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CAMPUS JUVINO OLIVEIRA
ÁREA DE CONCENTRAÇÃO: ENGENHARIA DE ALIMENTOS

MATEUS PEREIRA FLORES SANTOS

EFEITO DE DIFERENTES TIPOS DE AGENTES MODIFICADORES DE SUPERFÍCIE NA IMOBILIZAÇÃO DE PROTEASES DE ORIGEM ANIMAL EM CARVÃO ATIVADO E SUA APLICAÇÃO NA HIDRÓLISE DE CASEÍNA BOVINA

ITAPETINGA – BA
FEVEREIRO 2022

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Tese apresentada como parte das exigências para obtenção do título de Doutor em Engenharia e Ciência de Alimentos, área de concentração em Engenharia de Alimentos, da Universidade Estadual do Sudoeste da Bahia – UESB, Campus Juvino Oliveira.

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Índice Sistemático para desdobramentos por Assunto:

1. Carvão ativado na imobilização de proteases animais
2. Hidrólise enzimática - Caseína bovina
3. Biocatalisador

DECLARAÇÃO DE APROVAÇÃO

PPGECAL ENGENHARIA E CIÊNCIA DE ALIMENTOS
UESB
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Título: EFEITO DE DIFERENTES TIPOS DE AGENTES MODIFICADORES DE SUPERFÍCIE NA IMOBILIZAÇÃO DE PROTEASES DE ORIGEM ANIMAL EM CARVÃO ATIVADO E SUA APLICAÇÃO NA HIDRÓLISE DE CASEÍNA BOVINA.

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Aprovada como parte das exigências para obtenção do Título de ***DOUTOR EM ENGENHARIA E CIÊNCIA DE ALIMENTOS, ÁREA DE CONCENTRAÇÃO: ENGENHARIA DE ALIMENTOS***, pela Banca Examinadora.

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Itapetinga-BA, 24 de fevereiro de 2022.

“Não é sobre ganhar, é sobre não desistir.
Se você tem um sonho, lute por ele.”
Lady Gaga

A Deus.

Aos meus pais.

A minha Avó.

À minha irmã.

Dedico!

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LISTA DE ABREVIAÇÕES

BET – Brunauer, Emmett e Teller

CA – Carvão Ativado

CAF – Carvão Ativado Funcionalizado com Glutaraldeído

CAG – Carvão Ativado Funcionalizado com Genipina

CAM – Carvão Ativado Funcionalizado com Íons Metálicos

CQM – Carvão Ativado Funcionalizado com Íons Metálicos na presença do Agente Quelante

DP^a – Diâmetro Médio de Poros

DPPH – 2,2-difenil-1-picrilhidrazilil

FRAP – Poder Antioxidante Redutor Férrico

IDA – Agente Quelante Ácido Iminodiacético

K_m – Constante de Michaelis-Menten

pH_{PCZ} – pH do Ponto de Carga Zero

PN – Pepsina Nativa

S_g – Área Superficial Especifica

TL – Tripsina Livre

V_{max} – Velocidade Máxima de hidrólise

V_{meso} – Volume de Mesoporos

V_{micro} – Volume de Microporos

RESUMO

SANTOS, M. P. F. **Efeito de diferentes tipos de agentes modificadores de superfície na imobilização de proteases de origem animal em carvão ativado e sua aplicação na hidrólise de caseína bovina.** Itapetinga – BA: UESB, 2020. 150 p. (Tese – Doutorado em Engenharia e Ciência de Alimentos). *

As proteases têm importância particular na ciência e tecnologia de alimentos, sendo bastante utilizadas na indústria de alimentos no preparo de hidrolisados proteicos, catalisando a hidrólise das ligações peptídicas presentes nas proteínas. Apesar da alta eficiência catalítica, o custo de utilização e sua baixa estabilidade em algumas condições limitam seu uso em alguns processos industriais. A imobilização enzimática se tornou alternativa para solucionar essas dificuldades, propiciando a sua reutilização por vários ciclos, além de proporcionar um aumento da sua vida útil. Dentre os suportes utilizados, os suportes sintéticos como o carvão ativado, vêm ganhando mais espaço por apresentarem baixo custo de produção e serem mais apropriados para utilização industrial, devido às suas propriedades físico-químicas. Neste contexto, o presente trabalho teve como objetivo principal levantar dados sobre a aplicação do carvão ativado na imobilização de proteases animais (pepsina e tripsina), bem como estudar as diferentes modificações (funcionalização com glutaraldeído, genipina, partículas de íons ferro com e sem agente quelante) nessa matriz para garantir melhores resultados de imobilização.Foi possível utilizar a genipina com substituto ao glutaraldeído, garantindo resultados superiores de capacidade de imobilização e atividade para ambas as enzimas testadas em comparação ao método do glutaraldeído. O carvão ativado modificado através da metalização com íons de ferro (Fe^{2+} e Fe^{3+}) levou a uma melhor eficiência de imobilização. (> 85 % para Tripsina e > 99 % para Pepsina). A metalização proporcionou uma atividade enzimática de 2,30 U para a pepsina, superior às demais modificações e mais próxima ao valor da enzima na forma nativa (3,32 U). Além disso, os peptídeos obtidos através da hidrólise da caseína apresentaram potencial como agentes antioxidantes. Para a Tripsina a funcionalização com íons ferro na presença de um agente quelante levou a uma capacidade de imobilização de 95,49 $mg.g^{-1}$ e uma atividade hidrolítica de 4,11 U, valor superior aos valores obtidos para a enzima na forma livre (3,76 U). Além disso, a imobilização proporcionou um aumento da estabilidade das enzimas imobilizadas e garantiu a sua reutilização por mais de 5 ciclos para ambas. De maneira geral, foi possível avaliar o efeito de diferentes modificações na superfície do carvão ativado, responsáveis por formar ligações covalentes entre o suporte e a enzima, melhorando sua atividade proteolítica quando comparado ao método de imobilização por adsorção, indicando que as novas modificações são promissoras para imobilização de proteases de origem animal.

Palavras chave: Biocatalisador; Hidrólise; Imobilização; Pepsina; Tripsina;

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ABSTRACT

SANTOS, M. P. F. **Effect of different types of surface modifying agents on the immobilization of animal origin proteases in activated carbon and their application in casein hydrolysis bovine.** Itapetinga – BA: UESB, 2016. 148 p. (Thesis - Doctorate in Engineering and Food Science).*

Proteases are of particular importance in food science and technology, being widely used in the food industry in the preparation of protein hydrolysates, catalyzing the hydrolysis of peptide bonds present in proteins. Despite the high catalytic efficiency, the cost of use and its low stability under some conditions limit its use in some industrial processes. Enzymatic immobilization has become an alternative to solve these difficulties, allowing its reuse for several cycles, in addition to providing an increase in its useful life. Among the supports used, synthetic supports such as activated carbon, have been gaining more space because they have low production costs and are more suitable for industrial use, due to their physicochemical properties. In this context, the main objective of the present work was to collect data on the application of activated carbon in the immobilization of animal proteases (pepsin and trypsin), as well as to study the different modifications (functionalization with glutaraldehyde, genipin, iron ion particles with and without chelator) in this matrix to ensure better immobilization results. It was possible to use genipin as a substitute for glutaraldehyde, guaranteeing superior results in immobilization capacity and activity for both enzymes tested compared to the glutaraldehyde method. Activated carbon modified by metallization with iron ions (Fe^{2+} and Fe^{3+}) led to better immobilization efficiency. (> 85 % for Trypsin and > 99 % for Pepsin). Metallization provided an enzymatic activity of 2.30 U for pepsin, higher than the other modifications and closer to the value of the enzyme in its free form (3.32 U). In addition, the peptides obtained through the hydrolysis of casein showed potential as antioxidant agents. For Trypsin, the functionalization with iron ions in the presence of a chelating agent led to an immobilization capacity of 95.49 $mg.g^{-1}$ and a hydrolytic activity of 4.11 U, a value higher than the values obtained for the enzyme in the free form (3.76 U). In addition, immobilization provided an increase in the stability of the immobilized enzymes and ensured their reuse for more than 5 cycles for both. In general, it was possible to evaluate the effect of different modifications on the surface of activated carbon, responsible for forming covalent bonds between support and enzyme, improving its proteolytic activity when compared to the adsorption immobilization method, indicating that the new modifications are promising. for immobilization of proteases of animal origin.

Keywords: Biocatalyst; Hydrolysis; Immobilization; Pepsin; Trypsin;

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* Advisor (a): **DSc**. UESB. Cristiane Martins Veloso; Co-advisors: DSc. UESB. Renata Cristina Ferreira Bonomo; DSc. UESB. Leandro Soares Santos

CAPÍTULO 1

Introdução Geral

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1. INTRODUÇÃO GERAL

As proteases têm importância particular na ciência e tecnologia de alimentos, sendo bastante utilizadas na indústria de alimentos. Elas são empregadas no preparo de hidrolisados proteicos, catalisando a hidrólise das ligações peptídicas presentes nas proteínas. Dentre as proteases obtidas de fontes animais, a pepsina e a tripsina estão entres as enzimas proteolíticas mais eficiente, sendo liberadas pelo estômago e pâncreas, respectivamente. Elas atuam em conjunto com a quimotripsina e a carboxipeptidase no processamento metabólico das hidrólises de proteínas em peptídeos e aminoácidos essenciais, de fácil absorção. Esses aminoácidos têm diversas funções no organismo, como crescimento muscular e produção hormonal (MORAN, 2016; SOUZA JR et al., 2020).

Apesar da alta eficiência catalítica das enzimas digestivas na sua forma nativa, fatores ligados ao custo e a baixa estabilidade em algumas condições limitam a sua utilização em processos industriais. Para contornar estes problemas a sua utilização na forma imobilizada se tornou alternativa para solucionar essas dificuldades, uma vez que, as enzimas imobilizadas apresentam vantagens como: utilização múltipla (reutilização), melhor controle dos processos e estabilidade mecânica em comparação com a enzima nativa e em alguns casos a imobilização melhora a eficiência catalítica da enzima (KULKARNI, 2016).

Wongrod et al. (2019), destacam a utilização de diferentes suportes na imobilização enzimática. Entretanto o carvão ativado se destaca dentre os demais devido à alta resistência química, mecânica e térmica, hidrofilicidade e por ser insolúvel. Apresenta uma elevada área superficial BET e porosidade bastante definida, além de oferecer maior facilidade de produção e menor custo quando comparado com outros tipos de adsorvente comerciais (DANISH & AHMAD, 2018; AMIRZA et al., 2017).

O carvão ativado (CA) é um material carbonáceo de estrutura porosa bem desenvolvida responsável por conferir uma elevada área superficial especifica, além de apresentar em sua superfície heteroátomos de oxigênio, nitrogênio e hidrogênio ligados aos átomos de carbono. Podem ser sintetizados a partir de quaisquer precursores, desde que apresentem alto teor de carbono e baixo teor de materiais inorgânicos em sua composição. Os resíduos agroindustriais vêm se destacando como materiais precursores na síntese de carvão ativado por serem provenientes de fontes renováveis e serem ricos em materiais lignocelulósicos (lignina, celulose e hemicelulose), apresentando um alto

teor de carbono. Além disso, estes materiais não têm mercado definido para sua comercialização, logo seu reaproveitamento está relacionado com a redução de custos de produção do carvão ativado, bem como a redução de possíveis danos ambientais que possam vir a ser causados pelo seu acúmulo dos mesmos no meio ambiente (SANTOS et al., 2020).

A superfície do carvão ativado ainda pode ser modificada por diferentes métodos para melhorar sua capacidade de imobilização enzimática. Estas modificações são feitas com o intuito de criar e/ou aumentar os grupos funcionais já existente em sua superfície, os quais variam com o tipo de ativação durante a etapa de síntese. O carvão ativado com superfície com superfície modificada pode ser classificado como um suporte heterofuncional, ou seja, um suporte com funcionalidades distintas, permitindo interações físicas e/ou químicas com as enzimas (RAMANI et al., 2012; SILVA, 2018; OKURA et al., 2020). Diversos tipos de modificações superficiais vêm sendo estudadas para melhorar as propriedades destes suportes: modificações usando glutaraldeído (BRITO et al., 2017; SANTOS et al., 2019; SOUZA JR et al., 2020), modificações com óxido de ferro (JAIN et al., 2018; OLIVEIRA et al., 2022; SANTOS et al., 2022), carbonização hidrotérmica (GONÇALVES et al., 2021), modificação com genipina (SANTOS et al., 2022), entre outras.

As alterações na estrutura superficial do carvão ativado podem ser feitas pela adição de certas etapas ou componentes químicos durante o processo de ativação ou posterior a sua síntese. Essas modificações envolvem principalmente alterações químicas da superfície e consequentemente em suas propriedades, tendo como principal interesse a inserção de vários grupos funcionais, com o intuito de tornar o suporte mais eficiente ao processo de imobilização de enzimas e com maior eficiência catalítica devido à minimização dos efeitos difusionais de substratos e produtos durante a reação. Além disso, a imobilização tem como intuito manter conformação enzimática, sua atividade e estabilidade operacional em processos contínuos e descontínuos, múltiplas utilizações despertando o interesse industrial e comercial contornando os obstáculos para sua utilização na forma nativa (BEZERRA et al., 2015; PEGO et al., 2019).

Diante do exposto, objetivou-se neste estudo avaliar diferentes modificações da superfície do carvão ativado sintetizado a partir do caroço de tamarindo utilizando os agentes: Glutaraldeído, Genipina e os íons metálicos (Fe^{2+} e Fe^{3+}), avaliando o efeito destas modificações na imobilização de proteases de origem animal (Pepsina e Tripsina).

Além disso os derivados obtidos foram empregados na hidrólise da caseína bovina para síntese de peptídeos com propriedades antioxidantes.

2. REVISÃO DE LITERATURA

2.1. Enzimas

As enzimas são biocatalisadores de origem proteica que participam em diversos processos fisiológicos em condições padrão do meio (valores brandos de pH e temperatura). São responsáveis por acelerar as taxas das reações químicas, reduzindo a quantidade de energia necessária para que os processos ocorram (BILAL et al., 2018). Além de desempenharem funções específicas com alta especificidade de substrato, seletividade e condições de reação leves, qualificando as enzimas como (bio)catalisadores revolucionários em diversas aplicações (REN et al., 2019). Na natureza existem uma grande diversidade de enzimas, sendo que estas participam de diferentes tipos de reações metabólicas em plantas, animais ou microrganismos (NEELAN et al., 2019).

A especificidade enzimática é a capacidade da enzima de reagir com apenas um tipo especifico de substrato, devido à disposição tridimensional de resíduos de aminoácidos específicos que formam seu sítio ativo. Esta atividade biológica particular, baseia-se nas interações entre o sítio ativo da enzima com a molécula do substrato/inibidor, chamados de reação enzima-substrato, chave-fechadura ou teoria do encaixe induzido. Já a condição de alta seletividade enzimática ao substrato está relacionada com a natureza quiral do sítio ativo, que é capaz de executar diversos tipos de interações não covalentes específicas, como: ligações de hidrogênio, interações de Van der Waals, interações eletrostáticas entre outras. (BEZERRA et al, 2015; BILAL & IQBAL, 2019).

As enzimas possuem várias aplicações e são utilizadas em diversos segmentos das indústrias alimentícias, têxteis, de papel e na agricultura. O emprego das enzimas em processos industriais resulta em reduções significativas de custos de produção, devido a maior especificidade de atuação e por agirem geralmente sob condições reacionais brandas e com menores volumes de matérias primas (JEGANNATHAN e NIELSEN 2013).

Dentre os vários tipos de enzimas, as proteases ou proteolíticas representam uma classe de enzimas que desempenham funções importantes em processos fisiológicos, sendo utilizadas nas mais diversas áreas como nas indústrias alimentícia, têxtil, de

detergentes, couro e cosméticos e farmacêutica. É a classe de enzimas com uma das maiores aplicações comerciais e responsáveis por movimentar 90% do comércio internacional de enzimas (BARBOSA et al., 2020).

2.1. Proteases

Proteases (peptidases) são enzimas que catalisam a hidrólise de ligações peptídicas presentes em proteínas ou peptídeos, resultando assim na liberação de peptídeos de tamanho variável (MURI, 2014). Estas enzimas têm importância particular na ciência de alimentos, sendo utilizadas na indústria de alimentos nos processos de: tenderização de carnes, clarificação de bebidas, indústria de panificação, produção de xaropes de alta maltose, realçar o sabor, tratamento de resíduos, preparo de hidrolisados proteicos, entre outros. Os hidrolisados proteicos, por exemplo, apresentam propriedades que os tornam atrativos como fonte de aminoácidos na nutrição humana, sendo fisiologicamente melhores do que as proteínas nativas, uma vez que os oligopeptídeos, principalmente di- e tripeptídeos, são melhores absorvidos pelo organismo e apresentam melhor balanço de aminoácidos quando comparados a misturas de aminoácidos livres (SOARES et al., 2004; RAVEENDRAN et al., 2018).

Na nomenclatura internacional de classificação de enzimas (EC), as peptidases pertencem à classe 3 e subclasse 3.4, que ainda se divide em dois grupos: as exopeptidases e endopeptidases. As exopeptidases catalisam a hidrólise das ligações peptídicas nas extremidades N ou C terminal das cadeias polipeptídicas e, são denominadas então de aminopeptidases e carboxipeptidases, respectivamente. As endopeptidases atuam preferencialmente nas regiões internas das cadeias polipeptídicas (CHANDRAWANSHI et al., 2022).

As proteases ainda podem ser classificadas de acordo com a natureza química do seu sítio catalítico ou mecanismo de ação, de modo que cada classe das proteases irá apresentar um conjunto particular de aminoácidos no seu sítio ativo. Sendo assim classificadas em 4 grupos: (i) serino proteases (EC 3.4.21), que apresentam a tríade catalítica composta por Ser, His e Asp; (ii) cisteíno proteases (EC 3.4.22), apresentam os aminoácidos Cys, Asp e His; (iii) endopeptidases ou asparticoproteases (EC 3.4.23), possuem dois aminoácidos de Asp; e (iv) metaloproteinases ou metaloproteases (EC 3.4.24), responsáveis por apresentam um íon metálico no sítio ativo (DORNELLES et al., 2018).

Dentre todas as proteases presentes nos processos naturais, a pepsina e a tripsina estão entres as enzimas proteolíticas mais eficiente. Elas ajudam a decompor grandes macromoléculas encontradas nos alimentos em moléculas menores de mais fácil absorção no intestino, apoiando assim a saúde intestinal e garantindo que os nutrientes sejam absorvidos pelo corpo (MORAN, 2016).

2.1.1. Pepsina

A pepsina (E.C. 3.4.23.1) é uma endopeptidases, ou seja, apresenta no seu sitio catalíticos resíduos de Asp, sendo conhecida com protease ácida. Ela é encontrada na mucosa do estômago juntamente com a quimotripsina e a tripsina, outras enzimas proteolíticas do sistema digestivo. Durante o processo de digestão, cada uma destas enzimas é responsável em clivar tipos particulares de ligações peptídicas, digerindo as proteínas da dieta em seus componentes: peptídeos e aminoácidos, os quais serão absorvidos pela mucosa intestinal (SZAŁAPATA et al., 2016).

O pepsinogênio, que é um zimogênio (pró-enzima), é a forma inativa da pepsina liberada inicialmente no processo digestivo, a qual se tornará na forma ativa (pepsina) em contato com ácido clorídrico do estômago. A ativação da pepsina ocorre através da digestão parcial dos segmentos de cadeias polipeptídicas do pepsinogênio. O ácido clorídrico além de ativar a pepsina é responsável pela manutenção do pH do meio na faixa ácida garantindo a função da pepsina, que não tem atuação em valores de pH neutros/básicos (MIURA et al., 2015).

A pepsina tem um tamanho aproximado de 35 kDa e apresenta seu ponto isoelétrico próximo ao pH 1,0. Por ser uma enzima encontrada no sistema digestivo, tem atuação em pH mais ácido, numa faixa variando de 2,0 a 4,0. Devido à esta propriedade é uma das enzimas mais utilizadas para análise de outras proteínas devido à sua eficiência em clivar ligações envolvendo aminoácidos aromáticos (fenilalanina, triptofano e tirosina). Na indústria de alimentos é utilizada como coagulante de leite para produção de queijos, e na preparação de hidrolisados de proteínas vegetais e animais que podem ser usados como agentes aromatizantes em alimentos ou bebidas, bem como a hidrólise de alergênicos oriundos da soja (RAVEENDRAN et al., 2018; TAVANO et al., 2018; SANTOS et al., 2019).

2.1.2. Tripsina

A tripsina (EC 3.4.21.4) é uma serina protease, ou seja, é caracterizada pela presença de três aminoácidos: histidina, aspartato e a serina, no seu sítio ativo, sendo responsáveis por desempenhar sua função catalítica. A presença destes três aminoácidos forma um sistema de retransmissão de carga que funciona por transferência de elétrons do grupo carboxila da Asp para o oxigênio da Ser, que então se torna um poderoso nucleófilo capaz de atacar o carbono da carbonila, responsável pela ligação peptídica do substrato. Por ser uma serino protease a tripsina pode ter sua atividade aumentada na presença de cofatores metálicos como Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, entre outros componentes não proteicos, que podem potencializar sua atividade (LIU et al., 2019; SOUZA JR et al., 2020).

Assim como a pepsina a tripsina é uma protease do sistema digestivo, sendo produzida e secretada no pâncreas na forma inativa (tripsinogênio) se tornando ativa no intestino delgado através da sua hidrólise parcial pela enteroquinase. Esta apresenta uma estrutura muito similar a quimotripsina, mas seus substratos são específicos para clivagem nas cadeias peptídicas no lado carboxílico dos aminoácidos de lisina (Lys) e arginina (Arg), exceto quando ambos são seguidos por prolina (Pro) (DORNELLES et al., 2018; SOUZA JR et al, 2020).

A tripsina é caracterizada como serino protease devido ao envolvimento chave da serina no processo de proteólise, tem um tamanho aproximado de 23 kDa e apresenta seu ponto isoelétrico próximo ao pH 10,5. Por ser uma enzima encontrada no sistema digestivo com atuação no intestino delgado, tem atuação em pH mais básico, numa faixa variando de 7,0 a 9,0. Na indústria de alimentos pode ser utilizada como coagulante de leite na produção de queijo, porém o seu foco de utilização é a síntese de hidrolisados de aromas alimentícios (principalmente substituído por proteinases microbianas) (TAVANO et al., 2018; LIU et al., 2019).

2.2 Imobilização enzimática

Embora as enzimas apresentem muitas vantagens em relação aos catalisadores químicos, como elevada atividade catalítica, especificidade por determinado substrato e elevada atividade em condições brandas de reação, sua utilização em alguns processos industriais têm sido limitados, devido ao seu alto custo associado a utilização de enzima com elevada pureza, além da sua baixa estabilidade operacional em determinadas condições operacionais. Existe ainda a dificuldade de separação da enzima do produto

final, impedindo a sua reutilização, tornando assim difícil sua utilização em processos contínuos e aplicações em larga escala. Contudo, a utilização das enzimas em sua forma imobilizada vem sendo uma das formas de aproveitar as vantagens da catálise enzimática bem como superar as deficiências apresentadas (NGUYENA & KIM, 2017).

A imobilização enzimática é um termo genérico empregado para descrever a retenção de uma biomolécula no interior de um suporte/matriz inerte com retenção de suas atividades catalíticas, podendo utilizá-las repetidamente e continuamente. Além de facilitar a sua separação do produto final e aumentar a sua estabilidade através da redução das alterações na sua estrutura nativa pela influência do meio operacional (temperatura, pH e solventes orgânicos), o que é atrativo para a aplicação de enzimas no setor industrial (BEZERRA et al., 2015).

Em 1916 foi feita a primeira observação científica que levou à descoberta da imobilização enzimática, através da imobilização da invertase por adsorção em carvão. Descoberta que posteriormente foi transformada e aprimorada em técnicas de imobilização atuais (CALIFANO and COSTANTINI, 2020). Os estudos das técnicas de imobilização enzimática se intensificaram durante os anos de 1950 e 1960, mas foi no ano de 1960 que diferentes métodos de imobilização por ligações covalentes foram desenvolvidos, além de serem estudados sua aplicação em processos químicos, os quais continuam a ser estudados até hoje, contando com mais de 6.000 publicações e patentes publicadas sobre as diferentes técnicas de imobilização de enzimas (HOMAEI et al., 2013; CHAUHAN et al., 2022).

Segundo Motevalizadeh et al. (2015), do ponto de vista comercial, o principal interesse em imobilizar uma enzima é obter um biocatalisador com atividade e estabilidade que não sejam afetadas durante o processo, em comparação à sua forma nativa. A utilização de suportes e/ou técnicas que evitem as alterações estruturais no sítio ativo deve ser favorecida, de modo a evitar perdas de atividade catalítica durante sua utilização. A imobilização possui as seguintes vantagens: reutilização das enzimas; os processos químicos podem ser continuamente operados e controlados; facilidade na separação dos produto-substrato; perdas de enzimas por dessorção minimizadas; repetibilidade do processo; alta estabilidade.

Entretanto, existe algumas desvantagens em relação aos procedimentos de imobilização como: mudanças conformacionais da estrutura da enzima, devido ao tipo de imobilização, levando a imobilização numa forma inativa; possível perda da atividade enzimática durante o processo de imobilização; e os efeitos difusionais causados pelo

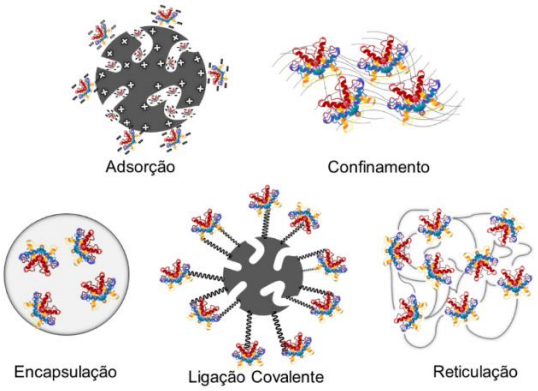
baixo transporte do substrato e do produto em decorrência de limitações do acesso do substrato ao sítio ativo da enzima. De modo que o custo da imobilização, bem como os métodos a serem utilizados devem ser compensados pela vida útil do biocatalisador. Para que estas desvantagens sejam minimizadas é necessário conhecer a natureza da enzima, do material usado como suporte para a imobilização e a técnica de imobilização (SHELDON & VAN PELT, 2013; FERNANDEZ-LOPEZ et al., 2017).

De maneira geral a utilização de suportes hidrofóbicos é vantajosa para aplicações práticas devido à conveniência no manuseio, facilidade de separação, maior estabilidade e reutilização, prevenção de interações com interfaces, entre outras. Já os métodos utilizados para a imobilização baseiam-se em tipos de interações entre o suporte e as enzimas, podendo ser considerados como métodos químicos ou métodos físicos. Os métodos químicos necessitam de alta energia de ligação como: ligação covalente, ligação cruzada e afinidade. Os métodos físicos apresentam baixa energia de ligação que pode envolver as forças de Van der Waals, ligações de hidrogênio, ligação iônica e interações hidrofóbicas. O método de imobilização deve garantir a estabilidade enzimática durante longos períodos de tempo, além de evitar sua dessorção, torná-la reutilizável e permitir a livre difusão de substratos e produtos da reação (CHOI et al., 2015; FERNANDEZ-LOPEZ et al., 2017; GONÇALVES et al., 2019).

2.3. Métodos de Imobilização

De acordo com Alnoch et al. (2020), a seleção do método de imobilização deve ser baseada em parâmetros como: suporte utilizado, eficácia da utilização da enzima, os custos do procedimento de imobilização, a toxicidade dos reagentes de imobilização e as propriedades finais desejadas do biocatalisador imobilizado, de modo que as enzimas podem ser imobilizadas por diferentes métodos, conforme ilustrados na Figura 1.

Figura 1. Esquema dos métodos de imobilização de enzimas.



2.3.1 Métodos físicos

2.3.1.1. Adsorção

De acordo com Furlani et al. (2020), a imobilização de enzimas pelo método da adsorção física foi uma das primeiras técnicas reportadas. Este método é conduzido de maneira simples e passível de reversão, podendo ocorrer por meio de interações hidrofóbicas, ligações de hidrogênio e forças de Van der Waals. Por ser um método simples, o método de imobilização por adsorção é um dos mais empregado devido aos poucos efeitos na estrutura conformacional da enzima, uma vez que a enzima é espontaneamente imobilizada em uma orientação que lhe é preferencial e energeticamente favorável, além de apresentar baixo custo e ser de fácil execução (TORRES et al., 2017).

Por ser um método espontâneo, este sofre o efeito da aleatoriedade da interação enzima-suporte e as forças envolvidas no processo de adsorção ocasionando assim uma desvantagem durante sua utilização. Essa aleatoriedade é ocasionada por diferentes parâmetros, como pH, temperatura, concentração salina, sendo responsáveis por influenciar na quantidade de enzima adsorvida e a força das interações. Além disso, as alterações destes parâmetros podem levar a dessorção das enzimas imobilizadas. Desta forma, a eficiência deste método irá depender das condições do meio bem como da relação entre a concentração da enzima e do suporte. Como alternativa para contornar essas desvantagens, diversos métodos foram desenvolvidos nos últimos anos, como por exemplo a modificação química do suporte e a utilização de agentes reticulantes e íons metálicos (SOUZA et al., 2017; FURLANI et al, 2020).

A utilização de íons metálicos nos suportes proporciona a adsorção por troca iônica, aumentando a força de interação entre as enzimas e o suporte, evitando as perdas por dessorção, uma vez que as interações das enzimas com o suporte neste método ocorrem por atrações eletrostáticas provenientes de cargas opostas presentes na superfície do suporte com a enzima. Embora as forças envolvidas neste método sejam mais fortes que em outras ligações envolvidas na adsorção física, as mesmas estão susceptíveis a interferência do meio externo, com a presença de íons provenientes dos tampões e da faixa de pH utilizadas, sendo fatores importantes a serem considerados para a imobilização por troca iônica e para a prevenção da dessorção da enzima (FURLANI et al., 2020).

2.3.1.2. Confinamento

Imobilização por confinamento ou aprisionamento é definido como um método físico e irreversível de imobilização onde a enzima ficará confinada na estrutura do suporte. A imobilização de enzimas por confinamento envolve a polimerização *in situ* da matriz porosa em torno dos biocatalisadores a serem imobilizados, tendo as enzimas ocluídas em redes poliméricas de baixo custo de modo a evitar a agregação das mesmas. Este método para a imobilização de enzimas pelo processo sol-gel é promissor, visto que o mesmo ocorre em meio aquoso e fornece condições ótimas de imobilização como pH, polaridade e afinidade, o que permite a manutenção da atividade catalíticas das enzimas confinadas na matriz, sem que essas sofram uma desnaturação significativa (SOUZA et al., 2017; BILAL & IQBAL, 2019; FURLANI et al., 2020).

O método de imobilização por confinamento tem como vantagens a manutenção da conformação enzimática original, proteção do contato direto com o meio reacional minimizando a inativação da enzima por solventes orgânicos, além de poder ser utilizado para imobilizar uma ampla variedade de enzimas, visto que o mesmo não promove alterações na sua estrutura, já que não existe a formação de ligações covalentes entre as enzimas e a matriz do suporte (LIU et al., 2018; FURLANI et al., 2020). As limitações deste método estão associadas especialmente aos problemas relacionados ao controle da porosidade do suporte, dificultando a transferência de massa do substrato para o sítio ativo da enzima, bem como perdas da enzima por dessorção através da matriz. Além disso, muitos precursores utilizados na polimerização das matrizes podem resultar na desnaturação das enzimas. Entretanto, precursores alternativos foram desenvolvidos a fim de minimizar este problema (BEZERRA et al., 2015; FERNÁNDEZ-FERNÁNDEZ et al., 2013; CAO et al., 2016).

2.3.2. Métodos químicos

2.3.2.1. Ligação covalente

A imobilização por ligação covalente tem se tornado um dos métodos mais empregados para a imobilização de enzimas. Baseia-se na formação de ligações químicas covalentes, que são, normalmente, estabelecidas entre os grupos amino, grupos amino residuais, grupos sulfidrila e grupo hidroxila do anel fenólico dos aminoácidos constituintes da enzima com os grupos reativos do suporte (amino alquila, aminoarila, 3-glicidoxipropil-carboxila, etc.). Os grupos funcionais dos suportes são gerados após a

funcionalização empregando agentes químicos, como por exemplo, o glutaraldeído, que introduz um grupo carbonila, susceptível a reações com os grupos nucleofílico da enzima. Em geral, para que ocorra estas reações deve ser feita a modificação da superfície, ou funcionalização do suporte através da inserção de reagentes espaçadores que têm como finalidade deixar a enzima em maior contato com o meio reacional (BEZERRA et al., 2015; BOUDRANT et al., 2020; FURLANI et al., 2020).

As vantagens desta técnica de imobilização são: proporcionar uma maior rigidez na estrutura da enzima, garantindo assim uma maior eficiência de imobilização e estabilidade a agentes desnaturantes; e os complexos formados não são tão suscetíveis a variação do pH, força iônica, solventes e temperatura (KHOOBI et al., 2014). A formação de múltiplas ligações covalentes reduz a flexibilidade conformacional e as vibrações térmicas e evita o desdobramento e desnaturação da proteína. A desvantagem do método é que ele não ocorre de maneira uniforme, e em alguns casos pode ocorrer a inativação da enzima e/ou redução da atividade catalítica devido à alteração em sua conformação nativa impostas pelas ligações entre as enzimas e os grupos reativos do suporte (BOUDRANT et al., 2020).

De acordo com Furlani et al. (2020), a atividade da enzima imobilizada covalentemente irá depender do suporte utilizado, natureza do método de acoplamento, composição do material e condições específicas durante a imobilização. Maiores atividades enzimáticas são obtidas quando os aminoácidos presentes no sitio ativo não estão envolvidos na interação com os suportes. Além disso, as enzimas imobilizadas covalentemente podem ser utilizadas em qualquer meio, devido a uma maior força de interação entre o suporte e a enzima quando comparado as enzimas imobilizadas por adsorção, que são mais susceptíveis as condições do meio, podendo apresentar perdas por dessorção.

Métodos de modificação química dos suportes com o objetivo de torná-los mais adequados para serem empregados na imobilização por formação de ligações covalentes vem sendo estudados (HANEFELD et al., 2009; MOHAMAD et al., 2015; ZDARTA et al., 2018). Além da utilização de agentes bifuncionais, como o glutaraldeído, estudos vêm sendo feito através da utilização de compostos naturais como a genipina que desempenha função de reticulação similar à do glutaraldeído e por apresenta uma toxicidade cerca de 10000 vezes menor que o glutaraldeído, além disso a utilização de íons metálicos como agentes modificadores superficiais vem sendo estudados (ACET et al., 2018; DU et al., 2020).

Dentre os íons metálicos utilizados destacam-se os íons de Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+} e Ca^{2+}, por apresentarem a capacidade de interagir com os resíduos específicos de aminoácidos presentes nas enzimas. Os princípios fundamentais da afinidade de biomoléculas por íons metálicos são conhecidos desde o início do século passado. Em 1974, Everson e Parker exploraram a interação entre espécies doadoras de elétrons presentes na superfície de biomoléculas em solução e íons metálicos quelatados imobilizados em um suporte sólido na cromatografia para separação e purificação biomoléculas, sendo conhecida com técnica IMAC (cromatografia por afinidade por metal imobilizado), que se baseia na técnica de imobilização enzimática usando ligações metálicas. Esta técnica vem sendo utilizada também para a imobilização de enzimas (BRESOLIN et al., 2009; UYGUN et al., 2015; ACET et al., 2018).

A técnica de IMAC baseia-se na afinidade dos íons metálicos suportados na matriz sólida pelos grupamentos presentes nos resíduos de aminoácidos das enzimas. Esta afinidade resulta de ligações de coordenação reversíveis formadas entre um íon metálico quelatado (o centro de adsorção) e certos resíduos de aminoácidos através da doação de elétrons, atuando como base de Lewis e consequentemente cria uma ligação coordenada. Estas interações ocorrem devido à sua capacidade para formar dipolos instantâneos das espécies envolvidas (polarizabilidade), eletronegatividade, estado de oxidação, tamanho, tipos de ligações (iônica ou covalente) e disponibilidade do doador de elétrons (BRESOLIN et al., 2009; HETTIARACHCHY et al., 2018).

2.3.2.2. Cross-linking

A técnica de ligação cruzada ou cross-link foi desenvolvida no ano 1960, porém devido a difícil utilização não se popularizou. 30 anos depois esta técnica começou a ser utilizada na imobilização de enzimas. É uma técnica aplicável a uma ampla variedade de enzimas usando diferentes agentes reticulantes. A técnica de reticulação com formação de agregados enzimáticos reticulados (*cross-linked enzyme aggregates* – CLEA) é a mais utilizada e que apresenta melhores estabilidades operacionais em comparação aos demais métodos: reticulados de enzimas (*cross-linked enzyme* – CLE), cristais de enzima reticulados (*cross-linked enzyme crystals* – CLEC) e enzimas atomizadas reticuladas (*cross-linked spray-dried enzyme* – CSDE). É um método de imobilização irreversível, devido as forças envolvidas no processo, e não é necessária a utilização de suportes, uma vez que as enzimas se ligam aos agentes de reticulação. Os agentes de reticulação são moléculas que tem pelo menos duas extremidades reativas, para que seja formado uma

estrutura tridimensional complexa (MOHAMAD et al., 2015; YAMAGUCHI et al., 2018).

Este método de imobilização se baseia em reações intermoleculares, ou seja, na formação de ligação covalente entre as enzimas e os reagentes bi- ou multifuncionais, com finalidade de torná-las insolúvel ao meio reacional. Uma vantagem desse método é a melhora na estabilidade da enzima durante a sua imobilização, possibilitando a sua reutilização. No entanto, esse método apresenta alguns inconvenientes, por ser uma técnica trabalhosa e lenta ela gera um alto custo, além de possuir baixa manutenção da atividade catalítica da enzima imobilizada (apresentando perdas de até 50% da atividade enzimática) durante o processo de reticulação, devido a possibilidade da formação de *clusters* (grandes aglomerados) ocasionando limitações difusionais do substrato até o sitio catalítico enzimático. Devido ao grande número de ligações formadas durante este processo, a enzimas ali disponíveis acabam apresentando alterações na sua configuração original e pelo fato de não ser utilizado nenhum suporte nesta técnica, a mesma apresenta uma baixa estabilidade mecânica, pouca reprodutibilidade, além da dificuldade do manuseio devido seu aspecto gelatinoso, especialmente quando utilizada uma grande quantidade de enzima (LIU et al., 2018; FURLANI et al., 2020).

2.4. Imobilização de Proteases

2.4.1. Métodos empregados na imobilização de protease

De acordo com Demirkan et al. (2017), a imobilização de proteases aumenta a sua vida útil, ou seja, garante sua utilização por mais tempo, facilita a separação da enzima dos produtos da reação, possibilita a utilização em condições que normalmente na sua forma nativa esta não teria tanta eficiência, além de evitar perdas da sua atividade catalítica associadas a modificação estrutural e fornecer resistência aos ataques desnaturantes e microbiano.

Na Tabela 1 são apresentados estudos em que foram utilizados os diferentes métodos para a imobilização de proteases.

Apesar de existirem diversas técnicas de imobilização de enzimas as mais utilizadas são adsorção (método físico), reticulação e ligação covalente (método químico). Na imobilização usando ligações covalentes, o agente modificador de destaque é o glutaraldeído. A química do glutaraldeído se destaca devido a sua eficiência e versatilidade, sendo utilizado para imobilizar uma série de enzimas em diversos tipos de

suportes (SIAR et al., 2017). Novos estudos visando encontrar agentes modificadores que melhorarem as propriedades das proteases imobilizadas por ligações covalentes vem sendo feitos.

Segundo Nadar & Rathod (2018), as matrizes metalizadas têm chamado grande atenção, pois além de poder empregar matrizes porosas já conhecidas a inserção desses grupos metálicos são responsáveis pela formação de ligações químicas com a enzima. Sendo observadas diversas melhorias quando comparados com outros agentes. Proteases imobilizadas por ligações covalentes em suportes metalizados mostraram maiores eficiências de ligação, melhorias na eficiência catalítica (melhor exposição dos sitio ativos por evitar o impedimento estérico), melhor estabilidade química/térmica, maior resistência à desnaturação provocadas por: mudanças de pH e temperatura, solventes orgânicos miscíveis e imiscíveis em água, detergentes entre outros (HUSAIN, 2018).

2.4.2. Suportes utilizados na imobilização enzimática e com potencial para aplicação na imobilização de proteases

Vários materiais podem ser utilizados como suportes, podendo os mesmos serem classificados como: orgânicos (naturais ou sintéticos), inorgânicos (naturais ou sintéticos) e híbridos, como apresentado na Tabela 1. A seleção do suporte depende das propriedades do material como: força mecânica, estabilidade física e química, caráter hidrofílico/hidrofóbico, capacidade da enzima de adsorção e o custo. Geralmente o suporte tem de satisfazer dois requisitos principais: (i) possuir uma quantidade suficiente de grupos funcionais na superfície que possam interagir com a enzima; e (ii) apresentar propriedades mecânicas e dimensões que devem permitir seu desempenho estável e a possibilidade de utilização repetida por muitos ciclos, ou aplicação de um processo contínuo (ZDARTA et al., 2018; Furlani et al., 2020; LIANG et al., 2020).

Os principais suportes utilizados no processo de imobilização são: quitosanas, carvões ativados; resinas poliméricas e sílicas, já que estes materiais apresentam características como seu tamanho de poro, sua elevada área de superfície BET, volume de poros e estruturas abertas, que favorecem a imobilização (MUBARAK et al., 2014). Segundo Wongrod et al. (2019), o carvão ativado se destaca dentre os demais suportes devido à alta resistência química, mecânica e térmica, hidrofilicidade e por ser insolúvel, além de apresentar uma elevada área superficial BET e porosidade bastante definida. Além disso, o mesmo pode ter a sua superfície modificada por diferentes métodos. Estas modificações são feitas com o intuito de criar e/ou aumentar os grupos funcionais já

existente em sua superfície, os quais variam com o tipo de ativação durante a etapa de síntese.

Pode-se observar na Tabela 1 que estudos voltados a imobilização por ligações químicas, utilizando suportes modificados, vem sendo mais recorrentes devido ao aumento na sua capacidade de imobilização e consequentemente no desempenho enzimático. Pode-se verificar ainda que estudos sobre imobilização de pepsina e tripsina não são tão recorrentes como de outras proteases, fazendo com que haja a necessidade de estudo mais aprofundados sobre a imobilização e utilização dessas enzimas na forma imobilizada. Principalmente pelo fato dessas enzimas apresentarem uma alta especificidade de hidrólise, ou seja, que fazem hidrólise apenas em algumas sequências específicas de aminoácidos, gerando sempre os mesmos peptídeos no final da hidrólise. Tavano et al. (2018), afirmam que a utilização dessas enzimas é bastante recorrente para identificação dos constituintes das proteínas usando análise de espectrometria de massa das proteínas hidrolisadas devido formação de peptídeos de cadeia curta com terminal C básico.

2.5. Utilização de carvão ativado como suporte para imobilização enzimática

O carvão ativado (CA) é um material carbonáceo de estrutura porosa bem desenvolvida, responsável por conferir uma elevada área superficial especifica BET. Apresenta em sua superfície heteroátomos de oxigênio, nitrogênio e hidrogênio ligados aos átomos de carbono. Este apresenta uma estrutura porosa bastante desenvolvida devido à presença de microporos (poros < 2 nm), mesoporos (poros variando de 2 a 50 nm) e macroporos (poros > 50 nm). Devido a suas características texturais o carvão ativado tem a capacidade de adsorver moléculas presentes tanto em fases gasosas quanto em fase liquida. Este ainda é um material com elevada resistência mecânica, com uma alta estabilidade química e é considerado um material não grafítico, devido seus átomos de carbono estarem dispostos em uma estrutura hexagonal de duas dimensões. Porém, o carvão ativado não é um material verdadeiramente amorfo, devido a presença de uma estrutura micro cristalina que difere da estrutura do grafite (SANTOS et al., 2020).

Tabela 1. Desempenho das proteases imobilizadas usando diferentes suportes e métodos de imobilização

Suporte	Enzima	Condições	Resultados alcançados	Referência
Método Físico (Adsorção)				
Celite	Ficina	pH 7 por 10 min	As enzimas imobilizadas apresentaram uma atividade de hidrólise da caseína de 160 U / mg	FADÝLOĞLU, 2001
Carvão ativado	Papaína	pH 7.5 por 0.5 h	Uma capacidade de imobilização de 97 mg/g foi alcançada e a enzima apresentou uma atividade na hidrólise de soro de leite de 75 mg Phe/100g	SILVA et al., 2007
Carvão ativado	Pancreatina	30 min a 25 ºC	As enzimas imobilizadas apresentaram uma atividade 84 % para a remoção de fenilalanina	SILVA et al., 2008
Composto quitosana / argila modificados com polímero	Papaína	pH 7, 20 ºC	Capacidade de imobilização de 34.47 mg/g com atividade catalítica residual para hidrólise de BAEE de 100%	METIN et al., 2016
Nanotubos de carbono com paredes múltiplas	Papaína	pH 7, 2 h, 200 rpm	A eficiência da imobilização foi de 4,2 mg/ml com uma atividade de hidrólise de caseína de 67 %	HOMAEI & SAMARI, 2017
Carvão ativado obtido a partir de resíduos agrícolas	Imobilização de Peroxidase	pH 3,0, 1 h, 40 °C	Eficiência de imobilização de 100 %. Enzima imobilizada ocasionou mais de 10 ciclos de reutilização	TORRES et al., 2017

Composto magnético de nanofibra de quitina	α- quimotripsina	2h, 20 ºC, 200 rpm	A capacidade enzima imobilizada foi de 92,4 mg/g com uma atividade relativa da hidrólise de caseína de 100%	HUANG et al., 2018
Quitina	Proteases	pH 7.5, 4 °C durante a noite	As enzimas imobilizadas apresentaram uma atividade recuperada na hidrólise da caseína 2.5 %	ÖZACAR et al., 2018
Carvão ativado	Pepsina	pH 3, 30 rpm por 2 h	Eficiência de imobilização de 93,6 % foi alcançada e a enzima apresentou uma atividade na hidrólise da caseína bovina de 1,3 $U.mg^{-1}$	SANTOS et al., 2019
Carvão ativado	Tripsina	pH 8, 30 rpm por 2 h	Uma eficiência de imobilização de 87,5 % foi alcançada e a enzima apresentou uma atividade na hidrólise da caseína caprina de 2,5 $U.mg^{-1}$	SOUZA JR. et al., 2020
		Método Químico (Ligação Covalente)		
Sílica ativada por glutaraldeído	Tripsina	pH 7.5; 4 ºC, 200 rpm por 1 h	Eficiência de imobilização de 63 % com uma atividade de hidrólise para BSA de 92 nmol/min/mg	DAGLIOGLU & ZIHNIOGLU, 2012
Nanopartículas de Fe_3O_4 revestidas com sílica	Papaína	pH 7,5 por 2 h	Eficiência de imobilização de 57,9 % com atividade na hidrólise da caseína bovina de 86 %	MOSAFA et al., 2013

Carbon revestido com nanopartículas	α- quimotripsina	-	Capacidade de imobilização de 50 mg/g com 25 % de atividade de hidrólise do substrato de éster etílico de N-benzoil-L-tirosina	ZLATESKI et al., 2014
Suporte poliacrílico com epóxi	Protease aspártica	pH 6, 4 ºC por 3 h	As enzimas imobilizadas apresentaram uma atividade de coagulação da K-caseína de 9,84 U	ESPOSITO, 2015
Nanopartículas magnéticas de quitosana	Pepsina	-	Capacidade de imobilização de 99 mg/g com uma atividade de 85 % para hidrólise de amidas	LAKOURAJ et al., 2015
Poli (tereftalato de etileno) (PET) com PVA	Tripsina	pH 5.5 por 2 h	A capacidade enzima imobilizada foi de 0,62 μmol pNA $min^{-1}g^{-1}$ mat para hidrólise de BAPNA	SILVA et al., 2015a
Quitosana modificada com glutaraldeído	Papaína	pH 8 por 5 h	As enzimas imobilizadas apresentaram uma atividade de 2,7 U/g para hidrólise de azo caseína sulfanilamida	SILVA et al., 2015b
Quitosana modificada com glutaraldeído	Bromelina	pH 3.2, 150, 20 ºC durante a noite	Eficiência de imobilização de 41 %	ZAPPINO et al., 2015
Compósitos de hidrogel PCMC / PVA / SBA-15	Papaína	pH 6.5 por 1.5 h	O hidrogel obteve 100% da capacidade de imobilização com uma atividade de hidrólise da caseína de 1800 U/g	DAI et al., 2017
Suporte glioxil-agarose	Ficina	pH 10, 25 ºC por 3 h	Eficiência de imobilização de 100 % foi alcançada e a enzima apresentou uma	SIAR et al., 2017

			atividade relativa na hidrólise da Benzoil-arginina-p-nitroanilida (BANA) de 40 %	
Nanopartículas magnéticas de quitosana	Tripsina	pH 7.5; 25ºC, 200 rpm por 1 h	Capacidade de imobilização de 149,25 mg/g com uma atividade residual de 100% para hidrólise de BAEE	SUN et al., 2017
Composto Magnético de Nanofibra de Quitina	α- quimotripsina	20 ºC, 200 rpm por 2 h	A capacidade enzima imobilizada foi de 581,84 mg/g com uma atividade relativa de 100% para a hidrólise da caseína	HUANG et al., 2018
Nanofibras eletrofiadas de PVA	Ficina	pH 8 por 1 h	A enzima imobilizada apresentou atividade relativa de 92 % para hidrólise de Nα-benzoil-L-arginina Cloridrato de 4-nitroanilida (BAPA)	ROJAS-MERCADO et al., 2018
Nanopartículas magnéticas porosas	Papaína	25 °C por 12 h.	Eficiência de imobilização de 82 % com uma capacidade de hidrólise da caseína de 4,95 mg/L·min	SHENG et al., 2018
Contas de agarose ativadas com glutaraldeído	Ficina	pH 7, 25 ºC por 4 h	Eficiência de imobilização de 100 % foi alcançada e a enzima apresentou uma atividade relativa na hidrólise da caseína de 40 %	SIAR et al., 2018
Quitina modificado com glutaraldeído	Protease	pH 7.5, 4 °C por 12 h	A enzima imobilizada apresentou uma atividade recuperada na hidrólise da caseína 38 %	ÖZACAR et al., 2018

Quitosana modificada com glutaraldeído	Tripsina	pH 8,5 a 25 ºC por 30 min	A enzima imobilizada apresentou atividade relativa de 100 % para hidrólise de Nα-benzoil-L-arginina Cloridrato de 4-nitroanilida (L-BAPA)	KIM & LEE, 2019
Matriz CNBr-Sepharose	Bromelina	pH 7.8, 4 ºC por 16 h	Eficiência de imobilização de 80,5 % foi alcançada e a enzima apresentou uma atividade relativa na hidrólise da caseína de 47.95 $U.mg^{-1}$	NGADIAH & FERDINAL, 2019
Carvão ativado modificado com glutaraldeído	Pepsina	pH 3, 30 rpm por 2 h	A eficiência de imobilização de 94,9 % foi alcançada e a enzima apresentou uma atividade na hidrólise da caseína bovina de 1,75 $U.mg^{-1}$	SANTOS et al., 2019
Carvão ativado modificado com glutaraldeído	Tripsina	pH 8, 30 rpm por 2 h	Eficiência de imobilização de 91 % foi alcançada e a enzima apresentou uma atividade na hidrólise da caseína caprina de 3 $U.mg^{-1}$	SOUZA JR et al., 2020

Fonte: Do Autor, 2022.

Na síntese do carvão ativado podem ser utilizados quaisquer compostos ricos em carbono, tais como: ossos, serragem, algas, resíduos agroindustriais, materiais lignocelulósicos, dentre outros materiais carbonáceos (ALAM et al., 2020). Os resíduos agroindustriais, como cascas e caroços apresentam altos teores de celulose, lignina e hemicelulose em sua composição. Devido a estes altos teores lignocelulósicos estes materiais podem ser convertidos em produtos comerciais de alto valor agregado, já que em sua grande maioria não possuem nenhum mercado definido, sendo muitas vezes descartado no meio ambiente de maneira inadequada. Na última década pesquisas têm focadas na síntese de carvões ativados a partir de diversos subprodutos agroindustriais, com atenção especial para aqueles que possam ser produzidos a partir da reutilização dos resíduos lignocelulósicos (biomassa), gerando assim materiais com baixos custos de produção (SANTOS et al., 2020).

Estes materiais são submetidos a etapas de ativação e carbonização, para que haja o desenvolvimento dos poros internos e a criação dos grupos funcionais superficiais. O processo de ativação pode ser físico, químico ou físico-químico. Na ativação física, o precursor é tratado termicamente em atmosfera suavemente reativa, tais como vapor de água ou gás carbônico, ativação e carbonização ocorrendo simultaneamente. Já a ativação química consiste na impregnação prévia do precursor com agentes químicos, tais como: ácido fosfórico (H_3PO_4), cloreto de zinco ($ZnCl_2$), hidróxido de potássio (KOH) entre outros, seguido da carbonização em altas temperaturas e em atmosfera inerte (FIERRO et al., 2010). Comparando com outros materiais adsorventes o carvão ativado apresenta grande versatilidade industrial associados a um baixo custo de produção, o que os torna uma opção importante e mais vantajosa para uma grande classe de aplicações quando comparados com outros materiais porosos (DANISH et al., 2018).

O carvão ativado tem sua aplicação datada de 2000 a.c., quando os egípcios passaram a utilizá-lo na purificação de água. Posteriormente, utilizados na forma granular durante a I Guerra Mundial, para a produção de máscaras de gás. Após a década de 50, foi desenvolvida a manufatura de carvão ativado em pó e o seu uso foi amplamente estendido para a purificação de água e no controle na emissão de poluentes (BUBANALE & SHIVASHANKAR, 2017). A partir do ano de 1974, ocorreu a primeira aplicação do carvão ativado de maneira industrial na Inglaterra. Sendo utilizado como agente de descoloração na indústria de produção do açúcar; utilizado em filtros de todos os sistemas de ventilação de esgotos para eliminar os odores desagradáveis e em máscaras gasosas nas indústrias químicas para impedir a inalação de vapores de mercúrio (BUBANALE &

SHIVASHANKAR, 2017). Devido ao fato de ser inerte o carvão ativado é aplicado também na purificação de compostos químicos, na clarificação e remoção de sabores e odores de óleos, bebidas alcoólicas, produtos químicos e farmacêuticos e no tratamento de águas residuária. Ele também é largamente utilizado em escala industrial como adsorvente principalmente na purificação/separação de líquidos e gases e como suporte catalítico (ZANELLA, 2014, YAHYA et al., 2015). Essas aplicações fazem do carvão ativado um produto de grande interesse para muitos setores econômicos nas mais diversas áreas, como alimentícia, farmacêutica, química, petrolífera, nuclear, automobilística, mineração, tratamento de água potável, água industrial e do ar atmosférico (FRANK et al., 2015; RANGABHASHIYAM e BALASUBRAMANIAN, 2019).

Segundo Bassan et al. (2016), além das áreas de aplicação citadas os carvões ativados são empregados como suportes em catálise inorgânica e como suporte para imobilização de enzimas, devido às suas propriedades como: elevada área superficial, boa estabilidade química, mecânica e térmica, hidrofilicidade e insolubilidade. Neste sentido, muitas pesquisas vêm sendo realizadas como o objetivo de avaliar a eficiência dos carvões ativados em diferentes processos de imobilização enzimática, conforme mostrado na tabela 2.

Conforme observado na Tabela 2, existem diversos estudo de imobilização de enzimas em carvão ativado, sendo que nestes estudos as principais justificativas de utilização desta matriz são suas características físicas e químicas (elevada resistência mecânica, alta estabilidade química, resistência a ataques microbianos, inércia química, biocompatibilidade, área superficial elevada, porosidade definida, presença de grupos funcionais superficiais, etc), além de ser um suporte de baixo custo de síntese. Ainda é possível observar que os derivados obtidos a partir de carvão ativado promoveram um número considerável de ciclos de reutilização, mantendo a atividade das enzimas imobilizadas. Considerando a aplicação em escala industrial, um suporte que apresente menor custo de síntese e ainda apresentar a capacidade de manter um alto número de ciclos é desejável, compensando os custos do processo de imobilização. Desta forma mais estudos envolvendo a imobilização de enzimas em carvão ativado e sua síntese de funcionalizado são necessários.

A imobilização por adsorção física é a mais usada para o carvão ativado devido as suas propriedades e a sua facilidade de aplicação e custo, porém conforme descrito anteriormente apresenta algumas desvantagens devido as forças de ligação atribuídas ao processo.

Tabela 2. Estudos realizados com carvão ativado como suporte para imobilização enzimática.

Enzima	Método	Aplicação	Atividade	Referências
Pancreatina	Adsorção	Hidrólise de soro de leite	100 % - enzima livre 100 % - enzima imobilizada (5 ciclos)	SILVA et al., 2005
Papaina	Adsorção	Hidrólise de soro de leite	100 % - enzima livre 100 % - enzima imobilizada (5 ciclos)	SILVA et al., 2005
Lipase	Adsorção	Hidrólise da emulsão de azeite	7,9 U/mg - enzima livre 2,8 U/mg - enzima imobilizada	BRITO et al., 2020A
Lipase	Ligação covalente	Esterificação de acetato de isoamila	91% - enzima imobilizada (5 ciclos)	BRITO et al., 2020B
Lipase	Ligação covalente	Hidrólise de óleo de cozinha usado	50% - enzima livre 100% - enzima imobilizada (21 ciclos)	RAMANI et al., 2012
Pepsina	Adsorção	Hidrólise da caseína bovina	41,67 U - enzima livre 245,02 U - enzima imobilizada (8 ciclos)	SANTOS et al., 2019
Pepsina	Ligação covalente	Hidrólise da caseína bovina	41,67 U - enzima livre 299,79 U - enzima imobilizada (8 ciclos)	SANTOS et al., 2019
Tripsina	Adsorção	Hidrólise da caseína de cabra	3,35 U - enzima livre 9,22 U - enzima imobilizada (4 ciclos)	SOUZA Jr et al., 2020
Tripsina	Ligação covalente	Hidrólise da caseína de cabra	3,35 U - enzima livre 10,45 U - enzima imobilizada (4 ciclos)	SOUZA Jr et al., 2020
Lipase	Adsorção	Hidrólise da emulsão de azeite	32,5 U – enzima livre 72,3 U – enzima imobilizada	OLIVEIRA et al., 2022
Lipase	Ligação covalente	Hidrólise da emulsão de azeite	32,5 U – enzima livre 84,0 U – enzima imobilizada	OLIVEIRA et al., 2022
Pepsina	Adsorção	Hidrólise da caseína bovina	3,32 U – enzima livre 1,04 U – enzima imobilizada	SANTOS et al., 2022
Pepsina	Ligação covalente	Hidrólise da caseína bovina	3,32 U – enzima livre 2,30 U – enzima imobilizada	SANTOS et al., 2022

Fonte: Do Autor, 2022.

Em contrapartida, estudos vêm sendo realizados com o objetivo de modificar a superfície destes suportes, com o intuito de melhorarem os processos de imobilização enzimática através da formação de ligações mais estáveis e irreversíveis, como as ligações químicas covalentes. Estas ligações são normalmente estabelecidas entre os grupos

amino, grupos amino residuais, grupos sulfidrila e grupo hidroxila do anel fenólico dos aminoácidos constituintes da enzima com os grupos reativos presentes na estrutura do suporte (SANTOS et al., 2020; SOUZA Jr et al., 2020).

Segundo Zdarta et al. (2018), modificações dos suportes para imobilização enzimática, podem melhorar sua eficiência catalítica devido à minimização dos efeitos difusionais de substratos e produtos durante a reação, além da melhoria da estabilidade operacional em processos contínuos e descontínuos. Consequentemente, vem sendo estudado métodos de modificação química dos suportes com o objetivo de torná-los mais adequados para serem empregados na imobilização por formação de ligações covalentes.

2.5.1. Modificação do carvão ativado

De acordo com Okura et al. (2020), a conversão de um suporte comum em um suporte heterofuncional, através da modificação superficial tende a garantir vantagens quando se trata de imobilização enzimática. Uma vez que os diferentes grupos funcionais formados na superfície do material são capazes de interagir com as enzimas por quimissorção (ligação covalente) e fisissorção (interações iônicas, ligações de hidrogênio e ativação interfacial), fornecendo aos derivados alta estabilidade, atividade catalítica e seletividade.

Segundo Bezerra et al. (2015), o aumento de grupos reativos na superfície de matrizes sólidas por modificações físicas, químicas e morfológicas dos suportes, podem produzir derivados com maior eficiência catalítica devido à minimização dos efeitos difusionais de substratos e produtos durante a reação, além da melhoria da estabilidade operacional em processos contínuos e descontínuos, e por esta razão desperta também o interesse industrial para estes biocatalisadores (RAMANI et al., 2012).

Durante a última década, a atenção científica tem sido direcionada para materiais híbridos e compostos, que combinam propriedades e, assim, maximizam suas vantagens. Diante disso, diversos tipos de modificações superficiais vêm sendo feitas como alternativa para melhorar as propriedades dos suportes. Modificações do carvão ativado são alterações na sua estrutura superficial, através da adição de certas etapas ou componentes químicos durante o processo de ativação ou posterior a sua síntese. Essas modificações envolvem principalmente alterações químicas da superfície e consequentemente em suas propriedades, tendo como principal interesse a inserção de vários grupos funcionais. As modificações do suporte podem tornar o processo de imobilização de enzimas mais eficiente, pois as interações suporte/enzimas são mais

fortes e estáveis podendo ocasionar melhorias na performance dos derivados (ZDARTA et al., 2018; PEGO et al., 2019).

Dentre os métodos de modificação de superfície em carvões ativados, destaca-se o método do glutaraldeído, que é o mais utilizado e responsável por formar ligações covalentes multipontual entre o suporte e a biomolécula. É um método eficaz em termos de estabilidade térmica e operacional das enzimas, aumentando sua rigidez e garantindo uma maior resistência à pequenas mudanças conformacionais causadas pela variação de temperatura, utilização de solventes orgânicos, agentes desnaturantes, entre outros. Esta estabilização ocorre devido à formação de ligações covalentes entre grupos aldeídos do suporte com os grupos amina (NH_2), tiol (SH), hidroxila (OH), entre outros resíduos de aminoácidos das proteínas presentes nas enzimas. Todavia, apresenta algumas desvantagens, tais como a polimerização espontânea do glutaraldeído em meio aquoso, podendo acarretar numa perda de grupos aldeídos para interação com as enzimas, além da toxicidade do glutaraldeído (RAMANI et al., 2012; SANTOS et al., 2019).

Apesar da funcionalização com glutaraldeído apresentar alta eficiência em processos de imobilização enzimática, novos métodos para modificação da superfície de suportes vêm sendo estudados. Entre os compostos em estudo para substituir o glutaraldeído destaca-se a genipina, um composto natural que apresenta uma toxicidade cerca de 10000 vezes menor que o glutaraldeído. Recentemente, vem-se utilizando genipina como agente de ativação e/ou reticulação em quitosana, gelatina, colágenos entre outros, para aplicação em diversos segmentos, principalmente na área da biotecnologia (DU et al., 2020).

Além de ser um composto natural de baixa toxicidade, a genipina reage espontaneamente com aminas primárias em glucosaminas e/ou proteínas formando ligações covalentes, aumentando assim a estabilidade das ligações formadas (DONG et al., 2014). Devido suas propriedades ativantes e reticulantes, esta pode ser utilizada na imobilização enzimática, encapsulamento de substâncias, quantificação de aminoácidos (em contato com aminoácidos produz pigmentos azuis), estabilização de emulsões (O/A), liberação gradativa de fármacos em regiões específicas, constituição de próteses, entre outras aplicações (BELLÉ et al., 2018).

Pego et al. (2019), estudaram modificações em carvões ativados utilizando um tratamento de alta tensão e descarga elétrica de frequência (corona). Este método tem como finalidade a oxidação da superfície do carvão ativado através da ionização de gases (O_3 e O_2) entre o eletrodo e o substrato por meio de descargas elétricas, levando a

formação de ligações covalentes especialmente entre os átomos de C e O, tornando a superfície mais reativa.

Além das modificações realizadas após a obtenção dos carvões ativados as mesmas podem ser feitas durante seu processo de síntese, através da carbonização em autoclaves, sendo esta modificação conhecida como carbonização hidrotérmica. Este método vem se sobressaindo devido à sua simplicidade e pelo fato de que na carbonização hidrotérmica ocorra um aumento de grupos funcionais carbonila na superfície do material. Durante o processo de síntese ocorre a inserção de átomos de oxigênio na matriz do precursor, elevando a acidez da matriz com aumento dos grupos superfícies oxigenadas polares superficiais. Estes novos grupos na superfície do adsorvente são responsáveis por favorecer as reações químicas do suporte com o adsorvato, como a formação de complexos superficiais, ligações cátions-π, atração eletrostática e troca iônica (ATTA-OBENG et al., 2015; PEGO et al., 2019).

Atualmente, os nanomateriais, materiais magnéticos e/ou nanocompósitos magnético vêm se destacando como novos agentes de imobilização enzimática. A modificação de suportes inertes com estas partículas metálicas também tem sido empregada, pois os grupos funcionais gerados e a sua elevada energia de ligação superficial favorecem o aumento da quantidade de enzima imobilizada e a sua estabilidade (ZHOU et al., 2020).

Diferentes métodos de modificação da superfície de materiais carbonáceos vêm sendo estudados com o intuito de melhorar sua performance frente a imobilização enzimática. Dentre eles, as modificações utilizando a metalização feitas com íons de ferro vêm se destacando como uma das formas mais eficientes, pois suas propriedades magnéticas são transferidas para o suporte após a impregnação. A modificação usando sais de ferro (Fe_3O_4; $(NH_4)_2Fe(SO_4)_2$; $Fe(NO_3)$; $FeCl_3$; $FeSO_4$) pode ser feita de duas maneiras, durante o processo de ativação ou depois da sua carbonização. Ambas as modificações têm como intuito a formação da magnetita (Fe_3O_4), devido a predisposição para exibir propriedades magnéticas, baixa citotoxicidade, boa biocompatibilidade e estabilidade em uma variedade de condições fisiológicas (OMAR et al., 2019; REIS et al., 2019; ZHOU et al., 2019).

A imobilização enzimática usando ligações metálicas é conhecida por técnica IMAC e se baseia na afinidade diferencial entre os íons metálicos bivalentes (Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} ou Fe^{2+}) suportados em uma matriz sólida pelos grupamentos expostos das enzimas. Esta afinidade resulta de ligações de coordenação reversíveis

formadas entre um íon metálico quelatado (o centro de adsorção) e certos resíduos de aminoácidos, tais como imidazol da histidina, tiol da cisteína e indol do triptofano, os quais doam elétrons para o íon metálico, ou seja, atuam como base de Lewis (BRESOLIN et al., 2009; ZLATESKI et al., 2014). De acordo com Ding et al. (2015), a imobilização utilização a modificação por íons metálicos bivalentes oferecem muitas vantagens, sendo que as principais são uma melhora na capacidade de imobilização e na atividade da mesma em alguns casos.

Na Tabela 3 são apresentados alguns estudos que destacam a imobilização enzimática utilizando carvões ativados modificados. É possível observar que não foram encontrados muitos trabalhos na literatura referentes a aplicação do carvão ativado modificado como suporte para imobilização de proteases, sendo o mesmo mais utilizado na imobilização de lipase. Sendo assim, uma investigação mais aprofundada sobre aplicação desta matriz e sobre as modificações que podem ser feitas na mesma para melhorar o processo de imobilização de enzimas deve ser realizado.

Tabela 3. Modificação de suportes carbonáceos para imobilização de enzimas

Material	Modificação	Aplicação	Referência
Carvão ativado comercial	Modificado com nitrocelulose	Imobilização de amilase	LIU et al., 2008
Carvão ativado mesoporoso funcionalizado	-	Imobilização de Protease ácida	KUMAR et al., 2009
Carvão ativado comercial	Glutaraldeído	Imobilização de Lipase	RAMANI et al., 2012
Carvão ativado comercial	Modificado com poli (álcool vinílico) (PVA)	Imobilização de Peroxidase	XU and CHEN, 2016
Carvão ativado de caroço de cajá	Glutaraldeído	Imobilização de Lipase	BRITO et al., 2017
Nanotubo de carbono	Modificação com grupamento amina	Imobilização de Lipase	DWIVEDEE et al., 2017
Nanotubo de carbono	Modificado com oxido de ferro	Imobilização de Lipase	FAN et al., 2017
Nanotubos de carbono	Modificação ácida, básica e física	Imobilização de Lipase	DIAS et al., 2018

Carvão ativado comercial	Magnetização com oxido de ferro	Tratamento de Efluentes	JAIN et al., 2018
Carvão ativado (resíduos agroindustriais)	Modificação com Glutaraldeído	Imobilização de Lacasse	LONAPPAN et al., 2018
Fibras de carvão ativado	Modificação com HNO_3	Imobilização de Lacasse	ZHANG et al., 2018
Nanotubo de carbono	Modificação com cobalto magnético	Imobilização de Lipase	ASMAT et al., 2019
Carvão Ativado	Magnetização com fosfato de cobre	Imobilização de Papaína	FENG et al., 2019
Carvão ativado comercial	Modificação com quitosana	Adsorção de corantes	GONÇALVES et al., 2019b
Carvão ativado nanoporoso	Modificado com glutaraldeído	Protease halotolerante	POUNSAMY et al., 2019
Carvão ativado de Bainha de pupunha	Glutaraldeído	Imobilização de Pepsina	SANTOS et al., 2019
Carvão ativado de esponja Luffa	Metalização ($Fe(NO_3)_3 \cdot 9H2O$)	Imobilização de Laccase	ZHANG et al., 2020
Carvão ativado de caroço de cajá	Glutaraldeído	Imobilização de Tripsina	SOUZA JR et al., 2020
Carvão ativado da bainha de pupunha	Glutaraldeído	Imobilização de Lipase	BRITO et al., 2020A
Carvão ativado de semente de tamarindo	Hidrotérmica	Imobilização de Lipase	GONÇALVES et al., 2021
Carvão ativado de sabugo de milho	Modificação com nanopartículas metálicas	Imobilização de Lipase	OLIVEIRA et al., 2022
Carvão ativado de semente de tamarindo	Modificação: Glutaraldeído; Genipina; Metalização (Fe2+ e Fe3+)	Imobilização de Pepsina	SANTOS et al., 2022

Fonte: Do Autor, 2022.

2.6. Importância de estudos sobre imobilização de proteases

As enzimas são biocatalisadoras que possuem várias aplicações e são utilizadas em diversos segmentos nas mais diferentes áreas, principalmente na indústria alimentícia. E dentre as classes enzimáticas as proteases são as de maior destaque devido a sua maior versatilidade. Porém, a sua utilização em escala industrial está associada com a baixa estabilidade em condições adversas, sendo assim consideradas como barreiras para o desenvolvimento de operações e aplicações em larga escala.

Para contornar tais problemas diversos estudos envolvendo a imobilização enzimática vem se destacando com o intuito de evitar os problemas associados à sua utilização na forma nativa. A imobilização é uma técnica que consiste em aprisionar ou ligar a enzima a um suporte de modo que estas enzimas continuem apresentando atividade catalítica, bem como a reutilização destes biocatalisadores. As possibilidades de aplicações de enzimas imobilizadas continuam a crescer. Por este motivo, a síntese de novos suportes bem como a modificação dos mesmos para melhorar a eficiência de imobilização, sem perda da atividade enzimática é de suma importância. Os suportes inorgânicos vêm se destacando nesse processo devido ao baixo custo de síntese, serem inertes e pelas suas características de elevada resistência mecânica, boa estabilidade térmica, resistência a solventes orgânicos e ao ataque por microrganismos.

Dentre os suportes, o carvão ativado vem se destacando como matriz para imobilização de enzimas, devido as suas propriedades físicas, como uma estrutura porosa altamente desenvolvida e elevada área superficial BET combinados a sua superfície composta por heteroátomos, que garantem aumento na imobilização por adsorção. Esse suporte, assim como os outros suportes inorgânicos e orgânicos, se destaca por serem passiveis de modificação, sendo assim classificados com heterofuncionais, devido a inserção de grupos reativos, que tem com intuito a formação ligações mais versáteis, ou seja são capazes de interagir com enzimas via quimissorção e fisissorção podendo garantir melhores estabilidades no suporte.

De maneira geral é possível observar que existem poucos estudos voltados para imobilização de proteases, principalmente em carvões ativados, fazendo com que um estudo mais aprofundado sobre esta matriz, que é susceptível à diversas modificações em sua superfície devem ser investigadas. Estudos voltados para as modificações com inserção de partículas metálicas e/ou grupos funcionais oxigenados se tornam

necessários, uma vez que estes grupos ocasionam melhores interações da enzima com o suporte promovendo melhores resultados de imobilização e atividade enzimática.

3. OBJETIVOS

3.1. Objetivo Geral

Funcionalização do carvão ativado com diferentes agentes modificadores de superfície: Glutaraldeído; Genipina e partículas metálicas (ferro bivalente e trivalente) e avaliação de sua eficiência na imobilização de enzimas para a obtenção de biocatalisadores suportados.

3.2. Objetivos Específicos

- Síntese e caracterização do carvão ativado do caroço de tamarindo;
- Extração e quantificação da genipina do jenipapo verde;
- Utilização da genipina como agente modificador de superfície;
- Impregnar a superfície do carvão ativado com nanopartículas magnéticas obtidas por co-precipitação na ausência e presença de agente quelante ácido iminodiacético (IDA);
- Verificar a eficiência das novas modificações em comparação com método de funcionalização do glutaraldeído;
- Estudar a influência do método de funcionalização sobre a atividade das enzimas imobilizadas (Pepsina e Tripsina);
- Comparar a eficiência das enzimas imobilizadas com sua forma nativa em solução em diferentes condições do meio reacional;
- Verificar se os suportes utilizados na imobilização influenciam a atividade dos derivados na hidrolise da caseína bovina;
- Obtenção de peptídeos bioativos com propriedade antioxidante através da hidrólise da caseína bovina.

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CAPÍTULO 2

ARTIGO 1

Pepsin Immobilization: Influence of Carbon Support Functionalization

Imobilização de Pepsina: Influência da Funcionalização do Suporte de Carbono

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Pepsin Immobilization: Influence of Carbon Support Functionalization

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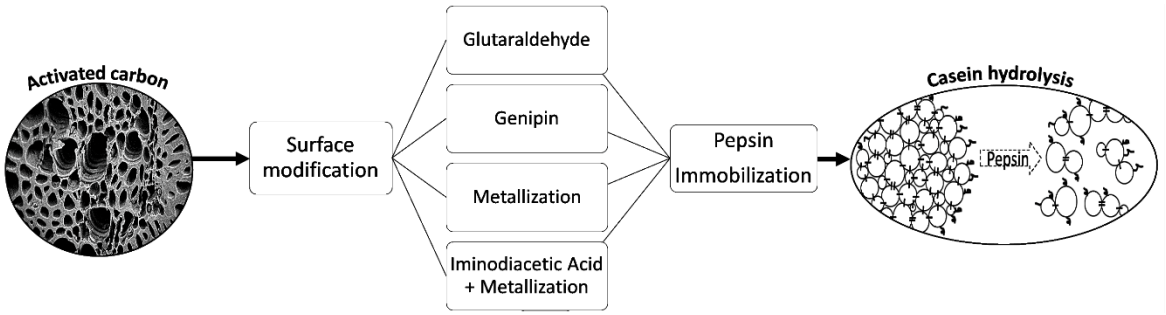
Abstract

Among the matrices for enzyme immobilization, activated carbon has been standing out in immobilization processes due to its properties and to its characteristics that provide superficial modification by inserting new functional groups capable of binding the enzymes forming covalent bonds. In this study the effect of different modification methods of activated carbon (functionalization with genipin, metallization, metallization in the presence of chelating agent, and functionalization with glutaraldehyde) on efficiency of pepsin immobilization was evaluated. The effect of immobilization pH and the reaction medium on hydrolysis activity of bovine casein was also evaluated. The functionalization of activated carbon using iron ions allowed an immobilization capacity of 98.93 $mg.g^{-1}$, with immobilization efficiency greater than 99%, and enzyme activity of 2.30 U, which was higher than the other modifications, and closer to the enzyme in the native form activity (3.32 U). In general, the carbon surface modifications were responsible for forming more stable bonds between support and enzyme, improving its proteolytic activity (from 1.84 to 2.30 U) when compared to traditional immobilization methods by adsorption and covalent binding using glutaraldehyde (from 1.04 to 1.1 U).

Keywords: Derivatives; Protease; Heterofunctional support.

Graphical abstract



1. INTRODUCTION

Proteases are hydrolases responsible for the hydrolysis of peptide bonds in proteins. They can be classified as exopeptidases (EC 3.4.11-19) when they cleave C-terminal peptide bonds, or endopeptidases (EC 3.4.21-19) when they cleave peptide bonds within the molecule. Endopeptidases can be subdivided according to the reactive group present at the active site, and are classified into serine proteases (EC 3.4.21),

cysteine proteases (EC 3.4.22), aspartichoproteases (EC 3.4.23), and metalloproteinases (EC 3.4.24) [1].

Pepsin (EC 3.4.23.1) is an aspartichoprotease, that is, it has an Asp residue in its catalytic site. It is produced in gastric mucosa in form of pepsinogen. Its inactive form is initially released in the digestive process, changing to active form (pepsin) when in contact with hydrochloric acid from the stomach. Due to this activation mechanism, it is considered an acid protease that catalyzes the hydrolysis of dietary proteins to peptides in the stomach, but it is also known as the agent responsible for damage in gastric mucosa, inducing cell damage and inflammation. Pepsin degrades proteins preferentially at carboxylic groups of phenylalanine (Phe), leucine (Leu), glutamic acid (Glu), and tyrosine (Tyr) [2].

Proteolytic enzymes have been used in industrial applications since 1940, mainly in the food, medical, pharmaceutical, and chemical sectors. In the food industry, these proteases are mainly used as a milk coagulant for cheese production, and in the production of plant and animal protein hydrolysates to be used as flavoring agents in foods or beverages, as well as in the hydrolysis of soy allergens [3]. Although it has a wide range of applications in different sectors, its use in industrial processes is still limited in some segments. This limitation is associated with the use of enzymes with high purity, as enzymes with a higher degree of purity require treatments with a higher production cost. In addition, there is a great challenge to maintain enzyme stability in certain processes. Native enzymes have low stability under some process conditions (high temperatures, medium rich in toxic solvents, outside the optimal pH range, and turbulent flow regime). There is also the difficulty of separating the enzyme from the final product, impairing its reuse or its use in continuous processes and large-scale applications. The use of enzymes in their immobilized form can be an effective alternative to use the advantages of enzymatic catalysis and overcome process deficiencies, especially in native form, as it changes with the reaction medium, in addition to the difficulty of recovering the enzyme from reaction system. Immobilized proteases can be used in synthesis of bioactive peptides with antioxidant, antihypertensive, and antimicrobial activity, hydrolysis of different proteins and structural analysis of proteins more efficiently than in their native form [4].

Enzymatic immobilization is defined as the confinement of enzymes in a defined region, physically confined, with the maintenance of its catalytic activities, and which

can be used repeatedly or continuously. Efforts have been made to develop supports for enzyme immobilization that enable their use in continuous processes, improvement in enzyme stability under different operating conditions, in addition to their reuse, providing more efficient control of large-scale processes [5].

The choice of an immobilization method is an important factor that affects enzyme activity. Therefore, it is extremely important to choose a matrix and an adequate method that allows the effective performance of the derivative. The matrix, the enzyme, and the immobilization method must be evaluated before enzyme immobilization. Many immobilization techniques have been developed, which can be classified as irreversible and reversible immobilizations. Irreversible immobilizations stand out for the use of covalent, ionic, and crosslinking chemical bonds. Reversible immobilization methods are characterized by ionic interactions, physical adsorption, encapsulation, metallic bonds or chelation, affinity bonds, and disulfide bonds [6–8].

The use of a support that allows the immobilization of enzymes without losing their activity is essential for improving bioprocesses. In this sense, several materials have been used as support for the production of derivatives, and activated carbon stands out for its adequate properties, including defined porous structure, high surface area, and resistance during the immobilization process. Another advantage of using activated carbon as a support material is the possibility of modifying its surface through the insertion of different functional groups, transforming it into a heterofunctional support. These functional groups can be inserted using different agents such as glutaraldehyde, genipin, metal ions, among others, to improve the enzyme immobilization capacity [9–11].

Glutaraldehyde is the chemical agent most used for surface modification in enzymatic immobilization, once it can react with different enzymatic fractions, mainly with primary amino groups of proteins, despite it can also react with other groups (thiols, phenols, and imidazole). However, there is a growing demand for search for natural crosslinking agents, and genipin stands out among the new crosslinking agents, as it is a naturally occurring crosslinker with low toxicity. Genipin spontaneously reacts with primary amines in glucosamines and/or proteins forming covalent bonds, increasing the stability of immobilized enzymes [12–14]. Modifications using metal ions, especially iron salts, have been used to modify the surface of activated carbons due to their low

cytotoxicity, good biocompatibility, and stability under various physiological conditions, in addition to providing an increase in the activity of some enzymes [15].

Given the above, this study aimed to evaluate the different modification methods of activated carbon (functionalization with genipin extracted from unripe genipap, metallization, and metallization in the presence of chelating agent) effect on efficiency of pepsin immobilization, and comparing the pepsin immobilization efficiency with the traditional modification method (glutaraldehyde).

2. METHODOLOGY

2.1 Genipin extraction and quantification

For genipin extraction, unripe genipap (*Genipa america* L.) were used. The fruits were washed with a brush to remove surface dirt and then sanitized with chlorinated water for 15 min. The solution was drained and the fruits were packed in polyethylene plastic bags and subjected to freezing at -18 °C until the extraction procedures.

Genipin extraction was carried out as described by Ribeiro et al. [16], with modifications. The fruits were initially separated into two fractions: epicarp with mesocarp (shell and pulp) and endocarp with seeds, and the first part was cut into slices that were used for the extraction. The slices were ground in a low-speed blender (1800 rpm, Philips RI 7762, Brazil) with 70% ethyl alcohol at a 1:3 ratio for 5 min. Subsequently, the extract was heated at 55 ºC for 30 min under mechanical agitation, followed by agitation in an ultrasonic bath (Sanders, Soniclean 6, MG, Brazil) with 40 kHz ultrasonic frequency and maximum temperature of 35 °C ± 5 °C for 30 min. After this period, it was filtered in organza and centrifuged at 3600 rpm for 5 min (SPLabor, SP-701, SP, Brazil), and then the supernatant was collected. The extract was concentrated in a rotary evaporator (IKA®HB Digital, RV8, USA) at 50 ºC until complete removal of solvent. The concentrated extract was stored in a freezer (-18 ± 1 ºC) until use.

The content of iridoids was determined as described by Náthia-Neves et al. [17], through high-performance liquid chromatography (HPLC) using a Shimadzu chromatograph (DGU-20A5R, Japan) with a diode array detector (Shimadzu, Japan). A stainless steel CLC (M) column (250 mm x 4.6 mm, Shimadzu) was used for chromatographic separation, which was maintained at 35 °C and a flow rate of 1.5 ml/min. The mobile phase consisted of ultrapure water (solvent A) and acetonitrile (solvent B).

An elution gradient was used to separate the compounds of interest: 0 min, 99% A; 9 min, 75% A; 10 min, 99% A. The total time for the separation of compounds was 15 min. The detection was performed at 240 nm and the injection volume was 20 uL.

2.2. Synthesis of activated carbons

2.2.1. Preparation and characterization of the precursor material

Tamarind seeds were used for the synthesis of activated carbon (AC), an abundant fruit in the region, used for the production of fruit pulp and sweets. They are produced by a healthy tree of 150-200 kg of fruit per year, and the average fruit weight varies between 10 and 15 g, consisted of pulp (30%), husk and fibers (30%), and seeds (40%) [18].

The tamarind seed was purchased from a fruit pulp agro-industry located in the city of Montes Claros – MG. The residues were washed and dried in an oven at 105 ºC for 24 h, milled in a knife mill, and sieved to 20 mesh, thus obtaining more homogeneous particles.

The residue was characterized for ash and moisture contents according to the methodologies 920.03 and 925.45, respectively, described by the AOAC [19]. Lignin, cellulose, and hemicellulose contents were determined using the neutral detergent fiber and acid detergent fiber (NDF and ADF) methodologies as described by Van Soest et al. [20]. Functional groups of precursor materials were evaluated by Fourier Transform Infrared Spectrophotometry (FTIR). Samples were directly analyzed using the attenuated total reflectance (ATR) technique in the infrared region of 4000-600 cm^{-1} in an Agilent, Cary 630 FTIR spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA).

2.2.2. Obtaining the activated carbon (AC) by chemical activation with phosphoric acid (H_3PO_4)

Synthesis of activated carbon from precursor material was carried out by the chemical activation method, using phosphoric acid as an activating agent, according to the methodology described by Santos et al. [21].

2.3. Functionalization of the activated carbon

Surface modifications of activated carbon were made using different modifying agents such as glutaraldehyde, genipin, and iron salts (Fe^{2+} and Fe^{3+}).

2.3.1. Modification with glutaraldehyde

Surface modification was performed using glutaraldehyde method suggested by Ramani et al. [22], with modifications. Glutaraldehyde functionalization was used as a standard modification. For that, 10 g of AC (previously dried) were used. The modification was divided into two steps, both carried out at room temperature (25°C). In first step, 10 g of AC was mixed with an amine solution (2.5% v/v), containing 0.85 mL of ethylenediamine (99%; CAS: 107-15-3) and 32.5 ml of acetone (CAS: 67-64-1), and kept under manual stirring for 10 min. After this period, activated carbon was kept at rest in the solution for 24 h, thus obtaining the carbon with an amino-functionalized surface. In second step, a glutaraldehyde solution containing 33.5 mL of glutaraldehyde (25%; CAS: 111-30-8) and 33.5 mL of acetone (CAS: 67-64-1) was added to the amino-functionalized carbon and kept under stirring (150 rpm) for 30 min using a magnetic stirrer (LABNET, PC-420D, New Jersey, USA). Then, the material was dried at 60 °C for 24. After solvent evaporation, functionalized carbon was washed with distilled water to remove unbound chemical compounds and dried at 60 °C for 6 h in an oven, in order to reduce diffusion resistance during the immobilization process (TECNAL, 393/1, Piracicaba – SP, Brazil). The glutaraldehyde-functionalized carbon (FAC) was sieved (40 mesh) and stored in an airtight container.

2.3.2. Modification with genipin

Surface modification using concentrated extract of genipin was performed as described in Section 2.3.1, with modifications in Step 2.

Amino-functionalized AC was placed in contact with a genipin solution containing 33.5 mL of concentrated genipin extract and 33.5 mL of acetone (CAS: 67-64-1), and kept under stirring (150 rpm), in a magnetic stirrer for 30 min. The material was dried at 60 °C for 24 h. After solvent evaporation, functionalized carbon was washed with distilled water to remove unbound chemical compounds and dried at 60 °C for 6 h in an oven (Tecnal 393/1, Piracicaba – SP, Brazil). Then the genipin-functionalized carbon (GAC) was sieved (40 mesh) and stored in an airtight container.

2.3.3. Modification with iron salts

Surface modification using iron salts was done by the method of co-precipitation of Fe^{2+} and Fe^{3+} ions in stoichiometric proportions, to incorporate magnetic particles on

the surface of activated carbon, responsible for the formation of cross-links with enzymes. For that, two methods were used: direct impregnation with iron particles, and carbon impregnation with a chelating agent (iminodiacetic acid – IDA) before impregnation with metal particles.

2.3.3.1. Direct impregnation of activated carbon with iron particles

For magnetic carbon synthesis, the modification methodology suggested by Mohan et al. [23] was used. Synthesis of magnetic particles was carried out through the co-precipitation of ferrous sulfate (Fe^{2+}) with ferric chloride (Fe^{3+}) in an alkaline medium rich in activated carbon. For that, 5 g of activated carbon (AC), previously dried, was mixed with 250 ml of ultrapure water and kept under stirring in a magnetic stirrer. The iron salts solution was prepared by mixing 200 mL of a solution containing 0.067 mol of trivalent iron ($FeCl_3.6H_2O$; CAS: 10025-77-1), 200 mL of a solution with 0.033 mol of bivalent iron ($FeSO_4.7H_2O$; CAS: 7782-63-0), and 2.5 mL of 37% v/v HCl. The ferrous sulfate and ferric chloride solutions were added to activated carbon and stirred vigorously for 15 min at 70 °C. After this period, 500 mL of a 4 $mol.L^{-1}$ sodium hydroxide (NaOH) was added to the initial solution to precipitate the particles, with magnetic stirring for 2 h. The precipitate was collected in Falcon tubes, washed 10 times with 40 ml of ultrapure water, and centrifuged at 4000 rpm for 5 min. After washing, the magnetic carbon (MAC) was dried in an oven at 60 ºC for 24 h.

2.3.3.2. Impregnation of activated carbon with iron particles and chelating agent

For the impregnation, 5 g of activated carbon, previously dried, were mixed with 20 mL of 1 $mol.L^{-1}$ sodium carbonate (Na_2CO_3) under magnetic stirring for 1 h. After this step, 20 mL of 0.5 $mol.L^{-1}$ iminodiacetic acid 98% ($HN(CH_2COOH)_2$; CAS: 142-73-4) were added, and the pH was adjusted to 11 with 1 $mol.L^{-1}$ sodium hydroxide (NaOH). The mixture was kept under agitation (20 rpm) at 40 °C for 14 h in an incubator chamber (SP-500, SPLabor, SP, Brazil). Then, the carbon was washed with an acetic acid solution (1% v/v), until the washing water become neutral.

After chelating insertion of the agent on the surface of the activated carbon, the metallization step was continued by the co-precipitation of iron salts according to the methodology previously described in Section 2.3.3.1, thus obtaining the metalized carbon with a chelating agent (MQC).

2.4. Supports characterization

The supports were characterized for pH of zero charge (pH_{PCZ}); Fourier Transform Infrared Absorption Spectrophotometry (FTIR); X-Ray Diffraction (XRD); Measurement of porosity and specific surface area; Scanning Electron Microscopy (SEM); and Energy Dispersive Spectroscopy (EDS).

The pH of the zero charge is defined as the pH at which carbon surface has a neutral charge, which was determined as described by Kuśmierek et al. [24]. Functional groups were directly evaluated by FTIR using the attenuated total reflectance (ATR) technique in the infrared region of 4000 - 600 cm^{-1} in a spectrophotometer Agilent Cary 630 FTIR (Agilent Technologies Inc., Santa Clara, CA, USA). X-ray diffractograms were obtained in diffractometers (D2 PHASER, Bruker, Germany) equipped with Cu Kα monochromatic radiation source (λ = 1, 54184 Å). The generator tension was 30kV and the current was 10mA. Support crystallinity was evaluated at room temperature in 2θ varying from 10º to 90 º with a speed rate of 4 ºC/min.

The adsorption and desorption isotherms of activated carbons were obtained in Micrometrics ASAP 2420 equipment (Georgia, USA). Before measurements, the samples were submitted to a pre-treatment step that consisted of heating at 60 ºC for 2 h. Subsequently, nitrogen adsorption and desorption isotherms were obtained at 77 K. Specific surface area was determined by the BET equation [25]. Pore distribution was obtained from the desorption isotherm using the BJH method [26], while micropore volume was determined by t-plot analysis from the adsorption isotherms [27].

Morphology and chemical composition of carbons were determined by Scanning Electron Microscopy (SEM) and X-ray Energy Dispersive Spectroscopy (EDS). Samples were placed on a conductive double-sided carbon tape and overlaid with a conductive material using the Desk V Gold Film Deposition System (Denton Vacuum LLC Moorestown, New Jersey, USA), equipped with the carbon accessory. Then, samples were analyzed in a Scanning Electron Microscope (SEM) JSM - 6610 (Jeol, Tokyo, Japan), equipped with EDS Thermo scientific (NSS Spectral Imaging, USA).

2.5. Pepsin immobilization on carbon supports

To investigate the support modification effect on pepsin immobilization, the effect of pH of pepsin solubilization was evaluated, and, the derivatives and the native enzyme

solution were used for enzymatic hydrolysis after immobilization, using bovine casein as a substrate solubilized at optimal pH of the enzyme activity (pH 3.0).

2.5.1. Effect of pepsin immobilization pH

Pepsin immobilization (pepsin from porcine gastric mucosa, Sigma; 87% protein, 3,200 - 4,500 U/mg, CAS: 9001-75-6) was evaluated by the adsorption method, using AC, and by covalent bonding using glutaraldehyde-functionalized carbon (FAC) at different pH values (3.0, 5.0, 7.0, and 9.0), to determine the optimal pH for immobilization. The variables studied were the amount of immobilized enzyme and the respective activity.

The experiment was carried out using conditions determined in preliminary tests. For that, 50 mg of carbon were mixed with 5 mL of solution with an initial pepsin concentration of 1 $mg.mL^{-1}$ in lactic acid/sodium lactate buffer 0.05 $mol.L^{-1}$, for pH 3.0, and in 0.05 $mol.L^{-1}$ sodium phosphate buffer for pH 5.0, 7.0, and 9.0. The tubes were kept under constant agitation (20 rpm) in an orbital shaker at room temperature (25°C). After 2 h, the tubes were centrifuged at 3500 rpm for 5 min, the supernatant was removed, and the non-adsorbed proteins were identified using the Bradford method [28]. Successive washes were performed on the samples to remove unbound enzymes. Then, 5 mL of the respective buffer was added to the samples, under agitation at 20 rpm for 10 min, followed by centrifugation at 3500 rpm for 5 min, and again the supernatant was removed and quantified. Process equilibrium was reached when the protein concentration values in supernatant solution were null. Derivatives were stored in a buffer solution at 4 ºC until analysis.

Immobilization capacity of each carbon was determined by the difference between the initial and final concentrations of proteins using Equation 1.

$$C_{Im} = \frac{V\,(C_{in} - C)}{m_S} \quad (1)$$

where: C_{Im} is the immobilization capacity ($mg.g^{-1}$); V is the volume of solution (mL); C_{in} is the initial concentration of the solution ($mg.L^{-1}$); C is the concentration of solution at equilibrium ($mg.L^{-1}$); m_S is the mass of the support (g).

2.5.2. Enzyme activity Determination

Enzymatic activity of the enzyme solution and the derivatives was determined using a 2% (w/v) bovine casein (casein from bovine milk - CAS 9000-71-9) as a substrate

for pepsin, in lactic acid/sodium lactate buffer 0.05 mol.L^{-1} (pH 3.0) [29]. In a centrifuge tube, 5 ml of the substrate and 1 ml of enzyme solution in native form were incubated at 40 °C for 60 min. After this period, 2 ml of trichloroacetic acid (6.5%, m/v) was added to terminate the reaction, and the tubes were centrifuged at 6000 rpm for 6 min at 4°C. Then, 1 ml of the supernatant was mixed with 5 ml of sodium carbonate (4% w/v) and 1 ml of Folin-Ciocalteu solution (20%, v/v). The mixture was kept at 40 °C for 20 min, and absorbance readings were performed in a spectrophotometer (UV-Vis Quimis, Q898UV2, SP, Brazil) at 660 nm. The activity proteolytic was calculated using the Equation 2. One unit of protease activity was defined as 100 µg of tyrosine equivalent released by the enzyme.

$$Activity\ (U) = \frac{(\mu g/mL \text{ of tyrosine equivalents released}) \times \text{Total volume of assay}}{\text{Volume of enzyme used (mL)} \times \text{Time of assay (minutes)}} \quad (2)$$

To determine the activity of derivatives, the previous methodology was followed with minor modifications. For that, 50 mg of derivative was mixed with 5 mL of casein solution (2% w/v) in centrifuge tubes and incubated at 40 ºC for 1 h. After this step, the tubes were centrifuged (3000 rpm/5 min) and all the supernatant was transferred to another tube containing 2 ml of trichloroacetic acid (6.5%, w/v). The tubes were centrifuged (5000 rpm /6 min /4 °C) and 1 mL of the supernatant was removed and placed to react, as described above.

2.5.3. Pepsin immobilization on functionalized supports

After determining the pepsin immobilization optimal on activated carbon (AC) and functionalized carbon (FAC), the immobilization was performed using genipin-functionalized carbon (GAC), metalized carbon (MAC), and metalized carbon in the presence of the chelating agent (MQC), to evaluate the effect of different functionalization methods on the pepsin immobilization and the respective activity. The methods for immobilization and determination of enzymatic activity are described in the previous Sections (2.5.1 and 2.5.2). Derivative activity was expressed in U. The supports without the immobilized pepsin were used as controls.

2.5.4. Stability of derivatives in high saline concentration and Tween 80

To confirm enzyme binding stability onto supports, the derivatives were incubated in a solution containing sodium chloride (1.5 mol.L^{-1}) and Tween 80 (1%) in

immobilization buffer. After a 2 h contact time, the supernatant was collected for protein quantification using the Bradford method [28].

2.6. Statistical analysis

All procedures were performed in triplicate. Data were subjected to analysis of variance and the differences between the means of hydrolytic capacity were analyzed by the Tukey's test ($p < 0.05$), using the SAS Studio and Origin Pro 2018 programs.

3. RESULTS AND DISCUSSION

3.1. Genipin extraction and quantification

For the genipin extraction using ethanol, 900 ml of the concentrated extract was obtained from 600 g of unripe genipap pulp. The results of the concentration of iridoids (genipin and geniposide) of extract are shown in Figure 1. The genipin concentration in the mesocarp of 600 g of unripe genipap was 21.54 $mg.g^{-1}$ (6.46 $mg.mL^{-1}$) which is greater than the findings of other authors. Ramos-de-la-Peña et al. [30] reported a concentration of 16.3 $mg.g^{-1}$; Náthia-Neves et al. [17] reported 20.7 $mg.g^{-1}$ of genipin in the mesocarp of unripe genipap, while Alves Camêlo et al. [31] obtained genipin levels around 16.75 $mg.g^{-1}$.

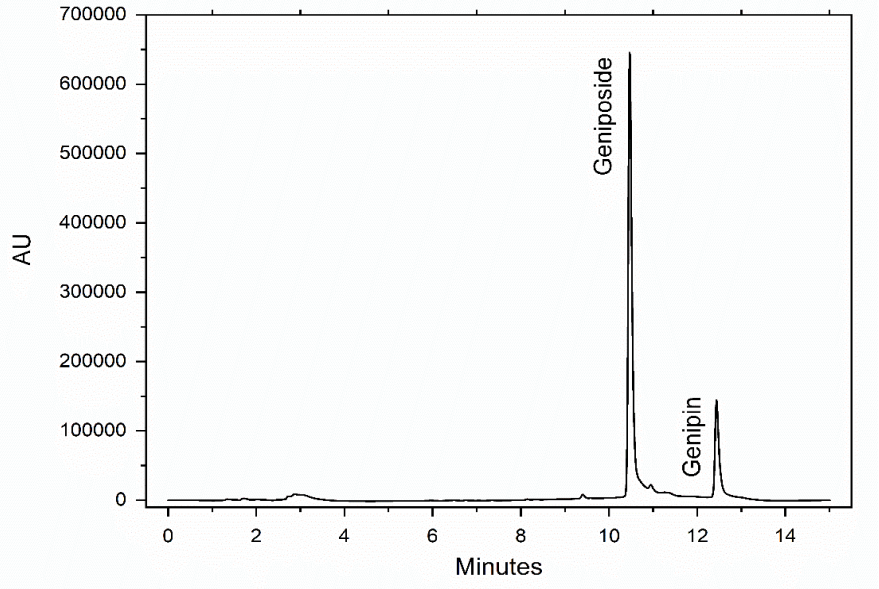


Figure 1. Chromatogram of genipin extract from the mesocarp of unripe genipap.

The difference in genipin concentration is related to multiple abiotic factors such as fruit variety, soil, growing conditions, climate, harvest time, storage conditions, and

extraction methods. Biological ripening is also responsible for the change in genipin contents, as the biochemical processes during fruit ripening can significantly affect the iridoid contents [32,33].

The chromatogram shows a high content of geniposide, which is associated with the region of the fruit used in the extraction. Náthia-Neves et al. [17], studied genipin extraction using different parts of the unripe genipap and observed that the mesocarp presents higher geniposide levels, while lower levels were observed in the endocarp of the fruit. According to the authors, it is possible to convert geniposides into genipin by enzymatic hydrolysis, which is carried out in the presence of enzymes from the glycosyl-hydrolases family.

3.2. Characterization of precursor material and supports

Precursor material obtained from tamarind seeds presented 35.3% cellulose, 33.1% lignin, and 10.0% hemicellulose, with a cellulose/lignin ratio of 1.07, and an ash content of 2.00%. Santos et al. [21] reported that the chemical composition of the residue has a direct effect on the yield of the activated carbon synthesis, as well as on its texture properties. Weber and Quicker [34] stated that activated carbon yield is related to the variation of lignocellulosic contents (lignin, cellulose, and hemicellulose) and the extraction compounds (alkaloids, essential oils, mucilages, pectins, among others) in the biomass. Species with high lignin contents lead to higher yields, while biomass with high cellulose contents generates activated carbon with larger surface areas. On the other hand, precursors rich in inorganic materials can lead to lower yields, due to the reduction of carbonaceous compounds in their structure [35]. Therefore, in general, tamarind seed has potential as a precursor material for synthesis of activated carbon due to its high lignocellulosic content (> 78.39 %), and low ash content.

The synthesis of activated carbon led to a process yield of 50.9%, which is related to lignocellulosic components of precursor material (78.39%), making it a rich carbon source [21,34]. In addition to the lignocellulosic composition, the yield is linked to activation method and activating agent used during the synthesis step. Chemical activation has higher yields, especially when phosphoric acid is used as an activating agent. Phosphoric acid interacts with biomass forming phosphate bonds between the biomass fragments, as well as cleavage reactions of the constituent bonds of biopolymers. Then, cross-links are formed within the precursor structure, generating an internal matrix

that decreases the shrinkage of precursor materials and losses of volatile material during heating [36,37].

Tamarind activated carbon had an ash content of 6.5%, formed by non-carbon mineral additives from precursor material, as well as components derived from the activating agents retained or bound to the carbonaceous material, such as phosphate groups from the reactions between precursor material and H_3PO_4, after pyrolysis, which are not leached in the washing process [38]. According to Patawat et al. [39], ash content of activated carbon should not exceed 10 %.

3.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to characterize precursor material (tamarind seed) and supports. The FTIR spectra (Figure 2) show the changes in both the precursor material when subjected to the activation process and the supports after the modifications.

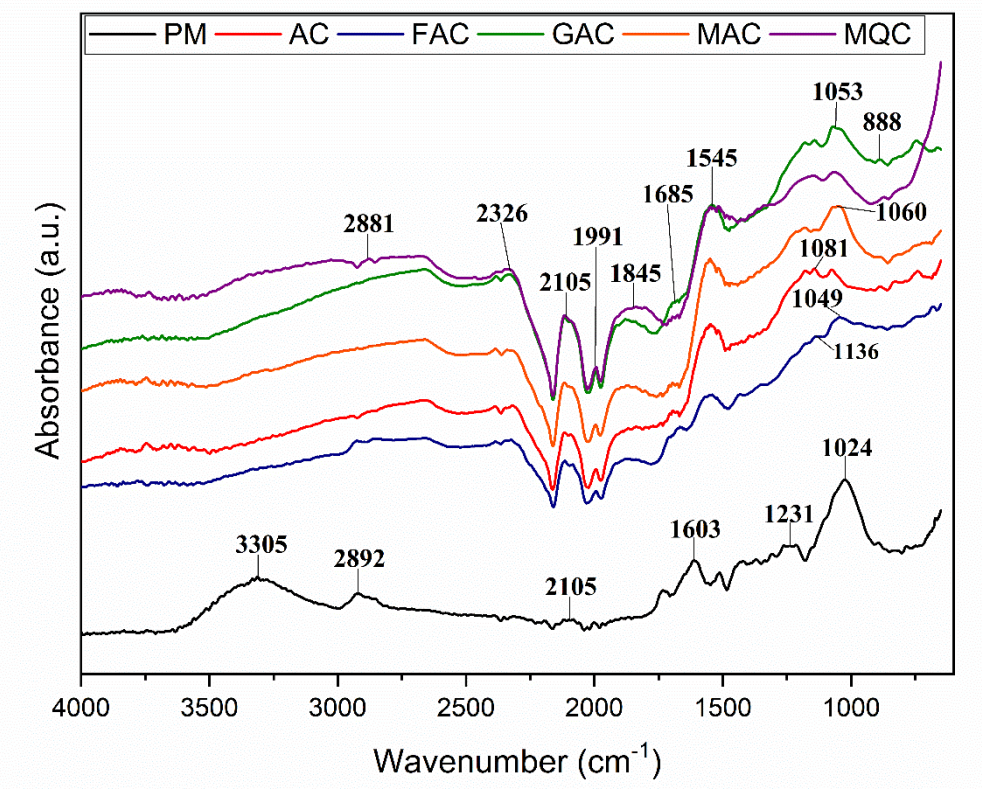


Figure 2. FTIR of precursor material (PM), activated carbon (AC) and Glutaraldehyde-modified supports (FAC); Genipin (GAC); Metal (MAC); IDA + metal (MQC).

The spectra of precursor material (*in natura*) show several functional groups such as esters, ethers, alcohols, aldehydes, ketones, phenols, and carboxylic acids,

characteristic of lignocellulosic materials [40]. Characteristic wavelengths of lignocellulosic compounds show vibrational modulus at 3305 cm^{-1}, attributed to the stretching of the hydroxyl groups (-OH); the bands at 1603 cm^{-1} are assigned to the C=O group of carboxymethyl cellulose; bands at 1231 cm^{-1} refer to the vibration of the guaiacylic rings from the lignin structure, and the bands at 1024 cm^{-1} refer to the stretching of the glycosidic bonds (C-O-C) present in the structure of cellulose and hemicellulose.

After chemical activation and carbonization process, several vibrational modules characteristic of lignocellulosic compounds (O-H, CH_3O, C-H, C=O, C-C, and C-O) disappeared due to thermal decomposition, and new functional groups were formed. Among the new bands, the bands at 1845 cm^{-1} and 1550 cm^{-1} stood out, referring to the stretching of C=O and C=C from carbonaceous materials and at 1081 cm^{-1} referring to phosphate ester bonds, due to the chemical activation with phosphoric acid, which are also related to increased ash content and process yields [41–43].

When comparing the spectra of activated carbon (AC) and glutaraldehyde-functionalized carbon (FAC), a reduction in the intensity and displacement of the peaks previously formed was observed, in addition to the formation of new peaks associated with glutaraldehyde. The wavelengths at 1136 cm^{-1}, which may be associated with the amine group (C-N), 1674 cm^{-1} referring to the vibrations of the aldehyde group (C=O), and 1442 cm^{-1} referring to the vibrations associated with the carboxylic acid (O-H) stood out. The presence of these functional groups is indicative of the chemical modification of activated carbon surface [22,44,45].

The spectra of genipin-functionalized carbon (GAC) and activated carbon (AC) showed an increase in peak intensity and the formation of new groups at 1685 cm^{-1} referring to the vibrations of the aldehyde group (C=O) present in the genipin molecule, at 1545 cm^{-1} referring to the vibration of the amine group (N-H), and at 1053 cm^{-1} referring to the vibration of the primary alcohol (C-O) [46]. Similar vibrational modes were observed when comparing the spectra of the genipin modification with glutaraldehyde, differing only in the peak intensity. These results indicate a possible replacement to the traditional glutaraldehyde method, once both modifications are formed by the groups responsible for the covalent bonding of the enzymes.

After the AC surface metallization, two spectra were obtained, one corresponding to the metalized carbon without the chelating agent (MAC) and the other with the addition

of the chelating agent (MQC). The modifications led to displacement and increase in the intensity of some peaks due to the interaction of Fe_3O_4 particles with the matrix functional groups, indicating that it was possible to make the surface modification of AC [47]. Furthermore, MAC and MQC presented the same vibrational modes, with variations only in the peak intensity. MQC showed higher peak intensities and an extra vibrational modulus at 2881 cm^{-1} related to the asymmetric vibrations of the amine group (N-H) from iminodiacetic acid (IDA) [47].

3.2.2. Point of zero charge (pHpcz)

The surface charge distribution of the carbonaceous supports was evaluated by determining the point of zero charge (pHpcz). The activated carbon (AC) had pHpcz of 4.84. The supports modified with glutaraldehyde (FAC), genipin (GAC), metal (MAC); and IDA + metal (MQC) had pHpcz of 5.45, 6.35, 7.63, and 8.76, respectively. The point of zero charge is defined as the pH at which the material's surface presents neutral charges, that is, the density of electrical charges on its surface becomes zero in the absence of specific adsorption.

More acidic pHpcz values of activated carbons are due to the dissociation of acidic surface oxygen complexes, such as carboxyl and phenolic groups. In addition, higher values may be due to the use of phosphoric acid as an activating agent [48,49]. The acid groups responsible for the low pHpcz value included COC (1991 cm^{-1}), C=O (1550 cm^{-1}), CO (1144 cm^{-1}), P+-O- and POP (1081 cm^{-1}), as shown in the FTIR spectra.

Carbon functionalized with glutaraldehyde (FAC) and genipin (GAC) presented pHpcz values lower than 7.0, and higher than that observed AC. This increase may be due to the amination of the carbon surface for insertion of glutaraldehyde and/or genipin. When comparing FAC and GAC, the functionalization with genipin was responsible for providing a more expressive increase in pHpcz of activated carbon, which may be associated with an increase in amine groups (C-N), as shown in the FTIR spectra.

Iron-modified carbons (Fe^{3+} and Fe^{2+}) had pHpcz greater than 7.0, which is common after this modification due to the reallocation of π electrons in the carbon plane as observed in some studies of activated carbon modification with iron particles [50]. According to Ren et al. [51], the increase in pHpcz is due to the modification process in a basic medium (NaOH solution), thus increasing the alkalinity of activated carbon surface. When comparing MAC with MQC, a higher pHpcz was observed for metalized

carbon in the presence of a chelating agent, probably due to the higher amount of iron oxide immobilized on the surface of the support [52].

3.2.3. XRD spectroscopy

Figure 3 shows the X-ray diffraction patterns of activated carbons. For the activated carbon (Fig. 3a) synthesized from tamarind seed, two amorphous halos were observed at 2θ = 22º and 2θ = 43º, as well as the absence of well-defined peaks, showing that CA has a predominantly amorphous structure.

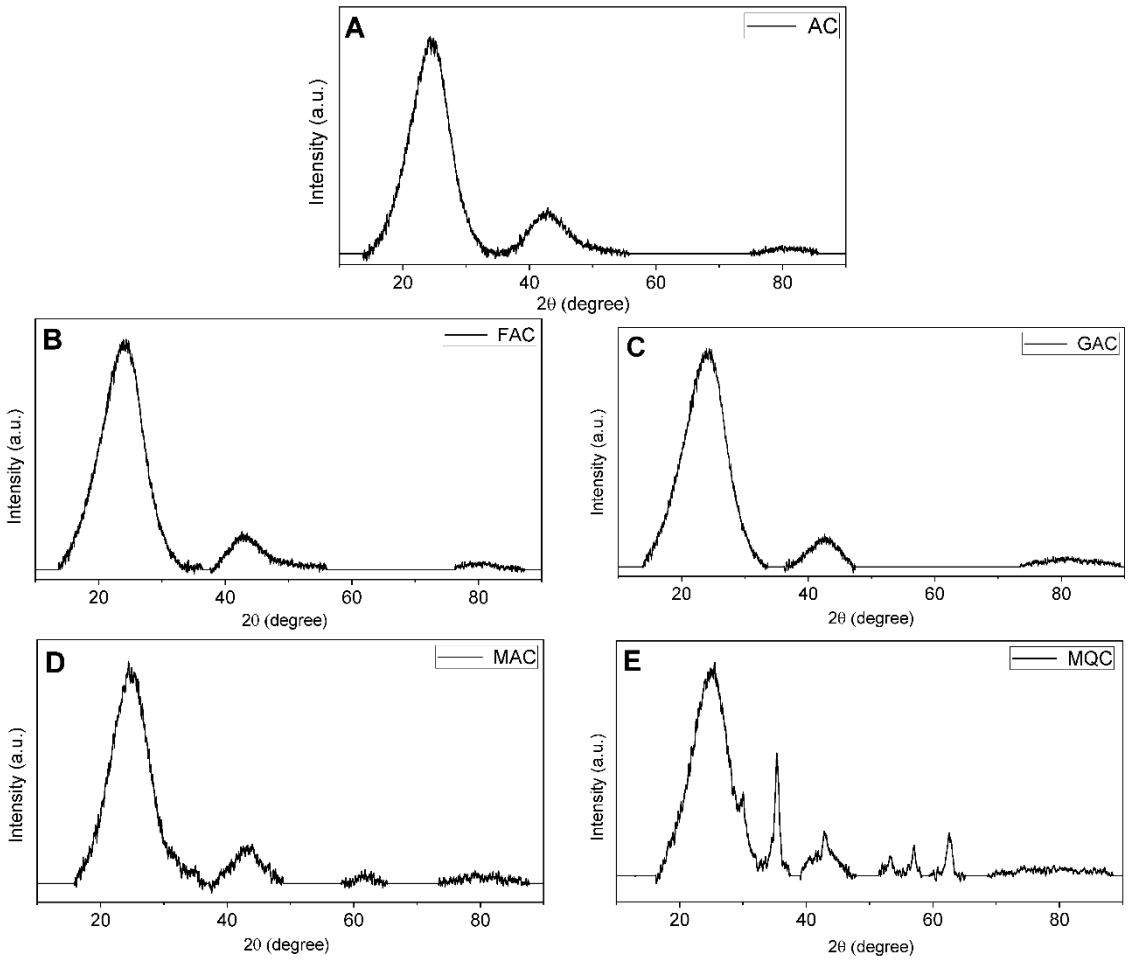


Figure 3. Diffractogram of activated carbon (a) and activated carbons modified with glutaraldehyde (b); Genipin (c); Metal (d); IDA + metal (e).

In diffractograms of modified carbons, two amorphous halos were observed at 2θ = 22º and 2θ = 43º, characteristic of activated carbon. This result demonstrates that the modifications did not change the amorphous structure of the activated carbon, leading only to the increased halo intensity for the modifications with glutaraldehyde (Fig 3-b) and genipin (Fig 3-c). The modifications made with iron salts (Fig 3-d and 3-e) led to a

reduction in the intensity of these halos, as well as the appearance of a new diffraction peak at 2θ = 62.3° corresponding to the diffraction of the magnetite and hematite structures (Fe_3O_4 and α-Fe_2O_3, at 440 and 214) respectively, both present in magnetic nanoparticles [53].

The activated carbon with iron salts in the presence of chelating agent (MQC) showed diffraction peaks at 2θ = 24.6º referring to hematite (α-Fe_2O_3 at 012); at 35.4° corresponding to magnetite diffraction (Fe_3O_4 at 311); at 41.8º corresponding to FeO (wustite at 200) and the peaks at 52.7 and 57.1º, both corresponding to the crystal diffraction of magnetite at 422 and 511, respectively [53].

The diffraction peaks of MAC and MQC indicate that it was possible to successfully modify the surface of AC through the incorporation of magnetic particles. The magnetization in the presence of iminodiacetic acid resulted in more intense crystalline peaks, as the chelating agent formed a chelating/metal complex, exposing the metal structure, leading to a graphitization of the activated carbon with better-defined peaks provided by iron compounds.

3.2.4. Porosity and specific surface area (BET)

The nitrogen (N_2) adsorption/desorption isotherms of activated carbons are shown in Figures 4a, 4c, 4e, 4g, and 4i, and the pore size distributions are shown in Figures 4b, 4d, 4f, 4h, and 4j.

For all analyzed supports, the isotherms showed an increase in the volume of N_2 adsorbed at low pressure, forming a hysteresis loop characteristic of type IV isotherms. According to IUPAC classification, type IV isotherms are characteristics of porous solids that have micro and mesopores in their structure. The hysteresis loop characterized as Type I is often associated with porous materials consisting of agglomerates or compacts of regular structure with a narrow pore size distribution, presenting a hysteresis curve closer to the adsorption curve, as observed for all synthesized carbons [54].

The results of carbons pore distribution showed a greater pore volume of AC, between 2.5 and 5 nm, with a tendency to form pores smaller than 2 nm (Figure 4b), classifying the material as micro-mesoporous. According to Zhang et al. [55], the presence of mesopores in the 2 to 6 nm range may be associated with the chemical treatment with phosphoric acid during the activation of precursor materials. The modified carbons (Figures 4d, 4f, 4h, 4j) presented a greater pore volume in the range of 5 nm, with a tendency to form micropores, which can also be classified as micro-mesoporous. When

comparing the activated carbon before and after the surface modifications, greater porosity was observed in the 2.5 to 5 nm range.

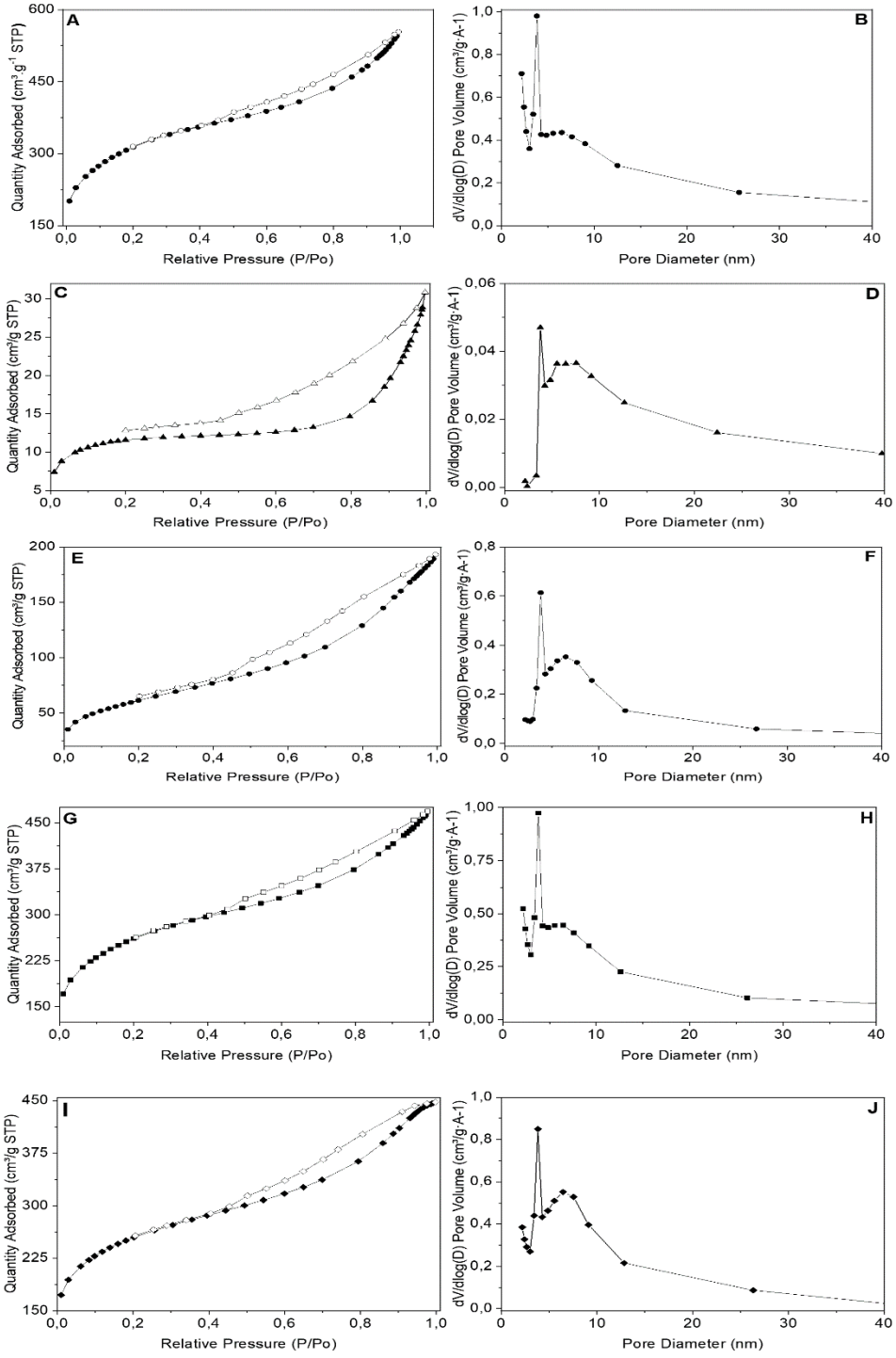


Figure 4. Nitrogen adsorption/desorption isotherms at 77 K, and pore distribution by the BJH method of activated carbon (A and B) and activated carbons modified with Glutaraldehyde (C and D); Genipin (E and F); Metal (G and H); IDA + metal (I and J).

In general, as can be seen in Table 1, all changes led to a reduction in the surface area and pore volume, confirming the structure changes. The reduction in the surface area of these materials is related to the pore volume, that is, the greater the reduction in pore volume, the smaller the surface area. Regarding the pore diameter, an increase in diameter was observed, which may be due to the distance between the molecules of the functionalizing agents inserted on the surface and the obstruction of the smaller pores.

Table 1. Texture properties of activated carbon (AC) and supports modified with glutaraldehyde (FAC); Genipin (GAC); Metal (MAC); IDA + metal (MQC)

Sample	Sg (m^2/g)	Dp (nm)[a]	V_{meso} (cm^3/g)	V_{micro} (cm^3/g)
AC	1082	4.88	0.452	0.166
FAC	40	8.12	0.031	0.009
GAC	221	5.94	0.262	0.004
MAC	924	5.20	0.442	0.117
MQC	892	5.24	0.416	0.151

[a] Maximum pore size distribution;

Regarding the texture properties of the functionalized carbons, there was a greater reduction in surface area when using glutaraldehyde due to greater pore obstruction caused by the high density of the groups introduced on the surface of the support (ethylenediamine and glutaraldehyde). These groups have high crosslinking power, leading to the formation of a coated cross-linked carbon. Genipin is a cross-linking agent, thus it also caused a reduction in the surface area of the activated carbon, slightly milder when compared to glutaraldehyde. This behavior may be due to the temperature used during the modification process since glutaraldehyde tends to increase its crosslinking power when subjected to higher temperatures, causing a self-crosslinking and consequently a greater pore obstruction [56].

The functionalization with Fe^{+2} and Fe^{+3} did not lead to a considerable reduction in the pore volume and surface area of the carbons. MQC, which was functionalized with a chelating agent, showed a greater reduction in the surface area when compared to MAC, probably due to the chelating agent provided greater incorporation of iron particles on the carbon surface [57], obstructing some smaller pores. These results allow us to infer that the iron particles were incorporated only on the carbon surfaces.

3.2.5. Surface morphology by SEM and Chemical Composition by EDS

Figure 5 shows the SEM images and the EDS spectra of the carbonaceous materials. Carbons presented a rough surface with a spongy appearance, with pores of different sizes and shapes.

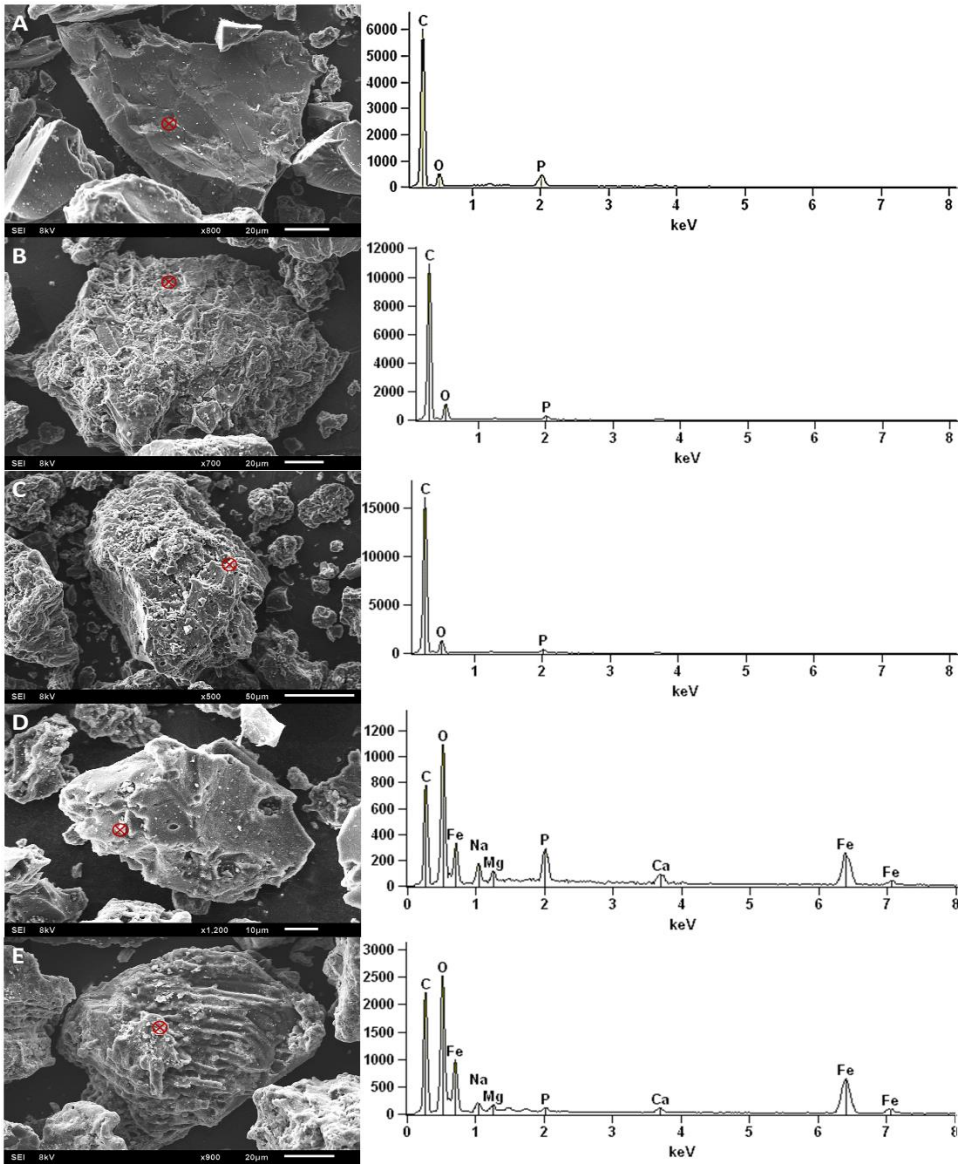


Figure 5. Scanning Electron Microscopy and Energy Dispersive spectroscopy of AC (A), FAC (B), GAC (C), MAC (D), and MQC supports (E).

Chemical composition determined by EDS (for the regions highlighted in red) shows that the activated carbon (AC) contains carbon, oxygen, magnesium, calcium, and phosphorus in its composition. These elements are associated with chemical structure of carbonaceous materials, precursor material, and activating agent, and can be observed in all supports after modification. There was an increase in the peak intensity associated

with carbon and oxygen in FAC and GAC, when compared to AC, probably due to the use of glutaraldehyde and genipin during functionalization. In addition to the elements already mentioned, the MAC and MQC surface showed a peak referring to sodium, and peaks associated with iron on the carbon surface.

3.3. Pepsin Immobilization Tests

3.3.1. Effect of enzyme solution pH on immobilization process

The study of enzyme solution pH effect on immobilization process on a given support is fundamentally important, as this parameter directly affects the enzyme charges and, consequently, the interactions with the support. The results of enzyme solution pH effect are shown in Table 2.

Table 2. Effect of initial pH of the pepsin solution on the amount of bound enzyme (M_{Lig}), immobilization capacity (C_{Im}), immobilization efficiency (Effic), and enzyme activity of activated carbon (AC), glutaraldehyde-functionalized carbon (FAC), and free enzyme (FP).

Sample	pH	M_{Lig} (mg)	C_{Im} (mg/g)	Effic (%)	Enzyme activity (U)
AC	3	4.96 ± 0.01	98.52 ± 0.24 a	99.20 ± 0.01%	1.04 ± 0.01 a
	5	4.93 ± 0.01	98.20 ± 0.42 a	98.60 ± 0.01%	0.62 ± 0.06 b
	7	4.51 ± 0.09	89.71 ± 1.96 b	90.12 ± 0.02%	0 ± 0 c
	9	4.31 ± 0.11	86.71 ± 2.24 b	86.14 ± 0.02%	0.04 ± 0.01 c
FAC	3	4.92 ± 0.02	97.57 ± 0.42 a	98.39 ± 0.01%	1.10 ± 0.01 a
	5	4.92 ± 0.01	97.03 ± 0.36 a	98.35 ± 0.01%	0.39 ± 0.01 b
	7	4.43 ± 0.03	87.70 ± 0.90 b	88.53 ± 0.01%	0.05 ± 0.01 c
	9	4.30 ± 0.10	86.79 ± 2.45 b	86.03 ± 0.02%	0.05 ± 0.01 c
FP	3	--	--	--	3.32 ± 0.01 a
	5	--	--	--	1.72 ± 0.01 b
	7	--	--	--	0.14 ± 0.01 c
	9	--	--	--	0.18 ± 0.01 c

* Means followed by the same lowercase letter in the columns for each sample do not differ significantly from each other by Tukey's test ($P < 0.05$).

Better immobilization conditions were observed at more acidic pH values (3 and 5) for both carbons used, with an efficiency above 98%, while in a neutral to basic environment (pH 7 and pH 9) the efficiency ranged from 86 to 90%.

The high immobilization capacity of AC is related to its porosity and pore diameter, once the enzyme under study has a low molecular mass (35 kDa), which facilitated immobilization. In addition to the texture characteristics of AC, its high immobilization capacity in the acidic pH range is related to the surface charges of the enzyme and AC. The pH determines the net charge of protein, affecting the electrostatic interactions in enzyme/activated carbon in the immobilization process.

AC had pH_{PCZ} close to 5.0, while pepsin had an isoelectric point (pI) close to 1.0 [58]. Therefore, at pH values above pI, the enzyme presents a greater density of negative charges on its surface. On the other hand, in solutions with a pH below its isoelectric point, its surface has a greater number of positive charges. In an acidic medium, the carbon surface has neutral (pH 5) and positive (pH 3) charges, while the enzyme had negative charges on its surface, due to its isoelectric point close to pH 1, favoring the immobilization due to electrostatic and hydrophobic interactions. In contrast, both carbon and enzyme had negative residual charges in the immobilization at pH 7 and 9, leading to a reduction in electrostatic interactions, thus the immobilization process is governed by hydrophobic interactions [29].

Even with a low surface area, glutaraldehyde-functionalized carbon (FAC) showed high immobilization efficiency, especially in an acidic medium (pH 3 and pH 5). This high immobilization capacity indicates that immobilization occurred through the spacer arms inserted in the support surface. Greater immobilization capacities in acidic media can be due to the incorporation of reactive groups (amine and aldehyde groups), which lead to the formation of a stable bond (Schiff base), mainly in acidic media due to the nucleophilic attack by the ε-amino group of lysine, responsible for facilitating the covalent interactions between enzyme and support, ensuring a high immobilization capacity. Furthermore, the glutaraldehyde molecules can form hydrophobic interactions, due to a hydrophobic portion formed by the glutaraldehyde dimer. They can also bind to one or two amino groups (cationic groups that can function as anion exchangers), forming an imine bond, showing that the immobilization on the support may also have occurred by ion exchange [12,59,60]. As FAC had lower BET surface area and pore volume when compared to other supports, the immobilization may have occurred preferentially through the formation of covalent bonds between the spacer arms and the enzyme.

Higher hydrolysis activity was observed for native enzyme (FP) and derivatives when solubilized and/or immobilized in more acidic conditions, mainly at pH 3. Increase in pH of the solution can lead to changes in enzyme structure, thus reducing the catalytic activity. According to Szałapata et al. [61], the reduction and/or loss of pepsin activity at pH values above 7.0 is expected, as the enzyme undergoes conformational changes in this pH range, transforming pepsin into pepsinogen, which leads to loss of activity. The enzymes immobilized at acidic pH showed higher activity when compared to immobilization at neutral/basic pH. This result is due to the optimal conformation of enzyme at acidic pH, especially in the pH range ranging from 2 to 4, with the active sites exposed, facilitating the hydrolysis process [29,62]. The present results showed that pH 3 can be considered as the optimum immobilization pH under the conditions studied.

3.3.2. Effect of carbon modification type on pepsin immobilization

After determining the optimal pH for pepsin immobilization, the effect of different carbon modifications on enzyme immobilization, and hydrolytic activity was investigated. The results for the enzyme immobilization capacity on different supports are shown in Table 3.

Table 3. Effect of activated carbon functionalization on enzyme immobilization at pH 3.0. Amount of bound enzyme (M_{Lig}), immobilization capacity (C_{Im}), immobilization efficiency (Effic), and enzymatic activity

Sample	M_{Lig} (mg)	C_{Im} (mg/g)	Efficiency (%)	Activity (U)
MQC	4.97 ± 0.05	99.01 ± 0.10 a	99.41 ± 0.001	1.91 ± 0.17 c
MAC	4.93 ± 0.01	98.93 ± 0.16 a	99.13 ± 0.001	2.30 ± 0.04 b
AC	4.96 ± 0.01	98.52 ± 0.24 a	99.20 ± 0.002	1.04 ± 0.01 e
GAC	4.91 ± 0.01	97.67 ± 0.39 b	98.25 ± 0.001	1.84 ± 0.01 d
FAC	4.92 ± 0.02	97.57 ± 0.40 b	98.39 ± 0.005	1.10 ± 0.01 e
FP	--	--	--	3.32 ± 0.01 a

* Means followed by the same letter in the columns do not differ significantly from each other by Tukey's test ($P < 0.05$).

Regarding the adsorptive capacity of the supports, all presented high pepsin immobilization capacity at pH 3.0, with immobilization efficiency higher than 98% for all materials. The highest immobilization capacities corresponded to metalized carbons (MAC and MQC) and AC, followed by genipin-functionalized carbon (GAC) and glutaraldehyde functionalized carbon (FAC), suggesting that the enzyme tends to be immobilized preferentially by adsorption (hydrophobic and ionic), followed by hydrogen

and covalent bonding. Figure 6 shows a schematic of the possible immobilization mechanism in the different supports.

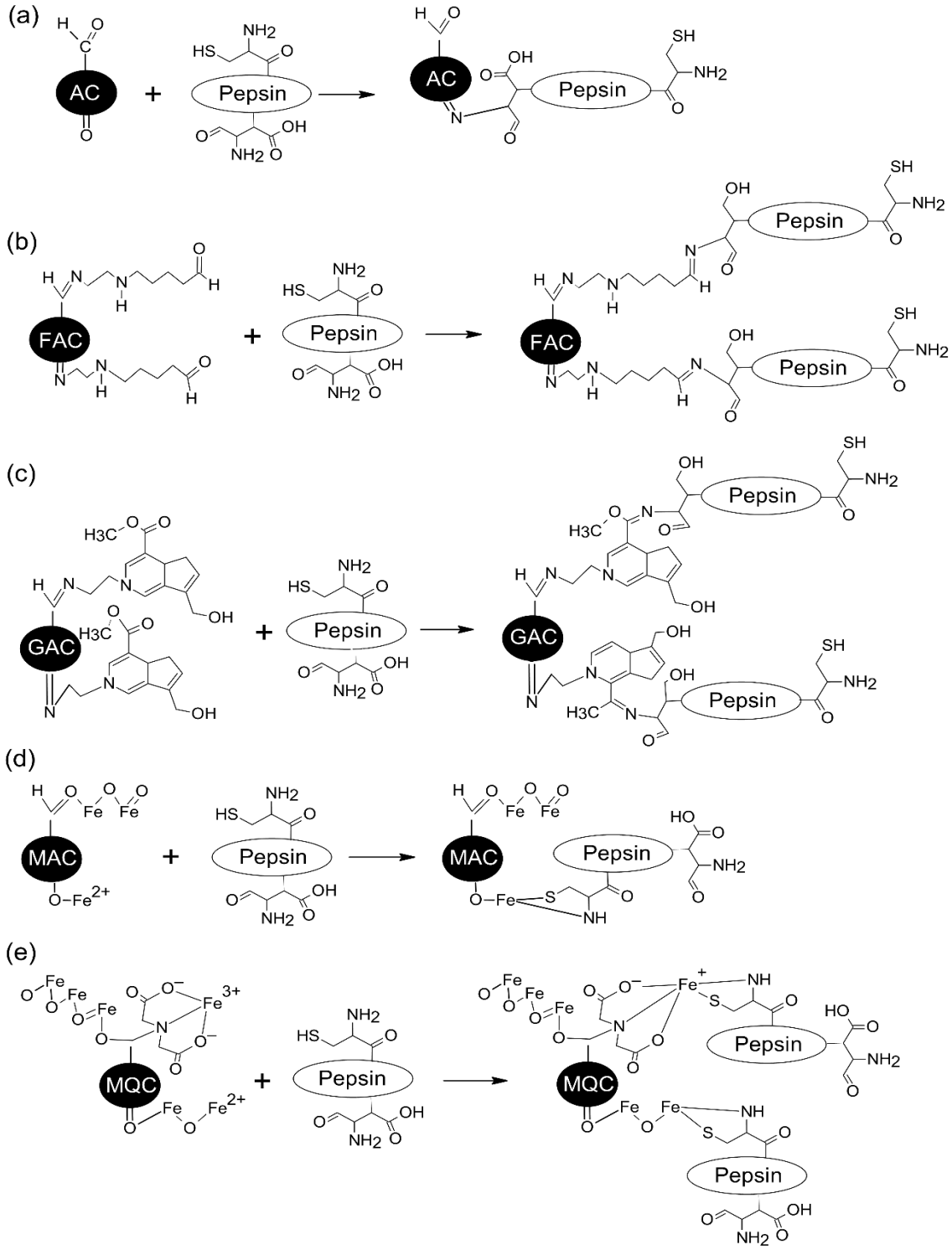


Figure 6. Possible mechanism of immobilization of pepsin in the different supports: AC (a), FAC (b), GAC (c), MAC (d) and MQC (e).

Lower immobilization capacity was observed for genipin-functionalized carbon (GAC) when compared to AC, which exhibited similar immobilization capacity of FAC. This result is satisfactory since the main function of genipin is replacement of glutaraldehyde for the formation of spacer arms, responsible for enzyme immobilization by covalent bonds, in addition to being a less toxic agent than glutaraldehyde. The effectiveness of genipin as a replacement for glutaraldehyde has been reported by some authors. Yu et al. [63] used genipin-crosslinked chitosan beads for berberine transport, and Flores et al. [64] used chitosan beads as catalytic support for enzyme immobilization. According to Flores et al. [64], the high immobilization capacity of genipin is related to ionic exchange followed by the formation of covalent bonds between the amino groups of the enzyme and the aldehyde groups of genipin. Under acidic conditions, the amino groups make a nucleophilic attack on carbon atom 3 (C-3), followed by opening the dihydropyran ring, which interacts with a secondary amino group on the newly formed aldehyde group. Therefore, genipin acts as a dialdehyde having two aldehyde groups available for binding, increasing its stability when compared to glutaraldehyde [64,65].

The incorporation of metallic particles into activated carbon, in the presence of a chelating agent (MQC) and without the use of chelating agent (MAC), was effective in enzyme immobilization onto the support, with immobilization efficiency close to 100%. According to Gennari et al. [66], the use of metallic particles in supports for enzyme immobilization confers greater immobilization power and a lower probability of precipitation losses. Enzyme interaction with the metalized support occurs through Van der Walls interactions, electrostatic interactions, as well as more specific interactions (covalent, coordinate, and hydrophobic interactions) between some enzyme amino acids and iron oxides from the carbon surface (hematite, magnetite, and/or maghemite) [67].

High immobilization capacity of these supports may also be due to the presence of the metal ions Fe^{2+} and Fe^{3+}, which act as cofactors in cysteine-proteases due to their strong interaction with the cysteine amino acids (Cys) from the pepsin structure, thus ensuring better enzyme stability [68].

In general, despite the lower pore volumes and BET surface areas of modified carbons, these materials were effective for pepsin immobilization. The results are also related to the immobilization pH (pH 3.0), once the acidic environment conferred positive charges on the surface of the supports. In contrast, the enzyme presented negative charges on its surface, favoring the immobilization by electrostatic interactions, especially in

metalized carbons, which had higher pHpcz values and metal groups that intensify such interactions [29].

Regarding enzyme activity, free enzyme (FP) showed greater hydrolytic activity due to its greater mobility, facilitating contact with the substrate. For derivatives, carbon modifications with genipin and metallization led to an increase in the hydrolytic capacity of the immobilized enzyme, especially for MAC, when compared to the traditional immobilization methods using AC and FAC.

Higher hydrolysis capacity of casein was observed for enzymes immobilized on genipin-functionalized carbon (GAC) when compared to traditional methods (AC and FAC), indicating that genipin can be used to replace glutaraldehyde, once it improved the catalytic activity of immobilized enzyme, in addition to being less toxic than glutaraldehyde. According to Klein et al. [13], immobilization using genipin has some advantages over the immobilization with glutaraldehyde, including lower cost, as it is a natural agent, and the maintenance of the enzyme activity, once glutaraldehyde can lead to the formation of stronger covalent bonds with a single enzyme, which can be linked by their active site, as well as compromising the enzyme structure.

Derivatives obtained using metalized supports (MAC and MQC) showed a higher hydrolysis capacity of casein. As reported by Khoshnevisan et al. [67], the enzyme immobilization in materials modified with metals improves the enzyme activity, and increases their stability, without changes in the structure. The higher activity may be due to the metal ions Fe^{2+} and Fe^{3+} acting as cofactors in cysteine-proteases due to their strong interaction with cysteine amino acids (Cys) from the pepsin structure. These ions provide immobilization in a more viable conformation, and expose the active site of the enzyme, due to the affinity between the cysteine groups of the enzyme and the ions on the support [68,69].

3.3.3. Stability of derivatives in high saline concentration and Tween 80

Results of derivatives stability by the Bradford assay were null for protein concentration in saline solution, indicating no enzyme desorption for all supports. The absence of enzymatic loss by desorption was also observed using Tween 80 for all derivatives obtained from functionalized carbons. The activated carbon (AC) showed a 4% loss of the immobilized enzyme, probably due to hydrophobic bonds between the support and the immobilized pepsin. These results show that the enzymes were

immobilized on the supports by strong interactions, such as covalent bonds and/or electrostatic interactions preventing enzyme losses by desorption.

4. CONCLUSION

Tamarind seeds have potential to be used as a carbon precursor material in the production of activated carbon, as the carbonaceous support presented a high BET surface area and average pore diameter in the mesopore range. It was possible to make different modifications in the activated carbon structure through the insertion of reactive groups, which were confirmed by FTIR, DRX, SEM, EDS, and texture analysis.

The optimal pH for pepsin immobilization on AC and FAC supports was pH 3. Supports functionalized by the methods proposed were effective for pepsin immobilization and provided an increase in its hydrolytic activity. The carbon modification with genipin showed greater immobilization capacity and hydrolytic capacity when compared to the functionalization with glutaraldehyde, indicating that it can be used as a substitute for glutaraldehyde, with the additional advantage of being less toxic. Modifications with metal ions presented the highest immobilization capacities and activity for the derivatives, providing better hydrolytic activity.

Declaration of competing interest

The authors declare that they do not have any conflict of interest

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CAPÍTULO 3

ARTIGO 2

Hydrolysis of casein by pepsin immobilized on heterofunctional supports to produce antioxidant peptides

Hidrólise da caseína pela pepsina imobilizada em suportes heterofuncionais para produzir peptídeos antioxidantes

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Hydrolysis of casein by pepsin immobilized on heterofunctional supports to produce antioxidant peptides

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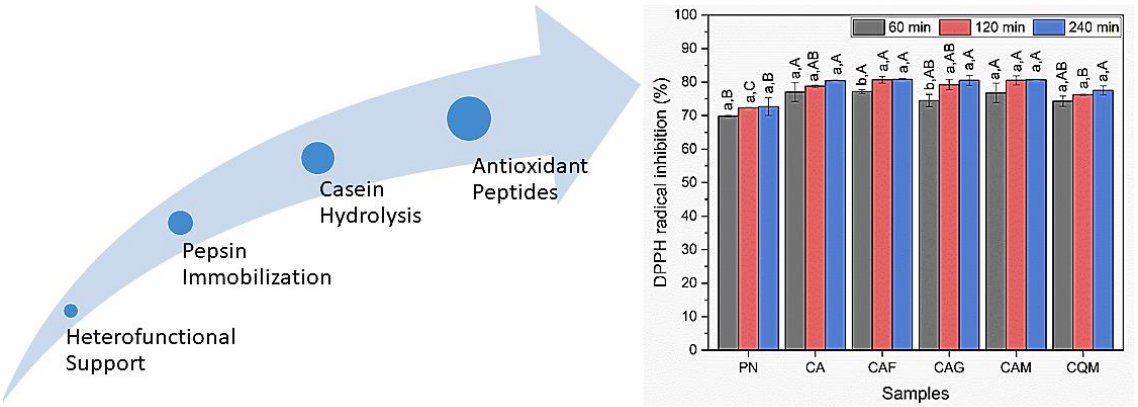
Abstract

Antioxidant hydrolysates from bovine casein were produced by enzymatic hydrolysis, using pepsin immobilized on activated carbon modified by different techniques (functionalization with glutaraldehyde, genipin, and iron ions with and without the presence of a chelating agent). The potential for reuse of the derivatives, storage stability, kinetic parameters, and antioxidant potential of peptides was evaluated. The pH 3.0 was selected to perform the hydrolysis reactions, due to the higher activity of the derivatives at this pH condition. Iron ion metallization was the modification method with the best results, providing an enzyme activity of 2.30 U, higher than the other modifications and closer to the activity of the native enzyme (3.32 U). Moreover, this derivative showed greater storage stability and allowed the reuse for 8 more cycles when compared to the native enzyme. In turn, the peptides from casein hydrolysis showed potential as antioxidant agents, with a DPPH radical scavenging activity higher than 70%, maximum protection against β-carotene oxidation close to 70%, and a maximum reducing power of Fe (III) into Fe (II) of 400 uM by the FRAP assay. The results showed that the new techniques for modification of activated carbon can be a promising approach for pepsin immobilization.

Keywords: Enzyme activity; Derivatives; Protease.

Graphical abstract



1. INTRODUCTION

Pepsin (E.C. 3.4.23.1) is a protease of animal origin that acts together with chymotrypsin and trypsin in metabolic processes in the hydrolysis of proteins to produce available nutrients in the form of peptides and easily absorbed essential amino acids [1]. It is one of the most widely used enzymes for analysis of other proteins due to its specificity in cleaving bonds involving aromatic amino acids (phenylalanine, tryptophan, and tyrosine) [2,3]. Pepsin is also widely used industrially, mainly in the food industry as a milk coagulant for cheese manufacture, in the production of animal and plant protein hydrolysates, as well as the hydrolysis of soybean allergens. Although it presents a wide range of applications in many different sectors, its use in the native form presents some limitations due to the costs of high purity enzymes, as well as the impossibility of separating the enzyme from the end product, thus impairing its reuse, and the low stability under certain operational conditions (pH, temperature, salt concentration, organic solvents, etc) that lead to the loss of its three-dimensional structure [4,5]. However, these limitations can be overcome by using immobilized enzymes.

The use of immobilized proteases has been widely studied to improve their operational stability after immobilization, with the exploitation of their catalytic advantages. Moreover, the immobilization contributes to higher enzyme stability in continuous processes, prevents leaching, makes the enzyme reusable, and allows the free diffusion of substrates and reaction products, providing more efficient control of large-scale processes [3].

Among the main supports used in the enzyme immobilization process, activated carbon has gained prominence for the immobilization of proteases for presenting ideal characteristics to be used as a support [1,3,6]. Activated carbon can have its surface modified by different methods to create and/or increase the functional groups already existing on its surface, improving the enzyme immobilization efficiency, as well as enhancing its catalytic efficiency [6,7]. In addition, the support functionalization can improve the operational stability of the derivatives in continuous and discontinuous processes, which is an interesting approach from the industrial point of view [8].

The derivatives obtained through the immobilization of digestive enzymes on activated carbon can be used in the hydrolysis of casein from different sources to produce bioactive peptides [1]. Besides their nutritional role, these peptides can also provide some beneficial biological activities, including antioxidant effects, anti-inflammatory,

antihypertensive, antimicrobial, and anticancer properties, with a potential role in promoting human health [1,9]. Among the bioactive peptides from casein hydrolysis, antioxidant peptides have gained attention due to their ability to remove excess free radicals, inhibit lipoxygenase activity, decompose oxides, and enhance anti-aging and disease resistance in the human body [10,11].

Studies of proteases immobilized on activated carbon have shown the efficiency of the derivatives from casein hydrolysis, with the production of peptides with antihypertensive properties [1,3]. Given these results, this study aimed to evaluate the catalytic activity of pepsin immobilized on activated carbon modified by different techniques (functionalization with glutaraldehyde, genipin, and iron ion particles with and without the presence of a chelating agent) in the hydrolysis reaction of casein. Its potential for reuse, the stability of the derivatives, the hydrolysis kinetic parameters, and the antioxidant potential of the bioactive peptides was also investigated.

2. METHODOLOGY

2.1. Materials

The following supports were used for immobilization: (i) activated carbon synthesized from tamarind seeds (AC); (ii) glutaraldehyde-functionalized carbon (FAC); (iii) genipin-functionalized carbon (GAC); (iv) metallized activated carbon (MAC); and (v) metallized activated carbon in the presence of a chelating agent (MQC). The methodologies used in the synthesis of the different supports as well as the results of characterization are described in a previous study [6]. Pepsin (pepsin from porcine gastric mucosa, 87% protein, 3,200 - 4,500 U/mg, CAS: 9001-75-6) and casein (casein from bovine milk - CAS 9000-71-9) were purchased from Sigma Aldrich. The other reagents used in the experiment were of at least analytical grade and were described throughout the methodology.

2.2. Pepsin immobilization on different supports

The pepsin immobilization was performed at pH 3.0 as described by Santos et al. [6]. pH 3.0 was selected for this study once previous studies have shown that this pH condition presents better results for pepsin immobilization on activated carbons, with immobilization efficiency higher than 98% [6].

2.3. Effect of reaction medium pH on enzyme activity

The enzyme activity, both for the native pepsin and the derivatives, was determined as described by Santos et al. [3], with modifications. For that, different solutions of bovine casein 2 % (w/v) were prepared at pH 3.0, 5.0, 7.0, and 9.0, to determine the best hydrolysis conditions.

Pepsin activity in its native form was determined using a 2 % (w/v) bovine casein solution as a substrate. Casein solution was prepared in lactic acid/sodium lactate buffer 0.05 mol.L^{-1} for the trial at pH 3.0, and sodium phosphate buffer 0.05 mol.L^{-1} for the trials at pH 5.0, 7.0, and 9.0. In a centrifuge tube, 1 mL of enzyme solution at pH 3.0 and 5 mL of substrate (solubilized in the different pH values) were incubated at 40 °C in a thermostatic bath (Tecnal, TE-205, SP, Brazil) for 60 min. After this period, 2 mL of trichloroacetic acid (6.5 %, w/v) was added under stirring to stop the reaction. Then, the tubes were centrifuged at 6000 rpm for 6 min at 4 °C (SPLabor, SP-701, SP, Brazil). After this step, 1 mL of the supernatant was removed and mixed with 5 mL of sodium carbonate solution (4 % w/v) and 1 mL of Folin-Ciocalteu solution (20 %, v/v). The mixture was kept at 40 °C for 20 min, and absorbance readings were performed at 660 nm in a spectrophotometer (UV-Vis Quimis, Q898UV2, SP, Brazil). One unit (U) of protease activity was defined as the amount of enzyme required to release 100 µg of tyrosine under the assay conditions.

To determine the activity of the immobilized enzyme, the standard derivatives (pepsin immobilized on AC and FAC supports) were used, according to the previous methodology with minor modifications. For that, 5 mL of casein solution (2 % w/v) were added to 50 mg of derivatives in centrifuge tubes and incubated at 40 ºC for 60 min. Then, the tubes were centrifuged (3000 rpm/5 min), and all the supernatant was transferred to another tube containing 2 mL of trichloroacetic acid (6.5 % w/v). The tubes were centrifuged (6000 rpm /6 min /4 °C) and 1 mL of the supernatant was sampled and set to react as described above. A control (blank) was also performed, using the supports without the enzyme.

2.4. Reuse of the derivatives

After determining the optimal pH of reaction medium for the standard derivatives, studies on the activity and cycles of reuse were performed with all derivatives, using the optimal pH condition. The purpose of this study was to evaluate the number of cycles of use the enzymes maintain their hydrolysis activity, thus determining the type of support

modification that provides a derivative with greater stability. The methodology described in a previous study [3] was used. After each hydrolysis cycle, 5 ml of the immobilization buffer (lactic acid/sodium lactate buffer 0.05 mol.L^{-1}, pH 3.0) was added to the tubes containing the derivatives, which were agitated (20 rpm/5 min) and centrifuged (3000 rpm/3 min) to remove casein residues, providing the derivatives for use in a new hydrolysis reaction.

2.5. Stability during the storage

Activity of the native pepsin and the derivatives was determined after storage for up to 30 days at 4 ºC, using test tubes containing the immobilization buffer (lactic acid/sodium lactate 0.05 mol.L^{-1} pH 3.0). The activity was determined at days 0, 7, 15, and 30 of storage, to assess whether the enzymes maintain their stability during storage. The methodology described in Section 2.3 was used, using the optimal pH of the reaction medium. The derivative that provided the best enzyme stabilization was used for the subsequent trials.

2.6. Effect of temperature of the reaction medium on the enzyme activity

The effect of temperature (20, 30, 40, 50, 60, and 70 °C) on the casein hydrolysis reaction was studied to evaluate whether the immobilization provides a wider range of use when compared to the native enzyme. The determination of enzyme activity was performed as described in Section 2.3 using the optimal pH of the reaction medium.

2.7 Determination of the kinetic parameters of hydrolysis reaction

The kinetic parameters were determined for the native enzyme and the derivative with the best results in the previous assays. For that, the enzyme activity was determined using different initial casein concentrations (0.1 - 2 % w/v). The results allowed the construction of a curve with the values of the initial reaction rates as a function of casein concentrations. The maximum velocity (V_{max}) and the Michaelis - Menten constant (Km) were determined from the Lineweaver-Burk equation (Equation 1).

$$\frac{1}{V_0} = \frac{K_m}{V_{max}+[S]_0} + \frac{1}{V_{max}} \quad (1)$$

Where V_0 is the reaction velocity, V_{max} is the maximum reaction velocity, K_m is the Michaelis-Menten constant, and $[S_0]$ is the substrate concentration (casein)

2.8. Antioxidant potential *in vitro* of peptides from hydrolysis of bovine casein

Whereas the peptides from casein hydrolysis present antioxidant potential, antioxidant activity tests were performed using the peptides from hydrolysis reaction at 1 h, 2 h, and 4 h, using the methods described below.

2.8.1. DPPH free radical scavenging activity

The scavenging activity of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was estimated as described by Li et al. [12], with modifications. An ethanolic solution of DPPH radical (0.2 mM) was used with initial absorbance adjusted to the range of 0.6 to 0.7 in a spectrophotometer at 515 nm. For the analysis, 500 μL of the DPPH solution was added to 500 μL of the sample in test tubes previously lined with aluminum foil. The mixture was homogenized and kept at room temperature for 30 min protected from light. The absorbance of samples was measured at 515 nm. Antioxidant activity was determined through the inhibition of the DPPH radical according to Equation 2.

$$\%DPPH\ inhibition = \left[\frac{(A_C - A_S)}{A_C}\right] \times 100 \quad (2)$$

Where, A_C and A_S are the absorbance readings of the control and the sample, respectively.

2.8.2. Inhibition of β-carotene-linoleic acid oxidation

Antioxidant activity using β-carotene-linoleic acid system was determined as described by Aminjafari et al. [13], with modifications. Initially, water was saturated with oxygen for 30 min. During the saturation process, the reactive mixture was made by adding 50 μg of linoleic acid, 200 mg of Tween 80 in 1 mL of β-carotene solution (1 $mg.mL^{-1}$ in chloroform). Then, the mixture was agitated in a rotary evaporator (IKA®HB Digital, RV8, USA) at 50 °C for 10 min for chloroform evaporation. After solvent evaporation, 50 mL of saturated water was added under vigorous stirring (750 rpm) using a magnetic stirrer. The reactive mixture presented absorbance values between 0.6 and 0.7 at 470 nm. For the blank, a reactive mixture was made without the addition of the β-carotene solution. Aliquots of 5 mL of the emulsion were transferred to tubes containing 500 μL of the sample, followed by immediate stirring and absorbance readings at 470 nm at two times, before and after incubation for 2 h in a water bath at 50 °C. The results were expressed as a percentage of protection against oxidation (Equation 3).

$$\% \, Protection = \, [1 - \left[\frac{\left(ABS_{0\ sample} - ABS_{120\ sample}\right)}{\left(ABS_{0\ control} - ABS_{120\ control}\right)}\right] \, x\, 100 \qquad (3)$$

2.8.3. FRAP assay (Ferric Reducing Antioxidant Power)

FRAP assay was performed as described by Liu et al. [14], with modifications. FRAP solution was prepared using 0.3 M acetate buffer (pH 3.6), 20 mM ferric chloride hexahydrate ($FeCl_3$-$6H_2O$) and 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) at a ratio of 10: 1: 1 (v / v / v), respectively. Then, 50 µL of the sample were mixed with 150 µL of distilled water and 1500 µL of the FRAP solution, vortexed, and kept in a water bath at 37 °C for 30 min. After this time, absorbance readings were performed at 595 nm. FRAP reagent was used as a blank to calibrate the spectrophotometer. A standard curve of ferrous sulfate heptahydrate ($FeSO_4$-$7H_2O$) was prepared at varying concentrations in the range of 50-2000 µM. The FRAP values of samples were determined using the standard curve and expressed as µM of Fe (II) equivalent per µl of sample.

2.9. Characterization of peptides from casein hydrolysis by chromatography

The peptides obtained from casein hydrolysis in native and derivatives presence at 1 h, 2 h, and 4 h were analyzed by reversed-phase liquid chromatography (RP-HPLC). A C18 column (250x4.6 mm ID, 5 mm particle size; ZORBAX Eclipse Plus C18) coupled to a pre-column (ZORBAX SB-C 18, 4.6 mm ID x 12.5 mm, 5 µm) connected to an Agilent 1260 Infinitty II HP system was used. Two mobile phases were used; the first (A) composed of a solution of acetonitrile (5 % v/v) (RIEDEL-DE-HAEN) and trifluoroacetic acid (0.1 % v/v) (Dynamics LTDA), and the second phase (B) formed by acetonitrile (50 % v/v) and trifluoroacetic acid (0.1 % v/v). A 20µL aliquot of sample was automatically injected into the column at a flow rate of 1mL/min of mobile phases A and B for 70 minutes. The analysis started with a flow rate of 100 % of mobile phase A and decreased linearly for 60 minutes until 100 % of mobile phase B; a new linear gradient was performed from 60 to 65 minutes to establish the flow rate at 100 % of A, which was maintained until the end of the analysis (70 minutes). The temperature of the run was kept at 30 °C. Peptides were detected at 280 nm in a UV-Vis detector, and the identification was carried out by comparing the retention times of the peaks and their UV spectra.

2.10. Statistical analysis

All experiments and analyses were performed in triplicate. Data were analyzed by analysis of variance and the differences between the means of hydrolytic capacity in the

different storage periods were analyzed by Tukey's test ($p < 0.05$), using the programs SAS Studio and Origin Pro 8.0.

3. RESULTS AND DISCUSSION

3.1. Support Properties

The main properties of activated carbon (AC), glutaraldehyde-functionalized activated carbon (FAC), genipin-functionalized carbon (GAC), metalized carbon (MAC), and metallized carbon in the presence of a chelating agent (MQC) are presented in Table 1 [6].

Table 1. Specific surface area (Sg); pore diameter (Dp); mesopore volume (Vmeso), micropore volume (Vmicro), and pH of zero charge (pH_{pcz}) of the supports

Sample	Sg (m^2/g)	Dp (nm)[a]	V_{meso} (cm^3/g)	V_{micro} (cm^3/g)	pH_{pcz}
AC	1082	4,88	0,452	0,166	4,84
CAF	40	8,12	0,031	0,009	5,45
CAG	221	5,94	0,262	0,004	6,35
CAM	924	5,20	0,442	0,117	7,63
CQM	892	5,24	0,416	0,151	8,76

[a] Maximum pore size distribution; Source: Santos et al. [6].

As reported by Santos et al. [6], the support modifications led to a reduction of the porosity and consequent reduction of the specific area due to the incorporation of the surface modifying agents. In addition, a change in the pH of zero charge of modified carbons was observed due to the modification of the functional groups present on their surface.

3.2. Effect of pH of the reaction medium on the activity of derivatives during the casein hydrolysis

pH effect of the reaction medium on pepsin activity during the casein hydrolysis reaction is shown in Table 2.

When evaluating the reaction medium pH effect on activity of native and immobilized pepsin during casein hydrolysis, an increase in the solubilization pH led to a decrease in hydrolysis activity for all samples. The best activities were observed in an acidic reaction medium (pH 3.0). This behavior may be due to the acidic character of pepsin, which tends to lose its catalytic activity when subjected to higher pH values

[3,15]. Similar results were observed by Santos et al. [3], with pepsin immobilized on activated carbon from pupunha palm heart sheaths. Hu et al. [16], studied the pepsin immobilization by covalent binding on PMMA microspheres and reported higher pepsin activities at pH values near 2.5. Zhang et al. [15], studied pepsin immobilization by covalent binding on monolithic support and reported that the optimal pH for immobilization and application of pepsin in casein hydrolysis was close to 3.0.

Table 2. Effect of the initial pH of casein solution on the activity of native pepsin (NP) and pepsin immobilized on activated carbon (AC) and glutaraldehyde-functionalized carbon (FAC)

Sample	Enzyme activity (U)			
	Initial pH of the reaction medium (casein)			
	3.0	**5.0**	**7.0**	**9.0**
AC	1.04 ± 0.01 a	0.62 ± 0.01 b	0.15 ± 0.01 c	0.09 ± 0.01 d
FAC	1.10 ± 0.01 a	0.63 ± 0.01 b	0.20 ± 0.04 c	0.08 ± 0.01 d
NP	3.32 ± 0.01 a	0.31 ± 0.01 b	0.96 ± 0.01 c	0.09 ± 0.01 d

*Médias seguidas de mesma letra minúscula, na horizontal (linhas), não diferem significativamente entre si pelo teste Tukey ($p < 0,05$).

Comparing the behavior of native enzyme and derivatives, the derivatives presented a higher activity at pH 5.0, probably due to less conformational change of the immobilized enzyme. For the other pH values, a lower activity was observed for the derivatives when compared to the native enzyme. This behavior was expected since native enzyme presents higher mobility, facilitating contact with the substrate. In turn, for immobilized enzymes, the substrate must be transferred (by diffusion) from within the solution to the catalytic sites. Another factor that affects the activity of the immobilized enzyme is the conformation during immobilization, which can lead to reduced activity when the active site is not accessible to the substrate.

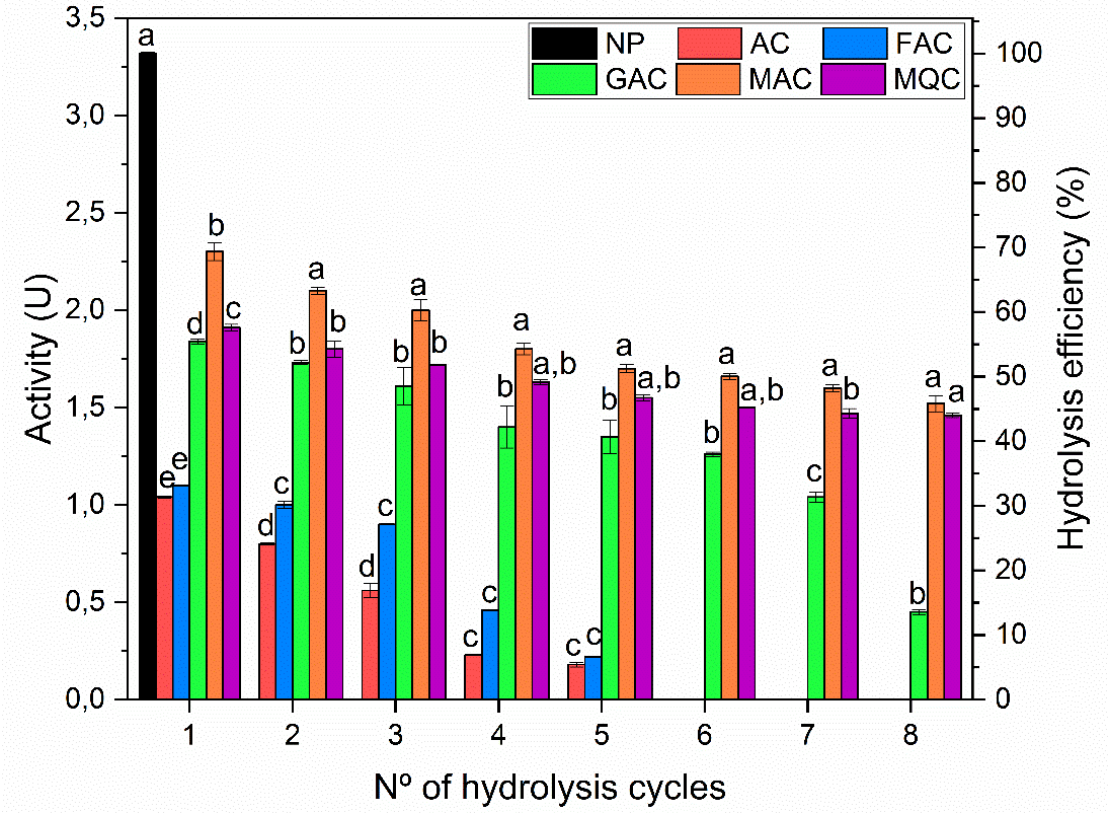
Based on the results, pH 3.0 was selected as the optimal condition to perform the casein hydrolysis reaction in the subsequent tests with the other supports.

3.3. Reuse of derivatives

After establishing pH 3.0 as the optimal pH to perform the casein hydrolysis, pepsin was immobilized on the other supports, and the activity of derivatives was evaluated in subsequent cycles of use. The results of enzyme activity and hydrolysis

efficiency for the cycles of reuse are presented in Figure 1. The hydrolysis efficiency of the native enzyme (3.32 U) was considered as 100% and used as a base to determine the hydrolysis efficiency of the derivatives. The results showed that it was possible to reuse some derivatives for up to 7 cycles with no loss of activity.

Figure 1. Activity of pepsin immobilized on activated carbon (AC), glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC); metalized activated carbon (MAC), and metalized carbon in the presence of a chelating agent (MQC) for the cycles of reuse.



*Means followed by the same letter for each cycle do not differ significantly by the Tukey's test ($P < 0.05$).

Regarding the cycles of reuse, the pepsin immobilized on AC had an initial hydrolysis efficiency of 31.5%, losing its activity over the cycles, reaching a total of 5 consecutive cycles of hydrolysis. The loss of activity may be due to enzyme desorption during the cycles, which was found in a previous study on the stability of the derivatives in saline (NaCl 1.5 mol.L^{-1}) and Tween 80 solutions [6]. The derivative immobilized on the support with glutaraldehyde showed an initial hydrolysis efficiency of 33.0 %. However, as also observed for AC, there were losses over the hydrolysis cycles, which was lower in the initial cycles, probably due to the stronger interactions involved in the immobilization process, once glutaraldehyde can lead to the formation of covalent bonds

between the support and pepsin. Although FAC-immobilized enzyme maintained a residual activity for up to 5 consecutive cycles of hydrolysis, all cycles together were equivalent to one cycle with the native enzyme. This result reinforces the need to study new methods of surface modification of activated carbons that provide increased activity of the immobilized enzyme.

When comparing the activity of standard derivatives (AC and FAC) and the derivatives obtained using the new modification methods, the latter maintained the enzyme activity for up to 8 consecutive cycles of casein hydrolysis. The GAC-immobilized pepsin exhibited higher activity when compared to the FAC-immobilized enzyme, indicating a potential substitution to glutaraldehyde. A previous study showed that the replacement of glutaraldehyde with genipin led to an increase in the immobilization capacity of pepsin, besides improving its activity. Therefore, it can be used as a substitute for glutaraldehyde in the surface modification of activated carbon, with promising results, in addition to the lower toxicity of genipin (10000 lower) when compared to glutaraldehyde [6,17]. The hydrolytic activity of the derivative was 55.7 % of the activity of the native enzyme in the first cycle, 31.5 % in the penultimate cycle, and 13.6 % in the last cycle. It is worth mentioning that the first two cycles were sufficient to overcome the activity of the native enzyme.

Higher hydrolytic capacity was observed for metalized carbons derivatives, allowing obtaining 8 consecutive cycles of hydrolysis. According to Liang et al. [18], the support metallization leads to higher stability of enzymes, preventing desorption during the cycles of use.

Hydrolysis capacity of MAC and MQC derivatives was 70.0 and 57.4 % of native enzyme activity in the first cycle, and 46.2 and 41.4 % in the last cycle, respectively. As observed for enzymes immobilized on genipin-functionalized carbon, only two cycles were required for the pepsin immobilized on metalized carbons to overcome the activity of native enzyme, thus offsetting the immobilization costs [3].

Concerning the cycles of metalized derivatives, the use of a chelating agent provided lower activity loss during the cycles due to the higher amount of magnetic particles in its structure, as reported by Santos et al. [6], who used X-ray diffraction (XRD) technique and stated that MAC showed a diffraction peak at 2θ = 62.3° corresponding to diffraction of magnetite and hematite structures (Fe_3O_4 and α-Fe_2O_3, with 440 and 214 planes) respectively, both present in magnetic nanoparticles. In turn, activated carbon with iron salts in presence of a chelating agent (MQC) showed

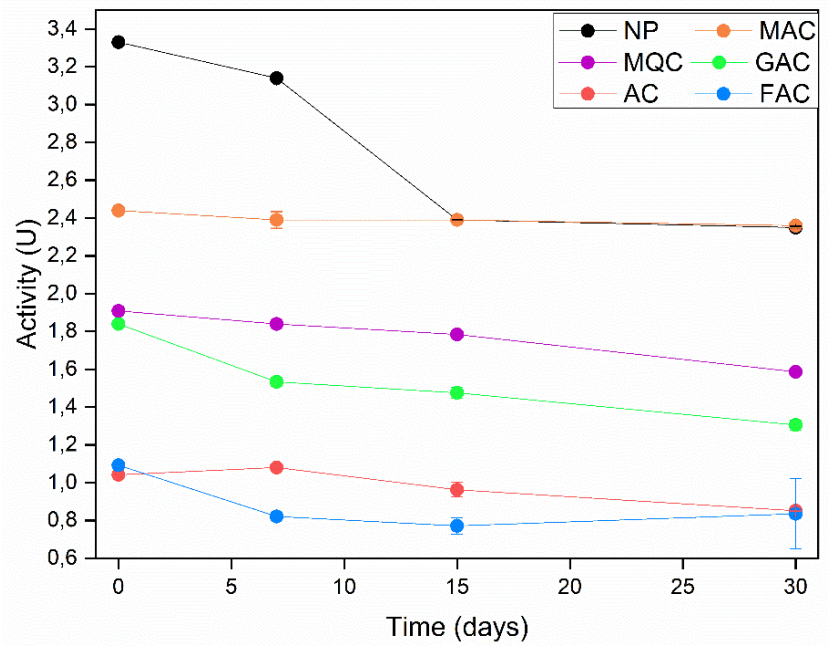
diffraction peaks at 2θ = 24.6° referring to hematite (α-Fe_2O_3, plane 012); at 35.4° corresponding to diffraction of magnetite (Fe_3O_4, plane 311); at 41.8° corresponding to FeO (wustite with plane 200) and peaks at 52.7 and 57.1º, both corresponding to magnetite crystal diffraction in planes 422 and 511, thus ensuring greater enzymatic stability. In addition to ensuring higher metal ion content, iminodiacetic acid protects metal particles from desorption [19]. However, the use of a chelating agent did not provide a significant increase in immobilization or pepsin activity when compared to MAC.

The results of blank for supports without enzyme showed no hydrolytic activity, thus the supports did not interfere with the results of enzyme activity.

3.4. Effect of hydrolysis temperature and storage stability

The results of native enzyme and immobilized enzyme stability to storage time are presented in Figure 2.

Figure 2. Stability of native pepsin (NP) and pepsin immobilized on activated carbon (AC), glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC), metalized activated carbon (MAC) and metalized carbon in the presence of a chelating agent (MQC).



* Means followed by the same uppercase letter for the hydrolysis time and lowercase letter for the methods (NP, AC, FAC, GAC, MAC, and MQC) do not differ significantly by Tukey's test ($P < 0.05$).

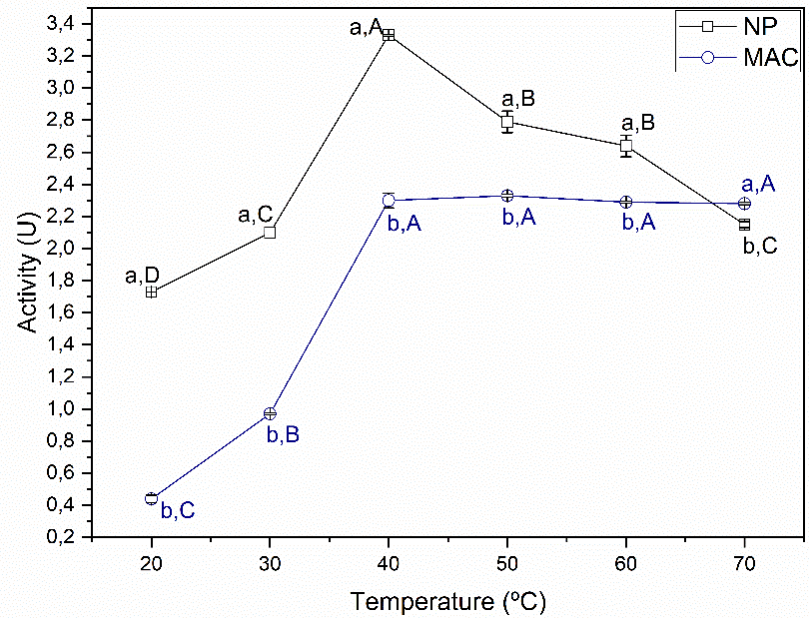
The results of stability during the storage at 4 °C showed a reduction in hydrolytic capacity of native enzyme with increasing storage time, with a reduction of 5.4 and 28.1

% after 7 and 15 days of storage, respectively. The loss of activity of native enzyme is a natural process and is dependent on the time that they remain in solution. In addition, protein-protein interactions (pepsin autolysis) can also lead to loss of activity [20].

Stability to storage time was also evaluated for derivatives aimed to select the most stable derivative for subsequent assays. Standards derivatives (enzymes immobilized on AC and FAC) showed a reduction in activity of 18.4 and 35.5 %, respectively, at the end of the storage time. Pepsin immobilized on GAC had a loss of 30.5 % and was close to pepsin immobilized FAC. These losses may be due to desorption losses of immobilized enzyme during the storage. The metallization-modified matrices MQC and MAC showed the lowest losses of activity, with values of 15.5 and 4.9 %, respectively, at the end of the storage. The greater stability to desorption is due to the presence of metal groups that increase the stabilization of proteases immobilized on the support [21]. Therefore, MAC was selected as the best support for pepsin immobilization, due to the number of recycles obtained, with the lowest loss of activity among them, and for remaining stable during the storage time (30 days), without significant loss in its activity.

The results of temperature effect on casein hydrolysis of derivative (MAC) when compared to the native enzyme (NP) are shown in Figure 3.

Figure 3. Effect of temperature on the hydrolysis reaction of native pepsin (NP) and pepsin immobilized on metalized carbon (MAC).



*Means followed by the same lowercase letter for the temperature between methods and uppercase letter for each method (NP and MAC) do not differ significantly from each other by Tukey's test ($P < 0.05$).

Native enzyme had an activity peak at 40 ºC followed by loss of activity with increasing temperature. Derivative from MAC showed higher activity with increasing temperature, probably due to increased diffusion of substrate on support, and remained constant from the temperature of 40 ºC. In general, besides allowing the reuse of the enzyme for several cycles, pepsin immobilization in metalized carbon led to improved thermal stability when compared to native enzyme, increasing its range of action and allowing its use under higher temperature conditions. According to Han et al. [22], the loss of activity of native enzyme is associated with its conformational flexibility with increasing temperature, while immobilized enzyme remains intact due to the bonds formed with the enzyme support.

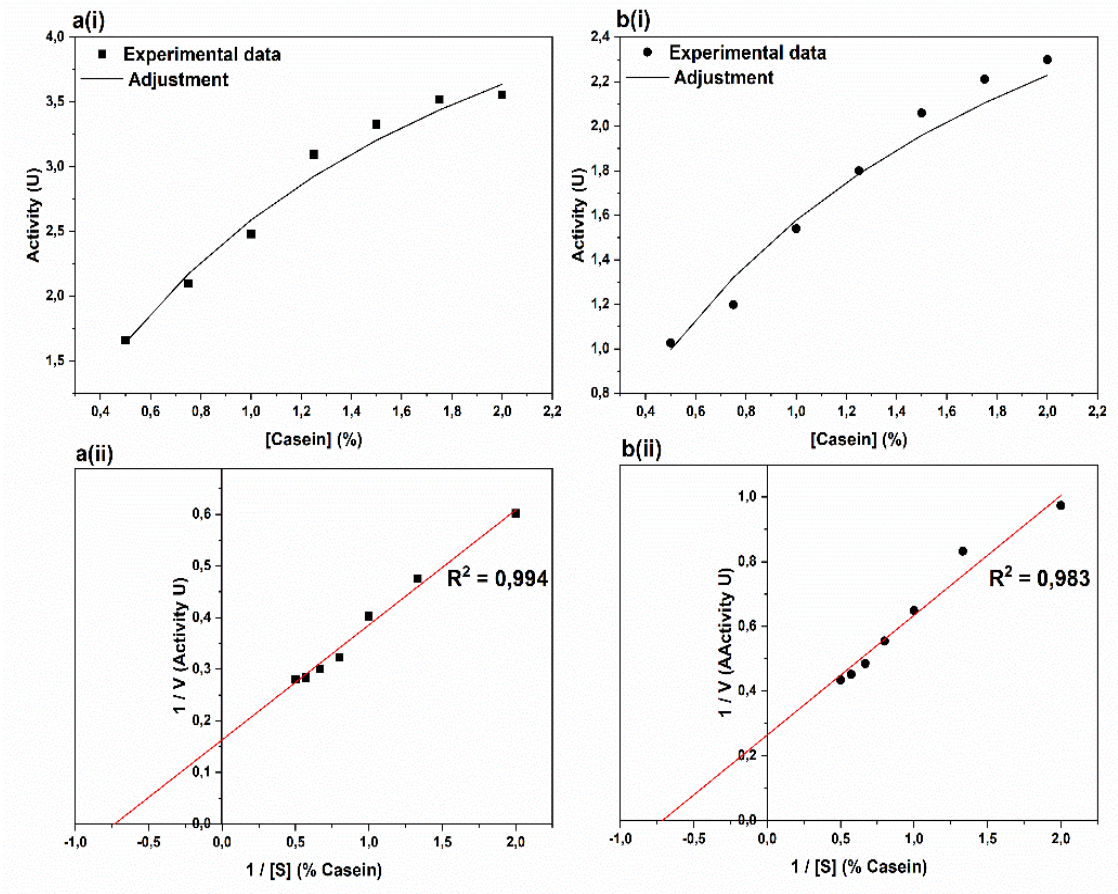
Pepsin immobilized on metalized carbon (MAC) maintained its activity of hydrolysis of bovine casein during the whole time of storage, besides presenting stability in a wide temperature range, which can compensate for the costs of the immobilization process [23].

3.3. Determination of kinetic parameters

Kinetic parameters (maximum velocity of bovine casein hydrolysis (V_{max}), and the respective Michaelis-Menten constant (Km) of native enzyme and pepsin immobilized on metalized carbon (MAC) were calculated according to the curves presented in Figure 4.

The graphs allowed determining the V_{max} and the Km values of native enzyme and derivative, with values of 6.18 and 3.79 U/min, and 1.37 and 1.40, respectively. According to Hegedüs et al. [24], V_{max} value measures the number of substrate molecules that are converted into a product by one enzyme molecule in a given time. Therefore, the higher Vmax of native enzyme was expected since native enzyme has a higher activity due to its higher mobility and easier access to the substrate. On the other hand, some enzymes are confined in the carbon pores of derivative, hindering the access of the substrate that must diffuse to the interior of the pores, reducing the reaction rate. Michaelis-Menten constant (Km) indicates the degree of affinity of enzyme for substrate. In this study, close values were observed, indicating that both native enzyme and derivative presented a similar affinity for substrate, and the higher the affinity, the lower the constant value. Furthermore, the constant Km of derivative was almost equal to Km of the native enzyme, which indicates that the immobilization did not cause structural changes in enzyme, maintaining the same catalytic active sites [25].

Figure 4. Changes in activity as a function of the initial casein concentration and plots after fitting the Lineweaver-Burk equation for native enzyme, a (i and ii), and enzyme immobilized on metalized carbon, b (i and ii).



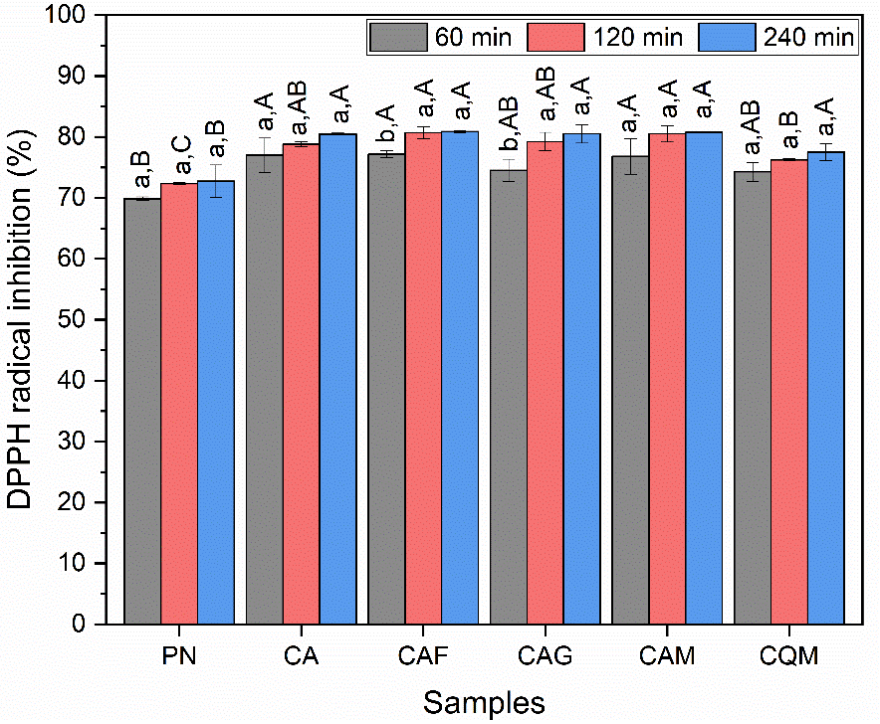
3.4. Determination of antioxidant activity of peptides from casein hydrolysis

Antioxidant activity measured by the DPPH radical inhibition of peptides from different hydrolysis times (1 h, 2 h, and 4 h) ranged from 69.9 to 80.9 % (Figure 5).

The results of DPPH radical inhibition (Figure 5) showed no significant effect of hydrolysis time to produce peptides using native enzyme (NP) and derivatives AC, MAC, and MQC, with similar inhibition potentials. In turn, derivatives immobilized on FAC and GAC showed higher antioxidant capacities in the DPPH assay for peptides from the hydrolysis times of 2 h and 4 h.

Regarding the effect of support, peptides from immobilized pepsin led to a greater and/or equal radical inhibition when compared to those obtained by native enzyme, therefore indicating a higher antioxidant potential.

Figure 5. DPPH antioxidant activity of peptides from casein hydrolysis at different hydrolysis times for native pepsin (NP) and pepsin immobilized on activated carbon (AC) glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC), metalized activated carbon (MAC), and metalized carbon in presence of a chelating agent (MQC).

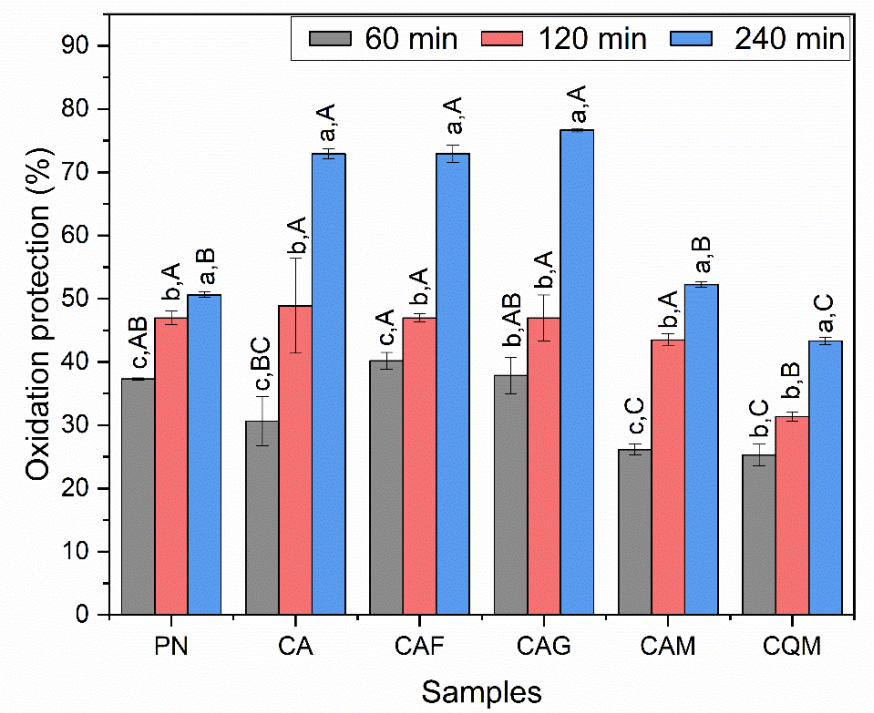


*Means followed by the same lowercase letter in the sample and uppercase letter in the hydrolysis time, for the same sample, do not differ significantly from each other by the Tukey's test ($P < 0.05$). *The bars at each point indicate the statistical deviations.

Antioxidant activity determined by the inhibition of β-carotene-linoleic acid oxidation of peptides from different hydrolysis times (1h, 2h, and 4h) ranged from 25.3 and 76.7% (Figure 6). Therefore, the increase in hydrolysis time led to a higher protection of peptides against oxidation of β-carotene and linoleic acid, with the highest protection observed for peptides from pepsin immobilized on AC, FAC, and GAC, hydrolysis time of 4 h.

As can be seen in Figure 6, derivatives with metals on their surface (MAC and QMC) provided lower protection when comparing antioxidant capacities of derivatives, probably due to leaching of these components during hydrolysis, which may lead to a reduction in the protective effect since these metals have pro-oxidant potential. Metal ions, especially copper and iron, tend to catalyze lipid oxidation, which may have led to the reduced antioxidant power of peptides obtained from metal supports [26].

Figure 6. Antioxidant activity by the β-carotene-linoleic acid system of peptides from casein hydrolysis at different hydrolysis times for native pepsin (NP) and pepsin immobilized on activated carbon (AC) glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC), metalized activated carbon (MAC) and metalized carbon in presence of a chelating agent (MQC).



* Means followed by the same lowercase letter in the sample and the same uppercase letter in the hydrolysis time, for the same sample, do not differ significantly from each other by the Tukey's test ($P < 0.05$). *The bars at each point indicate the statistical deviations.

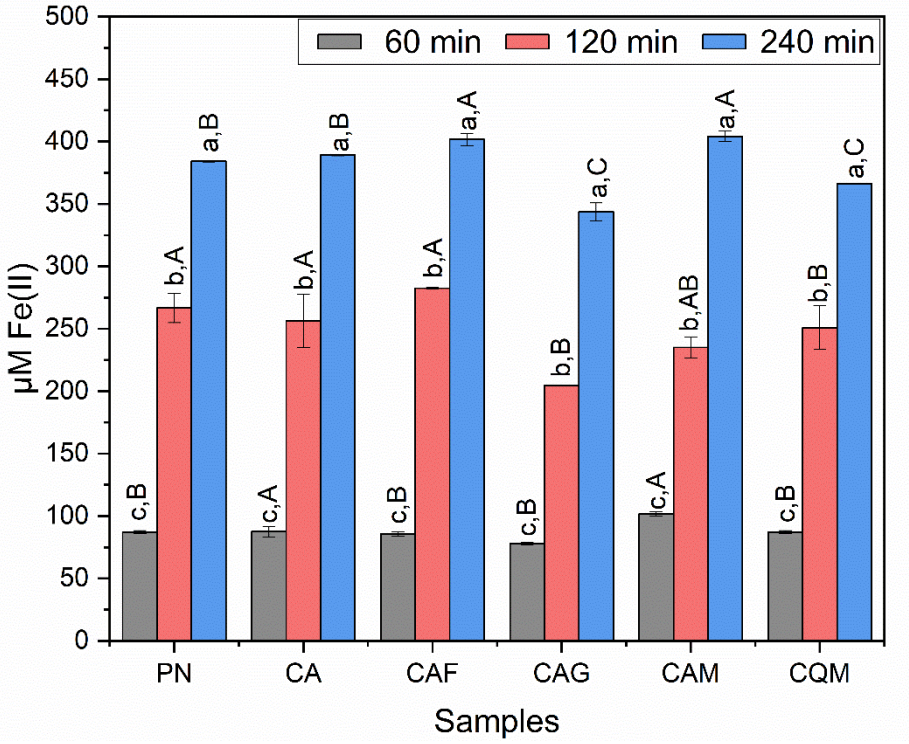
Antioxidant activity measured by FRAP assay of peptides from different hydrolysis times (1, 2, and 4 h) ranged between 79.2 and 406.7 uM Fe (II). As shown in Figure 7, the longer the hydrolysis time of casein, the higher the reducing power of peptides, indicating the formation of new peptides with higher antioxidant capacities with increase in hydrolysis time. Furthermore, some derivatives (FAC and MAC) were able to generate peptides with a better response to the reducing power than peptides obtained from other derivatives and native enzyme. Therefore, results showed that pepsin immobilization may be a very promising approach to produce antioxidant peptides from casein hydrolysis.

Banihashemi et al. [27], reported that casein-derived peptides exert multifunctional activities, including antioxidant activity. Antioxidant activity of proteins and peptides may be due to ability of proteins to chelate, retain, or transfer important active metals during food peroxidation. The proposed mechanism for antioxidant activity

of peptides occurs by capture of free radicals through oxidation of free amino acids present in their structure.

Antioxidant properties are directly related to amino acids present in structure of peptides, therefore all amino acids have an oxidation potential. However, some peptides show greater capacity than others, such as the reduction of antioxidant activity through sulfhydryl groups (cysteine and methionine) or by electron donation from aromatic sequences to radicals (tryptophan, tyrosine, and phenylalanine) [27].

Figure 7. Antioxidant activity by FRAP assay of the peptides from casein hydrolysis at different hydrolysis times for native pepsin (NP) and pepsin immobilized on activated carbon (AC) glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC), metalized activated carbon (MAC) and metalized carbon in presence of a chelating agent (MQC).



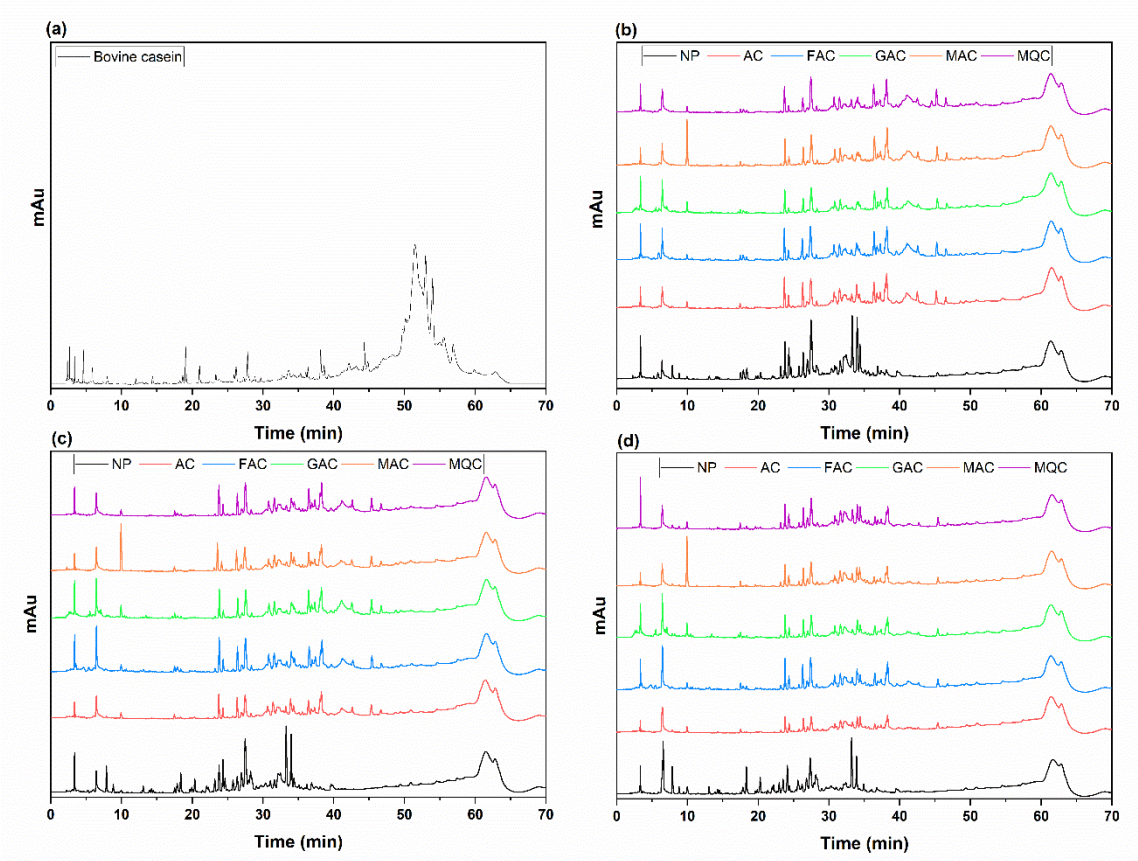
* Means followed by the same lowercase letter in the sample and the same uppercase letter in the hydrolysis time, for the same sample, do not differ significantly from each other by the Tukey's test ($P < 0.05$). *The bars at each point indicate the statistical deviations.

Peptides from casein hydrolysis by native pepsin and pepsin immobilized on different supports showed primary antioxidant capacity by the DPPH assay, secondary capacity by FRAP assay, and β-carotene/linoleic acid system. Furthermore, it can be pointed out that the higher hydrolysis time of casein led to an increase in secondary antioxidant capacity, which may be due to the degree of hydrolysis of peptides formed. This result indicates that peptides from hydrolysis of bovine casein by pepsin may be an effective tool to reduce food oxidation in several segments of the food industry.

3.5. Characterization of peptides by chromatography

Chromatograms of hydrolysates are presented in Figure 8. Bovine milk casein showed peaks with a higher frequency above 40 min (Figure 8-a), indicating the presence of larger molecules in its composition. After hydrolysis, the presence of peptides was observed mostly from 10 to 40 min (Figures 8-b, 8-c, and 8-d), indicating casein hydrolysis and conversion into peptides of smaller molecular size. Moreover, it was responsible for hydrolyzing casein at the same sites regardless of the type of immobilization during hydrolysis, generating mostly same peptides, varying only the number of peptides.

Figure 8. Chromatograms of casein (a) and peptides from casein hydrolysis by native pepsin and immobilized derivatives at 1 h (b), 2 h (c), and 4 h (d).



According to Santos et al. [3], similar peptide profile is due to the specificity of pepsin in cleaving bonds involving aromatic amino acids phenylalanine, tryptophan, and tyrosine. Different amounts of peptides were observed by peak intensity in chromatograms. Native pepsin was responsible for generating a greater variety of peptides mainly in the range of 30 to 40 min, due to its greater mobility in the substrate. For derivatives, the highest intensity was observed in the range of 10 min and 30 min,

and derivative of metalized carbon also showed two peaks of higher intensity at 10 min, which may be due to the higher catalytic activity of enzyme immobilized on this carbon.

As shown in Figures 8-b, 8-c, and 8-d, some changes were observed in chromatograms for different hydrolysis times. Increasing the hydrolysis time of casein led to hydrolysis of higher molecular weight peptides into lower molecular weight peptides. This event is shown by reduction of peaks in range of 30 to 50 min and an increase in the peaks at shorter retention times, mainly at 6.5 min, for all samples.

When comparing chromatographic peaks with the results of the antioxidant activity by the DPPH assay, it was observed that the reduction in concentration of high molecular weight peptides and the increase in smaller peptides did not affect the DPPH inhibition. This inhibition may be associated with peptides of intermediate molecular weights that remained unchanged with increasing hydrolysis time. However, regarding the protection against oxidation of the β-carotene/linoleic acid system, the increase in lower molecular weight peptides may be associated with higher antioxidant power. This relationship can be seen in Figure 8-d, most clearly for derivatives obtained with AC, FAC, and GAC, which exhibited a greater increase in peptide concentration at 6.5 min, thus showing greater protection for peptides obtained at 4 h of hydrolysis. In contrast, metallization-modified matrices (MQC and MAC) showed lower protection against oxidation of the β-carotene/linoleic acid with increase in hydrolysis time, as well as a smaller increase in peptides concentration.

Peptides obtained from pepsin immobilized on MAC and MQC presented a peak at 3.5 min, which increased with the increase in hydrolysis time. A similar peak was also observed for hydrolysis with native pepsin (NP), which also showed a large number of peptides at 6.5 min, with no response to protection against the β-carotene/linoleic acid when compared to derivatives (AC, FAC, and GAC). A possible reason for this phenomenon is that peptides found at 3.5 min in NP, MAC, and MQC acted as pro-oxidants on the β-carotene/linoleic acid system.

When comparing antioxidant activity by FRAP assay with chromatograms, the increase in the hydrolysis time led to an increase in the antioxidant power for all samples, probably due to the increase in low molecular weight peptides.

4. CONCLUSION

Results confirmed the pH 3.0 as the optimal pH condition for hydrolysis of bovine casein using immobilized pepsin. The new methods for modification of activated carbon (genipin functionalization and metallization) allowed an increase in enzyme activity when compared to traditional methods.

The use of different carbon functionalization methods (GAC, MAC, and MQC) guaranteed more than 8 cycles of use for immobilized pepsin. Modification with genipin provided more hydrolysis cycles than modification with glutaraldehyde, and also enhanced activity of immobilized pepsin, thus showing a potential substitute for modification of carbonaceous supports. MAC provided better stability of derivative to storage time and hydrolysis temperature when compared to native form. Regarding kinetic parameters, it was possible to determine reaction velocity (V_{max}) and the enzyme's affinity to substrate (Km) for native enzyme and metalized derivative (MAC), indicating again that new modifications are promising for pepsin immobilization.

HPLC analysis showed peptides similar to those obtained from casein hydrolysis by native enzyme, regardless of immobilization method and hydrolysis time, with concentrations varying with increasing hydrolysis time. Furthermore, these peptides showed potential as primary and secondary antioxidant agents by DPPH, β-carotene-linoleic acid, and FRAP assays. The results demonstrated potential of new modification methods proposed in this study, and further studies are required to improve immobilization efficiency and activity of immobilized pepsin.

CRediT authorship contribution statement

Mateus Pereira Flores Santos: Conceptualization**;** Methodology; Writing - Original Draft; Validation; Investigation. **Evaldo Cardozo de Souza Junior:** Funding acquisition; Resources. **Leandro Soares Santos:** Methodology: Resources. **Renata Cristina Ferreira Bonomo:** Funding acquisition; Resources; Supervision; Writing - Review & Editing. **Cristiane Martins Veloso:** Project administration; Funding acquisition; Resources; Supervision; Writing - Review & Editing;

Declaration of competing interest

The authors declare that they do not have any conflict of interest

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CAPÍTULO 4

ARTIGO 3

Activated carbon functionalization for trypsin immobilization: Effect of modifying agent and application in bovine casein hydrolysis

Funcionalização de carvão ativado para imobilização de tripsina: efeito de agente modificador e aplicação na hidrólise de caseína bovina

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*Manuscrito será submetido na revista Food and Bioproducts Processing (INSS: 0960-3085) – Classificação Qualis-Capes: A1

Activated carbon functionalization for trypsin immobilization: Effect of modifying agent and application in bovine casein hydrolysis

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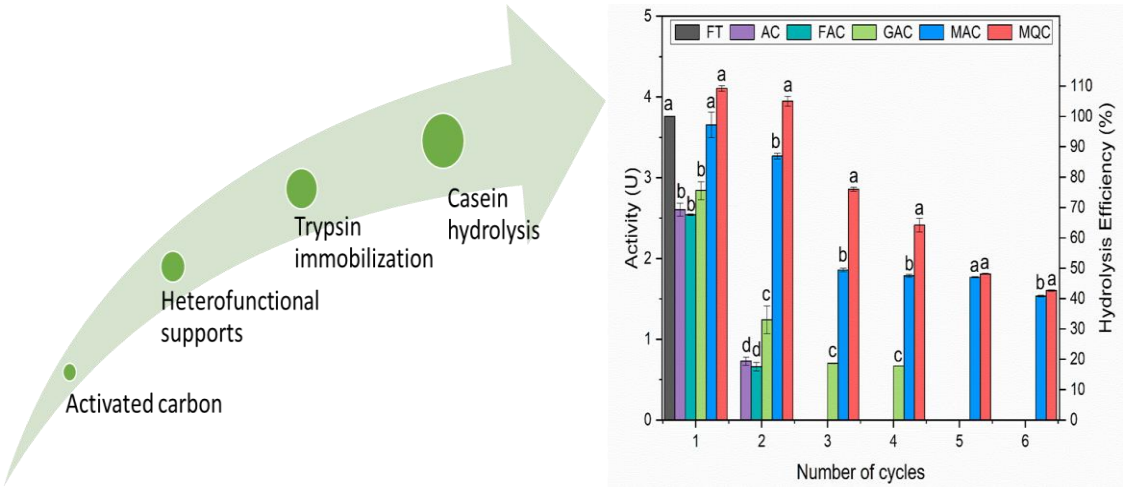
Abstract

This study aimed to immobilize trypsin on activated carbon subjected to different modification methods: functionalization with glutaraldehyde, genipin, and metallization with iron particles (heterofunctional supports) for application in the bovine casein hydrolysis. The potential for reuse of derivatives, storage stability, reaction kinetic parameters, and characterization of peptides were evaluated. Trypsin immobilization on heterofunctional supports may be an alternative to biocatalysis, aimed at reducing the costs of biocatalytic processes. The results showed that pH 5.0 was optimal pH for immobilization, while pH 9.0 was optimal condition for hydrolysis reaction. The functionalization of activated carbon with iron ions in presence of a chelating agent led to an immobilization capacity of 95.49 $mg.g^{-1}$ and a hydrolytic activity of 4.11 U, which was higher than those obtained for free enzyme (3.76 U). This derivative also proved to be effective for reuse, and can be used for more than 8 cycles, besides being stable to storage for 30 days, and temperatures above 40 ºC. The results demonstrated that the support under study can be a promising alternative for enzyme immobilization, with an emphasis on the advantages of immobilization from the biocatalytic and economic point of view.

Keywords: Enzyme activity; Biocatalyst; Modification; Protease; Heterofunctional support.

Graphical abstract



1. INTRODUCTION

Trypsin (EC 3.4.21.4) is an enzyme that is part of serine protease group due to involvement of Ser in the proteolysis process. It has an approximate size of 23 kDa and isoelectric point close to pH 10.5 (LIU et al., 2019). It is produced and secreted in pancreas in an inactive form (trypsinogen) becoming active in the small intestine through partial hydrolysis by enterokinase. It works in the small intestine, with optimum activity at pH range from 7.0 to 9.0. Its structure is very similar to chymotrypsin, but its substrates are specific for cleavage at the carboxyl side of arginine and lysine (Lys) and arginine (Arg) unless either residue is followed by proline (Pro) (DORNELLES et al., 2018; SOUZA JR. et al., 2020). In food industry, it can be used in synthesis of food flavor hydrolysates, replacing microbial proteinases. However, it stands out for use in laboratory-scale, in protein fragmentation for further identification and characterization (MORAN, 2016; LIU et al., 2019; TAVANO et al., 2018).

In addition to use of trypsin in its free form, its use in immobilized form on solid supports is one of its catalytic advantages. The advantages of immobilization include improvements in stability and proteolysis efficiency, separation and recovery of derivatives, preventing losses of catalytic activity associated with structural modification, resistance to denaturing and microbial attacks, and the possibility of reuse for a few cycles. These aspects provide more efficient control of large-scale processes, making them more economical and with better application in industrial-level processes (DEMIRKAN et al., 2017; NGUYENA & KIM, 2017; LIU et al., 2019).

The main supports used in immobilization process include chitosan, activated carbons, polymeric resins, and silica, as these materials have suitable porosity and high surface area, which favor immobilization (ASHKAN et al., 2020). Activated carbon stands out among the other supports due to its high chemical, mechanical, and thermal resistance, hydrophilicity, insolubility, besides presenting a high surface area and well-defined porosity. In addition, activated carbon can be subjected to surface modification by different methods, leading to formation and/or increase in functional groups already existing on its surface, making it a heterofunctional support (SANTOS et al., 2022).

As reported by Okura et al. (2020), the conversion of a common support to a heterofunctional support occurs through surface modification. These modifications offer advantages for enzyme immobilization, once different functional groups formed on surface of the material can interact with enzymes by chemisorption (covalent bonding)

and physisorption (ionic interactions, hydrogen bonds, and interfacial activation). The increase of reactive groups on surface of matrices can produce immobilized biocatalysts with higher catalytic efficiency due to the minimization of diffusion effects of substrates and products during reaction, in addition to the improvement of operational stability in continuous and batch processes, raising an industrial interest for these biocatalysts (RAMANI et al., 2012; BEZERRA et al., 2015).

The present study evaluated the trypsin immobilization on activated carbon submitted to different modification methods (functionalization with glutaraldehyde, genipin, and metallization with iron particles), determining the best pH conditions for immobilization and hydrolysis of bovine casein. Optimum hydrolysis temperature, stability of derivatives to cycles of reuse, storage stability of derivative with highest activity, and characterization of peptides obtained from the casein hydrolysis were also determined.

2. METHODOLOGY

2.1. Supports used in immobilization process

Activated carbons from a previous study by Santos et al. (2022) were used. Activated carbon (AC) was synthesized from tamarind seeds and its surface was functionalized with glutaraldehyde (FAC); genipin (GAC); metallization (MAC); and metallization in presence of a chelating agent (MQC). The methodologies used in synthesis of different heterofunctional supports as well as their characterizations are described by Santos et al. (2022).

2.2. Effect of pH on trypsin immobilization

First, the optimal pH of trypsin immobilization (*Trypsin from bovine pancreas*, TPCK Treated, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein, CAS: 9002-07-7, Sigma, USA) was determined using activated carbon (AC) and glutaraldehyde-functionalized carbon (FAC) as supports. The two supports were selected as standard supports to evaluate the immobilization by adsorption and covalent binding. For that, trypsin solutions at different initial pH values (3.0, 5.0, 7.0 and 9.0) were used. Lactic acid/sodium lactate buffer 0.05 $mol.L^{-1}$ was used for the trials at pH 3.0, and 0.05 $mol.L^{-1}$ sodium phosphate buffer was used in the trials performed at other pH values.

Immobilization was performed according to the conditions determined in preliminary tests. 50 mg of each support (AC and FAC) and 5 mL of trypsin solution (1 $mg.mL^{-1}$) solubilized in buffer solutions were placed in centrifuge tubes. The tubes were kept under constant agitation (20 rpm) on an orbital shaker at room temperature (25 ºC). After 2 h, the tubes were centrifuged at 3500 rpm for 5 min (MPW Med. Instruments, MPW-350, Warsaw, Poland) and the supernatant was completely removed for quantification of non-adsorbed proteins by the Bradford method (1976). Samples were then washed successively to remove non-immobilized enzymes. Then, 5 mL of buffer was added in the tubes containing the immobilized enzyme and kept under agitation at 20 rpm for 20 min, followed by centrifugation at 3500 rpm for 5 min, and removal and supernatant protein concentration was quantified. Process equilibrium was reached when protein concentration of supernatant solution was null. Derivatives were then stored in buffer solution at 4 °C until use.

The immobilization capacity of each support was calculated from the difference between the initial and final protein concentration, according to Equation 1.

$$C_{Im} = \frac{V\,(C_{in} - C)}{m_S} \quad (1)$$

where: C_{Im} is the immobilization capacity ($mg.g^{-1}$); V is the volume of the solution (mL); C_{in} is the initial concentration of the solution ($mg.L^{-1}$); C is the concentration of the solution ($mg.L^{-1}$) at equilibrium; and m_S is the mass of the support (g).

2.3. Effect of pH of the reaction medium on enzyme activity

Activity of free trypsin and derivatives was determined according to the bovine casein hydrolysis method as described by Santos et al. (2019), with minor modifications. Bovine casein (*casein from bovine milk* - CAS 9000-71-9, Sigma, USA) at 2 % (w/v) solutions were prepared using the same buffer solutions used in immobilization step (pH 3.0, 5.0, 7.0, and 9.0) and used as a substrate to determine the best hydrolysis conditions.

In a centrifuge tube, 1 mL of trypsin solution solubilized in different buffers and 5 mL of substrate (bovine casein solution), 1 mL of trypsin solution were incubated at 40 °C in a thermostatic bath (Tecnal, TE-205, SP, Brazil) for 60 min. After this period, 2 mL of trichloroacetic acid (6.5% w/v) was added to stop the reaction, and the tubes were centrifuged at 6000 rpm for 6 min at 4 °C (SPLabor, SP-701, SP, Brazil). Then, 1 mL of the supernatant was mixed with 5 mL sodium carbonate (4% w/v) and 1 mL of Folin-

Ciocalteu solution (20 %, v/v). The mixture was incubated at 40 °C for 20 min, and absorbance was measured in a spectrophotometer (UV-Vis Quimis, Q898UV2, SP, Brazil) at 660 nm. One unit (U) of trypsin activity was defined as the amount of enzyme required to release 100 µg of tyrosine under the assay conditions.

To determine activity of derivatives, standard derivatives (AC and FAC) were used, as reported in the previous methodology, with minor modifications. For that, 5 mL of the respective casein solutions (2% w/v) and 50 mg of derivatives were placed in tubes and incubated at 40 ºC for 60 min. After this step, the samples were centrifuged (3000 rpm/ 5 min) and the supernatant was transferred to another tube containing 2 mL of trichloroacetic acid (6.5%, w/v), stirred, and centrifuged (6000 rpm /6 min /4 °C). Then, 1 mL of the supernatant was removed and allowed to react as previously described.

2.4 Trypsin immobilization on genipin-modified carbons and iron ions

After determining the optimal immobilization pH, as well as the pH of hydrolysis of bovine casein in the standard derivatives (AC and FAC), studies were performed using the other carbon supports: genipin functionalized activated carbon (GAC); iron ion metallized carbon (MAC); and iron ion metallized carbon in presence of a chelating agent (MQC), to evaluate the effect of different surface modifications on trypsin immobilization. For that, the methodologies described in Sections 2.2 and 2.3 were used, according to optimal pH determined in each step.

Enzyme activity was determined using a control group of experiments, that is, the supports without immobilized trypsin, to assess possible interferences during the assays.

2.5. Stability of derivatives in saline solution and in presence of a surfactant

The study of stability of immobilized enzymes on the supports was carried out as described by Santos et al. (2022) to determine the binding force involved in immobilization process.

2.6 Reuse of derivatives

Cycles of reuse were determined for all derivatives at the optimal pH of reaction medium to determine the number of cycles of use that the enzymes maintain their hydrolysis activity, thus determining the type of support modification that provides a derivative with greater stability in the reaction medium of immobilized trypsin. For that, the methodology previously described by Santos et al. (2019) was used. After each

hydrolysis cycle, 5 ml of the immobilization buffer was added to tubes containing derivatives, which were agitated (20 rpm/5 min) and centrifuged (3000 rpm/3 min) to remove casein residues present in medium, providing the derivatives suitable for use in a new hydrolysis reaction. Then, derivative with the lowest loss of activity between cycles was selected for subsequent trials.

2.7. Effect of temperature of reaction medium on enzyme activity

Effect of temperature on casein hydrolysis reaction was determined using free enzyme and derivative with the best performance in the previous tests. Different hydrolysis temperatures were studied (20 °C, 30 °C, 40 °C, 50 °C, and 60 °C) to evaluate the effect of temperature on activity of the immobilized enzyme.

2.8. Stability during storage

Activity of free trypsin and derivative with the best performance in previous assays was determined after storage at 4 ºC, using test tubes containing immobilization buffer for up to 30 days, and the activity was measured at 0, 7, 15, and 30 days to assess whether the enzymes maintain their stability during storage.

2.9. Kinetic parameters of hydrolysis reaction

Kinetic parameters were determined for both free trypsin and derivative with the best results in previous assays. For that, enzyme activity was determined using casein solutions with different initial concentrations (0.1 % w/v- 2 % w/v). A graph was plotted with the values of the initial reaction rates as a function of casein concentrations. Maximum velocity (V_{max}) and the Michaelis - Menten constant (Km) were determined from the Lineweaver-Burk equation (Equation 2).

$$\frac{1}{V_0} = \frac{K_m}{V_{max}+[S]_0} + \frac{1}{V_{max}} \quad (2)$$

Where V_0 is the reaction velocity, V_{max} is the maximum reaction velocity, K_m is the Michaelis-Menten constant, and $[S_0]$ is the substrate concentration (casein)

2.10. Characterization of peptides from casein hydrolysis by high performance liquid chromatography (HPLC)

The biopeptides from casein hydrolysis in the presence of free enzymes and derivatives were analyzed by reverse phase liquid chromatography (RP-HPLC). A C18

column (250x4.6 mm ID, 5 mm particle size; ZORBAX Eclipse Plus C18) coupled to a pre-column (ZORBAX SB-C 18, 4.6 mm ID x 12.5 mm, 5 μm) connected to an Agilent 1260 Infinitty II HP system was used. Two mobile phases were used, the first (A) composed of acetonitrile solution (5 %, v/v) (RIEDEL-DE-HAEN) and trifluoroacetic acid (0.1 %, v/v) (Dynamics LTDA) and the second phase (B) was formed by acetonitrile (50 %, v/v) and trifluoroacetic acid (0.1 %, v/v). A 20 μL aliquot of sample was automatically injected into the column at a flow rate of 1 mL/min for mobile phases A and B for 70 minutes. The analysis started with 100 % flow of the mobile phase A and decreased linearly for 60 minutes until 100 % of the flow was represented by phase B. A new linear gradient was performed for 60 to 65 minutes to reach the flow rate with 100 % of A, which was maintained until the end of the analysis (70 minutes). The temperature of the run was kept at 30 °C, detection of the peptides was performed at 280 nm on a UV-Vis detector, and the identification was performed by comparing the retention times of the separated peaks and their UV spectra.

2.11. Statistical analysis

All experiment was performed in triplicate. Data were submitted to analysis of variance and the differences between the means of hydrolytic capacity at the different storage periods were analyzed by Tukey's test ($p < 0.05$), using the software SAS Studio and Origin Pro 8.0.

3. Results and Discussion

3.1. Effect of pH on trypsin immobilization

pH is one of the factors that most affect enzyme immobilization process, once it changes properties of amino acid side groups and chemical properties of insoluble support. Trypsin was initially solubilized in solutions at different pH values, and immobilized on AC and FAC aiming to evaluate the effect of carbon properties on enzyme immobilization and hydrolytic activity. The results of immobilization capacity and efficiency are presented in Table 1.

After immobilization, successive washes were performed to remove unbound and/or weakly bound enzymes. Enzymes at more acidic conditions (pH 3.0) were removed first, followed by enzymes at pH 7.0, leading to an immobilization efficiency

close to 80% and 91% for AC and FAC, respectively. For both carbons, the highest immobilization capacities were obtained at pH 5.0 and 9.0. Similar values were reported by Souza Jr et al. (2020), who studied trypsin immobilization on activated carbon and glutaraldehyde-functionalized carbon obtained from caja seeds.

Table 1. Effect of the pH of initial trypsin solution on mass of bound enzyme (M_{Lig}), immobilization capacity (C_{Im}), and immobilization efficiency (Efic) for AC and FAC.

Sample	pH of enzyme solution	M_{Lig} (mg)	C_{Im} (mg/g)	Effic (%)
AC	3.0	4.03 ± 0.07	80.31 ± 1.54 C	80.58 ± 0.01 C
	5.0	4.63 ± 0.01	91.79 ± 0.34 B	92.62 ± 0.01 B
	7.0	4.58 ± 0.03	90.62 ± 0.73 B	91.70 ± 0.01 B
	9.0	4.85 ± 0.02	96.80 ± 0.51 A	96.99 ± 0.01 A
FAC	3.0	4.01 ± 0.03	79.80 ± 0.61 c	80.19 ± 0.01 c
	5.0	4.71 ± 0.01	93.71 ± 0.47 a	94.18 ± 0.01 a
	7.0	4.51 ± 0.05	88.83 ± 1.20 b	90.20 ±0.01 b
	9.0	4.79 ± 0.05	95.38 ± 1.22 a	95.85 ± 0.01 a

*Averages followed by the same upper-case letter in the column for AC, and the same lower-case letter for FAC do not differ significantly by Tukey's test ($P < 0.05$).

The high immobilization capacity of the activated carbon is due to its high porosity and pore diameter, once the enzyme under study has low molecular mass (23.3 kDa), favoring immobilization by adsorption. Besides the textural characteristics of AC, its higher immobilization capacity at pH 5.0 and 9.0 may also be due to the net surface charge of enzyme and support.

pH interferes with the net charge of protein, affecting the contributions of electrostatic interactions on enzyme/activated carbon interaction associated with adsorption process. According to Santos et al. (2022), AC presents a pH of zero charge (pH_{PCZ}) equal to 4.84, while trypsin has an isoelectric point (pI) around 10.0 (VOYTEK and GJESSING, 1971; SAHIN et al., 2020). Therefore, at pH values near the isoelectric point, the enzyme has a density of neutral charges while a higher density of negative charges on the surface are observed at pH values above the pI. In solutions with pH below its isoelectric point, its surface has a higher number of positive charges. Similar behavior was observed for AC in medium with pH value below or above its pH_{PCZ}.

For both supports, the higher immobilization capacity was obtained at pH 9.0, close to the pI of trypsin, in which trypsin is positively charged. Thus, immobilization may have been governed by electrostatic and hydrophobic interactions between trypsin

and supports. Although trypsin also has a higher density of positive charges on its surface at pH 7.0, a lower immobilization capacity was observed for both supports, indicating that hydrophobic and other interactions were responsible for governing the process. At pH 5.0, the supports were close to their pH_{PCZ} not favoring electrostatic interactions and reducing immobilization capacity of AC. However, FAC presented an immobilization capacity similar to that obtained at pH 9.0, once the incorporation of reactive groups, including amine and aldehyde groups, were responsible for formation of a stable bond with the primary amino groups of the enzymes, facilitating covalent interaction between enzyme and support, ensuring a high immobilization. In addition, each glutaraldehyde molecule can contain one or two amino groups, indicating an initial immobilization through ion exchange on support before covalent reaction and formation of Schiff base, which occurs at weakly acidic pH ($4.0 < pH < 5.0$) between the amino group and the amino acids of enzyme. As expected, lower immobilization values were obtained at pH 3.0, once both surfaces were protonated, favoring electrostatic repulsion and negatively influencing immobilization (ZAAK et al., 2017; SANTOS et al., 2019; BRITO et al., 2020; MO et al., 2020).

According to Sahin et al. (2020), at more alkaline pH values, trypsin immobilization is favored by the activation of lysine present in its structure, and as the pH approaches neutral/acidic values immobilization occurs mainly through the most reactive and exposed amine groups, terminal amino group.

3.2. Effect of pH of reaction medium on trypsin activity

The results of the effect of reaction medium pH on the trypsin activity during casein hydrolysis are shown in Table 2. Trypsin in free form (FT) showed activity in all pH ranges tested, with a lower activity observed when using the casein solution in more acidic media, mainly at pH 5.0, probably due to conformational change of enzyme and low casein solubilization, which leads to a low substrate content for enzymatic hydrolysis and consequently low activity (POST et al., 2012).

When trypsin was solubilized at higher pH (7.0 and 9.0), an increase in trypsin activity was observed, regardless of the solubilization pH, i.e., solubilization of enzyme at pH values different from the optimal pH range did not cause irreversible changes in its structure. However, higher activity values were observed for the enzyme solubilized in the most acidic pH, using the soluble casein in a more alkaline pH range. Seabra and Gil (2007) studied the stability of trypsin and reported a hydrolytic activity in a wide pH

range, while maximum activity was observed in more alkaline pH range, due to better enzyme conformation.

Table 2. Effect of initial pH of free trypsin solution (FT), trypsin immobilized on AC, and trypsin immobilized on FAC on hydrolysis of solubilized bovine casein at different pH values.

Sample	pH of enzyme immobilization solution	Activity (U)			
		pH of casein solution			
		3.0	5.0	7.0	9.0
AC	3.0	0.26 ± 0.01 c	0 ± 0 d	1.18 ± 0.01 b	1.51 ± 0.02 a
	5.0	0.32 ± 0.01 c	0 ± 0 d	1.77 ± 0.03 b	2.60 ± 0.08 a
	7.0	0.04 ± 0.01 c	0 ± 0 d	1.73 ± 0.01 b	2.33 ± 0.01 a
	9.0	0.09 ± 0.01 b	0 ± 0 b	0.86 ± 0.07 a	1.10 ± 0.10 a
FAC	3.0	0.33 ± 0.01 c	0.02 ± 0.01 d	0.72 ± 0.06 b	1.21 ± 0.02 a
	5.0	0.44 ± 0.01 c	0.04 ± 0.01 d	0.77 ± 0.03 b	2.54 ± 0.01 a
	7.0	0.08 ± 0.01 c	0 ± 0.01 c	1.01 ± 0.02 b	1.46 ± 0.02 a
	9.0	0.13 ± 0.01 c	0.03 ± 0.02 c	0.35 ± 0.07 b	0.58 ± 0.01 a
FT	3.0	0.28 ± 0.01 b	0.03 ± 0.01 b	4.06 ± 0.05 a	4.12 ± 0.10 a
	5.0	0.24 ± 0.01 c	0.15 ± 0.01 d	3.68 ± 0.01 b	3. 76 ± 0.01 a
	7.0	0.21 ± 0.01 c	0.09 ± 0.01 d	2.99 ± 0.01 b	3.51 ± 0.01 a
	9.0	0.40 ± 0.01 c	0.21 ± 0.01 d	3.86 ± 0.01 b	3.69 ± 0.01 a

*Means followed by the same lower-case letter in the rows do not differ significantly for each treatment (AC, FAC, and FT) by Tukey's test ($P < 0.05$).

Regarding hydrolytic activity of derivatives, a reduction in casein hydrolysis rate was observed when compared to free enzyme, which was expected since free enzyme has greater mobility, facilitating contact with substrate. On the other hand, immobilized enzymes are often confined in pores of the material, with no direct access to substrate, which must be transferred (by convection and/or diffusion) from solution to catalytic sites. However, even with lower enzyme activity, similar behavior was observed as a function of the pH of casein solution. Hydrolytic activity of derivatives for casein solubilized at acidic pH values was lower when compared to casein solubilization at higher pH, regardless of immobilization pH Therefore, pH of casein solubilization affects

more significantly the activity of derivatives and free enzyme when compared to pH of trypsin solubilization.

Based on these results, pH values 5.0 and 9.0 were considered the optimal immobilization pH, and pH 9.0 was the optimal pH for casein solubilization. However, pH 5.0 was selected as immobilization pH due to the formation of Schiff base between support and the enzyme, which guarantees a stronger interaction, preventing enzyme desorption. According to Mo et al. (2020), the use of an acidic pH range in immobilization, especially at pH values close to 5.0, tends to generate stronger bonds between support and enzyme, mainly in supports with residual amino group, favoring the formation of Schiff base.

3.3. Effect of type of AC modification on trypsin immobilization

After determining the immobilization pH (pH 5.0) and the pH of casein solution (pH 9.0), which together led to the highest hydrolytic activity of trypsin, a study was performed to evaluate the effect of different modifications methods of activated carbon on hydrolytic activity of derivatives. The results of enzyme immobilization are presented in Table 3.

Table 3. Effect of AC functionalization on enzyme immobilization at pH 5. Mass of bound enzyme (M_{Lig}), Immobilization capacity (C_{Im}), Immobilization efficiency (Efic).

Sample	M_{Lig} (mg)	C_{Im} (mg/g)	Efic (%)
AC	4.63 ± 0.01	91.80 ± 0.34 a	91.70 ± 0.01 a
FAC	4.71 ± 0.02	93.71 ± 0.47 b	90.20 ± 0.01 a
GAC	4.36 ± 0.01	85.71 ± 0.06 c	87.26 ± 0.01 b
MAC	4.26 ± 0.01	85.16 ± 0.03 c	85.33 ± 0.01 b
MQC	4.76 ± 0.03	95.49 ± 0.40 d	95.15 ± 0.01 c

* Means followed by the same lower case letter for Immobilization capacity (Cim) do not differ significantly by Tukey's test ($P < 0.05$).

Concerning the adsorptive capacity of supports (Table 3), all showed high capacity of trypsin immobilization, with immobilization efficiency above 85% for all materials. Therefore, even presenting lower pore volume and surface area, as reported by Santos et al. (2022), modified carbons showed high immobilization capacity, demonstrating positive effect of the modifications under study. The highest

immobilization capacity was observed for the carbon with incorporation of iron particles in presence of iminodiacetic acid (MQC).

When correlating immobilization capacity with pHpcz of carbons, all modified carbons at pH 5.0 showed positively charged surface as well as the enzyme surface, thus the electrostatic interactions may have not been the main responsible for immobilization.

Genipin-functionalized carbon (GAC) showed an immobilization efficiency of 85 %, with a lower immobilization capacity when compared to traditional immobilization methods (AC and FAC) (Table 3). This lower immobilization capacity may be associated with electrostatic interactions as a function of point of zero charge (pH_{PCZ}) of GAC (6.35). As immobilization was conducted at pH 5.0, both enzyme and support (GAC) exhibited positive surface charges, leading to a small electrostatic repulsion that interfered with immobilization. For immobilization using genipin as a crosslinking agent, ion exchange first occurs between the amine groups present on the enzyme with the genipin groups, followed by formation of covalent bonds (FLORES et al., 2019).

Functionalization of activated carbon with iron ions, both in presence of a chelating agent (MQC) or not (MAC) led to high immobilization efficiency, with values of 95.15% and 85.33%, respectively. The lower immobilization values of MAC when compared to MQC may be due to the lower presence of metal ions on support surface, generating fewer active sites for enzyme interaction, as reported by Santos et al. (2022). In this case, electrostatic interactions are responsible for driving immobilization between the amino acids of enzymes with the iron oxides present on the surface of support (ZDARTA et al., 2018; KHOSHNEVISAN et al, 2019). In turn, the higher immobilization capacity of MQC when compared to MAC may be due to the presence of chelating agent, iminodiacetic acid (IDA), which in addition to conferring an increase in amount of supported particles, ensures greater stability to metal particles, making them more exposed.

3.4. Hydrolytic activity of trypsin in casein hydrolysis reaction

Activity of trypsin, both in its free form and immobilized form on different supports, can be seen in Table 4. Blanks of supports were also analyzed and presented no hydrolytic activity, showing that the different supports did not interfere in activity of immobilized enzymes.

As can be seen in Table 4, the higher hydrolysis capacity was obtained for enzymes immobilized on the metallized supports (MQC and MAC) and free enzyme

(FT), followed by the enzymes immobilized on other supports GAC, AC, and FAC. However, the specific activity of immobilized enzymes was lower than that of the free enzyme, indicating few enzymes with active sites available to hydrolyze casein or accessible to the substrate. In turn, these active enzymes showed high casein hydrolysis activity (2.54 to 4.11 U), similar to the free enzyme (3.76 U) in some cases.

Table 4. Effect of activated carbon functionalization on casein hydrolysis at pH 9.0

Sample	Activity (U)	Specific activity (U/mg)
FT	3.76 ± 0.01 A	3.76 ± 0.01 a
AC	2.60 ± 0.08 B	0.58 ± 0.02 b
FAC	2.54 ± 0.01 B	0.57 ± 0.01 b
GAC	2.84 ± 0.11 B	0.65 ± 0.03 b
MQC	4.11 ± 0.04 A	0.87 ± 0.01 c
MAC	3.65 ± 0.16 A	0.86 ± 0.04 c

* Means followed by the same upper case letter (activity) and lower case letter (specific activity) do not differ significantly by Tukey's test ($P < 0.05$).

Regarding casein hydrolysis capacity of enzymes immobilized on genipin-functionalized carbon (GAC), similar activity was observed when compared to traditional immobilization methods (AC and FAC). When correlating immobilization capacity with enzyme activity, GAC showed a lower immobilization capacity than AC and FAC, with a higher hydrolytic activity, thus indicating that the immobilized enzymes were in a more favorable conformation. According to Klein et al. (2016), immobilization using genipin brings some advantages over immobilization with glutaraldehyde, with emphasis on maintenance of enzyme activity since glutaraldehyde can lead to the formation of covalent bonds with active site of enzyme or cause deformation in structure. Therefore, genipin can be used to replace glutaraldehyde to modify the carbon surface, ensuring satisfactory results of immobilization and enzyme activity. In addition, genipin has toxicity 10000 times lower than glutaraldehyde (SANTOS et al., 2022).

Functionalization of activated carbon with metal ions was responsible for providing higher hydrolytic capacity when compared to traditional immobilization methods (AC and FAC). Regarding the specific activity of derivatives, the metallized supports allowed the higher values, and MAC presented high activity values even presenting one of the lowest immobilization capacities. MQC also provided higher hydrolytic activity for immobilized enzymes, probably due to the better accessibility of

immobilized enzymes. For AC, which had the largest surface area and pore volume, and less functional groups on its surface, some enzymes were confined in carbon pores, hindering the access of substrate to catalytic sites.

In general, Fe^{2+} and Fe^{3+} ions present on supports improved the activity of immobilized trypsin. According to Liu et al. (2018), trypsin is part of cysteine proteases, that is, it is characterized by a nucleophilic cysteine thiol at active site (Cys, His, and Asn), which is one of the most abundant groups of this protease. Therefore, these enzymes can have their activity increased in presence of a cofactor, a non-protein component, which can enhance their activity. The cofactors can be metal ions such as Ca^{+2}, Mg^{+2}, Co^{+2}, Fe^{+2}, Fe^{+3}, and others.

3.5. Stability of derivatives in high salt and Tween 80 concentrations

The Bradford readings of derivatives supernatants were null after contact with both saline and Tween 80 solutions, indicating absence of trypsin in medium, thus proving no desorption on supports. These results confirm that the enzymes were immobilized on supports by strong interactions, such as hydrophobic interactions, covalent bonds and/or electrostatic interactions, preventing enzyme losses by desorption.

3.6. Reuse of derivatives

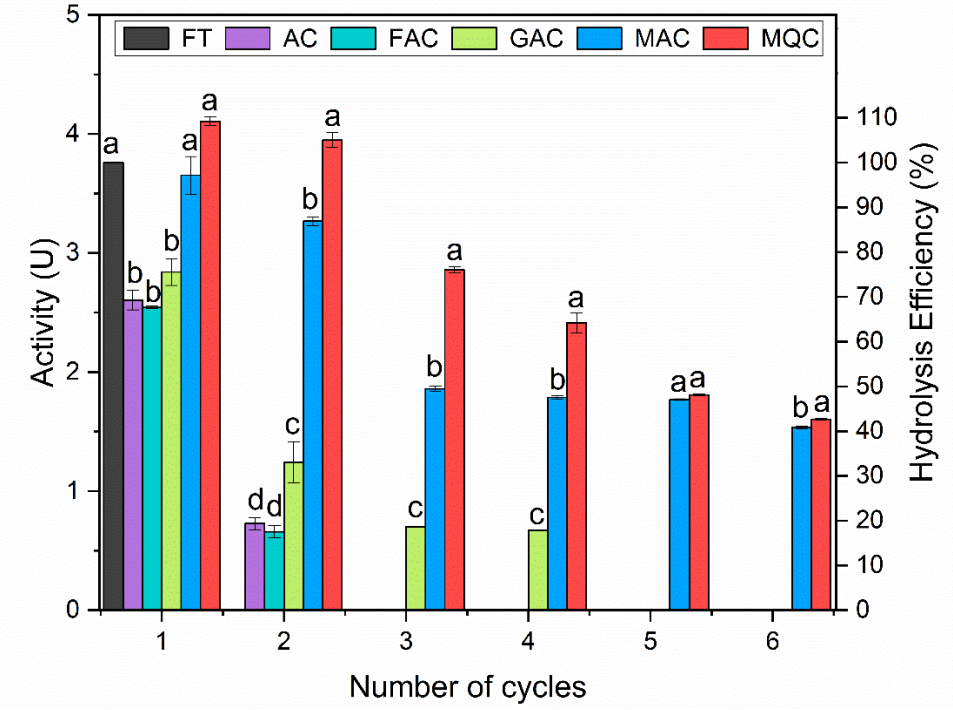
The results of enzyme activity for the cycles of reuse are presented in Figure 1. Hydrolysis efficiency of free enzyme was assumed to be 100% and used as a base to determine the hydrolysis efficiency of derivatives. The results showed that it was possible to perform casein hydrolysis for several cycles using derivatives from the study.

Regarding the cycles of reuse, metallized supports presented the best results, once they were able to hydrolyze the bovine casein for more than 5 cycles, while other supports presented less cycles of use. The low repeatability of use of these supports may be due to possible losses by desorption that may be related to pH of the substrate, or leaching of enzyme over the cycles.

Derivatives obtained with activated carbon (AC) and glutaraldehyde-functionalized carbon (FAC) remained active for only 2 consecutive cycles of hydrolysis. The use of the derivative with genipin-functionalized carbon (GAC) provided an increase in hydrolytic activity of immobilized enzymes, and also a greater number of cycles of reuse. This derivative was active for 4 consecutive cycles, with a decrease in activity during the cycles, which may indicate enzyme loss during its use. The results demonstrate

the importance of studying new immobilization methods, as well as optimizing the existing methods to provide better activities for immobilized enzyme (SUN et al., 2017).

Figure 1. Activity of free trypsin (FT) and derivatives: activated carbon (AC), glutaraldehyde-functionalized carbon (FAC), genipin-functionalized carbon (GAC); metalized activated carbon (MAC), and metalized carbon in the presence of a chelating agent (MQC) for the cycles of reuse.



* Averages followed by the same letter for each cycle do not differ significantly by Tukey's test ($P < 0.05$).

Derivatives obtained with metal-functionalized carbons (MAC and MQC) showed the best hydrolytic capacities, as well as a higher stability to recycles, allowing 6 consecutive hydrolysis cycles. The carbons modified with metal ions improved the activity of immobilized enzymes, providing activities similar to the values observed for free enzyme in the first cycle. According to Sun et al. (2017), support metallization leads to a greater enzyme stability, preventing desorption or inactivation during the cycles of use. Trypsin immobilized on MAC and MQC showed a casein hydrolysis capacity of 40 and 43 % in the last cycle, respectively, when compared to the first cycle. Therefore, the use for more cycles may compensate for immobilization costs (SANTOS et al., 2019).

When comparing metallized supports with each other, the cycles performed with derivative from metallized carbon in presence of a chelating agent (MQC) provided lower loss of activity, due to the greater presence of magnetic particles in its structure, thus ensuring greater enzyme stability. The presence of metal ions is associated with the presence of iminodiacetic acid (IDA), which prevents leaching of metal particles from

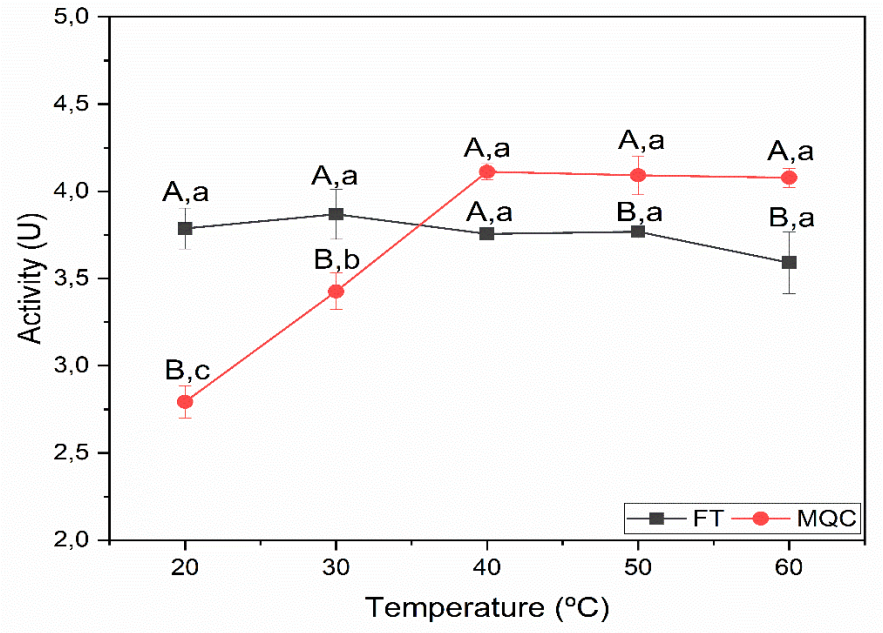
support, ensuring a greater number of particles on the surface and consequently higher enzyme activities (CHANG et al., 2017). Therefore, MQC was selected to perform subsequent trials due to the best results when compared to other supports (number of cycles with the lower loss of activity).

3.7. Effect of hydrolysis temperature and storage stability of free and immobilized trypsin

The results of the effect of temperature on trypsin activity in its free form and the best derivative obtained with MQC are presented in Figure 2.

The activity of free enzyme was constant at all temperatures studied, indicating that the temperature led to no changes in trypsin conformation. In turn, the derivative showed an increase in activity with increasing temperature, reaching maximum hydrolysis activity at 40 ºC, remaining constant until the maximum temperature studied of 60 ºC. The increase in activity of derivative may be due to an improvement in the access of substrate to enzyme since higher temperatures can favor the transfer of substrate to catalytic site of trypsin immobilized on pores.

Figure 2. Effect of temperature on hydrolysis reaction of free trypsin (FT) and trypsin immobilized on metalized carbon in presence of a chelating agent (MQC).

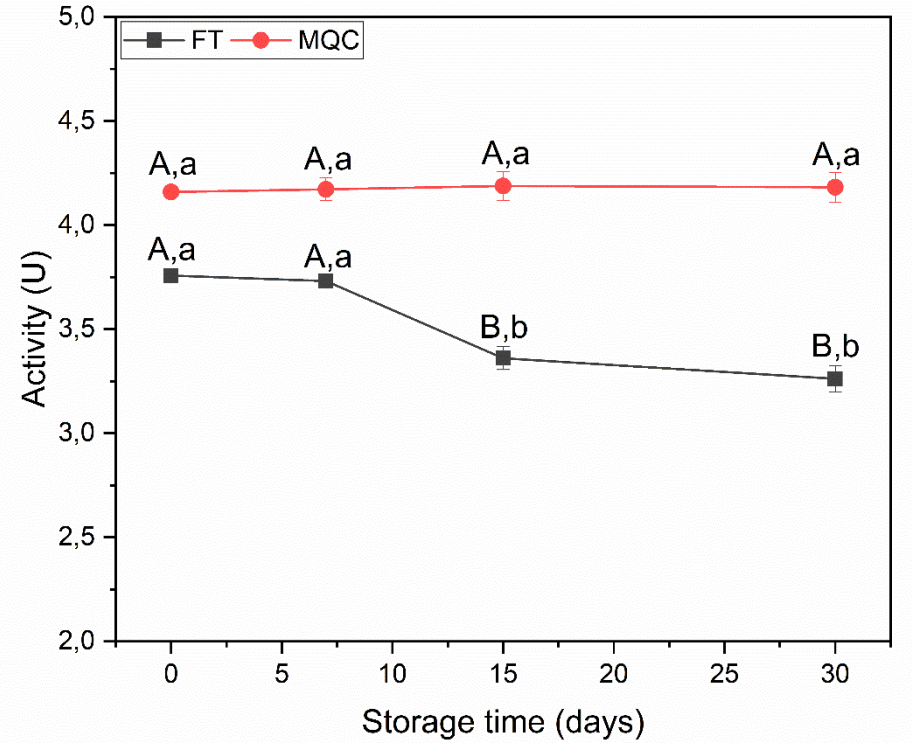


* Means followed by the same lower case letter for the temperature and upper case letter for the method (FT and MQC) do not differ significantly by Tukey's test ($P < 0.05$).

Doğan et al. (2021) studied trypsin of animal origin in free and immobilized form and reported little effect of the temperature on the relative enzyme activity of free enzyme, with the higher values observed at 45-65 °C, while an increase in activity was observed for immobilized enzyme at 40 °C. According to Han et al. (2015), the maintenance of the activity of derivative with increasing temperature is expected, once the immobilization ensures fewer conformational changes in the enzyme structure caused by temperature.

The results of stability to storage at 4 °C (Figure 3) showed that free trypsin (FT) showed a 10.56 % reduction in activity after storage for 15 days, and a total loss of activity of 13.19 % after 30 days. This loss of activity is a natural process caused by trypsin autolysis (protein-protein interaction), and the speed of this interaction depends on various factors, including the conditions of the medium, the enzyme used, and the storage time (SIDDIQUI et al., 2020). Unlike free enzyme, derivative remained stable during 30 days of storage, with no significant loss of activity. The storage stability of derivative was due to the protective microenvironment provided by metal ions inserted into support, responsible for preventing losses by desorption, conformational changes, and the autolysis of enzyme (DOĞAN et al., 2021).

Figure 3. Stability of free trypsin (FT) and trypsin immobilized on MQC support during storage at 4°C.



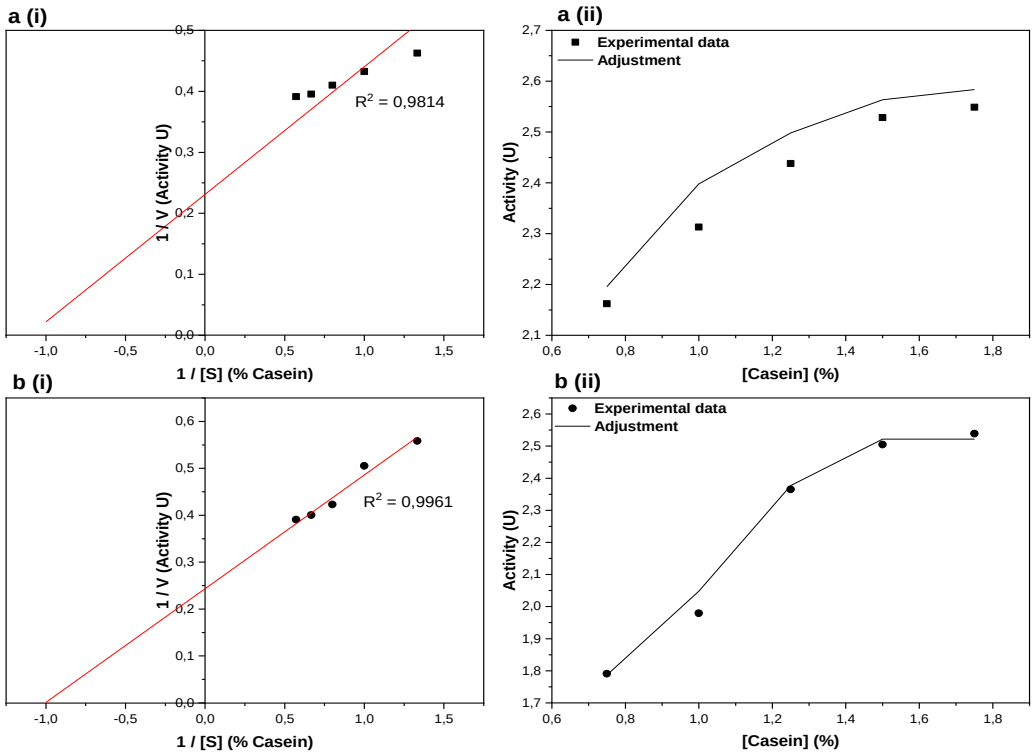
* Means followed by the same upper case letter for the storage time and lower case letter for the methods (FT and MQC) do not differ significantly from each other by Tukey's test ($P < 0.05$).

In general, trypsin immobilization on MQC allowed the reuse of enzyme for more than 5 cycles, with no significant loss of hydrolytic activity in the first 2 cycles. The support maintained the thermal stability of enzyme at the temperatures studied, and the hydrolytic activity remained constant during the 30 days of storage. According to Doğan et al. (2021), studies on enzyme stability and reuse are crucial parameters to investigate the economic advantages of immobilization, that is, the immobilization costs. Thus, the present results showed that the trypsin immobilization on activated carbon modified by metallization using a chelating agent is a very promising technique. Furthermore, immobilization prevents autocatalysis of enzyme since it restricts the contact between them (ASLANI et al., 2018).

3.8. Determination of kinetic parameters

Kinetic parameters (V_{max} and K_m) related to bovine casein hydrolysis of free enzyme and the derivative obtained with MQC were calculated according to the curves in Figure 4.

Figure 4. Changes in activity as a function of the initial casein concentration and plots after fitting the Lineweaver-Burk equation for the native enzyme, a (i and ii), and the enzyme immobilized on metalized carbon in the presence of a chelating agent, b (i and ii).



The graphs allowed determining the V_{max} and K_m values of free enzyme and derivative, with values of 4.26 and 4.09 U/min, and 0.88 and 0.92, respectively. The Michaelis-Menten constant (K_m) indicates the degree of affinity of enzyme for substrate, with close values for free and immobilized enzyme. According to Narayan et al. (2018), small differences in Km values for both free and immobilized enzyme can be negligible, i.e., they can be considered equal, indicating that immobilization did not cause structural changes in enzyme, keeping active the catalytic sites available for hydrolysis.

V_{max} value measures the number of substrate molecules that are converted into a product by one enzyme molecule in a given time (Hegedüs et al., 2020). Again, a small difference was observed between V_{max} values of free enzyme and immobilized enzyme. This difference may be due to the higher mobility of free enzyme and easier access to the substrate, while the diffusional effects and steric hindrance reduce reaction speed of immobilized enzymes (ASLANI et al., 2018). Narayan et al. (2018) pointed out that close V_{max} values indicate that the conversion of substrate to product was not affected by immobilization process, indicating that immobilization has no detrimental effects on the catalytic conversion.

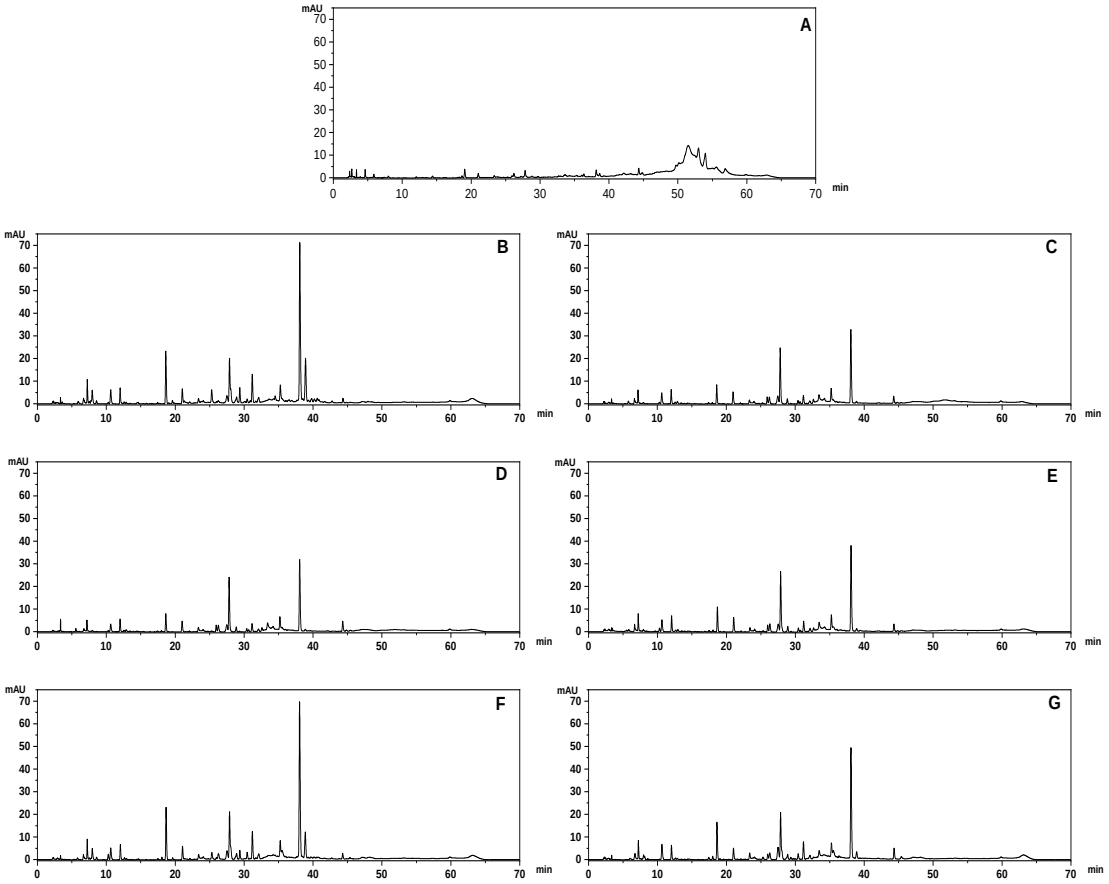
3.9. Characterization of peptides obtained from casein hydrolysis by chromatography

The chromatograms of hydrolysates are presented in Figure 5. For bovine milk casein (Sigma Aldrich CAS 9000-71-9), peaks with higher frequency in time range above 40 min were observed, indicating the presence of larger molecules in its composition. After hydrolysis, higher peaks were observed from 10 to 40 min, indicating casein hydrolysis and conversion to low molecular weight peptides. Moreover, regardless of derivative used in hydrolysis reaction, it was responsible for cleaving the casein molecules in the same sites, generating mostly the same peptides, varying only their concentration.

The occurrence of the same peptide profile (Figure 5) is due to specificity of trypsin to cleave peptide chains from carboxyl side of amino acids lysine (Lys) or arginine (Arg), except when both are followed by a proline (Pro) (SOUZA JR et al., 2020). The variation in peptide concentration upon hydrolysis can be seen in the intensity of peaks formed in chromatograms. Free trypsin (Figure 5-b) was responsible for generating a greater variety of peptides identified at 30 to 40 min, due to its higher mobility in the substrate. For derivatives, the higher intensity was observed between 10 min and 30 min,

while derivative of metallized carbon (Figure 5-f) also showed the presence of two peaks of higher intensity at 10 min, which may be related to the enzyme immobilized on this support, which has a higher catalytic activity and generate other types of peptides.

Figure 5. Chromatograms of casein (a) and peptides from casein hydrolysis with free trypsin (b) and trypsin immobilized on activated carbon (c), glutaraldehyde-functionalized carbon (d); genipin-functionalized carbon (e); metalized activated carbon (f); and metalized carbon in the presence of a chelating agent (g).



4. CONCLUSION

The present study established pH 5.0 as the optimal pH condition to perform immobilization, while pH 9.0 was the best condition for casein hydrolysis on the supports studied. The different modification methods of activated carbon were considered efficient, promoting a high immobilization capacity, with efficiency higher than 85%, besides forming more stable bonds with the enzyme, preventing enzyme desorption. The new modifications studied, such as functionalization with genipin and metallization, provided an increase in enzyme activity when compared to traditional immobilization methods.

The carbon modification with genipin increased activity of immobilized enzyme when compared to functionalization with glutaraldehyde, indicating its potential to replace glutaraldehyde in the modification of carbonaceous supports. The activated carbon metallization provided a heterofunctional support capable of enhancing the enzyme activity, ensuring more than 6 cycles of use. MQC provided higher stability of derivative to storage time when compared to free trypsin. The results showed that the metallization with a chelating agent can generate a promising support for trypsin immobilization, with great advantages from an economic point of view.

Regarding the peptides generated and analyzed by HPLC, the same peptides generated in casein hydrolysis by free enzyme were observed, regardless of the support used. Further studies on application of these peptides are required to investigate their antioxidant properties.

Declaration of competing interest

The authors declare that they do not have any conflict of interest

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CAPÍTULO 5

Considerações Finais

A partir do resíduo da tamarindo foi possível produzir um carvão ativado com uma elevada área superficial BET e diâmetro médio de poros na faixa de mesoporos. Além disso, pode ser conduzida quatro modificações na estrutura desse suporte. A primeira delas, e mais conhecida, foi realizada utilizando o agente glutaraldeído. Fez-se também modificações usando a metalização pela co-precipitação dos sais de ferro na presença e ausência de um agente quelante (ácido iminodiacético). E por fim foi feita uma modificação inédita neste tipo de suporte utilizando a genipina extraída no jenipapo verde. De modo quea eficieência das modificações foram comprovadas por análises texturais, MEV com EDS, FTIR e DRX. Todos estes suportes foram utilizados para imobilizar as proteases de origem anima (Pepsina e Tripsina), sendo responsáveis por proporcionar uma elevada capacidade de imobilização enzimática. Estas novas modificações também foram responsáveis por potencializar a atividade das enzimas imobilizadas em comparação aos métodos de imobilização tradicionais utilizados, garantindo vários ciclos de reutilização, bem como elevada estabilidade ao armazenamento e temperatura de hidrólise. A pepsina imobilizada quando empregada na reação de hidrólise da caseína bovina levou a produção de peptídeos similares ao produzido ao se utilizar a enzima na forma livre, com potenciais antioxidantes. Por fim, com base nos resultados obtidos neste estudo verifica-se o potencial da utilização do carvão ativado funcionalziado como suporte na imobilização de proteases de origem animal para o uso na síntese de peptídeos bioativos com poder antioxidante.