively charged amino acids close to their active sites (Figure 6). Similarly, sulfopropyl-agarose was not adequate for the immobilization of other fungal peptidases [26]. Since the immobilization of CpCPs on sulfopropyl-agarose was inefficient, due to the insignificant recovered activity, we performed new assays using different anion-exchange supports (DEAE- and MANAE-agarose).

Figure 7 – Immobilization course of *Calotropis procera* cysteine peptidases (CpCPs) on different ionic supports. Immobilization was performed at 25 °C with different pH levels and ionic strengths (buffer concentration, 5 mM or 200 mM). The yield (protein content in the supernatants) was measured using the Bradford reagent and compared with the initial CpCPs solution. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.

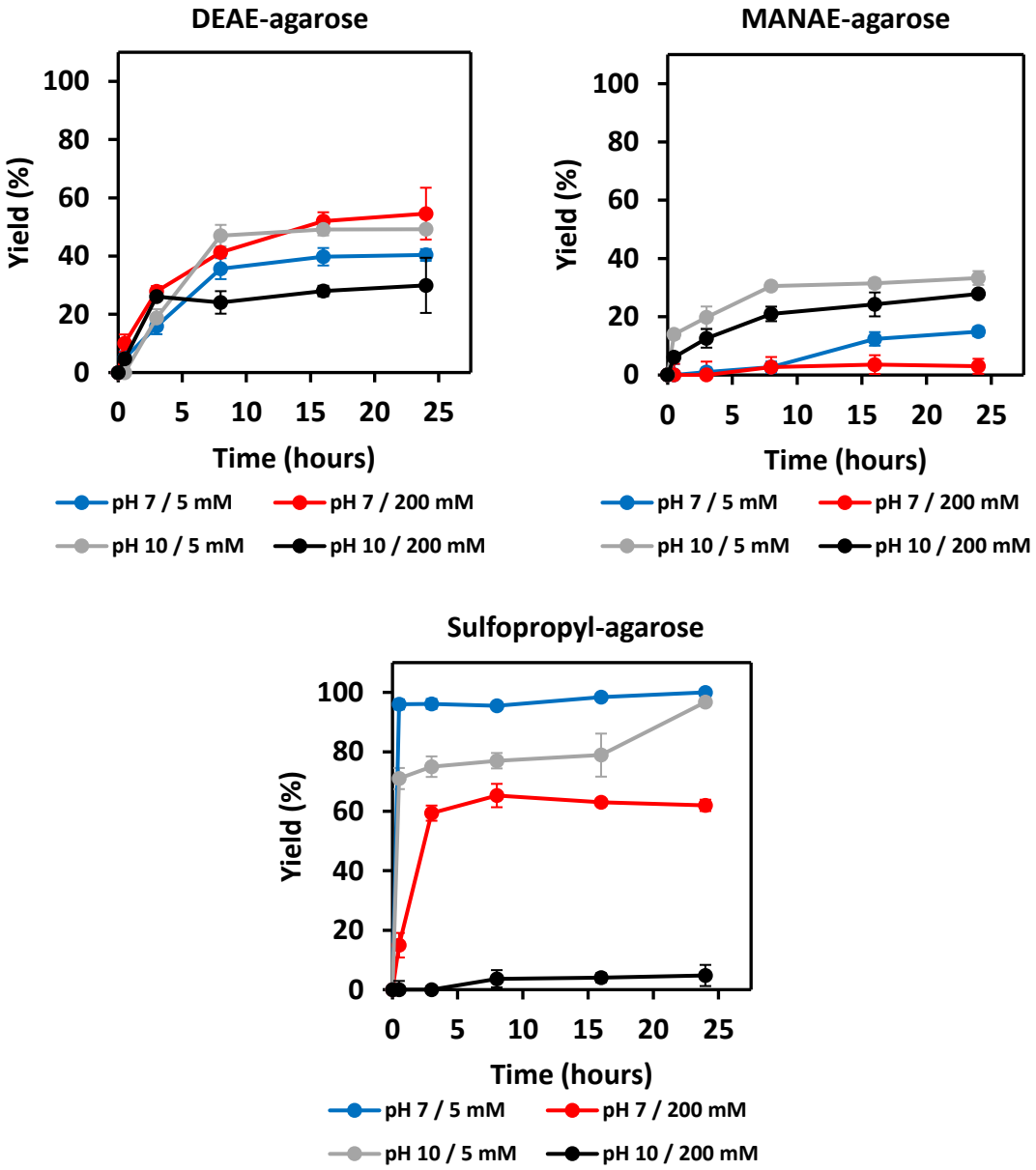


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Table 1 – Yield and recovered activity of immobilized *Calotropis procera* cysteine peptidases (CpCPs) using different ionic supports at different pH values and ionic strengths. Immobilization was performed during 24 h and the enzymatic activity was measured using 1% azocasein as substrate.

Supports	Conditions	Yields (%)	Recovered activity (%)
DEAE-agarose	pH 7.0/5 mM	40.39 ± 2.01	5.22 ± 0.42
	pH 7.0/200 mM	54.57 ± 8.89	3.65 ± 0.59
	pH 10.0/5 mM	49.21 ± 0.77	9.08 ± 0.08
	pH 10.0/200 mM	35.94 ± 5.47	20.81 ± 7.87
MANAE-agarose	pH 7.0/5 mM	14.88 ± 1.54	8.56 ± 0.96
	pH 7.0/200 mM	2.99 ± 2.56	11.29 ± 1.29
	pH 10.0/5 mM	33.30 ± 4.29	18.98 ± 0.36
	pH 10.0/200 mM	32.60 ± 1.56	23.33 ± 1.86
Sulfopropyl-agarose	pH 7.0/5 mM	99.01 ± 1.13	4.01 ± 0.06
	pH 7.0/200 mM	61.99 ± 0.74	3.98 ± 0.57
	pH 10.0/5 mM	85.44 ± 1.98	4.46 ± 0.08
	pH 10.0/200 mM	4.77 ± 0.13	0

Source: elaborated by the author.

The immobilization courses of CpCPs on DEAE- and MANAE-agarose are also shown in Figure 7 and Table 1. Although the immobilization on DEAE and MANAE supports was slower and less pronounced compared with sulfopropyl, they produced more appropriate biocatalysts since the recovered activities were higher. Although DEAE-CpCPs exhibited higher immobilization yields than MANAE-CpCPs, the recovered activities using MANAE were better under all conditions.

Among all ionic immobilization protocols, the best biocatalyst was that using MANAE-agarose at pH 10.0 (200 mM buffer), because it exhibited the highest recovered enzymatic activity (~23%) (Table 1). In comparison, ficin isoforms ionically immobilized on MANAE-agarose exhibited recovered activity close to 10% [13]. Since high ionic strength enables the production of biocatalysts with more intensely adsorbed enzymes [27], MANAE-CpCPs produced at pH 10.0 using 200 mM buffer were chosen for further analysis.

3.3 Covalent immobilization using glyoxyl-agarose

Table 2 shows that CpCPs were almost completely immobilized on glyoxyl-agarose at pH 10.0 (5 or 200 mM buffer) after 3 h of incubation. Although the buffer concentration did not affect the immobilization yields at pH 10.0, the recovered activity was better using higher ionic strength. Immobilization using glyoxyl supports may require a high density of reactive amino groups on the protein surface [28]. As shown in Figure 6, CpCP1, CpCP2, and CpCP3 have 8, 6, and 18 Lys residues (marked in black), respectively. Some of these Lys residues in the peptidases are located close to their active sites, so if the immobilization occurs through these residues, steric hindrance might be observed during hydrolysis of large substrates, such as azocasein. This result may explain the low recovered activity obtained with this support (3-11%) (Table 2). CpCPs also were immobilized on glyoxyl-agarose at pH 7.0, with lower yields and recovered activities than at pH 10.0 (Table 2). At pH 7.0, immobilization occurs only by N-terminal residues, which must be exposed on the peptidase surface. Therefore, this one-point immobilization can result in biocatalysts with lower stability and recovered activity [28].

Table 2 – Immobilization of *Calotropis procera* cysteine peptidases (CpCPs) on glyoxyl-agarose at different pH and ionic strength values. Immobilization was performed during 3 h and the enzymatic activity was measured using 1% azocasein as substrate.

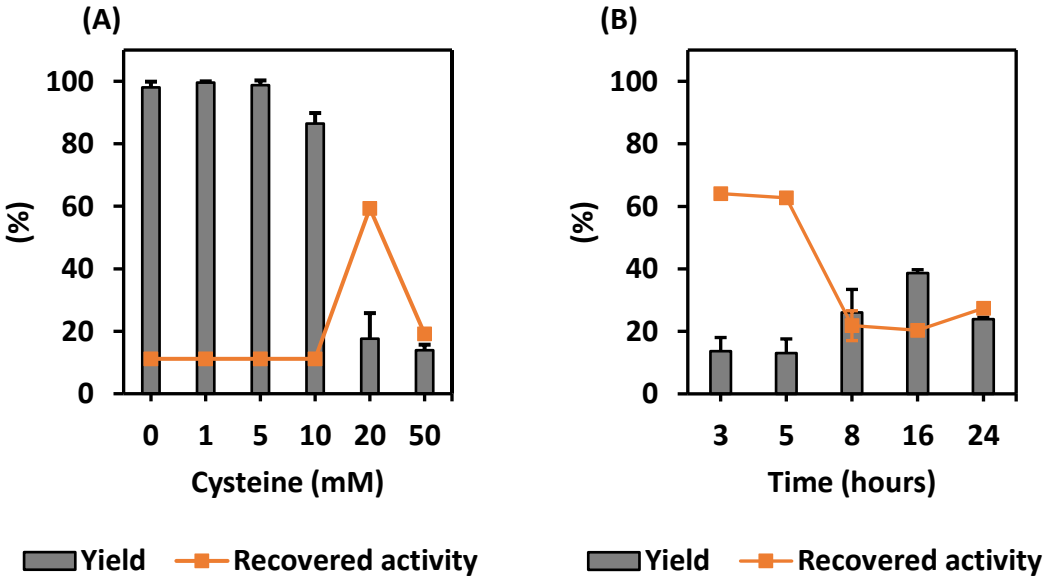
Immobilization condition	**Yield (%)**	**Recovered activity (%)**
pH 7.0/5 mM	70.9 ± 1.1	2.8 ± 0.4
pH 7.0/200 mM	45.0 ± 2.4	3.3 ± 0.3
pH 10.0/5 mM	97.2 ± 2.1	4.5 ± 0.5
pH 10.0/200 mM	97.3 ± 2.7	11.1 ± 0.8

Source: elaborated by the author.

To increase the recovered activity of CpCPs immobilized on glyoxyl-agarose at pH 10.0 (200 mM buffer), new assays were performed in the presence of L-cysteine, a reducing agent used as an activator of cysteine peptidases. Figure 8A shows that the immobilization yield was not affected when using low L-cysteine concentration (up to 10 mM), but drastically decreased using concentrations of 20 or 50 mM. This result suggests that L-cysteine, in high concentrations, may compete for glyoxyl groups of the support. On the other hand, a very

significant enhancement of the recovered activity (60%) was obtained using 20 mM L-cysteine in comparison with CpCPs immobilized without L-cysteine (11%). In this respect, a previous study showed that immobilization in the presence of L-cysteine enhanced the activity of papain [29]. Considering that 20 mM of L-cysteine increased the recovered activity but decreased the immobilization yield of CpCPs on glyoxyl-agarose, we analyzed the immobilization course of this preparation during 24 h instead of 3 h. The yields were higher at longer incubation times, but the recovered activity drastically decreased after 5 h (Figure 8B).

Figure 8 – Immobilization of *Calotropis procera* cysteine peptidases (CpCPs) on glyoxyl-agarose using 200 mM bicarbonate buffer (pH 10.0). (A) Immobilization performed during 3 h with different concentrations of L-cysteine. (B) Immobilization performed with 20 mM L-cysteine during different time intervals. The yield (protein content in the supernatants) was measured using the Bradford reagent, while the enzymatic activity was quantified using 1% azocasein as substrate. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.



Source: elaborated by the author.

Furthermore, other tests were performed to improve the recovered activity of CpCPs on glyoxyl-agarose without L-cysteine (Table 3). Initially, CpCPs were immobilized in the presence of sodium tetrathionate (a reversible cysteine peptidase inhibitor) to fill the active

sites of peptidases and prevent their interaction with the support. Nevertheless, no difference was observed in the immobilization parameters (Table 3). When the immobilizations were performed with lower enzyme loads, the recovered activities were higher compared to the control (2 mg/g). These were 1.9-fold and 2.7-fold higher, respectively, using 0.5 and 0.1 mg/g. That result suggests that diffusion limitation might have masked the enzyme activity [21].

Table 3 – Yield and recovered activity of *Calotropis procera* cysteine peptidases (CpCPs) immobilized on glyoxyl-agarose under different conditions.

Immobilization condition	**Yield (%)**	**Recovered activity (%)**
Control	97.36 ± 2.7	11.17 ± 0.8
Inhibitor*	97.36 ± 2.7	11.17 ± 0.8
Enzyme load		
0.5 mg/g	90.15 ± 0.1	21.95 ± 1.1
0.1 mg/g	84.67 ± 0.9	30.52 ± 3.3
Urea		
2 M	87.85 ± 1.0	14.78 ± 0.1
4 M	54.80 ± 0.4	24.13 ± 1.5

All the tests were compared with the control: CpCPs immobilized on glyoxyl-agarose in 200 mM bicarbonate buffer, pH 10.0, with an enzyme load of 2 mg/g and without urea. *Immobilization in the presence of 30 µM of sodium tetrathionate. Enzymatic activity was measured using 1% azocasein as substrate. Source: elaborated by the author.

When enzymes are removed from their native environment, small structural changes can result in protein aggregation, which reduces their efficiency [21]. To overcome this problem, CpCPs were immobilized in the presence of the chaotropic agent urea. Although this reagent is used as a protein denaturant, some studies have shown its preventive effect on protein aggregation [30]. Here, different concentrations of urea were tested as an additive in the proteolytic activity of soluble CpCPs as well as of its effect during immobilization. In the presence of urea, the proteolytic activity of soluble CpCPs improved in a dose-dependent manner at 2 M (123%) and 4 M (156%) of urea (data not shown). Similar behavior was reported for another cysteine peptidase [31]. In addition, procerain (another peptidase from *C. procera* latex) was stable in 8 M of urea [3]. Regarding immobilized CpCPs on glyoxyl-agarose, the immobilization yields were 87 and 54% and recovered activities were 1.3-fold and 2.1-fold

higher using urea at 2 M and 4 M, respectively, compared to the controls (CpCPs immobilized without urea) (Table 3).

Among all results, the best condition for immobilization using glyoxyl-agarose was 200 mM buffer (pH 10.0), containing 20 mM L-cysteine, and 3 h of incubation at 25 ºC, since the recovered activity was around 60% (Figure 8). That sample was named glyoxyl-CpCPs(Cys) and was used in further assays. CpCPs immobilized on glyoxyl-agarose, in the same conditions but without L-cysteine, were used as control and named glyoxyl-CpCPs.

3.4 Covalent immobilization using MANAE-glutaraldehyde (MANAE-GLU)

Table 4 shows the immobilization parameters, under different conditions, of CpCPs on glutaraldehyde-preactivated MANAE-agarose (named MANAE-GLU). Since MANAE-GLU is a heterofunctional support, all the immobilizations were performed at high ionic strength (200 mM buffer) to prevent ionic interaction between the enzyme and the support, and thus favor only covalent immobilization.

Table 4 – Immobilization parameters of *Calotropis procera* cysteine peptidases (CpCPs) on 5% glutaraldehyde pre-activated MANAE-agarose (MANAE-GLU) at 200 mM bicarbonate buffer (pH 10.0) or 200 mM sodium phosphate (pH 7.0) with or without 20 mM L-cysteine.

	No Cysteine		**20 mM L-Cysteine**	
	pH 7.0	pH 10.0	pH 7.0	pH 10.0
	Yield (%)			
	96.6 ± 2.8	97.4 ± 1.1	5.0 ± 0.9	7.4 ± 0.8
	Recovered activity (%)			
Azocasein	1.3 ± 0.1	3.6 ± 0.1	96.7 ± 3.3	95.7 ± 1.5
BANA	5.3 ± 1.2	21.6 ± 2.1	222.7 ± 4.1	228.3 ± 6.1

*Immobilizations were performed during 3 h at 25 °C and recovered activity was obtained using azocasein and BANA as substrates. Source: elaborated by the author.

Immobilization on glutaraldehyde-activated supports usually occurs by the action of amino acid residues containing primary amino groups, such as lysine, asparagine, glutamine, arginine, and the N-terminal amino [32]. The number of these residues in CpCPs able to react

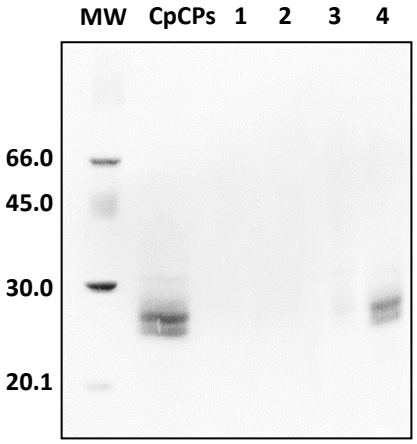
with glutaraldehyde was large (30 for CpCP1 and CpCP2, and 46 for CpCP3), and they were well distributed on the protein surface, allowing different enzyme orientations on the support (Figure 6). After 3 h, CpCPs were almost completely immobilized at both pH 7.0 and 10.0, without L-cysteine, but low recovered activities (below 4%) were obtained using azocasein as substrate. However, when a small substrate (BANA) was used, the biocatalysts exhibited higher recovered activity (about 21%, at pH 10.0). These results suggest that the three peptidases were randomly oriented toward the support, hindering the access of large molecules to their active sites, since the residues that react with glutaraldehyde are distributed over the protein surface and some are close to the active site. Similarly, some immobilization conditions also negatively affected the recovered activity of immobilized ficin [13]. On the other hand, when 20 mM L-cysteine was used during immobilization, the yields were drastically reduced (5-7%) and the enzymatic activity was almost fully recovered when using azocasein, while enzyme hyperactivation (> 220%) was observed with BANA as the substrate (Table 4). Improvements in enzyme activity can be achieved upon immobilization by the stabilization of a hyperactivated form of the peptidases [33].

3.5 Characterization of immobilized CpCPs

3.5.1 Enzyme-support interaction

We expected ionically attached enzymes to desorb from the support and be detectable by SDS-PAGE, as was observed for CpCPs immobilized on MANAE support (Figure 9). On the other hand, no protein band was detected in the gel when CpCPs were immobilized on both glyoxyl- and MANAE-GLU supports, confirming that the enzyme-support interactions were stablished by covalent bonds (Figure 9).

Figure 9 – SDS-PAGE (12.5%) of the *Calotropis procera* cysteine peptidases **(**CpCPs) immobilized on different supports. MW: molecular weight marker; CpCPs: soluble CpCPs; 1: Glyoxyl-CpCPs; 2: Glyoxyl-CpCPs(Cys); 3: MANAE-GLU-CpCPs; 4: MANAE-CpCPs.

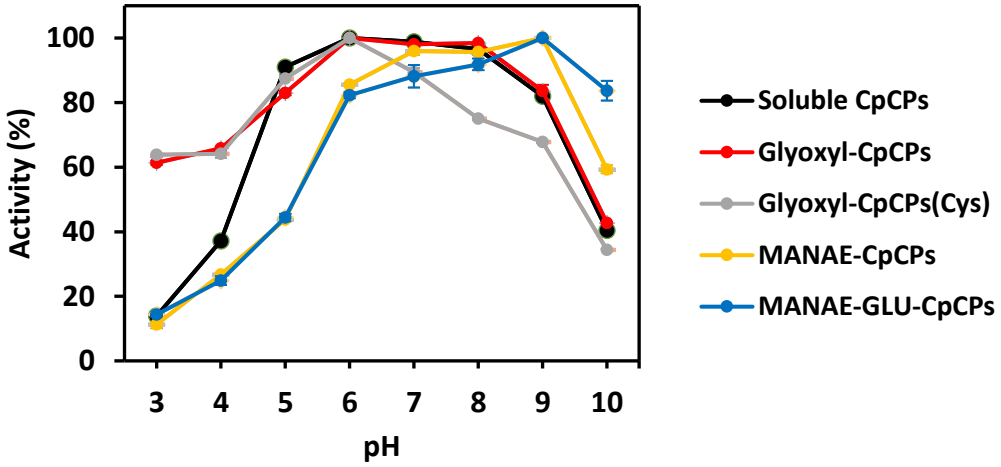


Source: elaborated by the author.

3.5.2 The pH effect on activity

The pH value is an important parameter that affects enzyme activity. It is known that immobilization can cause enzyme conformational changes, affecting their optimum pH level for activity. In addition, the immobilization can also affect the microenvironment and cause a partition effect away or towards the enzyme [33]. In turn, this can influence optimum pH values of the enzyme. As shown in Figure 10, the maximum proteolytic activity was achieved between pH 5.0 and pH 8.0 for soluble CpCPs and glyoxyl-CpCPs, and pH 7.0 and 9.0 for MANAE-CpCPs and MANAE-GLU-CpCPs. That shift in the optimum pH toward alkaline values has been reported for other peptidases, caused by the influence of support surface charge [34]. In addition, in more acidic conditions, such as pH 3.0 and 4.0, glyoxyl biocatalysts exhibited higher activity than soluble CpCPs, which may have been caused by enzyme stabilization.

Figure 10 – Effect of pH on the proteolytic activity of soluble and immobilized *Calotropis procera* cysteine peptidases (CpCPs). The maximum activity was considered to be 100% and was measured using 1% azocasein as substrate. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.

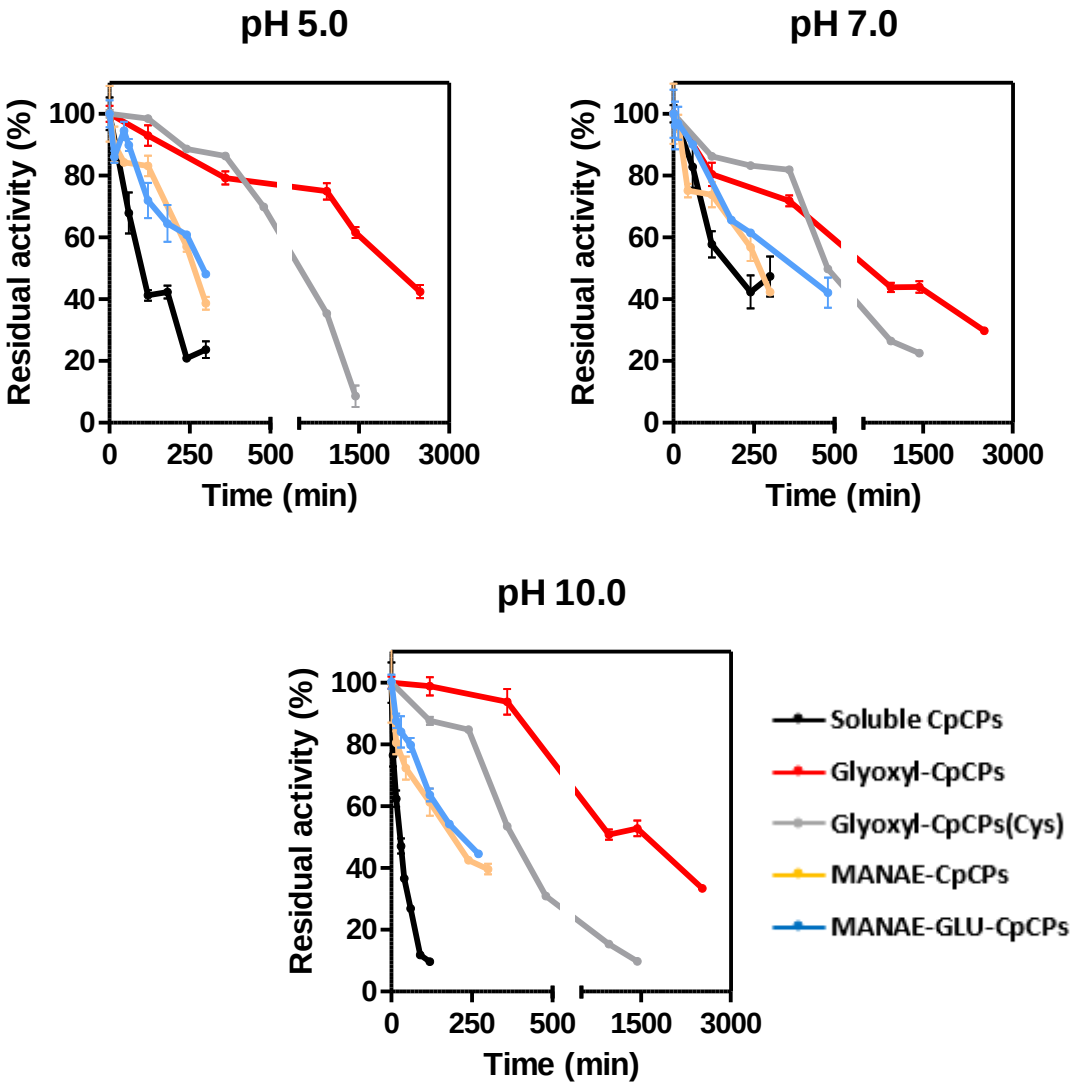


Source: elaborated by the author.

3.5.3 Thermal stability

Thermal stability of soluble and immobilized CpCPs at different pH values (5.0, 7.0 or 10.0) was determined incubating the samples at 60 °C for different times. As shown in Figure 11, soluble enzymes were more stable at pH 7.0, followed by pH 5.0 and pH 10.0.

Figure 11 – Thermal inactivation course of soluble and immobilized *Calotropis procera* cysteine peptidases (CpCPs). Inactivation was performed at 60 °C at different pH values. Enzymatic activity was measured using 1% azocasein as substrate. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.



Source: elaborated by the author.

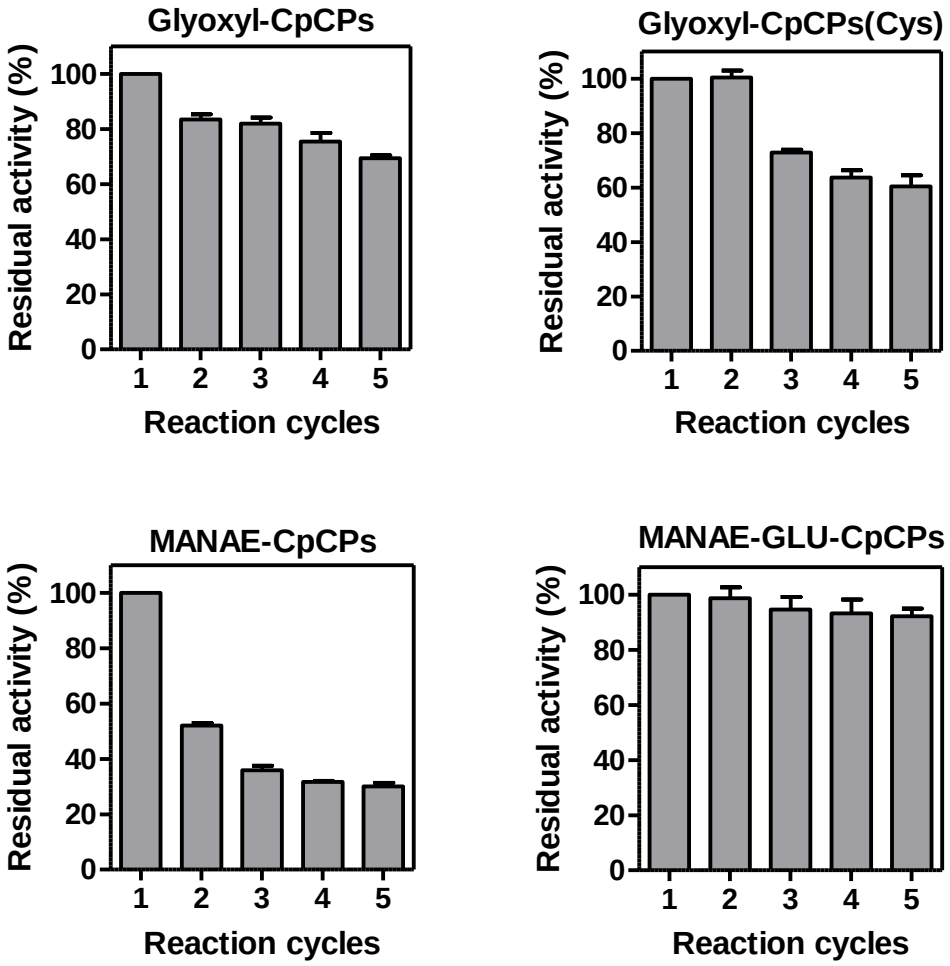
In general, all immobilization techniques improved the stability of the soluble peptidases. The half-life values showed that the enhancement levels of the stability of MANAE-CpCPs were 2.7-fold (pH 5.0) and 6.7-fold (pH 10.0) compared to soluble CpCPs. However, at pH 7.0, no significant increment in stability was observed for immobilized enzymes on MANAE-agarose. The enhanced stability levels of MANAE-GLU-CpCPs were 2.8-fold (pH

5.0), 1.7-fold (pH 7.0), and 9.8-fold (pH 10.0) in comparison with soluble CpCPs (Figure 11). Similarly, the stability of the peptidases from other plant extracts was also improved when immobilized using the same support [13]. The highest stabilization of CpCPs was achieved using glyoxyl-agarose as support, better than when co-immobilized with 20 mM L-cysteine [glyoxyl-CpCPs (Cys)]. The enzyme-support multi-interaction through covalent immobilization can increase the rigidity of the enzymes and improve their stability [35]. Glyoxyl-CpCP(Cys) exhibited stabilization factors of 8-fold (pH 5.0), 2.5-fold (pH 7.0), and 16-fold (pH 10.0), while the values for glyoxyl-CpCPs were 20-fold (pH 5.0), 3.7-fold (pH 7.0), and 60-fold (pH 10.0) more stable compared to the soluble form. In comparison, ficin immobilized on glyoxyl was 40-fold more stable than the soluble enzyme [11].

3.5.4 Operational stability

Reuse is one of the main objectives of enzyme immobilization. This is an important feature in large-scale applications by making the process more economical [36]. Thus, the capacity to reuse immobilized CpCPs on different supports was evaluated. Figure 12 shows that MANAE-GLU-CpCPs could be reused for five cycles with minimal decrease of initial activity. In contrast, MANAE-CpCPs retained only 30% of the initial activity after five consecutive cycles. Additionally, glyoxyl-CpCPs and glyoxyl-CpCPs (Cys) retained around 70% and 60% of the initial activity, respectively, after five cycles.

Figure 12 – Operational stability of *Calotropis procera* cysteine peptidases (CpCPs) immobilized on different supports. The reactions were performed using 1% azocasein as substrate with cycles of 40 min reaction, at 37 °C and 120 rpm stirring. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.



Source: elaborated by the author.

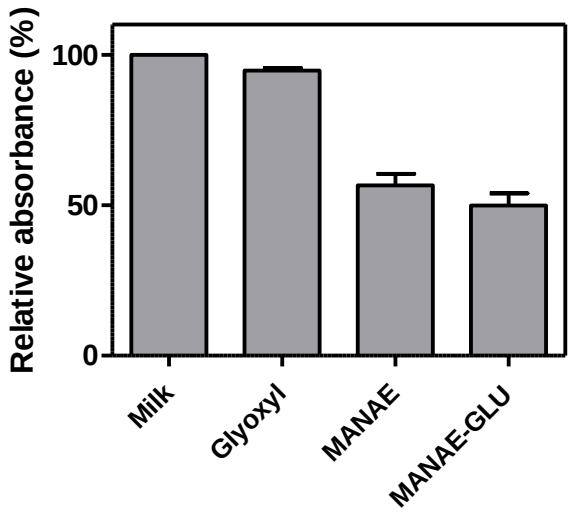
As expected, the immobilized CpCPs obtained by covalent attachment exhibited the best stability after multiple reaction cycles. As shown in Figure 6, glutaraldehyde-activated supports have more binding sites than glyoxyl supports, so the greater stabilization of MANAE-GLU-CpCPs may be due to the formation of multipoint interaction with the carrier. On the other hand, MANAE-CpCPs were obtained by ionic interaction and the lower reusability may be explained by enzyme leakage after the consecutive cycles (Figure 9).

3.6 Hydrolysis of milk proteins

Cow's milk proteins are a common cause of food allergy during infancy and childhood. Caseins are the most abundant proteins in milk (about 80%) and are the major allergens. Furthermore, whey proteins such as α-lactalbumin and β-lactoglobulin are also reported to be important allergens [37]. Enzymatic hydrolysis is one of the most common strategies to reduce the allergenicity of milk proteins, by the cleavage of protein epitopes, preventing immune responses. Interestingly, few studies have focused on using immobilized enzymes to reduce milk allergenicity [16]. Since soluble CpCPs were previously demonstrated to achieve efficient hydrolysis of milk proteins [7], here we assessed whether immobilized CpCPs would be able to digest caseins and whey proteins and thus be suitable for use to produce hydrolyzed milk formulas.

In milk, caseins form colloidal structures called micelles. Casein micelles have an external κ-casein hairy layer with negative charges [38], which may interact with the positive charge of MANAE and MANAE-GLU. To investigate this hypothesis, we incubated milk proteins with the supports and measured the protein content of the supernatants after 2 h. As shown in Figure 13, about 50% of the milk proteins were adsorbed on both MANAE supports, while glyoxyl did not interact with milk proteins. Thus, MANAE-CpCPs and MANAE-GLU-CpCPs were not used in the hydrolysis assays of milk proteins.

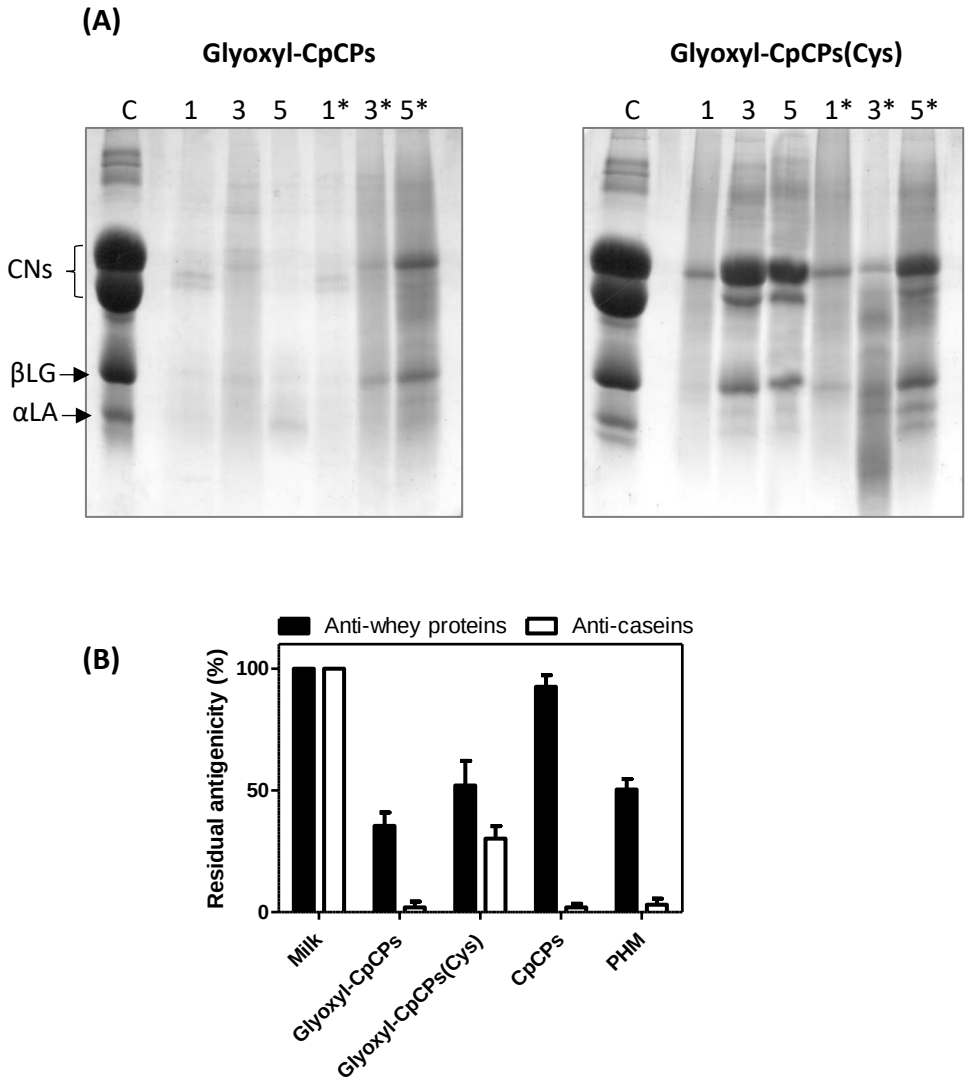
Figure 13 – Adsorption of the milk proteins by different supports. Milk proteins were incubated with each support at 37 °C and 150 rpm stirring, simulating the reaction conditions performed in Fig 6. The protein content in the supernatants was measured after 2 h of incubation using Bradford method. The vertical bars correspond to standard deviation (SD) of the mean of three replicates.



Source: elaborated by the author.

As observed in the SDS-PAGE (Figure 14A), the caseins were completely hydrolyzed by glyoxyl-CpCPs. In addition, the ELISA (Figure 14B) showed that anti-casein antibodies poorly detected the peptides in the hydrolysates and the residual antigenicity after 2 h of proteolysis was 2%, similar to milk proteins hydrolyzed by the soluble enzymes (2%) and a commercial partially hydrolyzed milk formula (PHM) (3%). In contrast, caseins were only partially hydrolyzed by glyoxyl-CpCPs(Cys) (Figure 14A). Antigenic peptides (30%) were detected in the hydrolysates, although at a lower level than in non-hydrolyzed milk (100%). This lower efficiency may be related to wrong enzyme orientation or diffusional limitations. In comparison, papain immobilized on glyoxyl-agarose showed difficulty to hydrolyze milk proteins [39].

Figure 14 – Cow's milk proteins hydrolyzed by soluble and immobilized *Calotropis procera* cysteine peptidases (CpCPs) evaluated by 12.5% SDS-PAGE (A) and ELISA (B). (A) The operational stability was evaluated with reaction cycles of 2 h for the hydrolysis of milk proteins (10 mg/mL) with the immobilized CpCPs (2 mg protein/g support), at 37 °C, pH 6.5, and 150 rpm stirring. (B) The residual antigenicity of milk proteins was determined for the first reaction cycle using polyclonal antibodies against casein (white bar) and whey proteins (black bar). The vertical bars correspond to standard deviation (SD) of the mean of three replicates. Legend: C (control/non-hydrolyzed milk); 1, 3 and 5 (1st, 3rd and 5th reaction cycles, respectively); CNs (caseins); βLG (β-lactoglobulin); αLA (α-lactalbumin); PHM (partially hydrolyzed milk, Enfamil Gentlease); *(reactions performed with milk proteins preheated at 85 ºC for 30 min).



Source: elaborated by the author.

Regarding whey proteins, they are known to be more resistant to enzymatic hydrolysis. Oliveira et al. [7] reported that after 24 h of hydrolysis by CpCPs, the hydrolysates exhibited 85% of their initial antigenicity. Here, both glyoxyl-CpCPs and glyoxyl-CpCPs(Cys) were able to hydrolyze whey proteins after 2 h of reaction (Figure 14A), exhibiting residual antigenicity values of 35% and 52%, respectively (Figure 14B). In comparison, the residual antigenicity was 92% for whey proteins hydrolyzed by soluble CpCPs and 50% for the commercial partially hydrolyzed milk formula (PHM) (Figure 14B). Additionally, other immobilized enzymes also showed significant activity to whey proteins [40]. As observed, hydrolysates produced by glyoxyl-CpCPs exhibited less immunoreactive peptides than the commercially available formula and the soluble enzyme. These results showed that immobilization might have improved CpCPs' activity against these substrates. Conversely, Pessato et al. [41] reported that immobilized alcalase was not as efficient as the soluble enzyme in reducing whey antigenicity.

The activity of glyoxyl-CpCPs and glyoxyl-CpCPs(Cys) was also evaluated against milk proteins after three and five reaction cycles. The results were in accordance with the operational stability found against azocasein (Figure 12). Glyoxyl-CpCPs could extensively hydrolyze caseins and whey proteins for at least five cycles. On the other hand, glyoxyl-CpCPs(Cys) was efficient only in the first cycle (Figure 14A).

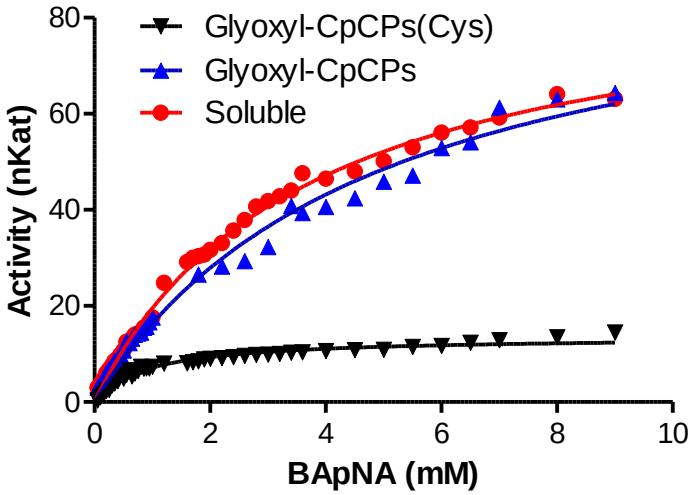
Some physical treatments, such as heat denaturation, can be used to improve the degree of hydrolysis of milk proteins. Oliveira et al. [7] showed that the hydrolytic action of CpCPs on whey protein was increased after heat treatment of the substrate. Here, the hydrolysis of the milk proteins, by immobilized CpCPs, tested under similar conditions, was not significantly enhanced compared to the results obtained with the unheated substrate (Figure 14A). However, the operational stability of glyoxyl-CpCPs(Cys) was slightly higher using denatured milk proteins as substrate, since it sustained hydrolysis for at least three cycles.

3.7 Enzyme kinetic parameters and structural changes

Because glyoxyl-CpCPs and glyoxyl-CpCPs (Cys) were the best biocatalysts to hydrolysis the milk proteins, new experiments were performed to evaluate other kinetic parameters and structural changes of enzymes on these supports. As observed in Figure 15, glyoxyl-CpCPs and glyoxyl-CpCPs (Cys) followed Michaelis-Menten kinetics. Glyoxyl-CpCPs and glyoxyl-CpCPs (Cys) exhibited V_{max} and K_m of 95.5 ± 2.7 nkat and 4.8 ± 0.2 mM

and 13.5 ± 0.1 nkat and 0.9 ± 0.03 mM, respectively, while the V_{max} and K_m for soluble CpCPs were 89.8 ± 1.0 nkat and 3.6 ± 0.08 mM. Interestingly, glyoxyl-CpCPs (Cys) presented notable decreases of V_{max} and K_m when compared to the soluble form. Lower K_m values were reported for immobilized Procerain B [10].

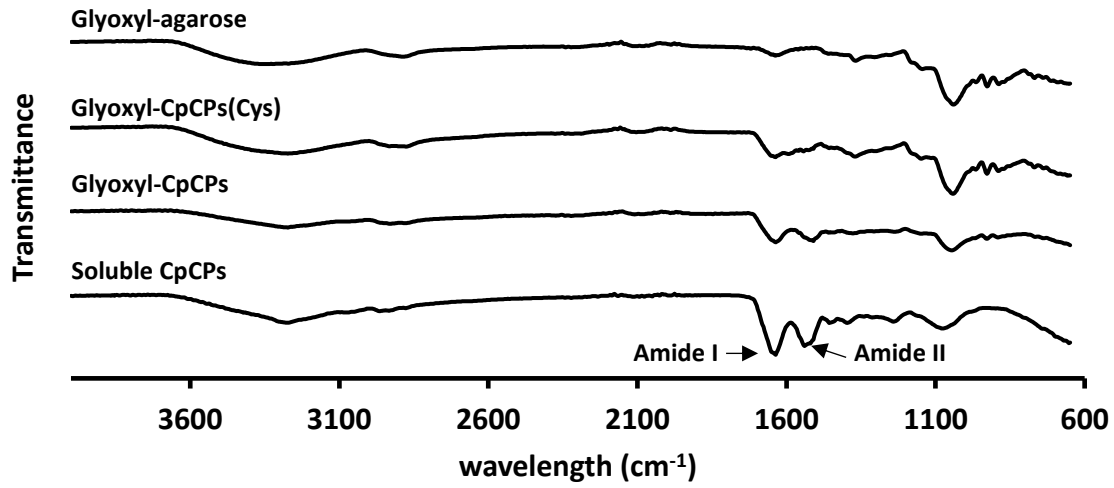
Figure 15 – Effect of substrate concentration on the proteolytic activity of soluble and immobilized *Calotropis procera* cysteine peptidases (CpCPs). The assays were performed using different concentrations of BApNA at pH 5.0 and 37 °C. K_m and V_{max} were determined from a nonlinear curve fit of the untransformed data using GraphPad Prism 7. The numbers used to define the kinetic parameters were best-fit estimate ± SD.



Source: elaborated by the author.

The K_{cat} and catalytic efficiency (K_{cat}/K_m) values for glyoxyl-CpCPs, glyoxyl-CpCPs(Cys), and soluble CpCPs were 0.40 s^{-1}, 0.11 s^{-1}, and 0.68 s^{-1} and 82.73 M^{-1} s^{-1}, 126.01 M^{-1} s^{-1}, and 189.07 M^{-1} $s^{-}1$, respectively. The decreased catalytic efficiency of both biocatalysts may be explained by enzyme conformation changes after immobilization. To test this hypothesis, the samples were analyzed by Fourier-transform infrared (FTIR) spectroscopy (Figure 5).

Figure 16 – FTIR spectra of glyoxyl-agarose beads, Glyoxyl-CpCPs, Glyoxyl-CpCPs(Cys) and soluble *Calotropis procera* cysteine peptidases (CpCPs).



Source: elaborated by the author.

FTIR allows the analysis of protein secondary structures by the absorption/transmittance of the infrared radiation at specific wavelengths. Each structural component has a specific band pattern in the infrared spectrum vibrations and the most prominent bands of the protein backbone are amide I and II. Amide I (1700 – 1600 cm^{-1}) is the band used most to determine the protein secondary structure components, and its stretching vibrations are associated with C=O regions, whereas amide II (1575 – 1480 cm^{-1}) bands are associated with N–H and C–N regions [42]. The amide I band observed in Supplementary Fig. 5 consists of an overlap of bands corresponding to α-helices, β-sheets, and turns. The amide I band in soluble CpCPs and glyoxyl-CpCPs was detected at 1640.0 cm^{-1}, while a shift was observed for glyoxyl-CpCPs(Cys) (1636.3 cm^{-1}). With respect to amide II, the band was similar to that of soluble CpCPs and glyoxyl-CpCPs (1513.3 cm^{-1}), and it was not detected in glyoxyl-CpCPs(Cys). In general, the changes observed in the region and intensity of bands suggest that the covalent bonds between the enzyme and glyoxyl-agarose beads led to structural changes of the peptidases. Major alteration of the protein structure in glyoxyl-CpCPs(Cys) may explain the lower activity in comparison with glyoxyl-CpCPs.

4. Conclusion

Although the yield of ionic immobilization using sulfopropyl-agarose (~99%) was much better and faster that on DEAE- and MANAE-agarose, its derivatives had negligible recovered activity (4%). On the other hand, MANAE-agarose produced biocatalysts with the highest recovered activity (23%). Regarding the covalent immobilization, glyoxyl-agarose was a better support than MANAE-GLU-agarose. Although the covalent interaction on glyoxyl-agarose induced some structural changes or altered some of its enzyme kinetic parameters, such as V_{max}, K_m, K_{cat}, as well as catalytic efficiency, the biocatalyst showed better pH stability (pH 3.0-10.0), 60-fold more stable at 60 °C, and retained 70% of its activity after five reaction cycles. It also was able to hydrolyze the major milk allergens better than soluble CpCPs, and reduced the milk protein antigenicity to levels as low as those of a commercially available formula. Therefore, all results show the promising potential of CpCPs immobilized on glyoxyl-agarose as new biocatalysts for the production of cow's milk hydrolysates with low allergenicity.

Conflict of interest

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

Contributions

JPBO, MVR, JSO, DCF and CDTF performed the latex peptidase purification, gel electrophoresis, proteolytic activity assays and hydrolysis assays. PFNS produced polyclonal antibodies and performed ELISA. JPBO, KPSA, BBP and LRBG performed all the immobilization assays. All authors contributed to data analysis, discussion and writing of the manuscript.

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4 CONCLUSÃO

Através deste estudo foi possível estabelecer protocolos eficientes para a imobilização das peptidases laticíferas de *C. procera,* produzindo biocatalisadores com diferentes atividades e estabilidades. Além disso, a aplicação dos biocatalisadores na hidrólise das proteínas do leite bovino apresentou uma performance satisfatória, produzindo hidrolisados com alergenicidade residual menor que uma fórmula hipoalergênicas disponível no mercado. Como perspectivas pode-se avaliar a aplicabilidade dos biocatalisadores obtidos para a produção de outros hidrolisados proteicos de importância alimentar.

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