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DEBORAH CARVALHO FREITAS

PEPTÍDEOS ANTIMICROBIANOS PRODUZIDOS COM A HIDRÓLISE DE PROTEÍNAS DO GLÚTEN POR PROTEASES VEGETAIS AUMENTAM O TEMPO DE PRATELEIRA DO PÃO

FORTALEZA
2021

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Tese apresentada ao programa de Pós-graduação em Bioquímica da Universidade Federal do Ceará, como parte dos requisitos para obtenção de título de Doutora em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas.

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“What’s the use of doing all this workif we don't get some fun out of it?”

Rosalind Franklin

RESUMO

O pão é um dos produtos alimentícios mais consumidos no mundo e sua contaminação por bolores e leveduras pode gerar perdas econômicas e insatisfação dos consumidores. A forma maiscomum de estender o tempo de prateleira do pão é o uso de aditivos químicos. Peptídeos antimicrobianos (AMPs) têm sido relatados como uma alternativa promissora a estes conservantes. O presente estudo teve como objetivo identificar, caracterizar e sintetizar AMPs, gerados a partir da hidrólise de proteínas do glúten, com atividade antifúngica. As proteínas do glúten foram hidrolisadas pelas frações proteolíticas dos látices de *Calotropis procera, Cryptostegia grandiflora* e *Carica papaya* gerando três hidrolisados que exibiram atividade contra todos os seis fungos testados. Pães foram produzidos com os hidrolisados e analisados quanto a parâmetros físicos. As médias de volume específico e de fator de expansão não foram afetadas com a adição de hidrolisados, enquanto o número de alvéolos foi favorecido e a dureza foi parcialmente afetada com a adição de 0,2 e 0,3 g kg^{-1}. Os hidrolisados estenderam a vida útil do pão em pelo menos três dias. A partir dos três hidrolisados, 31 peptídeos foram sequenciadose caracterizados com base em critérios atribuídos aos AMPs e, em seguida, os quatro melhores peptídeos foram sintetizados. Ensaios antifúngicos contra *Penicillium sp* mostraram que os peptídeos sintéticos foram eficazes em inibir o crescimento fúngico. Análises defluorescência e microscopia eletrônica de varredura mostraram que todos os hidrolisadose peptídeos sintéticos foram capazes de induzir danos à membrana plasmática dos fungos.Todos os resultados corroboram com o potencial dos peptídeos provenientes do glúten como conservantes naturais para inibir o crescimento fúngico e estender a vida útil do pão.

Palavras-chave: *Calotropis procera*; *Carica papaya*; *Cryptostegia grandiflora;* peptídeos antimicrobianos; peptídeos do glúten; *Penicillium*.

ABSTRACT

Bread is one of the most consumed food products in the world and its contamination byfungi can generate economic losses and consumers dissatisfaction. The most common way to extend bread's shelf time is the use of chemical preservatives. Antimicrobial peptides (AMPs) have been reported as a promising alternative to these preservatives. The present study aimed to identify, characterize and synthesize AMPs, generated from the hydrolysis of gluten proteins, with antifungal activity. The gluten proteins were hydrolyzed by the proteolytic fractions of the latex of *Calotropis procera, Cryptostegia grandiflora* and *Carica papaya* generating three hydrolysates that exhibited activity against all six fungi tested. Breads were produced with the hydrolysates and analyzed for physical parameters. The averages of specific volume and expansion factor were not affected with the addition of hydrolysates, while the number of alveoli was favored and the hardness was partially affected with the addition of 0.2 and 0.3 g kg^{-1}. The hydrolysates extended the shelf life of the bread by at least three days. From the three hydrolysates, 31 peptides were sequenced and characterized based on criteria attributed to AMPs, and then the four best peptides were synthesized. Antifungal assays against *Penicillium sp* showed that synthetic peptides were effective in inhibiting fungal growth. Fluorescenceand scanning electron microscopy analysis showed that all hydrolysates and synthetic peptides were able to induce damage to the fungal plasma membrane. All resultscorroborate with the potential of peptides from gluten as natural preservatives to inhibit fungal growth and extend the shelf life of bread.

Keywords: antimicrobial peptides; *Calotropis procera*; *Carica papaya*; *Cryptostegia grandiflora;* gluten peptides; *Penicillium*.

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LISTA DE ABREVIATURAS E SIGLAS

AMPs	Peptídeos antimicrobianos
C. grandiflora	*Cryptostegia grandiflora*
C. papaya	*Carica papaya*
C. procera	*Calotropis procera*
CapLP	Proteínas do látex de *Carica papaya*
CgLP	Proteínas do látex de *Cryptostegia grandiflora*
CpLP	Proteínas do látex de *Calotropis procera*
GP	Proteínas do glúten
Pep1	Peptídeo 1
Pep2	Peptídeo 2
Pep3	Peptídeo 3
Pep4	Peptídeo 4
PI	Iodeto de propídio

SUMÁRIO

1 INTRODUÇÃO

1.1 Pão

O pão consiste em um alimento que começou a ser produzido há pelo menos 5000 anos na região da Mesopotâmia ou Egito Antigo, e registros sugerem que surgiu antes mesmo do advento da agricultura (KIM, 2013; DE BONI *et al*., 2019). Atualmente, há uma grande variedade de pães que diferem em tipos, formas, tamanhos e texturas (MELINI, V; MELINI, F., 2018; JEROME; SINGH; DWIVEDI, 2019). Os pães podem ser classificados como artesanais, que são geralmente produzidos em padarias locais, ou como pães processados, que são embalados e possuem um tempo de prateleira maior (JAYASHREE; VAISHALI; MOSES, 2018).

O pão desempenha um papel importante na dieta diária da maioria da população mundial, porque além do ótimo sabor, ele é rico em macro e micronutrientes que são vitais para o ser humano (ROSELL, 2011; DAS; SARKAR; HOSSAIN, 2020). Os pães podem ainda ser enriquecidos com diversos ingredientes ou preparados com diferentes farinhas que irão proporcionar maior concentração de proteínas, antioxidantes, fibras alimentares e outros nutrientes essenciais (BIJLWAN *et al*., 2019).

O pão se destaca não apenas pela sua importância nutricional, mas também devido a sua importância econômica. Pesquisas realizadas pela Associação Brasileira da Indústria de Panificação e Confeitaria (ABIP), em parceria com o Instituto Tecnológico de Panificação e Confeitaria (ITPC), mostraram que o setor de panificação brasileiro registrou um crescimento da ordem de 2,65% em 2019, o que é equivalente a um faturamento de R$ 95,08 bilhões (AFNEWS, 2020). De acordo com a BusinessWire, o mercado mundial de produtos de panificação deve crescer, com o segmento de pães apresentando potencial para crescer acima de 5,8%, sendo previsto atingir mais de US $ 214,8 bilhões até o ano 2025 (BUSINESSWARE, 2020).

Apesar destes produtos da panificação continuarem sendo amplamente consumidos, observa-se mudanças nos padrões de consumo. O consumidor se tornou mais exigente ao longo dos anos passando a ter também um grande interesse em pães que usam menos aditivos químicos (KUBICOVÁ; KÁDEKOVÁ, 2011; KUBICOVÁ *et al*., 2020). Muitos destes aditivos químicos adicionados ao pão têm como objetivo aumentar sua vida útil.

Contudo, eles podem levar ao endurecimento da migalha e diminuição da textura, tornando o pão desagradável para o consumidor (DASHEN *et al*., 2016; AXEL *et al*., 2016; MELINI, V; MELINI, F., 2018).

1.2 Bolores e leveduras na Panificação

Desde que as pessoas deram início às práticas de cultivo e armazenamento de alimentos, problemas de deterioração e perdas desses produtos passaram a ser constantes. Devido à incrível capacidade de adaptação dos fungos em utilizar diferentes substratos, incluindo carboidratos, ácidos orgânicos, proteínas e lipídios, eles se destacam como o principal grupo de microrganismos que contaminam produtos alimentares (GARNIER; VALENCE; MOUNIER, 2017; RIBES *et al*., 2018).

Os produtos da panificação são excelentes substratos para o desenvolvimento de bolores e leveduras, não somente por causa da sua composição (GEREZ *et al*., 2015; FAPARUSI; ADEWOLE, 2019), mas também pela sua estrutura porosa que facilita a fixação dos micélios, o alto teor de umidade (cerca de 40%), alta atividade de água (0,94 a 0,98), pH ligeiramente ácido (pH = 5,5-6,0) e temperatura de armazenamento (20–35 ° C) (DAGNAS *et al*., 2017; GARCIA *et al*., 2019a; 2019b). Por conta destas características, o pão é um dos alimentos mais descartados pela indústria e por consumidores devido à contaminação fúngica (MELIKOGLU; WEBB, 2013; TORRIJOS *et al*., 2019).

Os fungos são responsáveis por provocar uma aparência indesejável, além de causarem alterações nas características sensoriais como odor e sabor desagradáveis, em virtude da produção de metabólitos e exoenzimas como lipases e proteases (GARNIER; VALENCE; MOUNIER, 2017; GARCIA *et al*., 2019c). Apesar de ser difícil mensurar as perdas atribuídas aos fungos, estima-se que estão entre 1 e 5% da produção (SARANRAJ; SIVASAKTHIVELAN, 2016). Em países de clima tropical, os prejuízos podem ser ainda maiores. Por exemplo, um estudo realizado por Freire (2011) em padarias do Brasil, revelou que as perdas podem chegar a valores próximos de 10% da produção.

Problemas de contaminação por bolores e leveduras podem estar presentes desde o início, devido ao uso de matérias-primas que foram contaminadas no campo ou durante o armazenamento (PITT *et al*., 2009). Contudo, na etapa de cozimento, o pão é exposto a temperaturas elevadas com o seu interior atingindo até 100 °C durante alguns minutos (NEVES

et al., 2019), inativando a maioria dos esporos de fungos e outros microrganismos associados à massa do pão (DEBONNE *et al*., 2018). Além disso, os problemas associados a contaminação fúngica são mais evidentes depois que o pão já foi produzido, por conta da distribuição dos esporos fúngicos no ambiente (DOS SANTOS *et al*., 2016).

Durante o armazenamento do pão ocorre perda de água em razão da transferência de umidade da superfície do pão para o espaço superior da embalagem e daí para a atmosfera. Bolores podem crescer rapidamente em uma atmosfera úmida e especialmente no pão dentro de uma embalagem. Pode-se observar que a perda de umidade aumenta com a temperatura de armazenamento e sua duração. Quando o pão é tirado do forno quente, gotículas de água condensam-se na superfície interna da embalagem promovendo o crescimento de bolores. Pão fatiado embrulhado é ainda mais suscetível à deterioração fúngica, pois uma superfície mais ampla fica exposta a infecções. Vários fatores podem influenciar a taxa de crescimento de fungos: o tipo de farinha, o método de processamento, a embalagem e as condições de armazenamento (NAJAFABADI *et al*., 2014; MELINI, V; MELINI, F., 2018).

Embora a deterioração por bolores e leveduras seja um grave problema devido às perdas econômicas, outro motivo de preocupação é a possibilidade da presença de micotoxinas nos produtos de panificação decorrente da contaminação por fungos em cereais dos quais o pão e os produtos de panificação são derivados (DE KOE; JUODEIKIENE, 2012; OLIVEIRA; ZANNINI; ARENDT, 2014). Micotoxinas são metabólitos secundários produzidos por uma grande variedade de patógenos de cultivo e fungos de deterioração de alimentos, incluindo espécies dos gêneros *Aspergillus* e *Penicillium*. As micotoxinas geralmente são termoestáveis (acima de 100 C), logo, podem ser transferidas para o pão, mesmo após a etapa de cozimento (OLIVEIRA *et al*., 2014; AXEL; ZANNINI; ARENDT, 2017).

Desta forma, evitar a ocorrência destes fungos em alimentos é um objetivo da indústria de panificação. Assim, várias estratégias têm sido aplicadas para prevenir e controlar as contaminações fúngicas nas instalações industriais. Métodos tradicionais, também chamados de tecnologias tradicionais de obstáculos, que incluem boas práticas de fabricação, tratamento do ar, procedimentos de limpeza e desinfecção são exemplos de procedimentos padrões importantes que permitem redução dos níveis de contaminação. Outra forma encontrada pela indústria é a utilização de conservantes químicos (ROSS; MORGAN; HILL, 2002; GUERRERO *et al*., 2017).

1.3 Aditivos Químicos e Naturais

Como dito anteriormente, a deterioração do pão ocorre principalmente devido à contaminação pós-produção. A deterioração visual do pão por bolores e leveduras é o motivo mais comum para a rejeição do pão pelo consumidor (LÓPEZ-ALARCÓN *et al.* , 2019). A principal estratégia adotada para inibir o crescimento fúngico e estender o prazo de validade tem sido a adição de conservantes químicos durante o processamento. Dentre os conservantes, propionato de cálcio ou sódio e ácido sórbico ou seus sais são os aditivos mais comumente utilizados (FSSAI, 2011; SALADINO *et al*., 2017; HU *et al*., 2019). De acordo com a Food and Drug Administration (FDA) e a European Food Safety Authority (EFSA), principais órgãos reguladores de segurança alimentar do mundo, a quantidade permitida desses aditivos é de até 0,2% e 0,3 % (peso/peso) de sorbato e propionato, respectivamente (STOPFORTH; SOFOS; BUSTA, 2005; LAVERMICOCCA *et al*., 2016).

O setor de alimentos enfrenta o desafio de se adequar a um mercado em constante transformação para atender consumidores cada dia mais informados e exigentes em relação aos produtos que consomem. Fatores como o aumento do poder de compra da população, maior acesso à informação, aumento da escolaridade, entre outros, modificam as percepções e as preferências em relação ao alimento a ser consumido e influenciam a busca por um estilo de vida mais saudável (BRASIL FOOD TRENDS, 2020).

Embora o uso de conservantes químicos favoreça a inibição de bolores e leveduras, o que proporciona o aumento da vida útil do pão, eles não têm valor nutricional e podem ser prejudiciais à saúde humana e ao meio ambiente (DEBONNE *et al*., 2019; SHEHATA *et al*., 2019). Com relação ao propionato, sua ingestão contínua pode causar efeitos colaterais como dores de cabeça e irritabilidade comportamental e distúrbios do sono em crianças (DENGATE; RUBEN, 2002). Além disso, o uso de aditivos químicos pode ser restringido pelo surgimento de cepas de fungos resistentes (NIONELLI *et al*., 2020). Assim, a necessidade de encontrar alternativas naturais e saudáveis aumentou nos últimos anos como resultado da maior conscientização dos consumidores sobre os riscos associados ao uso de conservantes químicos (AXEL *et al*., 2016; LUZ *et al*. 2017). A demanda por produtos naturais obrigou a indústria a se esforçar na busca por produtos isentos de conservantes químicos (MOTARJEMI; MOY; TODD, 2013; SAMAPUNDO *et al*., 2017) para atender a consumidores cada vez mais exigentes e preocupados com saúde e bem-estar e que buscam alimentos rotulados como naturais (CALEJA *et al*., 2016; CÍSAROVÁ *et al*., 2019).

Conservantes naturais podem ser obtidos de plantas, animais e microrganismos (CAROCHO; MORALES; FERREIRA, 2015; SHARIF *et al*., 2018). Óleos essenciais, ervas, especiarias e produtos de fermentação são exemplos de bio-conservantes que vêm sendo estudados (JHANDAI *et al*., 2019). Os microrganismos mais usados para a biopreservação de alimentos incluem as bactérias do ácido lático (LAB) devido às suas propriedades probióticas e por serem reconhecidos como seguros (GRAS). Além disso, podem ser encontrados em abundância na natureza (SKARIYACHAN; GOVINDARAJAN, 2019; HAMMAMI; ISMAIL; CORSETTI, 2019).

As LABs já são aplicadas na panificação como agentes de preservação, consistindo de uma alternativa adequada aos aditivos químicos (ZANNINI *et al*., 2012, AXEL; ZANNINI; ARENDT, 2017). As cepas LAB produzem diferentes substâncias antimicrobianas capazes de inibir o crescimento fúngico e degradar micotoxinas, incluindo ocratoxinas e aflatoxinas (ALVAREZ-SIEIRO *et al*., 2016; SADIQ *et al*., 2019). Apesar dos benefícios das LABs, algumas cepas podem transportar e expressar genes associados à virulência e resistência a antibióticos, comprometendo seu uso como biopreservativos de alimentos (DE CASTILHO; NERO; TODOROV, 2019).

Compostos antifúngicos de origem vegetal vêm sendo utilizados como estratégias de preservação natural em produtos de panificação (TAKWA *et al*., 2018; DEBONNE *et al*., 2019). Óleos essenciais são utilizados há muito tempo para diversos fins, inclusive como antimicrobianos. Alguns estudos relatam sucesso na inibição de microrganismos no pão com o uso de vapor oriundo da extração dos óleos, com aumento da inibição proporcional ao aumento das concentrações de vapor (DEBONNE *et al*., 2018; MANI LÓPEZ *et al*., 2018).

Embora já se tenha conhecimento sobre muitos conservantes naturais, nem todos são capazes de manter todas as propriedades organolépticas dos produtos (SHARIF *et al*., 2017). Além disto, eles encontram limitações como baixo rendimento e o alto custo, o que representam perdas econômicas para as indústrias, visto que aditivos químicos têm menor custo de produção e muitas vezes conseguem melhores resultados na inibição do crescimento de microrganismos e consequentemente maior vida útil de prateleira dos produtos (MAKKAR; CAMEOTRA; BANAT, 2011; GARCÍA-GARCÍA; SEARLE, 2016).

1.4 Peptídeos Antimicrobianos

Peptídeos antimicrobianos (AMPs), também conhecidos como peptídeos de defesa do hospedeiro (HDPs), têm sido relatados como uma alternativa promissora aos conservantes químicos (RAI *et al*., 2016; TANG *et al*., 2018). Os AMPs correspondem a sequências de aminoácidos que são potentes antibióticos de amplo espectro contra microrganismos (REDDY; YEDERY; ARANHA, 2004). Esses peptídeos são abundantes na natureza, podendo estar presentes em microrganismos, animais e plantas (KUMAR; KIZHAKKEDATHU; STRAUS, 2018).

Os AMPs compartilham características em comum como carga líquida positiva e estrutura anfipática e são compostos por L-aminoácidos definidos em estruturas secundárias formadas por α-hélices, folhas β ou ambos. Eles variam no número de aminoácidos (5 a 50 aminoácidos), além de apresentarem diferentes propriedades físico-químicas, solubilidade e mecanismo de ação (SIRTORI; MOTTA; BRANDELLI, 2008; BISWARO *et al*., 2018). Parâmetros como hidrofobicidade, anfipaticidade e presença de resíduos carregados positivamente são essenciais para a atividade antimicrobiana dos AMPs, pois permitem que os peptídeos possam interagir com as membranas fosfolipídicas dos microrganismos, e essa interação é crítica para a especificidade e atividade desses peptídeos (CHEN *et al*., 2007; WANG *et al*., 2016).

Embora o mecanismo de ação dos AMPs não seja totalmente elucidado, as explicações variam desde formação de poros à ruptura da membrana (SCOCCHI *et al*., 2016; LEI *et al*., 2019). As complexas interações dos AMPs com a membrana celular dos microrganismos demonstram que os mecanismos podem variar consideravelmente entre as diferentes classes de peptídeos antimicrobianos (LEE *et al*., 2016).

A princípio foi proposto que o mecanismo de ação dos peptídeos antimicrobianos contra fungos compreendia lise celular fúngica e interferência na síntese de parede celular (DE LUCCA; WALSH, 1999). Porém, alguns estudos sugerem que peptídeos antifúgicos podem atuar também sobre diferentes alvos (WANG *et al*., 2016), como inibição do crescimento de hifas (GALVEZ *et al*., 1993), perturbação da integridade da membrana do fungo, translocação para atingir alvos intracelulares ou alteração do metabolismo fúngico (THERY; LYNCH; ARENDT, 2019).

Apesar da atividade de AFPs geralmente envolver mecanismos relacionados à membrana, já foi demonstrado que atuam também na inibição de enzimas ou na síntese de

componentes da parede celular (THERY; LYNCH; ARENDT, 2019). Além disso, alguns peptídeos são capazes de induzir a produção de espécies reativas de oxigênio (ROS) e, portanto, ativar a morte celular programada (KHAN *et al*., 2019).

O interesse em peptídeos antifúngicos tem aumentado devido aos seus múltiplos modos de ação, o que limita o risco de fungos desenvolverem resistência. Os peptídeos antimicrobianos parecem ser mais ativos que os ácidos fracos comumente usados como conservantes químicos nos alimentos (PREMA; PRUTHVI, 2012; THERY; LYNCH; ARENDT, 2019). Embora haja um número crescente de relatos de AMPs em aplicações alimentares, muitos têm focado principalmente em peptídeos com ação contra bactérias Gram-positivas, como é o caso do peptídeo antibacteriano nisina, obtido do *Lactococcus lactis* (TAJKARIMI; IBRAHIM, 2012; RAI *et al*., 2016). Alguns AMPs foram aprovados e certificados para uso pela EFSA, no entanto a quantidade limitada de pesquisas sobre AMPs e seus efeitos em alimentos restringem a aplicação desses peptídeos (THERY; LYNCH; ARENDT, 2019).

1.5 Proteínas do Glúten

As proteínas do glúten formam uma das redes de proteínas mais complexas da natureza, em virtude de seus inúmeros componentes, tamanhos e diversidade, correspondendo a uma fonte abundante de proteínas vegetais que podem ser usadas em formulações de alimentos (WIESER, 2007; ELMALIMADI *et al*., 2017). O glúten tem um grande impacto na nutrição humana, pois é responsável por muitas propriedades da farinha de trigo usada na fabricação de pães e massas (SHEWRY, 2019).

O glúten é definido como uma massa proteica obtida após lavagem suave da farinha de trigo sob água corrente para remover o excesso de amido e componentes solúveis (CERESINO *et al*., 2019). Ele consiste em uma rede complexa de proteínas, composta principalmente pelas proteínas gliadina e glutenina, que são responsáveis pelas propriedades viscoelásticas da massa, que se desenvolvem durante a mistura (CAO; BAUMERT; DOWNS, 2020). Estas proteínas são as principais responsáveis pela capacidade da massa de reter gás durante a fermentação. As ligações dissulfeto intermoleculares da glutenina são responsáveis por seu comportamento altamente elástico, enquanto as ligações dissulfeto intramoleculares da gliadina resultam em comportamento viscoso. A viscoelasticidade da rede do glúten é

conhecida por depender dessas interações intermoleculares (WIESER, 2007; YAZAR *et al*., 2017).

As proteínas do glúten são o principal grupo de proteínas de armazenamento nos grãos, no entanto, os grãos de cereais contêm centenas de diferentes componentes proteicos que são tradicionalmente agrupados em quatro frações, de acordo com a classificação desenvolvida por Osborne (1924), com base em séries de extrações por diferentes solventes. As frações correspondem às albuminas (solúveis em água), globulinas (solúveis em solução salina), prolaminas (solúveis em álcool) e glutelinas (insolúveis em outros solventes, mas podem ser extraídas com ácidos ou álcalis) (SCHALK *et al*., 2017; SHEWRY, 2019). Albuminas e globulinas são encontradas em menor proporção (20–25%) e compreendem principalmente proteínas metabólicas e enzimas, enquanto prolaminas e glutelinas (75–80%) atuam como proteínas de armazenamento (WIESER, 2007).

As prolaminas compõem um grupo de proteínas de armazenamento com alto conteúdo de nitrogênio e prolina. Elas recebem nomes específicos de acordo com as espécies em que são encontradas, tais como: gliadina no trigo, hordeína na cevada, secalina no centeio e avenina na aveia (FOK *et al*., 2016, LIAO *et al*., 2017). As gliadinas são divididas nos tipos α, γ e ω com base em suas sequências de aminoácidos e mobilidade na eletroforese, e as subunidades reduzidas de glutenina são divididas em grupos de alto peso molecular (HMW), médio peso molecular (MMW) e baixo peso molecular (LMW) (TATHAM *et al*., 2000).

Para ampliar a utilização do glúten de trigo é necessário melhorar suas propriedades funcionais. Modificações enzimáticas estão entre as principais estratégias para modificar a estrutura nativa dessas proteínas (SHARIF *et al*., 2018). Vários peptídeos com diversas atividades biológicas já foram isolados a partir de proteínas alimentares após hidrólise enzimática (RANI; POOJA; PAL, 2017; NONGONIERMA ; FITZGERALD, 2018), e o glúten pode representar uma fonte promissora de peptídeos que podem ser usados na panificação.

1.6 Hidrólise Proteolítica

A maneira mais conhecida de produzir peptídeos com atividade biológica é através da hidrólise por proteases (AGUILAR; GRNATO CASON; DE CASTRO, 2019). As proteases, também chamadas de proteinases ou peptidases, são o grupo de enzimas hidrolíticas que realizam a hidrólise de ligações peptídicas em proteínas e peptídeos. Elas envolvem

principalmente dois grupos de enzimas, que são classificadas como exo- e endo-proteases (COOPER, 2002; MAZORRA-MANZANO; RAMÍREZ-SUAREZ; YADA, 2018). As exo-proteases atuam nas extremidades amino- ou carboxil-terminal das proteínas, enquanto as endo-proteases atuam no interior da sequência proteica (GONZÁLEZ-RÁBADE *et al*., 2011). As propriedades e funções dessas enzimas têm atraído a atenção da comunidade científica e da indústria há muitos anos, e têm sido essenciais em muitas aplicações biotecnológicas na indústria de alimentos (TAVANO *et al*., 2018; BINOD *et al*., 2019).

As propriedades catalíticas das proteases já permitiram sua introdução em vários processos industriais e os recentes avanços na produção de hidrolisados de proteínas proporcionaram um importante desenvolvimento nessa área. A hidrólise enzimática permite o uso de diferentes fontes de proteínas alimentares que, após a hidrólise, também podem ser utilizadas como fontes de peptídeos bioativos, com propriedades biológicas e funcionais variadas que podem ser empregadas industrialmente (AVRAMENKO; LOW; NICKERSON, 2013; DOS SANTOS AGUILAR; SATO, 2018).

A maioria das preparações enzimáticas comerciais frequentemente usadas são de fontes animais (tripsina, pepsina e quimotripsina) e microbianas (alcalase, flavourzima e neutrase). Um levantamento na literatura científica indica que as proteases mais citadas para produção de peptídeos bioativos giram em torno de 75% para animais e microbianas e apenas 15% para vegetais (MAZORRA-MANZANO; RAMÍREZ-SUAREZ; YADA, 2018). O uso de proteases vegetais ainda é muito restrito à papaína e bromelina, que são extraídas do mamão e abacaxi, respectivamente. No entanto, o interesse por proteínas vegetais de alta qualidade para aplicações nos setores de alimentos e rações está aumentando (SCHLEGEL *et al*., 2019). O uso de proteases vegetais cria possibilidades de obtenção de novos peptídeos bioativos, pois elas podem apresentar especificidades hidrolíticas diferentes, resultando em diferentes sequências de peptídeos (LIU *et al*., 2018).

1.7 Látex e suas enzimas proteolíticas

O látex é um fluido biológico de aspecto leitoso produzido por mais de 40 famílias de plantas (KONNO, 2011; RODRIGUES *et al*., 2012). Esse fluido é constituído por uma suspensão coloidal composta por uma fase sólida (cis-1, 4-poliisopreno ou borracha) e uma fase líquida (soro) (PICKARD, 2008). Grande variedade de proteínas e metabólitos secundários tais como alcalóides, terpenóides, cardenólidos, fenóis, glicosídeos, taninos e borracha, já foram

identificados no látex de diversas plantas (HAGEL; YEUNG; FACCHINI, 2008). Ao sofrer alguma injúria mecânica, ataque de patógenos ou insetos, o látex é liberado, que se encontra armazenado nos laticíferos, células altamente especializadas que produzem látex e se encontram espalhadas por todo o corpo da planta (PICKARD, 2008, RAMOS *et al*., 2019).

As proteases, especialmente as cisteínicas, são proteínas abundantes nos fluidos laticíferos, sendo relacionadas a papéis importantes na defesa das plantas. Estas proteínas são alvos de muitos estudos em razão das diversas possibilidades de aplicações (FREITAS *et al*., 2007; RAMOS *et al*., 2014, 2019). Alguns trabalhos têm explorado o potencial dessas proteases na produção de peptídeos bioativos. Proteases cisteínicas do látex do jaracatiá (*Jacaratia corumbensis*) foram utilizadas para hidrólise das caseínas. O hidrolisado exibiu atividade contra *Enterococcus faecalis, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia e Staphylococcus aureus* (ARRUDA *et al*., 2012). Ficina, um extrato enzimático composto por diversas proteases, obtido a partir do látex da figueira (*Ficus carica*), é capaz de hidrolisar uma grande variedade de ligações peptídicas. O hidrolisado de gelatina de *Uroteuthis duvauceli* (uma lula indiana) com propriedades anti-câncer de mama foi produzido usando ficina (MORELLON-STERLING *et al*., 2020).

1.8 Plantas Laticíferas usadas neste estudo

1.8.1 Cryptostegia grandiflora

C. grandiflora popularmente conhecida como videira-da-borracha, é uma trepadeira lenhosa perene da família Apocynaceae, nativa de Madagascar. É uma espécie invasiva, adaptável em ambientes áridos e semiáridos, onde a água é limitada. Também pode tolerar uma variedade de condições de solo, incluindo sódico e salino (INVASIVE SPECIES COMPENDIUM, 2014; LUIZZA *et al*., 2016) (Figura 1). Seu uso medicinal já foi relatado, sendo empregada como purgante, analgésico, curativo, além de atividades anticâncer, antiinflamatória, antioxidante e antiviral (PRABAKARAN *et al*., 2016; PRASAD; LAVANYA, 2017). Atividade proteolítica já foi detectada no látex de *C. grandiflora* (CgLP). Freitas *et al*. (2010) constataram que proteases cisteínicas são responsáveis por quase toda a atividade proteolítica de CgLP. Uma peptidase cisteínica purificada de CgLP foi capaz de inibir o crescimento de fungos fitopatogênicos, evidenciando o papel defensivo das peptidases cisteínicas do látex contra fitopatógenos. De fato, peptidases cisteínicas parecem predominar na maioria dos laticíferos estudados (RAMOS *et al*., 2014; 2019).

Figura 1 - Aspecto geral da planta, flores e coleta do látex de *Cryptostegia grandiflora.*

Fonte: Silva, 2019; Davesgarden.

1.8.2 Calotropis procera

C. procera é um arbusto perene da família Apocynacea, relatada como invasora, sendo nativa da Ásia e da África, e amplamente distribuída em diversas partes do mundo (HASSAN *et al*. 2015; MENGE *et al*., 2017). No Brasil, é encontrada principalmente na região Nordeste onde predominam biomas de Cerrado e Caatinga. Popularmente é conhecida como leiteiro, algodão-de-seda, flor-de-seda, ciúme, hortência, entre outros, dependendo da região onde se encontra. Suas características permitem fácil propagação, devido sua habilidade de se adaptar e crescer em condições severas como a falta de água e solos salinos (FIGUEIREDO *et al*., 2014; FERNANDES *et al*., 2020) (Figura 2).

Ela é conhecida popularmente como leiteiro, em função da grande quantidade de látex liberado pelas folhas ou caule (RAHMAN; WILCOCK, 1991). O látex tem sido bastante estudado e uma grande quantidade de atividades farmacológicas foram identificadas, tais como antidiabética, hepatoprotetora, antiartrítica, anticancerígena (SINGHAL; KUMAR, 2009), analgésica (PARIHAR; BALEKAR, 2016), cicatrizante (RAMOS *et al*., 2016) e anti-inflamatória (OBESE *et al*., 2018). Relatos demonstram sua atividade contra fungos fitopatogênicos (SOUZA *et al.,* 2011).

Pesquisas concentradas na atividade proteolítica de proteínas do látex de *C. procera* (CpLP) revelam que consiste em uma fonte rica nesta atividade. Freitas *et al*. (2007) caracterizaram as proteínas laticíferas dessa planta evidenciando a presença principalmente de proteases cisteínicas. Em estudo recente foram relatadas que suas proteases cisteínicas apresentaram atividade antifúngica, apoiando as evidências de que essas enzimas desempenham papel defensivo da planta contra infecções fúngicas (FREITAS *et al*., 2020). O potencial proteolítico do extrato desse látex tem sido explorado em aplicações biotecnológicas como na depilação de couro (LOPÉZ *et al*., 2017) e fabricação de queijo (SILVA *et al*., 2020).

Figura 2 - Aspecto geral da planta, flores, fruto e sementes de *Calotropis procera*.

Fonte: Rocha, 2019; Plantas Flores.

1.8.3 Carica papaya

A espécie laticífera *C. papaya*, comumente conhecida como mamão, é uma planta tropical nativa da América Central e cultivada em várias regiões tropicais e subtropicais por seus frutos comestíveis e seu látex (Figura 3) (LIYONGO *et al*., 2020; ADEDAYO *et al*., 2020). É uma planta herbácea perene que pode atingir até 12 m e se destaca como a espécie mais popular e economicamente importante entre a família Caricacae (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017; SANTANA *et al*., 2019). As propriedades farmacológicas de *C. papaya* são conhecidas tradicionalmente como antioxidante, anti-inflamatória e antibacteriana. A literatura descreve seu uso como fito-fármaco no manejo de várias doenças, com relatos de atividades contra malária, diabetes, hiperglicemia, entre outras (LIYONGO *et al*., 2020). Satrija *et al*. (1995) relataram o potencial anti-helmíntico do látex desta espécie contra nematóides intestinais de hospedeiros mamíferos. O látex de *C. papaya* (CapLP) é uma rica fonte de proteases cisteínicas (KONNO *et al*., 2004). Estudos revelaram que a atividade proteolítica desse látex está notadamente relacionada com as proteases cisteínicas presentes. A análise proteômica de CapLP previu que várias dessas proteínas estavam envolvidas em respostas ao estresse da planta (RODRIGUES *et al*., 2012).

Há uma alta demanda mundial pela papaína. Esta enzima extraída do látex de *C. papaya* é uma das proteases mais estudadas e amplamente utilizadas na indústria, com aplicações como ingrediente na fabricação de cerveja, amaciamento de carnes, indústrias farmacêuticas e cosméticas (MAMBOYA, 2012). A papaína é um exemplo de enzima proteolítica obtida de látex vegetal (FEIJOO-SIOTA; VILLA, 2011) que vem se consolidando na produção de peptídeos bioativos (COTABARREN *et al*., 2019; SITANGGANG; LESMANA; BUDIJANTO, 2020), sendo uma boa candidata para atuar na hidrólise do glúten. Os potenciais dos extratos provenientes do látex de *C. procera* e de *C. grandiflora* também podem ser explorados, visto que são ricos em atividade proteolítica (FREITAS *et al*., 2010; RAMOS *et al*., 2013). Algumas proteases extraídas do látex dessas plantas já foram purificadas e caracterizadas (DUBEY; JAGANNADHAM, 2003; RAMOS *et al*., 2014; KWON *et al*., 2015). Estudos realizados com as proteases presentes nas frações de CpLP e CgLP demonstraram que elas foram eficientes na hidrólise da k-caseína do leite produzindo peptídeos (FREITAS *et al*., 2016). Portanto, as proteases de látex de *C. papaya*, *C. procera* e *C. grandiflora* podem ser eficientes na hidrólise das proteínas do glúten para produção de AMPs que podem ser usados como aditivos naturais para estender a vida útil do pão.

Figura 3 - Aspecto geral da planta, frutos e látex de *Carica papaya*.

Fonte: Melo, 2019; Embrapa; Sítio da Mata.

2 OBJETIVOS

2.1 Objetivos gerais

Hidrolisar as proteínas do glúten através do uso de proteases dos látex de *C. papaya*, *C. procera* e *C. grandiflora* para obtenção de peptídeos antimicrobianos (AMPs), assim como avaliar a atividade antifúngica dos hidrolisados e AMPs, testando a eficiência dos mesmos em estender a vida útil do pão de forma.

2.2 Objetivos específicos

1 - Obter hidrolisados a partir das proteínas do glúten usando proteases dos látex de *C. papaya*, *C. procera* e *C. grandiflora*;
2 - Realizar ensaios para avaliar o potencial antifúngico dos hidrolisados;
3 - Utilizar os hidrolisados como aditivos naturais no preparo de pães de forma;
4 - Avaliar parâmetros físicos de qualidade dos pães de forma processados com os hidrolisados, bem como avaliar a eficácia em estender a vida útil dos pães de forma;
5 - Obter sequências de peptídeos a partir dos hidrolisados;
6 - Caracterizar as sequências de peptídeos identificadas;
7 - Identificar e sintetizar os peptídeos que apresentem maior potencial antifúngico;
8 - Testar a atividade antifúngica dos peptídeos contra bolores e leveduras importantes na panificação e indústria de alimentos.

Latex peptidases produce peptides capable of delaying fungal growth in bread

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Abstract

Antimicrobial peptides (AMPs) have been reported to be promising alternatives to chemical preservatives. Thus, this study aimed to characterise AMPs generated from the hydrolysis of wheat gluten proteins using latex peptidases of *Calotropis procera*, *Cryptostegia grandiflora*, and *Carica papaya*. The three hydrolysates (obtained after 16 h at 37 °C, using a 1:25 enzyme:substrate ratio) inhibited the growth of *Aspergillus niger*, *A. chevalieri*, *Trichoderma reesei*, *Pythium oligandrum*, *Penicillium* sp., and *Lasiodiplodia* sp. by 60–90%, and delayed fungal growth on bread by 3 days when used at 0.3 g/kg. Moreover, the specific volume and expansion factor of bread were not affected by the hydrolysates. Of 28 peptides identified, four were synthesised and exhibited activity against *Penicillium* sp. Fluorescence and scanning electron microscopy suggested that the peptides damaged the fungal plasma membrane. Bioinformatics analysis showed that no peptide was toxic and that the antigenic ones had cleavage sites for trypsin or pepsin.

Keywords: antimicrobial peptides; *Calotropis procera*; *Carica papaya*; *Cryptostegia grandiflora;* gluten peptides; *Penicillium*.

1. Introduction

Bread is among the most consumed foods worldwide (Asri, Muhialdin, Zarei, & Saari, 2020). Although some technologies have been developed to improve its characteristics, bread still spoils very quickly, mainly due to fungal contamination, leading to consumer dissatisfaction and economic losses for the bakery industry (Garcia et al., 2019). In addition, fungal colonisation of bread leads to undesirable changes in taste and the production of harmful substances such as mycotoxins (Rizzello, Verni, Bordignon, Gramaglia, & Gobbetti, 2017). This contamination is mainly caused by fungal spores in the environment where bread is produced (Garcia et al., 2019). Several species are responsible for bread contamination and deterioration. However, species of the genera *Penicillium* and *Aspergillus* stand out (Nionelli et al., 2020). Other common fungi related to spoilage of baked goods belong to the genera *Cladosporium, Fusarium, Wallemia, Monilia, Mucor, Rhizopus*, and *Endomyces* (Asri et al., 2020; Garcia et al., 2019).

Researchers have been seeking new technologies to increase the shelf life of bread. The main alternative has been the application of chemical preservatives. Nevertheless, there are problems associated with the use of these additives, such as the resistance developed by fungi, the induction of some collateral effects, and changes in the nutritional level (Rai, Pandit, Gaikwad, & Kövics, 2016). Thus, the development of innovative methods is an important concern in the food industry, which has been looking mainly for natural alternatives to satisfy consumers who want healthier foods (Feng, Li, Wang, Deng, & Zeng, 2020; Luz, Izzo, Ritieni, Mañes, & Meca, 2020).

The use of biological molecules instead of synthetic chemicals for food preservation is an eco-friendly alternative to inhibit microorganism growth (Rai et al., 2016). In this respect, antimicrobial peptides (AMPs) have gained attention as alternative molecules for food preservation (Muhialdin, Hassan, Bakar, & Saari, 2016) because they are natural, active against various food-borne pathogens, and pose low health risks, as well as not changing the taste and nutritional quality of food (Asri et al., 2020; Rai et al., 2016).

AMPs can be produced from food proteins after digestion by different enzymes. For example, trypsin has been used to hydrolyse goat milk whey, generating 27 peptides with activity against 10 toxigenic fungi from the genus *Penicillium*, extending the shelf life of bread by 2 days (Luz et al., 2020). In this context, gluten proteins, which are the main storage proteins of wheat grains, consisting mainly of gliadins and glutenins (Wei, Helmerhorst, Darwish,

Blumenkranz, & Schuppan, 2020), are also interesting sources of AMPs. Therefore, we hypothesised that the hydrolysis of gluten proteins by latex peptidases could give rise to AMPs able to increase the shelf life of bread.

Plant latex fluids have been described as rich sources of peptidases (Ramos, Demarco, Souza, & Freitas, 2019). For instance, papain, which is purified from *Carica papaya* latex, is the best-known and studied plant proteolytic enzyme because of its numerous applications (Feijoo-Siota & Villa, 2011). Although latex peptidases have exhibited various biotechnological potential in the food industry, such as improvement of cheese (Silva et al., 2020), allergenicity reduction of cow's milk proteins (Oliveira et al., 2019a), and tenderisation of meat (Rawdkuen, Jaimakreu, & Benjakul, 2013), they have not been studied to produce AMPs from hydrolysis of gluten proteins. In addition, some studies have reported the food safety of latex peptidases. For instance, *Calotropis procera* latex peptidases (CpLP) are not toxic or allergenic in different animal models. CpLP are totally digested by digestive peptidases, not detected in the faecal material of rats, and do not cause death or toxic effects (Ramos et al., 2006). Moreover, CpLP does not induce allergy by the oral route (Ramos et al., 2007) or have any toxicity (Bezerra et al., 2017). Accordingly, the goal of this study was to evaluate the ability of latex peptidases from *Calotropis procera*, *Cryptostegia grandiflora*, and *Carica papaya* to hydrolyse gluten proteins and produce AMPs to be used as natural additives to extend the shelf life of bread.

2. Materials and methods

2.1. Reagents

Azocasein, L-cysteine, trichloroacetic acid (TCA), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), acetonitrile (ACN), trifluoroacetic acid (TFA), yeast peptone dextrose (YPD), dimethylsulphoxide (DMSO), and propidium iodide (PI) were obtained from Sigma-Aldrich (SP, Brazil). RapiGest™ SF (catalogue number 186001861) was obtained from Waters Corp. (MA, USA). The Resource RPC column, Sephadex G-25 column, and molecular mass markers were purchased from GE Healthcare (SP, Brazil). Commercial sodium propionate (E-281) and the yeast *Saccharomyces cerevisiae* were obtained at local markets (Fortaleza, CE, Brazil). All other chemicals were of analytical grade.

2.2. Plant materials and proteolytic activity

The latex fluids from *C. procera* and *C. grandiflora* were collected from the end branches of the plants, while the *C. papaya* latex was obtained from its green fruits. All latex fluids were mixed with distilled water (1 : 1 ratio) and the rubber was separated by centrifugation (10,000 × *g* at 4 °C for 10 min). The supernatants were dialysed against distilled water for 2 days at 4 °C using membranes with an 8 kDa cut-off, and then centrifuged again (10,000 × *g* at 4 °C for 10 min) (Freitas et al., 2010). The resulting supernatants were lyophilised and are called here *C. procera* latex peptidases (CpLP), *C. grandiflora* latex peptidases (CgLP), and *C. papaya* latex peptidases (CapLP).

The proteolytic activity was determined using 1% azocasein (a non-specific substrate for peptidases) at pH 5.0 (optimum pH for latex peptidases) (Freitas et al., 2010), containing 1 mM L-cysteine (a reducing agent used to activate cysteine peptidases). The activity was quantified by absorbance at 420 nm, where one unit of activity was defined as the amount of enzyme capable of increasing the absorbance by 0.01 after 60 min at 37 °C (Freitas et al., 2010). The three latex fractions presented very similar proteolytic activity (around 3.0 ± 0.2 AU/μg of protein).

2.3. Hydrolysis of gluten proteins by latex peptidases

The gliadin and glutenin fractions (here called gluten proteins, GP) were extracted from commercial wheat flour according to Tatham, Gilbert, Fido, and Shewry (2000). Briefly, 50 g of flour was submitted to continuous extraction with 500 mL of water-saturated butan-1-ol for 1 h at 20 °C, and centrifuged (5,000 × *g*, 10 min, 20 °C), after which the supernatant was discarded. The pellet was washed three times with 500 mL of 0.5 M NaCl for 1 h at 20 °C, and then with distilled water. Finally, 500 mL of 50% (v/v) aqueous propan-1-ol containing 2% mercaptoethanol and 1% acetic acid was added to the pellet. After 1 h at 20 °C under stirring, the material was centrifuged, and the supernatant (total reduced gliadin and glutenin subunits) was dialysed extensively against distilled water and then lyophilised.

The hydrolysis assays were carried out by incubating different aliquots (25, 50, or 100 μL) of CpLP, CgLP, and CapLP (4 mg in 1 mL of 50 mM sodium acetate buffer, pH 5.0, containing 1 mM L-cysteine) with 1 mL of the gluten proteins (10 mg/mL, which were dissolved in 50 mM sodium acetate buffer, pH 5.0, after heating at 80 °C for 5 min) to achieve

different enzyme: substrate ratios (1:100, 1:50, and 1:25, mass: mass). After 1, 2, 4, 6, and 16 h at 37 °C, the reactions were stopped by heating the samples at 80 °C for 5 min.

All hydrolysates (1 mg) were dissolved in 500 µL of 5% ACN containing 0.1% TFA. After centrifugation (10,000 × *g*, 10 min, 20 °C), the supernatants were used to evaluate the degree of hydrolysis by reverse-phase chromatography, using a Resource RPC column (Medium SOURCE™ 15RPC, matrix monodisperse underivatised polystyrene/divinyl benzene beads, bed volume of 3 mL, column dimensions 6.4 × 100 mm, particle size 15 µm, and pore volume 1.9 mL/g; GE Healthcare, Instructions 71-7174-00) coupled to an AKTA chromatography system, previously equilibrated with 5% ACN containing 0.1% TFA. The protein elution was performed using a linear gradient from 5% to 80% ACN, containing 0.1% TFA, at a flow rate of 1 mL/min for 45 min. The absorbance was monitored at 280 nm.

2.4. Antifungal activity of hydrolysates

The antifungal potential of the three gluten protein hydrolysates was assessed as described by Souza et al. (2020). First, *Aspergillus niger*, *A. chevalieri*, *Trichoderma reesei*, *Pythium oligandrum*, *Penicillium* sp., and *Lasiodiplodia* sp. were grown on potato dextrose agar medium for 14 days at 25 °C. Afterward, spore suspensions were obtained in a laminar flow cabinet by adding 5 mL of sterile 0.15 M NaCl solution and scraping the surface with a sterile Drigalski spatula. The suspension was filtered to remove any hyphae, and then adjusted to 2×10^5 spores/mL, using a Neubauer chamber. Then, 10 µL of the spore suspension was incubated with 90 µL of YPD medium in 96-well plates for 16 h at 37 °C. Next, 100 µL of the hydrolysates (obtained after 16 h of digestion by different latex peptidases, 1: 25 enzyme:substrate ratio) was added and the fungal growth was monitored by measuring the turbidity at 630 nm for 48 h, using an automated microplate reader (ELx800 Absorbance Microplate Reader, BioTek Customer Care) (Souza et al., 2020). Hydrogen peroxide (1 mM), sodium propionate (1 mg/mL) and sodium acetate buffer (50 mM, pH 5.0) were used as controls.

Likewise, the antifungal activity of the three hydrolysates was determined against the yeast *Saccharomyces cerevisiae*. Aliquots (100 µL) of *S. cerevisiae* cell suspensions (0.5×10^3 to 2.5×10^3 CFU/mL) in potato dextrose broth (PDB) were incubated in flat-bottomed 96-well microtitre plates with 100 µL of the three hydrolysates (obtained after 16 h of digestion by different latex peptidases, 1:25 enzyme:substrate ratio). Plates were incubated

in the dark at 37 °C, for 48 h, and the yeast growth was evaluated by absorbance readings taken at 630 nm using an automated absorbance microplate reader (Oliveira et al., 2019b).

2.5. Bread processing

The gluten proteins hydrolysed by latex peptidases were used as additives to evaluate their effectiveness either to inhibit or delay fungal proliferation. The breads were made as described by Zambelli et al. (2017), using the following formulation: 150 g of commercial wheat flour enriched with iron and folic acid (composed of 72.5% carbohydrates, 12.5% protein, 14.0% water, 0.6% fat, and 0.4% ash), 7.5 g of refined sugar, 5.5 g of dry yeast (*S. cerevisiae*), 4.5 g of hydrogenated vegetable fat, 3 g of refined salt, and 90 g of water. The amount of water added was a function of the degree of water absorption by the wheat flour and the same amount was used for all formulations, since the quantity of hydrolysates added was not enough to change the degree of water absorption of the flour.

The hydrolysates (obtained after 16 h of digestion, 1:25 enzyme : substrate ratio) were lyophilised and added to the breads in proportions corresponding to 0.1, 0.2, and 0.3 g/kg of wheat flour weight. A negative control was prepared without the addition of the hydrolysates, while sodium propionate (positive control) was used in the proportions of 0.1 and 2 g/kg. All ingredients were mixed in a semi-industrial mixer and then allowed to rest at 26 ± 1 °C. After 5 min, the doughs were divided into portions of approximately 250 g, moulded into ellipses by hand, and placed in moulds made of galvanised iron sheet. They are suitable for producing bread without a lid and have a bottom dimension of 13.5 cm × 6.0 cm and a top dimension of 15.0 cm × 7.5 cm × 4.5 cm. For the microbiological tests only, the breads were exposed to the free growth profile. The fermentation was performed in a fermentation chamber at 28 ± 2 °C (70% relative humidity) for 90 min, and then the dough was baked without steam at 220 °C for 20 min and cooled to room temperature (28 ± 2 °C).

2.6. Bread characterisation

2.6.1. Mould and yeast count

The qualitative fungal growth inhibition was evaluated visually using the following codes, as described by Humar and Pohleven (2005):

0: mycelium growth more intense than in the control

1: normal growth, insignificant retardation (area of colony ≥ 90% of area of control)

2: visible signs of retardation (area of colony < 90% and ≥ 60% of area of control)

3: pronounced retardation (area of colony < 60% and ≥ 25% of area of control)

4: very marked retardation (area of colony < 25% of area of control)

5: no growth

Quantitative microbiological analysis of the breads was performed using homogenised samples (10 g) in 50 mL of sterile peptone water (0.1%). After serial dilutions (1:10), the samples were plated in depth on standard agar for total aerobic mesophyll counting. The anaerobic count was performed using an anaerobiosis generator. Superficial plating on acidified potato dextrose agar was used to count yeasts. The plates were incubated at 27 °C, and the number of viable colonies was counted after incubation for 72 h (Dal Bello et al., 2007).

Moreover, controlled specific fungal application on the breads containing the three hydrolysates (0.3 g/kg, obtained after 16 h of digestion, 1:25 enzyme : substrate ratio) was performed according to Luz et al. (2020). For this, *Penicillium* sp. spores (10 μL, 1×10^2 spores/mL) were added to the surfaces of the bread samples, which were stored in plastic bags (15 cm × 10 cm) at 25 °C. These surfaces were examined visually for 5 days. Sodium propionate (0.1 and 2 g/kg) was used as a positive control.

2.6.2. Maximum expansion factor

The maximum expansion factor was calculated considering dough shape as a truncated ellipse, assuming that during fermentation the relative ratios between each geometric parameter of the ellipse remained constant. The dough samples were sliced into elliptic forms and the volume expansion was evaluated according to vertical and horizontal expansion. The bread was weighed after cooling and its specific volume (cm^3/g) was determined by the rapeseed displacement method (Gabric, Ben-Aissa, Le-Bail, Monteau, & Curic, 2011).

2.6.3. Bread hardness

Bread hardness was determined using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell and a 35 mm aluminium cylindrical probe. Three bread slices (25 mm thickness) taken from the centre of each loaf were used to evaluate the physical texture. The settings used were a test speed of 5 mm/s with trigger force of 0.98 N to compress the middle of the bread sample to 50% of its original height. Waiting time between first and second compression cycle was 5 s (Licciardello, Cipri, & Muratore, 2014).

2.6.4. Bread porosity

The bread was sliced transversely with a slicing machine and the images were captured using a colour camera (Nikon P610, Nikon Inc., Japan) with resolution of 4272 × 2848 pixels. The camera was located 20 cm above the samples, which were illuminated with two 36 W fluorescent light tubes (model T8 G13, Philips, Brazil) and enclosed in a black cardboard box (Al-Rahbi, Manickavasagan, Al-Yahyai, Khriji, & Alahakoon, 2013). The inside walls of the box were covered with black paper to reduce reflectance, and the lid was covered inside with white paper to enhance light scattering and reduce shadows (Al Ohali, 2011). The sliced bread samples were positioned on a black background to provide the greatest contrast between the background and sample. The camera was connected to a computer running the remote shooting software Nikon Wireless Mobile Utility (WMU) (version 1.5.0, Nikon Inc, Japan). ImageJ software (National Institute of Health, Bethesda, MD, USA) was employed to analyse and produce the final images. The images were divided into regions of cells and surrounding cell wall material. The analysis was performed on a slice area of 600 × 600 pixels and the porosity measurements were performed as described by Martínez, Román, and Gómez (2018). The results were expressed as the number, total area, and mean size of alveoli.

2.7. Peptide sequencing by mass spectrometry

First, the three hydrolysates obtained from the gluten protein hydrolysis (after 16 h of digestion, 1:25 enzyme:substrate ratio) were submitted to size exclusion chromatography to isolate the peptide fraction from the intact gluten proteins and latex peptidases, according to the instruction manual of the column (Sephadex G-25; particle size range: 85 to 260 μm; packed

bed dimensions: 1.45 × 5.0 cm (8.3 mL); maximum sample volume: 2.5 mL; GE Healthcare Instructions 52-1308-00). For this, the hydrolysates (2.5 mL) were loaded into a Sephadex G-25 column (equilibrated with 50 mM sodium acetate buffer, pH 5.0) and the proteins/peptides were eluted with distilled water at a flow rate of 1 mL/min. The absorbance was monitored at 280 nm and the fractions were evaluated by 12.5% SDS-PAGE as previously described (Freitas et al., 2010).

The fractions containing only peptides were lyophilised, suspended (100 µg) in 0.05% (w/v) RapiGest SF solution, and incubated with 5 mM DTT for 15 min at 65 °C, followed by the addition of 10 mM iodoacetamide for 30 min at 25 °C. Afterward, the samples were submitted to reverse-phase (RP) chromatography in a BEH300 C18 column (100 µm × 100 mm) using a nanoAcquity™ system at a flow rate of 600 µL/min with a gradient (5–85%) of ACN containing 0.1% formic acid. The liquid chromatography system was connected to a nanoelectrospray mass spectrometer source (SYNAPT HDMS system, Waters Corp., Milford, MA, USA). The mass spectrometer ran in positive mode using a source temperature of 90 °C and capillary voltage of 3.5 kV. The instrument's calibration was performed with infusion of phosphoric acid solution (0.1%, in 50:50 ACN:water solution). Phosphoric acid clusters from 100 to 1500 *m/z* were used to fit the calibration curve. Mass values were accepted up to 5 ppm. The LC–MS/MS procedure was performed according to the data-dependent acquisition (DDA) method, selecting MS/MS doubly to triply charged precursor ions. Ions were fragmented by collision-induced dissociation using argon as the collision gas, with ramp collision energy that varied according to the charge state of the selected precursor ion (Araujo et al., 2021).

Data acquisition was performed at an *m/z* range of 300–2100 for the MS analysis (1 scan/s) and at an *m/z* range of 50–2500 for MS/MS. Data were collected with MassLynx 4.1 software and processed using the Protein Lynx Global Server 2.4 (Waters Corp.). The data were submitted to a database search using the MASCOT search engine against the NCBI non-redundant database using a significance threshold of $p < 0.05$, and the Protein Lynx Global Server 2.4 search engine against a local database of gluten protein sequences.

2.8. Characterisation and synthesis of peptides

All peptides sequenced by mass spectrometry were characterised and their antifungal potential was predicted using appropriate bioinformatics tools, considering: (1) positive net charge; (2) Boman index ≤ 2.5; (3) total hydrophobic ratio (40–60%); and (4) low

molecular mass. These criteria were evaluated using the Antimicrobial Peptide Database (ADP) server (http://aps.unmc.edu/AP/).

The iAMPpred tool (http://cabgrid.res.in:8080/amppred/server.php) was used to evaluate the probability of peptides having antimicrobial activity, considering a score higher than 0.5 as indicating that a peptide has a high probability of exhibiting activity. The Peptide Cutter tool (http://web.expasy.org/peptide_cutter/) was used to assess whether the peptides had cleavage sites for trypsin as well as pepsin at pH values of 1.3 and > 2. The isoelectric point and molecular mass were calculated using the Compute pI/MW tool (http://web.expasy.org/compute_pi/). The peptides were characterised for their antigenic and toxic potential using the servers Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl) and Prediction of Toxic Peptides (ToxinPred) (https://webs.iiitd.edu.in/raghava/toxinpred/design.php), respectively.

The four best peptides (those presenting the highest probability of being antifungals) were synthesised by the company Chempeptide (Shanghai, China). Purification was performed using reverse-phase liquid chromatography (RP-HPLC) with a Kromasil C18 column. Eluent A consisted of 0.1% TFA in 100% ACN and eluent B corresponded to 0.1% TFA, using a flow rate of 1 mL/min. The peptides were detected at 220 nm. Electrospray mass spectrometry (LCMS-2010 EV spectrometer, Shimadzu, Japan) was used to confirm the purity and the molecular mass of peptides.

2.9. Antifungal activity of synthetic peptides

The antifungal activity of the synthetic peptides was tested against *Penicillium* sp. by evaluating spore viability using the reagent MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Life Technologies, USA). Aliquots (25 µL) of spore suspensions (2×10^5 spores/mL) in PDB medium were incubated in 96-well flat plates with 25 µL of the hydrolysates (10 mg/mL) or peptides (2 mg/mL) dissolved in 0.15 M NaCl containing 5% DMSO (DMSO-NaCl). The DMSO-NaCl and sodium propionate (2 mg/mL) were used as negative and positive controls, respectively. The treated spores were incubated for 0, 1, 2, 4, and 6 h, at 37 °C, and then 50 µL MTT (4 mg/mL in DMSO-NaCl) was added. After 3 h in the dark, 100 µL of DMSO-NaCl was added and the mixture gently shaken. Absorbance at 495 nm was read using an automated absorbance microplate reader (Epoch, BioTek Instruments, Inc., USA).

2.10. Action mechanism of hydrolysates and synthetic peptides

2.10.1. Propidium iodide (PI) uptake assay

The integrity of the fungal cell membrane was assessed by the PI uptake assay (Oliveira et al., 2019b). A spore suspension (20 μL) of *Penicillium* sp. was incubated with the hydrolysates (10 mg/mL) or synthetic peptides (2 mg/mL) (20 μL) in DMSO-NaCl for 6 h, at 37 °C. Next, the treated cells were incubated with 10 μL of PI (0.1 mg/mL) in 1.5 mL tubes for 15 min at 25 °C in the dark. The cell suspensions were washed with 0.15 M NaCl solution (500 μL) and centrifuged at 5000 × *g* for 10 min at 10 °C. The excess supernatants were discarded and cells were resuspended in 15 μL of 0.15 M NaCl and observed using a fluorescence measurement system (Olympus microscope system; excitation wavelength 400–500 nm, emission wavelength 600–700 nm).

2.10.2. Scanning electron microscopy (SEM)

SEM analysis was performed to detect possible morphological changes of the spores. The samples were prepared according to Oliveira et al. (2019b). The spore suspension (20 μL) of *Penicillium* sp. was incubated for 6 h at 37 °C, with the hydrolysates (10 mg/mL), synthetic peptides (2 mg/mL), sodium propionate (2 mg/mL), or DMSO-NaCl (20 μL). The spores were centrifuged at 3000 × *g* for 5 min at 10 °C, the supernatants were discarded, and the cell pellets were fixed for 16 h at room temperature (22–25 °C) with 50 μL of the fixation solution (1% glutaraldehyde containing 4% (v/v) formaldehyde in 0.15 M sodium phosphate buffer at pH 7.2). After centrifugation as mentioned above, the cells were recovered, washed three times with 0.15 M sodium phosphate buffer, pH 7.2, with centrifugation between washes (3000 × *g*, 5 min, at 10 °C). Next, sample dehydration was conducted by incubation with increasing ethanol concentrations (30%, 70%, and 100% v/v) for 10 min each, followed by centrifugation as previously described. Finally, the samples were dried by immersion in 50:50 ethanol:hexamethyldisilazane (HMDS) for 10 min, centrifuged, and then the pellet was washed with 100% (v/v) HMDS and transferred to a coverslip previously treated with 0.1% (m/v) gelatin. After complete drying, the coverslips were assembled on stubs and coated with a 20 nm gold layer using a positron-emission tomography (PET) coating machine (Emitech-Q150T ES, Quorum Technologies, England). Images were captured with an FEI Inspect™ 50 scanning electron microscope (Oregon, USA), equipped with a low-energy detector (Everhart-Thornley) using 25,000× magnification.

2.11. Statistical analysis

Three independent experiments were performed for all assays, in triplicate, and the data were expressed as mean ± SD. ANOVA was performed for multiple comparisons followed by Tukey's multiple comparison test, using GraphPad Prism version 7.0. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Gluten protein hydrolysates exhibited antifungal activity

The gluten proteins were partially resistant to the proteolytic action of CpLP, CgLP, and CapLP, even after 16 h of incubation and using the 1:25 ratio (enzyme:substrate ratio, mass:mass) (Fig. 4). Likewise, the gastrointestinal enzymes pepsin, trypsin, and chymotrypsin do not break down gluten proteins efficiently (Wei et al., 2020). A previous study showed that gluten proteins are resistant to extensive proteolysis due to their high content of proline and glutamine (Wei et al., 2020).

On the other hand, some enzymes capable of degrading gluten proteins have already been identified in plants. For example, EP-B2, a cysteine peptidase from barley, acts in the hydrolysis of gluten proteins, preferentially cleaving peptide bonds after glutamine (Scherf, Wieser, & Koehler, 2018). Although the enzymes of the papain family have broad specificity, they preferentially cleave the peptide bonds after the carboxylic acid group of Lys or Arg (Wang et al., 2019), which may explain the low action on gluten proteins by CpLP, CgLP, and CapLP.

Some studies have reported the effectiveness of protein hydrolysates as antifungals (Luz et al., 2020; Nionelli et al., 2020). Nionelli et al. (2020) showed that bread hydrolysate, produced by the proteolytic action of metallo- and serine peptidases from *Lactobacillus brevis* AM7, exhibits a broad inhibitory spectrum against the fungal species tested and antifungal activity ranging from 20% to 70%.

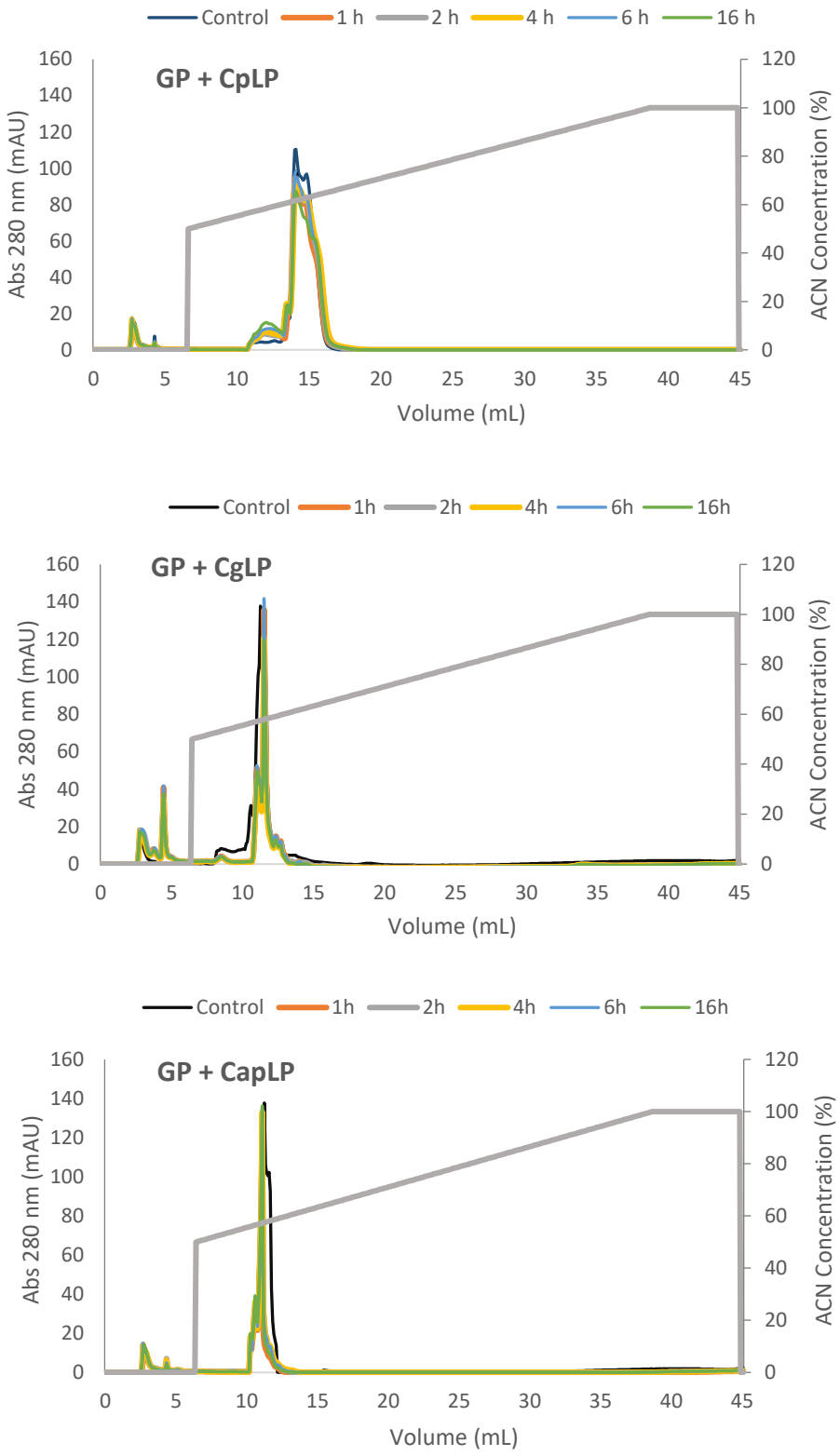


Fig. 4. Reverse-phase chromatography, using a Resource RPC column, of gluten proteins (GP) hydrolyzed by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), or *Carica papaya* (CapLP) after different times (1:25, enzyme: substrate ratio), at pH 5.0, and 37 ºC. Samples were eluted using an acetonitrile (ACN) gradient in 0.1% aqueous trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The chromatogram was recorded at 280 nm. Gluten proteins (GP) were used as control.

Likewise, the results obtained by Luz et al. (2020) demonstrated the antifungal activity of goat whey hydrolysates after action by trypsin against *Penicillium* sp. Here, the non-hydrolysed gluten proteins (GP) exhibited antifungal activity, inhibiting the growth of the six fungi tested by 40% to 60% (Fig. 5). This effect can be related to the presence of antifungal proteins in this protein fraction, since pathogenesis-related proteins (PR-proteins) have been reported in wheat (Caruso et al., 2001). However, the hydrolysates obtained from the gluten proteins after the action of CpLP, CgLP, and CapLP were able to enhance such activity, reaching values of around 90% inhibition (*Penicillium* sp., *Aspergillus niger*, and *Lasiodiplodia* sp.), very similar to hydrogen peroxide and better than sodium propionate, which were used as positive controls. For the other fungi (*Aspergillus chevalieri*, *Trichoderma reesei*, and *Pythium oligandrum*), inhibition ranged from 60–90% (Fig. 5). In particular, the proteolytic fractions (CpLP, CgLP, and CapLP), used at the tested final concentration, did not have antifungal activity (Fig. 5).

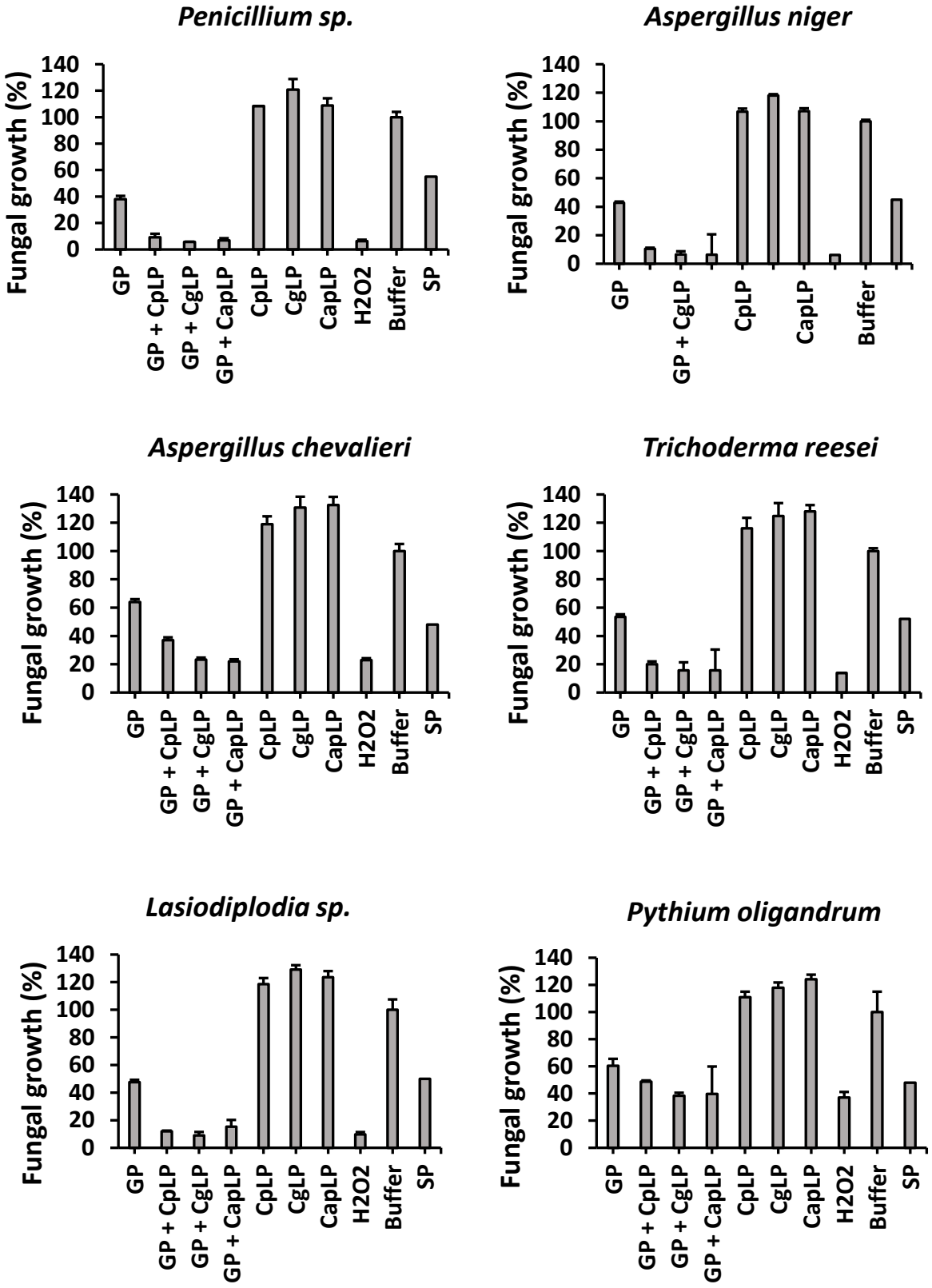


Fig. 5. Effect of the peptides obtained from hydrolysis of gluten proteins (GP) by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP) and *Carica papaya* (CapLP) on the vegetative growth of different fungi. For hydrolysis, GP were incubated with the latex peptidases (1:25, enzyme: substrate ratio, mass:mass) for 16 h, at pH 5.0 and 37 ºC. CpLP, CgLP, and CapLP, at the same concentration used for GP hydrolysis, were used as controls. H_2O_2 (1 mM), 50 mM sodium acetate buffer, pH 5.0 (Buffer), and sodium propionate (SP, 1 mg/mL) were used controls.

3.2. Gluten protein hydrolysates delayed the fungal growth and changed some physical characteristics of breads

The presence of visible mould on the surface of bread is a critical point of spoilage evaluation by consumers (Garcia et al., 2019). Thus, the spontaneous fungal contamination of bread samples containing the gluten protein hydrolysates was evaluated visually for 6 days of storage at 25 °C (Fig. 6 and Table 1). In general, the first signs of fungal growth were observed only after 4 days on the controls and breads containing sodium propionate at 0.1 g/kg. After 6 days, there was extensive mould growth only on the control breads (Fig. 6 and Table 1). In contrast, slight and no contamination were observed on the breads containing the hydrolysates at 0.2 and 0.3 g/kg, respectively (Fig. 6 and Table 1). These results were better than those obtained with sodium propionate (a commercial preservative used as control), since it was effective only at 2 g/kg, around 10-fold more concentrated than the gluten protein hydrolysates (Table 1).

Furthermore, the most efficient hydrolysate was that produced by CpLP digestion, since no sign of fungal contamination was observed even at the lowest concentration tested (0.1 g/kg). Only after 7 days was a slight fungal growth observed on the surfaces of the bread samples containing 0.3 g/kg of hydrolysates, suggesting an increase of the shelf life of bread by at least 3 days, similar to the results obtained for sodium propionate at 2 g/kg (Fig. 6). Other assays should be performed, because shelf life is affected by more diverse parameters and needs more critical measurements, including safety issues and conditions of packaging, storage, and handling.

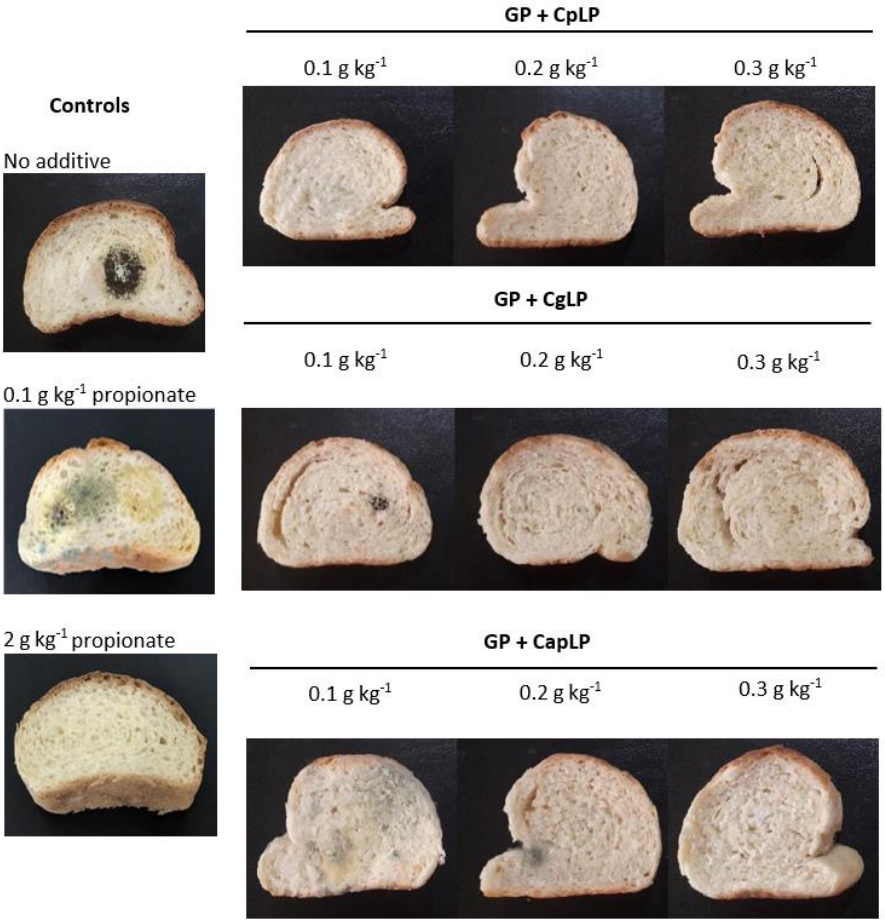


Fig. 6. Effect of the hydrolysates obtained from proteolysis of gluten proteins (GP) by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) on the fungal contamination of breads. Controls were breads without any treatment (no additive) and breads with sodium propionate (0.1 and 2 g/kg). Photos were taken after six days at 25 °C.

The microbiological quantification of breads confirmed the antifungal action of the three gluten protein hydrolysates (Table 1). After the fourth day, there was a statistically significant difference between the control and all breads containing hydrolysates. On the sixth day, the control bread had a fungal population of 9.8 log CFU/g, while for the breads treated with hydrolysates this ranged from 4.0 to 6.4 log CFU/g ($p < 0.05$). The lowest population (4.0 ± 0.02 log CFU/g) was found for the breads treated with gluten protein hydrolysates (0.3 g/kg) obtained by the action of CpLP, which was very similar to that obtained using sodium propionate at 2 g/kg ($p > 0.05$) (Table 1).

Table 1. Mold and yeast growth evaluation of breads treated with different concentrations of gluten proteins (GP) hydrolyzed by latex peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *C. papaya* (CapLP).

Samples (g kg^{-1})	**Visual Observation***						**Mold and yeast quantification** (Log CFU g^{-1})**		
Days	1	2	3	4	5	6	2	4	6
Control	5	5	5	4	2	2	2.3^{a} ±0.05	5.3^{a}±0.04	9.8^{a}±0.03
GP + CpLP									
0.1	5	5	5	5	5	5	2.2^{a}±0.02	3.7^{b}±0.03	4.4^{b}±0.03
0.2	5	5	5	5	5	5	2.2^{a}±0.04	3.8^{b}±0.05	4.6^{b}±0.02
0.3	5	5	5	5	5	5	2.1^{c}±0.05	3.6^{b}±0.02	4.0^{c}±0.02
GP + CgLP									
0.1	5	5	5	4	4	3	2.2^{a}±0.03	4.9^{c}±0.02	6.3^{d}±0.03
0.2	5	5	5	5	4	3	2.2^{a}±0.04	4.7^{c}±0.03	6.2^{d}±0.05
0.3	5	5	5	5	5	5	2.1^{a}±0.03	3.6^{b}±0.03	4.5^{b}±0.02
GP + CapLP									
0.1	5	5	5	4	4	3	2.2^{a}±0.03	3.9^{d}±0.03	6.4^{d}±0.04
0.2	5	5	5	5	4	3	2.2^{a}±0.04	3.6^{b}±0.02	4.7^{b}±0.03
0.3	5	5	5	5	5	5	2.2^{a}±0.02	3.7^{b}±0.02	4.4^{b}±0.03
Sodium proprionate									
0.1	5	5	5	4	4	3	2.2^{a}±0.02	4.9^{c}±0.04	6.2^{d}±0.02
2	5	5	5	5	5	5	2.1^{a}±0.04	3.5^{b}±0.02	4.2^{c}±0.02

*****The fungal growth inhibition was evaluated by the naked eye during six days using the following codes: **0**: mycelium growth intense; **1**: normal growth, insignificant retardation (area of colony ≥ 90%); **2**: visible signs of retardation (area of colony < 90% and ≥ 60%); **3**: pronounced retardation (area of colony < 60% and ≥ 25%); **4**: very marked retardation (area of colony < 25%); **5**: no growth. ****** The quantification of molds and yeasts was performed after two, four, and six days. Three independent experiments were performed for all assays and in triplicate. Data were expressed as mean ± SEM and then submitted to one-way ANOVA ($p<0.05$) followed by Tukey's multiple comparison test. Same letters indicate that there was no significant difference between the means.

Moreover, spores of *Penicillium* sp. were artificially inoculated on the surface of the breads to challenge the antifungal activity of the hydrolysates under extreme contamination conditions. Fungal growth was observed on the control breads after 2 days and on the breads containing the hydrolysates (0.3 g/kg) and sodium propionate (0.1 g/kg) after 5 days. After 6 days, there was extensive fungal growth on the control breads and slight contamination on the breads containing the hydrolysates (Fig. 7). Again, the hydrolysate produced by CpLP was the best at inhibiting fungal contamination, exhibiting results similar to those of sodium propionate at 2 g/kg. These results confirm the assay of spontaneous fungal contamination on breads (Fig. 6).

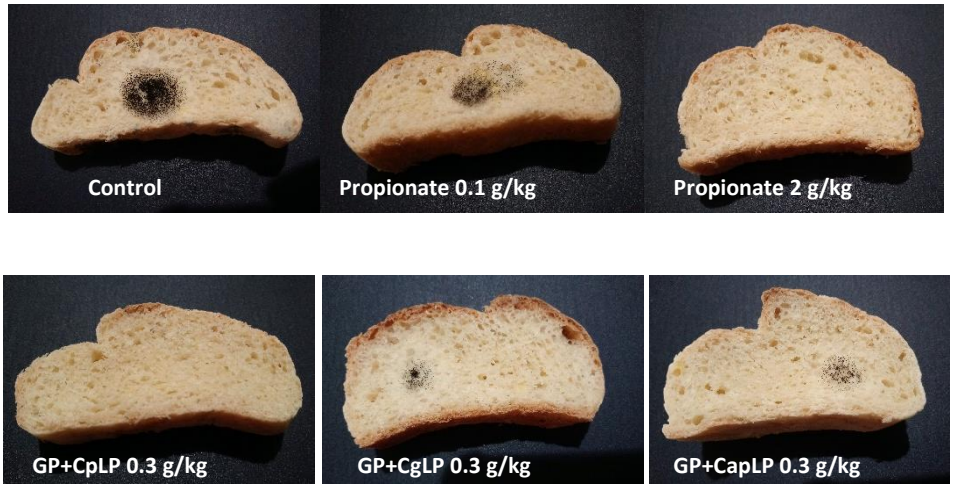


Fig. 7. Effect of the hydrolysates obtained from proteolysis of gluten proteins (GP) by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) on breads contaminated with *Penicillium sp.* spores. Controls were breads without any treatment (Control) or breads with sodium propionate (0.1 and 2 g/kg). Photos were taken after six days at 25 °C.

Some studies have reported a longer shelf life for bread treated with different protein hydrolysates. However, they used high concentrations of hydrolysates. Rizzello et al. (2017) obtained a 7-day shelf-life extension for breads supplemented with 1.5 g/kg of a mixed flour from lentils (*Lens culinaris*), peas (*Pisum sativum*), and fava beans (*Vicia faba*) hydrolysed by a commercial neutral peptidase (Veron PS) from *Aspergillus oryzae*. Similarly, Asri et al. (2020) reported that the hydrolysate from palm kernel cake, generated by enzymatic hydrolysis via lacto-fermentation using *Lactobacillus casei*, successfully delayed fungal growth, thus extending the shelf life of bread by up to 10 days when added to the dough at 2.0 g/kg. Although our most promising result was prolonging the shelf life of breads for 3 days, a very small concentration of hydrolysate (0.3 g/kg) was used. This positive effect at low concentration opens a perspective for real application, since hydrolysates can change some physical characteristics as well as the taste, and thus the acceptance of bread by consumers.

We studied whether the hydrolysates could modify some physical parameters of bread. In general, the specific average volumes of bread containing the gluten protein hydrolysates were very similar to those of control breads (around 3 cm^3/g) ($p > 0.05$), even when the highest dose of hydrolysates was used. Similarly, no hydrolysate affected the expansion factor of bread, which was close to 2.2 cm^3 ($p > 0.05$) (Table 2). These results suggest that the incorporation of hydrolysates as new additives does not compromise the fermentation process and gluten network. That is extremely positive, since it has been extensively reported in the literature that bread quality decreases after the addition of non-gluten-forming ingredients

to the dough, causing damage to the gluten network (Gabric et al., 2011). Fitzgerald et al. (2014) reported that the addition of seaweed (*Palmaria palmata*) protein hydrolysates (40 g/kg) to breads resulted in a decreased loaf volume. They reported that this result could be due in part to the additional protein added which could compete with the wheat starch for free moisture, thereby restricting the hydration and swelling of starch granules. Franco-Miranda, Chel-Guerrero, Gallegos-Tintoré, Castellanos-Ruelas, and Betancur-Ancona (2017), working with hydrolysates of protein concentrates of lima beans (*Phaseolus lunatus*) and cowpeas (*Vigna unguiculata*), added to the dough at 10 g/kg or 30 g/kg, reported that the incorporation of exogenous proteins weakened the gluten network, creating competition between the hydrolysates and the dough proteins to bind water molecules. It is important to note that these works used a high amount of protein hydrolysate. Thus, it is expected that a small amount of hydrolysate will not compete with gluten for water absorption and so will not alter the gluten protein network.

Table 2. Physical properties of breads treated with different concentrations of gluten proteins (GP) hydrolyzed by latex peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *C. papaya* (CapLP).

Samples (g/kg)	Specific Volume (cm^3/g)	Expansion Factor (cm^3)	Hardness (N)	Number of Alveoli (cells)	Area of alveoli (mm^2)	Mean size of alveoli (mm)
CONTROL	3.05^{a}±0.04	2.27^{a}±0.12	2.78^{a}±0.16	1688^{a}±33	1825.45^{c}±85	1.08^{b}±0.23
GP + CpLP						
0.1	2.95^{a}±0.05	2.26^{a}±0.13	2.88^{a}±0.15	1683^{a}±30	1832.21^{c}±80	1.08^{b}±0.17
0.2	3.02^{a}±0.04	2.25^{a}±0.15	3.27^{b}±0.18	1699^{a}±44	1840.39^{c}±79	1.08^{b}±0.19
0.3	3.00^{a}±0.02	2.24^{a}±0.11	3.35^{b}±0.12	1844^{b}±38	2501.52^{b}±83	1.35^{a}±0.23
GP + CgLP						
0.1	2.94^{a}±0.04	2.23^{a}±0.12	2.74^{a}±0.19	1699^{a}±29	1833.38^{c}±85	1.07^{b}±0.19
0.2	3.01^{a}±0.03	2.27^{a}±0.15	3.21^{b}±0.11	1689^{a}±36	1821.77^{c}±80	1.07^{b}±0.21
0.3	3.02^{a}±0.02	2.25^{c}±0.09	3.33^{b}±0.12	1877^{b}±32	2609.23^{b}±88	1.38^{a}±0.20
GP + CapLP						
0.1	3.01^{a}±0.02	2.25^{a}±0.11	2.74^{a}±0.15	1669^{a}±38	1803.29^{c}±75	1.08^{b}±0.15
0.2	3.08^{a}±0.04	2.24^{a}±0.10	3.22^{b}±0.12	2120^{c}±49	3055.48^{a}±82	1.44^{a}±0.19
0.3	3.03^{a}±0.03	2.27^{a}±0.12	3.38^{b}±0.09	2195^{c}±35	3210.96^{a}±89	1.46^{a}±0.22

Three independent experiments were performed for all assays and in triplicate. Data were expressed as mean ± SEM and then submitted to one-way ANOVA ($p<0.05$) followed by Tukey's multiple comparison test. Same letters indicate that there was no significant difference between the means.

Another physical parameter tested was the hardness of the bread, which is related to the force necessary to deform the sample to a certain extent (Rizzello et al., 2017). The addition of 0.1 g/kg of the hydrolysates did not affect the bread hardness compared to the control ($p > 0.05$). However, a significant increase (around 20%) was observed when 0.2 and 0.3 g/kg of the hydrolysates were added compared to the control ($p < 0.05$). It is important to note that hardness did not differ significantly between the two concentrations ($p > 0.05$) (Table 2). Additionally, the number, area, and mean size of alveoli were altered by the concentration of hydrolysates. When the hydrolysates were added at 0.3 g/kg ($p < 0.05$), there were increases of between 11% and 30% in number, 37% and 75% in area, and 25% and 35% in the mean size of alveoli (Table 2). These results suggest that the hydrolysates, even at the highest concentration, did not affect the fermentation process. Madruga, Rocha, Fernandes, and Salas-Mellado (2021) reported that chia (*Salvia hispanica* L.) hydrolysate (3 g/kg) influenced the formation of a greater number of smaller and denser alveoli. They observed that the addition of hydrolysate can influence dough elasticity, increasing the foaming capacity, thus generating a greater number of smaller and more compact alveoli, strengthening the crumb structure. Moreover, the gluten peptides produced by the action of CpLP, CgLP, and CapLP did not exhibit antifungal activity against the yeast *S. cerevisiae* (data not shown), confirming that the hydrolysates do not affect the fermentation process.

Although many natural preservatives are already known, not all are able to maintain all organoleptic properties of bread (Nionelli et al., 2020; Rai et al., 2016). Even though some characteristics, such as hardness, are affected by the addition of hydrolysates, this does not necessarily reduce acceptability by consumers. For example, in the study carried out by Franco-Miranda et al. (2017), the breads containing lima bean (*Phaseolus lunatus*) and cowpea (*Vigna unguiculata*) hydrolysates, which showed reduced hardness, were well accepted by consumers. Therefore, the sensory analysis of breads containing the hydrolysates from gluten proteins after

digestion by latex peptidases should be performed in a further study to examine their acceptance by consumers.

3.3. Characterisation of gluten peptides

All three gluten protein hydrolysates were fractioned by size exclusion chromatography (Sephadex G-25) for peptide purification. The first peak (fractions 4–8) corresponded to partially hydrolysed gluten proteins, while the second peak (fractions 9–13) corresponded to the gluten peptides (Fig. 8), which were further lyophilised and sequenced by mass spectrometry. In general, 14, 11, and 8 peptides were obtained from gluten proteins after digestion by CpLP, CgLP, and CapLP, respectively (Tables 3, 4, 5, and 6). Remarkably, two common peptides were produced by the action of the three peptidases (LPYPQPQPFRPQ and VPLYR) and one by CpLP and CgLP (QQQLIPCR). The peptides presented molecular masses and pI ranging from 600 to 3391 Da and 4.0 to 11.0, respectively. The net charge and hydrophobic ratio ranged from −1 to +5 and 7% to 62%, respectively (Table 3). In addition, CpLP, CgLP, and CapLP produced nine, seven, and seven peptides with antimicrobial potential > 50%, respectively (Table 3). These results can be explained by the hydrolysate obtained from digestion by CpLP being the most efficient at inhibiting fungal contamination, even at the lowest concentration tested (Fig. 6).

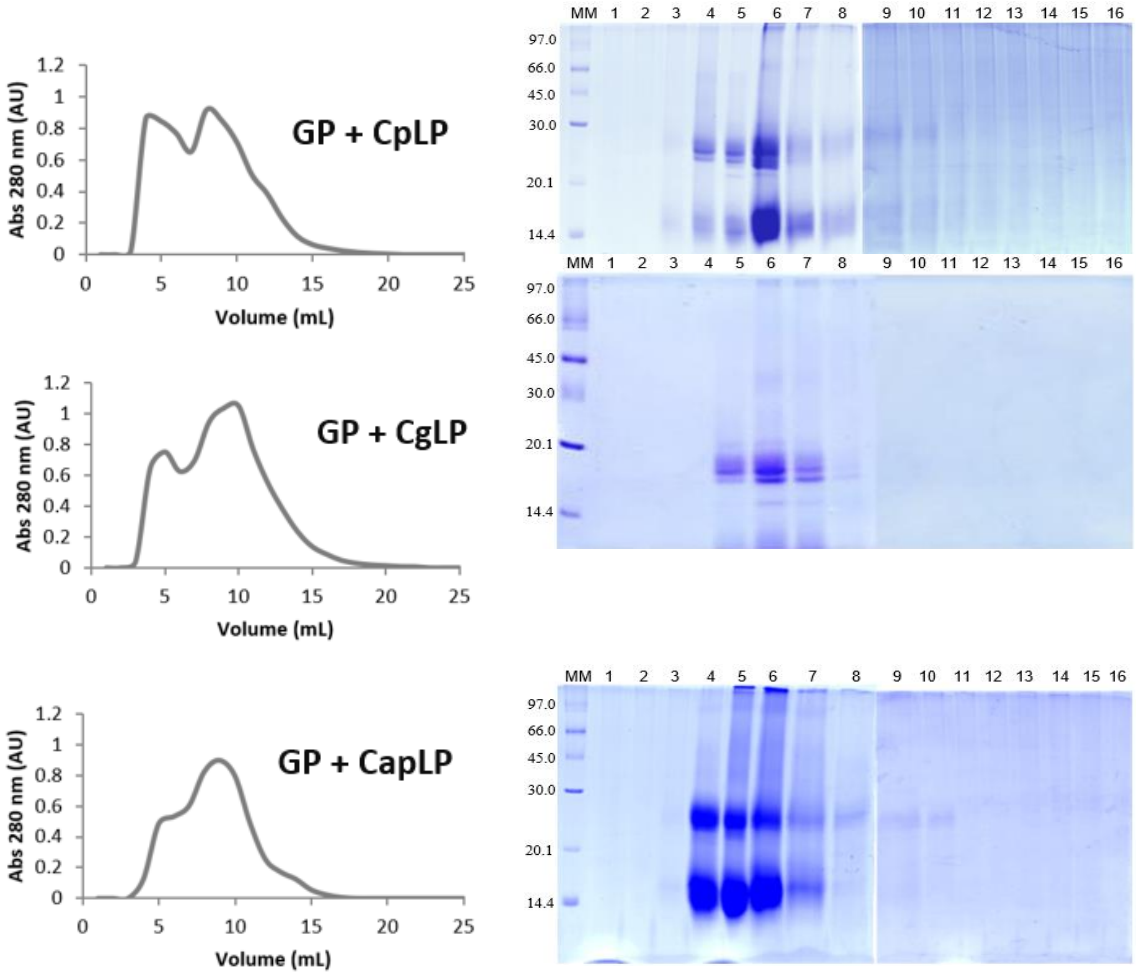


Fig. 8. Size exclusion chromatography and SDS-PAGE of gluten proteins hydrolyzed by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP). MM: Molecular mass markers. 1-16 fractions from chromatography. Fractions 4-8 and 9-13 refer to protein and peptide fractions, respectively, according to the column's instructions.

Table 3. Some physicochemical parameters and antimicrobial potential of all peptides derived from gluten proteins hydrolyzed by latex peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *C. papaya* (CapLP).

Latex Peptidase	Peptide	[a]Molecular Mass (Da)	[b]pI	Net Charge	[c]Hydrophobicity (%)	[d]Boman index	[e]Antimicrobial Potencial (%)	
							Antifungal	Antibacterial
CpLP/CgLP/CapLP	LPYPQPQPFRPQ	1467.694	8.75	+1	16	1.98	31	56
CpLP, CgLP/CapLP	**VPLYR***	**646.789**	**8.72**	**+1**	**40**	**1.22**	**47**	**76**
CpLP/CgLP	QQQLIPCR	985.178	8.25	+1	37	2.55	40	51
CpLP	QPFPPRQPYPQPQ	1579.782	8.75	+1	7	2.63	35	58
	QQLIPCR	857.047	8.25	+1	42	2.12	46	39
	QLIPCR	728.916	8.25	+1	50	1.55	67	46
	QQVISQPQQPFPQ	1524.7	5.52	0	23	1.89	35	52
	QQPQQQPFPQPQ	1450.577	5.52	0	8	2.98	42	64
	LPQQQIPFVHPSIL	1616.928	6.74	+1	42	-0.14	15	8.2
	IPFVHPSIL*	**1022.258**	**6.74**	**+1**	**55**	**-1.52**	**80**	**80**
	WGIPALLR	925.144	9.75	+1	62	-0.61	4.1	5.6
	REPPKVPPTPTKRIK	1744.105	11.1	+4	13	3.29	28	63
	MHDGGEIDARACYTAIF	1870.101	4.54	-1	47	1.34	5.5	11
	FATLAAAYIGESMEGHH	1805.004	5.23	0	47	0.27	10	33
CgLP	QVFIPPQ	827.4541	5.52	0	42	-0.12	32	44
	LPFPQ	600.3271	5.52	0	40	-0.47	44	60
	QPQPFRPQ	997.124	9.75	+1	12	3.57	35	59
	AQGFVQPQQPPQ	1324.462	5.57	0	25	1.49	29	41
	FPQPQLPFPQ	1198.39	5.52	0	30	0.57	39	60
	TLPTMCRVN	1034.271	7.91	+1	44	1.56	15	41
	QPHQIAQLEVMTSIALPILPTMCSVNV	2934.546	5.24	0	51	-0.16	1.4	0.4
	DLRKILQTGMFICKYLGRLSGSKVA VAYKM*	**3391.164**	**10.02**	**+5**	**46**	**0.76**	**92**	**79**

CapLP	LPYPQPQPFRPQ	1467.694	8.75	+1	13	1.98	31	56
	PQVQQPQPFPPQ	1390.565	5.96	0	16	1.72	37	57
	IILPR*	**610.8**	**9.75**	**+1**	**60**	**0.03**	**69**	**84**
	QQPQQPFSQSQQPQRPFPQ	2281.477	9.75	+1	10	3.45	35	59
	LILPR	610.4166	9.75	+1	60	0.03	72	73
	YGGRRFGEPQ	1165.5627	8.75	+1	10	3.65	47	41

[a] Molecular mass was calculated using the Antimicrobial Peptide Database (APD).
[b] Theoretical isoelectric point was calculated using the Compute pI/MW tool.
[c] Total percentage of hydrophobic amino acid residues present in the peptide.
[d] Boman index was generated using Antimicrobial Peptide Database (APD), indicates the protein-binding potential.
[e] The antimicrobial potential was calculated using the iAMPpred tool.

* Peptides selected to further analysis and antifungal activity.

Table 4. Peptide sequences identified by mass spectrometry of the gluten proteins hydrolyzed by peptidases from *Calotropis procera* (CpLP).

Calotropis procera			
Acession Number	**Protein**	**Specie**	**Peptide**
[1]Q6J160	LMW glutenin subunit	*Triticum aestivum*	70LPQQQIPFVHPSIL83 75IPFVHPSIL83 226VPLYR230
[1]A0A0K2QJ89	Alpha/beta-gliadin	*Triticum aestivum*	86LPYPQPQPFRPQ97 138QQQLIPCR144 139QLIPCR144
[1]I3RXX8	Alpha-gliadin	*Secale cereale*	55QPFPPRQPYPQPQ67 143QQLIPCR149 144QLIPCR149
[1]K7WJI1	Gamma secalin	*Secale strictum*	147QQVISQPQQPFPQ159
Q7X900	HMW glutenin subunit	*Triticum spelta*	95WGIPALLR102
[1]B6DQC6	Gamma gliadin	*Elymus elongatus*	11QQPQQQPFPQPQ22
[2]NP_114284	Photosystem II protein	*Triticum aestivum*	24REPPKVPPTPTKRIK38
[2]ABI74692	Farnesyltransferase beta subunit	*Triticum aestivum*	185MHDGGEIDARACYTAIF201
[2]CDM81675	Unnamed protein product	*Triticum aestivum*	270FATLAAAYIGESMEGHH286

*HMW, high molecular weight; LMW, low molecular weight.
*Species corresponds to the species in which the peptide was identified.
[1]Sequence identified by the UniprotKB database.
[2]Sequence identified by the NCBI GenBank database.

Table 5. Peptide sequences identified by mass spectrometry of the gluten proteins hydrolyzed by peptidases from *Criptostegia grandiflora* (CgLP).

Criptostegia grandiflora			
Acession Number	**Protein**	**Specie**	**Peptide**
[1]A0A0K2QJ89	Alpha/beta-gliadin	*Triticum aestivum*	86LPYPQPQPFRPQ97 137QQQLIPCR144 90QPQPFRPQ97
[1]D3UAL7	LMW glutenin subunit	*Triticum aestivum*	143TLPTMCRVN151 152VPLYR156
[1]A0A346R8U2	LMW glutenin subunit	*Aegilops comosa*	266QPHQIAQLEVMTSIALPILPTMCSVNV292 292VPLYR296
[1]A0A287EFG2	AAI domain-containing protein	*Hordeum vulgare*	283VPLYR287 57LPFPQ61
[1]F5C1U8	Gamma gliadin	*Dasypyrum hordeaceum*	52FPQPQLPFPQ61 57LPFPQ61
[1]I6V8J5	Alpha-gliadin	*Triticum dicoccoides*	262AQGFVQPQQPPQ273 145QQQLIPCR152
[1]I4EP57	Avenin	*Avena murphyi*	198QVFIPPQ204
[2]CDM82754	Unnamed protein	*Triticum aestivum*	397DLRKILQTGMFICKYLGRLSGSKVAVAYKM426

*LMW, low molecular weight.
*Species corresponds to the species in which the peptide was identified.
[1]Sequence identified by the UniprotKB database.
[2]Sequence identified by the NCBI GenBank database.

Table 6. Peptide sequences identified by mass spectrometry of the gluten proteins hydrolyzed by peptidases from *Carica papaya* (CapLP).

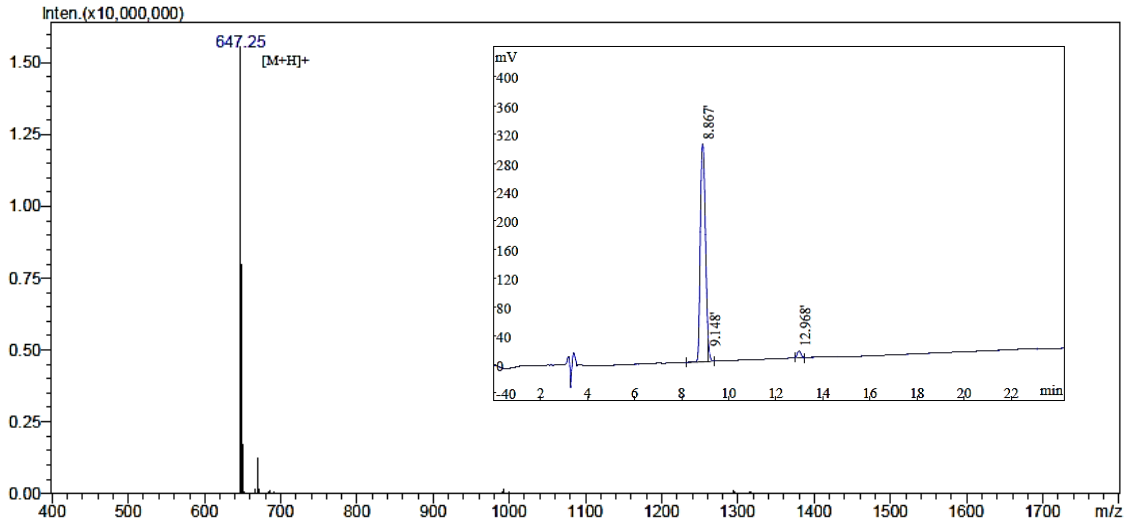
Carica papaya			
Acession Number	**Protein**	**Specie**	**Peptide**
[1]Q30DX6	Gamma-gliadin/LMW-glutenin chimera	*Triticum aestivum*	68VPLYR72
[1]Q94G95	Gamma-gliadin	*Triticum aestivum*	92IILPR96
[1]H8Y0P5	Gamma prolamin	*Secale cereale*	125LILPR129
[1]A0A0K2QJ89	Alpha/beta-gliadin	*Triticum aestivum*	86LPYPQPQPFRPQ97
[1]W8E192	Gamma-gliadin	*Aegilops tauschii*	104QQPQQPFSQSQQPQRPFPQ122
[1]E5FF13	Alpha-gliadin	*Thinopyrum bessarabicum*	91LPYPQPQPFPPQ102
[1]E5FF16	Alpha-gliadin	*Pseudoroegneria spicata*	95PQVQQPQPFPPQ106
[1]A0A0D9WQQ8	AAI domain-containing protein	*Leersia perrieri*	107YGGRRFGEPQ116

*Species corresponds to the species in which the peptide was identified.
[1]Sequence identified by the UniprotKB database.

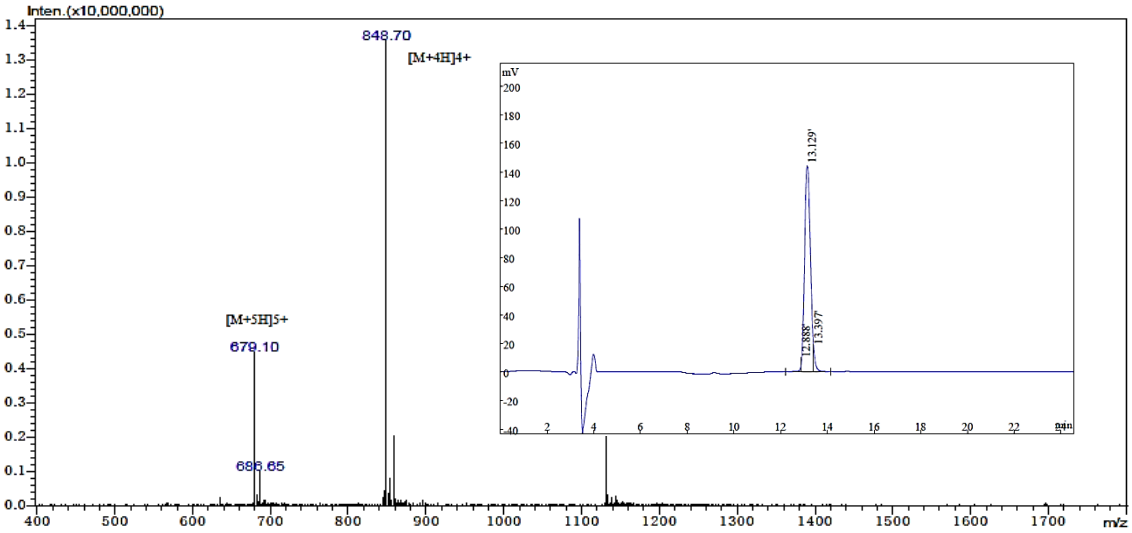
Based on the essential criteria for antimicrobial activity (Souza et al., 2020), such as a positive net charge, hydrophobic ratio around 40–60%, low Boman index (< 2.5), and predicted antifungal or antibacterial activity > 60%, the four most promising peptides were selected: VPLYR (Pep 1), DLRKILQTGMFICKYLGRLSGSKVAVAYKM (Pep 2), IPFVHPSIL (Pep 3), and IILPR (Pep 4), for further characterisation, synthesis, and measurement of antimicrobial activity. All four peptides presented a positive net charge, which is important for antimicrobial activity because it drives the initial electrostatic attraction of the peptide to the negative membrane of microorganisms (Oliveira et al., 2019b). Moreover, except for Pep 3, all peptides contained the amino acid arginine in their sequences. It is a strong positive amino acid, important for antimicrobial activity (Seyedjavadi et al., 2019). Moreover, valine, a hydrophobic amino acid, was present in Pep 1, Pep 2, and Pep 3 (Table 3). The presence of both valine and arginine may indicate a positive effect on antifungal activity, since their presence has been shown to have a linear relationship with antimicrobial potency (Ma et al., 2012).

Hydrophobicity is another essential characteristic for interaction with the phospholipid membranes of microorganisms (Souza et al., 2020). The antimicrobial potential of peptides can also be predicted by the Boman index. Values lower than 2.5 are considered low and may be indicative of greater antimicrobial activity without side effects (Oliveira et al., 2019b). The results observed in our study are in line with those observed by Seyedjavadi et al. (2019), who isolated and characterised a new peptide from *Matricaria chamomilla* flowers with remarkable antifungal activity. That peptide has net charge, hydrophobicity, and Boman index of +3, 47%, and 0.68, respectively. Thus, all the analyses indicate that the characteristics of the four chosen peptides (VPLYR, IPFVHPSIL, DLRKILQTGMFICKYLGRLSGSKVAVAYKM, and IILPR) are within the desired values to act as antifungal and/or antibacterial agents (Table 3). The four peptides were synthesised and evaluated by RP-HPLC and mass spectrometry (Fig. 9). The degree of purity was > 95%. The molecular mass values obtained were compatible with those predicted by the bioinformatics tools, confirming their sequences (Fig. 9).

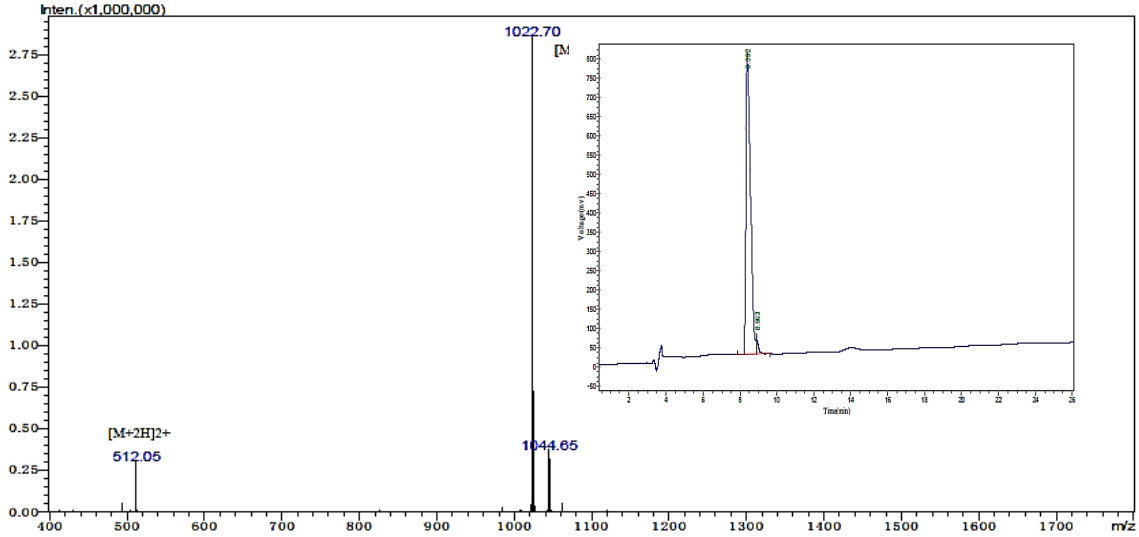
VPLYR



DLRKILQTGMFICKYLGRLSGSKVAVAYKM



IPFVHPSIL



IILPR

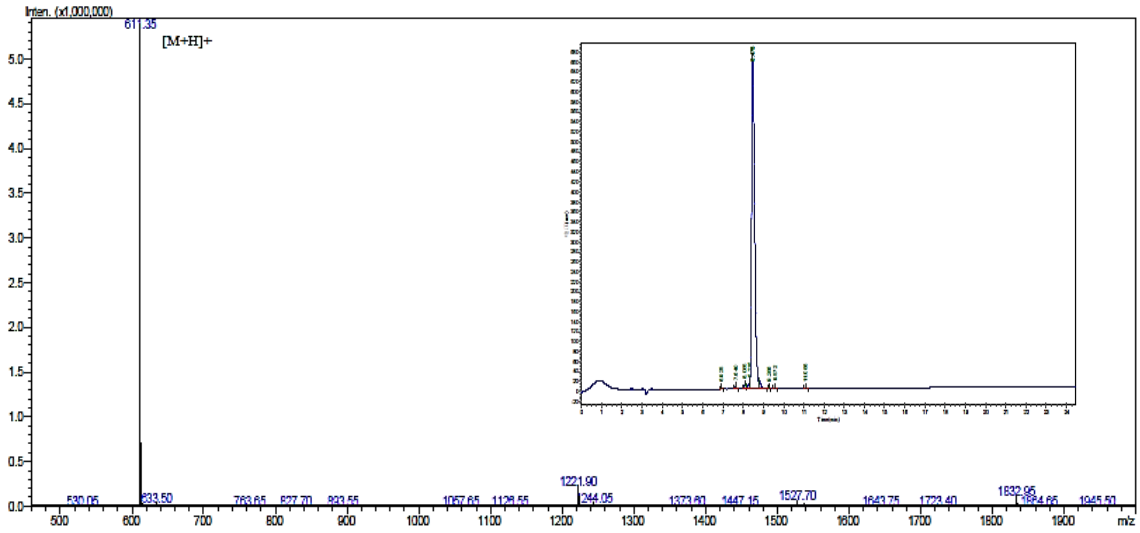


Fig. 9. Determination of purity of the synthetic peptides produced from gluten proteins hydrolyzed by latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) by mass spectrometry and reverse-phase high-performance liquid chromatography (insert), which was measured at 220 nm.

Because the three hydrolysates or the four selected peptides can be applied in bread or other types of food, it was relevant to evaluate if some human digestive enzymes could digest them. Therefore, cleavage sites for pepsin and trypsin were evaluated by bioinformatics (Table 7). The results revealed that only two peptides (QQVISQPQQPFPQ and PQVQQPQPFPPQ) had no cleavage site for pepsin and trypsin, while all others had at least one cleavage site for both enzymes (Table 7). Bioinformatics analysis also revealed that only three peptides showed antigenic potential. Nevertheless, these had cleavage sites for both trypsin and pepsin, suggesting low antigenic potential of the resulting peptides after proteolysis. Moreover, no peptide showed toxic potential (Table 7).

Table 7. Properties of peptides derived from gluten protein hydrolysis by latex peptidases obtained by bioinformatics.

Peptide	[a]Cleavage sites			[b]Antigenic Potential	[c]Toxic potential
	Pepsin (pH1.3)	Pepsin (pH>2)	Trypsin		
LPYPQPQPFRPQ	Yes	Yes	No	No	No
QQQLIPCR	Yes	Yes	Yes	No	No
QPQPFRPQ	Yes	Yes	No	No	No
AQGFVQPQQPPQ	Yes	Yes	No	No	No
FPQPQLPFPQ	Yes	Yes	No	No	No
TLPTMCRVN	Yes	Yes	Yes	No	No
VPLYR	Yes	Yes	Yes	No	No
QPHQIAQLEVMTSIALPILPTMCSVNV	Yes	Yes	No	Yes	No
DLRKILQTGMFICKYLGRLSGSKVAVAY KM	Yes	Yes	Yes	Yes	No
LSAVEVPSING	Yes	Yes	No	No	No
QPFPPRQPYPQPQ	No	No	Yes	No	No
QQLIPCR	Yes	Yes	Yes	No	No
QLIPCR	Yes	Yes	Yes	No	No
QQVISQPQQPFPQ	No	No	No	No	No
QQPQQQPFPQPQ	No	No	No	No	No
LPQQQIPFVHPSIL	Yes	Yes	No	No	No
IPFVHPSIL	Yes	Yes	No	No	No
WGIPALLR	Yes	Yes	Yes	No	No
REPPKVPPTPTKRIK	No	No	Yes	No	No
MHDGGEIDARACYTAIF	Yes	Yes	Yes	No	No
FATLAAAYIGESMEGHH	Yes	Yes	No	No	No
PQVQQPQPFPPQ	No	No	No	No	No
IILPR	Yes	Yes	Yes	No	No
QQPQQPFSQSQQPQRPFPQ	Yes	Yes	No	Yes	No
LPFPQ	Yes	Yes	No	No	No
QVFIPPQ	Yes	Yes	No	No	No
LILPR	Yes	Yes	Yes	No	No
YGGRRFGEPQ	No	Yes	Yes	No	No

[a] The cleavage sites were predicted using the Peptide Cutter tool.
[b] Antigenic potential was predicted using the Antigenic Prediction tool.
[c] Toxic potential was predicted using the ToxinPred tool.

3.4. Gluten peptides induced cell membrane disturbance

A cell viability assay against *Penicillium* sp. was performed to evaluate the antifungal activity of the four synthetic peptides. That fungus was chosen due to its importance as a bread contaminant and because the hydrolysates showed the best activity against it (Fig. 5). The peptides (2 mg/mL) exhibited antifungal activity with inhibition rates ranging from 45 to 85% (Fig. 10), standing out in relation to sodium propionate (2 mg/mL), which inhibited fungal growth by about 40%. The antifungal activity of the peptides (2 mg/mL) also exceeded the activity of all hydrolysates, even when tested at 10 mg/mL (Fig. 10). The antifungal activity demonstrated by the peptides in this study was in the same order of magnitude as that obtained by Feng et al. (2020) who investigated the antifungal effects of the peptide thanatin against *Penicillium digitatum* and observed inhibition of approximately 53%. Muhialdin et al. (2016) isolated a peptide from the cell-free supernatant of *Lactobacillus plantarum* and showed that it was able to inhibit the growth of *Penicillium roqueforti* by 60%, a similar rate to those obtained in our study. However, to achieve this activity they used a higher concentration of peptide (5 mg/mL).

We performed new tests to evaluate the mechanism of action of the four synthetic peptides and gluten protein hydrolysates (Fig. 11). First, the fluorescence of the fungal spores treated or not (control) with the peptides or hydrolysates and then with PI was used to evaluate the plasma membrane integrity (Fig. 11). PI binds to DNA, releasing red fluorescence, but only for cells with damaged membranes. Healthy membranes do not allow PI to enter the cell. Therefore, PI is considered an indicator of pore formation or alteration of membrane integrity (Souza et al., 2020). The membrane is considered a crucial target for AMPs, since it is difficult to acquire resistance. Unlike other targets such as proteins, which are easily changed and thus make drugs useless, alterations in the membrane composition are very expensive in energy

terms and can also be dangerous to the cell (Souza et al., 2020). Because of that, peptides that act on the cell membrane have strong potential for application.

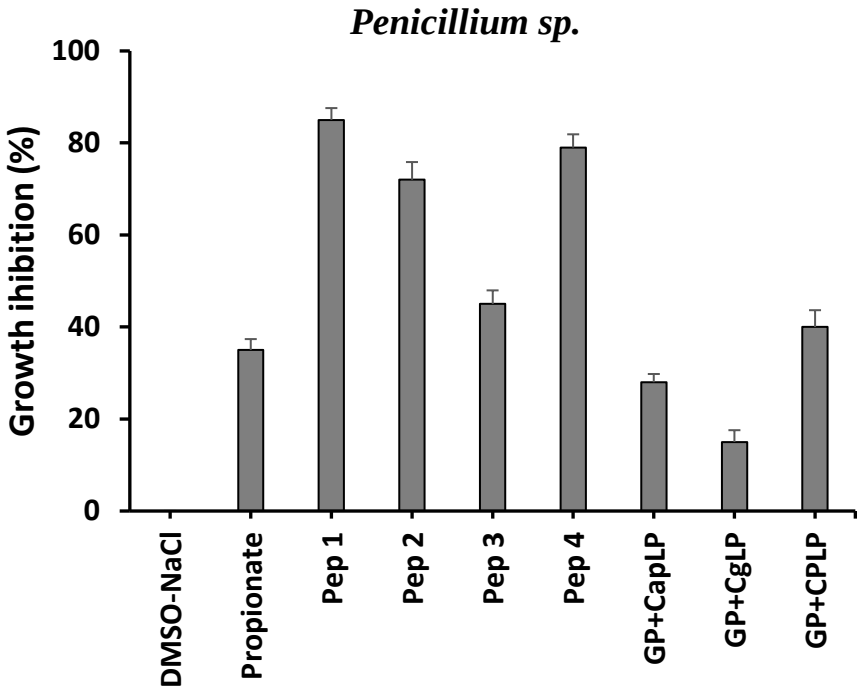


Fig. 10. Effect of the four synthetic peptides and hydrolysates obtained from proteolysis of gluten proteins (GP) by latex peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *C. papaya* (CapLP) on the *Penicillium sp*. The fungal cell viability was determined using MTT reagent.

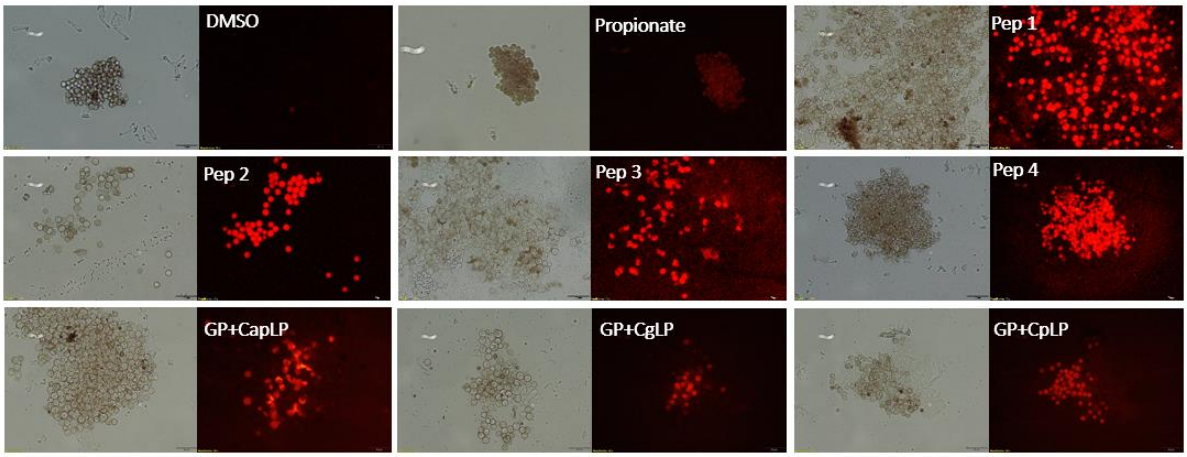


Fig. 11. Fluorescence images showing membrane disturbance in *Penicillium sp* spores. The peptides (2 mg/mL) and GP hydrolysates (10 mg/mL) were incubated with the fungal spores for 6 h at 37 °C. DMSO-NaCl solution (Control) and sodium propionate (1 mg/mL) were used as negative and positive controls, respectively. Bars: 20 µm. VPLYR (Pep 1), DLRKILQTGMFICKYLGRLSGSKVAVAYKM (Pep 2), IPFVHPSIL (Pep 3), and IILPR (Pep 4).

Fluorescence microscopy revealed that all peptides and hydrolysates induced strong red fluorescence in spores. Sodium propionate and the control caused, respectively, weak and no red fluorescence in spores. Based on how PI works, it is feasible to suggest that peptides induced damage in the spore membrane, allowing PI to pass through and reach the DNA (Souza et al., 2020). The synthetic peptides analysed here have all the features needed to interact with membranes, such as hydrophobicity and a positive charge, causing ionic interaction with the negative charge of microbial membranes, allowing insertion in the membranes (Souza et al., 2020). Recently, Lima et al. (2021) reported that peptides with the same characteristics and mechanism of action induce strong red fluorescence in spores of *Penicillium digitatum*.

SEM was used to confirm the results obtained by fluorescence microscopy. All peptides and hydrolysates drastically changed the morphology of the spores compared to the control (Fig. 12). While control spores were intact and had a normal cell volume, the spores treated with peptides or hydrolysates had clearly decreased cell volume and loss of cell content, caused by the formation of pores on the cell surface. Some of these spores also had buds with reticular ridges (Fig. 12). Accordingly, cell damage was visible after incubation of *A. niger, A. chevalieri,* and *Penicillium lanosocoeruleum* spores with sodium propionate. Microscopic analysis of these fungal biofilms revealed extensive damage to the cell membrane and showed distorted intracellular structures (Dijksterhuis, Meijer, Doorn, Houbraken, & Bruinenberg, 2019). Altogether, the results provided by both microscopic techniques strongly suggest that the mechanism of action of peptides targets the plasma membrane. This finding is in line with many other studies of synthetic peptides (Lima et al., 2021; Souza et al., 2020). Lima et al. (2021) reported that the peptides that induced strong red fluorescence also led to a reduction in the spore volume when analysed by atomic force microscopy. The pores in the membrane caused by peptides can lead to electrolyte imbalance or leakage of cytoplasm contents. Additionally, once the pores are formed, the peptide can enter the cells and interact with intracellular components, enhancing the antifungal effect (Oliveira et al., 2019b).

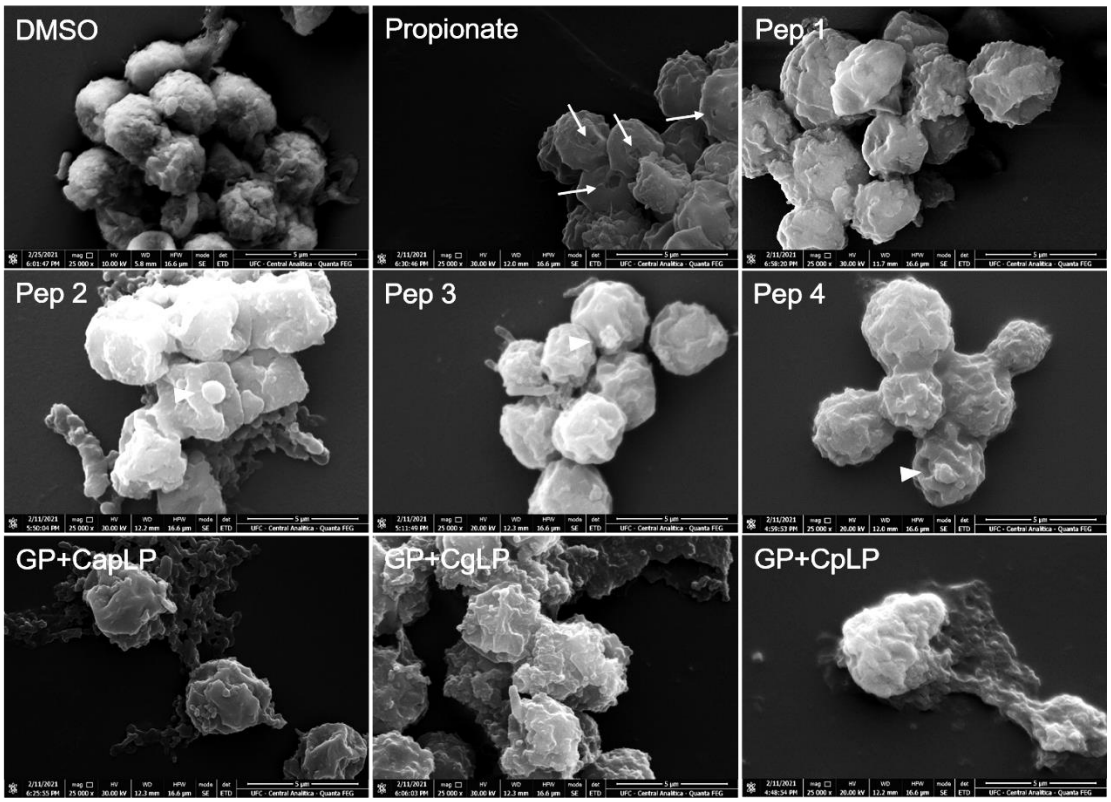


Fig. 12. Scanning electron microscopy (SEM) images of *Penicillium* sp. spores treated with synthetic peptides and hydrolysates obtained from proteolysis of gluten proteins (GP) by latex peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *C. papaya* (CapLP). Arrows indicate spores on the surface. Arrowheads show the presence of buds in spores with reticular surface ridges. VPLYR (Pep 1), DLRKILQTGMFICKYLGRLSGSKVAVAYKM (Pep 2), IPFVHPSIL (Pep 3), and IILPR (Pep 4).

4. Conclusion

The present study showed the biotechnological potential of latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) to partially hydrolyse wheat gluten proteins and produce antifungal peptides able to delay fungal growth on bread, without altering the specific volume and expansion factor, but increasing the number, area, and mean size of alveoli. Different peptides were obtained after digestion by each latex peptidase. CpLP produced the most antifungal peptides, explaining the longer shelf life of the bread samples containing these peptides. No peptide exhibited toxic potential and only three were antigenic. Nevertheless, the antigenic peptides had cleavage sites for trypsin or pepsin. Of 28 peptides sequenced, four were selected and synthesised. Their antifungal activity was better than that of the three hydrolysates and sodium propionate, causing damage to the cell membrane structure. Of these four gluten peptides, only one (DLRKILQTGMFICKYLGRLSGSKVAVAYKM) exhibited antigenic potential, but it has cleavage sites for the digestive enzymes. Although the results showed that the hydrolysates or purified peptides are promising alternatives for use as natural preservatives to delay fungal growth, new tests are needed to evaluate other quality and/or sensory parameters of bread.

Conflict of interest

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

Contributions

DCF, MVR, JPBO, GBMS, and CDTF performed the latex peptidase purification, chromatography, gel electrophoresis, and hydrolysis assays. CSN and JSO sequenced the peptides by MS/MS. DCF, LPB, AFBS, and PFNS performed the fluorescence and SEM assays. All authors contributed to data analysis, discussion, and writing of the manuscript.

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4 CONCLUSÃO

Em conclusão, os resultados mostrados neste estudo evidenciaram o potencial das peptidases do látex de *Calotropis procera*, *Cryptostegia grandiflora* e *Carica papaya* em hidrolisar as proteínas do glúten do trigo e gerar peptídeos antifúngicos. Dos 31 peptídeos sequenciados, quatro foram sintetizados e apresentaram atividade antifúngica, causando danos às estruturas da membrana celular fúngica. Além disso, os hidrolisados retardaram o crescimento dos fungos no pão, prolongando a vida útil em até três dias, sem alterar o processo fermentativo e parâmetros físicos importantes. Visto que o glúten é amplamente utilizado na indústria alimentícia e que seus peptídeos derivados apresentaram baixo potencial alergênico, espera-se que estes AMPs sejam seguros para o consumo humano, podendo ser uma alternativa promissora para uso como conservantes naturais.

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