Universidade Federal de Pernambuco
Programa de Pós-Graduação em Biologia Aplicada à Saúde
Laboratório de Imunopatologia Keizo Asami

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Imobilização de protease de *Penicillium aurantiogriseum* URM4622 em nanopartículas magnéticas e sua aplicação na produção de peptídeos com atividade antioxidante

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à minha família, em especial meus pais, irmã e avós,
que sempre estiveram do meu lado desde o princípio e à cada etapa.

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“Para TER algo que você nunca TEVE, é preciso FAZER algo que você nunca FEZ”.

Chico Xavier

Resumo

Proteases são enzimas que agem sobre substratos proteicos e são largamente utilizadas na medicina, na indústria química e na indústria de alimentos. Peptídeos bioativos, com propriedades de interesse biotecnológicos podem ser obtidos através da hidrólise da caseína utilizando proteases microbianas, como a produzida pelo *Penicillium aurantiogriseum* URM 4622. A utilização de enzimas imobilizadas pode trazer muitas vantagens em relação ao uso da enzima nativa, como isolamento, possibilidade de reuso, aumento de estabilidade e redução dos custos do processo. O uso de nanopartículas magnéticas como suporte acrescenta vantagens à imobilização, como fácil separação do meio de reação aplicando um campo magnético, e maior área superficial. O presente trabalho teve como objetivo otimizar a imobilização da protease produzida pelo *P. aurantiogriseum* URM 4622 em nanopartículas magnéticas para obtenção de peptídeos com propriedades antioxidantes a partir da hidrólise da caseína bovina comercial. O processo de imobilização foi aperfeiçoado utilizando um planejamento fatorial completo (2^4) seguido de um planejamento central composto (2^2). A protease livre e imobilizada foi utilizada para a hidrólise de caseína. Os peptídeos gerados foram analisados em espectrômetro de massa e suas atividades antioxidantes foram avaliadas. Os resultados do planejamento completo 2^4 indicaram que as variáveis mais significativas para a imobilização da protease foram o pH e o tempo de ativação, o primeiro exerceu efeito negativo enquanto o segundo efeito positivo. A análise dos resultados do planejamento central composto (2^2) utilizado para otimizar o processo de imobilização demonstrou que as condições ótimas para imobilização foram um tempo de imobilização de 2 horas, proteína ofertada de 200 µg/mL, pH de 6,3 e tempo de ativação de 7,3 h. O perfil de peptídeos obtido por espectrometria de massa mostrou que embora algumas diferenças tenham sido encontradas entre picos de massa de peptídeos dos hidrolisados das enzimas livre e imobilizada, ambos apresentaram perfils similares. Os seguintes peptídeos foram sequenciados: DVPSERYLGY; GLPQEVLNENLLRF; LSLSQSKV, YQEPVLGPVRGPF e LLYQEPVLGPVR. Estes peptídeos foram capazes de reduzir o radical ABTS (atividade antioxidante de 44,35%) e de capturar radicais H_2O_2 (atividade antioxidante de 644,5 µmol de trolox/mg de amostra). Os resultados validam a capacidade da protease do *P. aurantiogriseum* de produzir peptídeos de caseína com potencial para ingredientes em alimentos funcionais e nutracêuticos.

Palavras chave: Glutaraldeído, Polianilina, Magnetita, ABTS, Peróxido de Hidrogênio.

Abstract

Proteases are enzymes that act over protein substrates and are widely used in medicine, chemical industry and food industry. Bioactive peptides, with properties of biotechnological interest, can be obtained by hydrolysis of casein using microbial proteases, such as the produced by *Penicillium aurantiogriseum* URM4622. The use of immobilized enzymes can add many advantages to native enzyme use, as isolation, possibility of reuse, increase stability and reduce process costs. The use of magnetic nanoparticles as support adds advantages to immobilization as easy separated from reaction medium by applying a magnetic field and higher surface area. This study aimed to optimize the immobilization of protease produced by *P. aurantiogriseum* URM4622 on magnetic nanoparticles in order to obtain peptides with antioxidant properties from casein hydrolysis. The immobilization process was optimized using a full factorial design (2^4) followed by a central composite design (2^2). The free and immobilized enzyme were used for commercial bovine casein hydrolysis. The peptides generated were analyzed in a mass spectrometer and their antioxidant activities were evaluated. The results of full factorial design (2^4) indicated that the most significant variables for protease immobilization were pH and the activation time, the first exerted negative effect while the second positive effect. The results of the central composite design (2^2) used to optimize the immobilization process showed that the optimum conditions for immobilization were an immobilization time of 2 hours, offered protein amount of 200 mg/mL, pH 6.3 and activation time of 7.3 h. The profile of the peptides obtained by mass spectrometry showed that although some differences were found between hydrolyzed peptide mass peaks of free and immobilized enzymes, both had similar profiles. The following peptides were sequenced: DVPSERYLGY, GLPQEVLNENLLRF, LSLSQSKV, YQEPVLGPVRGPF and LLYQEPVLGPVR. The hydrolysates were able to reduce ABTS radicals (antioxidant activity of 44.35%) and to capture H_2O_2 radicals (antioxidant activity of 644.5 µmol of trolox/mg of sample). The results validate the ability of protease from *P. aurantiogriseum* to produce casein peptides with potential for ingredients in functional foods and nutraceuticals.

Key words: Glutaraldehyde, Polyaniline, Magnetite, ABTS, Hydrogen Peroxide.

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Lista de Abreviaturas e Siglas

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	Angiotensin-converting enzyme
CCD	Central Composite Design
CMI	Commonwealth Mycological Institute
ECA	Enzima Conversora de Angiotensina
ERRO	Espécies Reativas de Oxigênio
FFD	Full Factorial Design
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
Mpani	Magnetized Polyaniline
MS	Mass Spectrometer
PANI	Polianilina
PMF	Peptide Mass Fingerprint
RA	Residual Activity
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
SF	Soybean flour
URM	Universidade Recife Micologia

Sumário

1. Introdução

Enzimas são responsáveis por um segmento de mercado global que faturou 3,6 bilhões de dólares em 2010, possui um crescimento anual de 9,1%, e estima-se um valor aproximadamente 6 bilhões de dólares para 2016 (Shalini and Dewan, 2012). Enzimas com capacidade de hidrolisar ligações peptídicas respondem por aproximadamente 60% deste mercado, e possuem principal aplicação no setor alimentício (Anitha e Palanivelu, 2013). As proteases são de grande importância para a manutenção da vida, sendo encontradas em todos os seres vivos e constituem o maior grupo de enzimas encontrado no corpo humano (Rogers et al. 2013). Estas enzimas desempenham funções fisiológicas complexas e são importantes em vias regulatórias e metabólicas. Já o papel de uma protease extracelular está diretamente relacionado ao catabolismo de grandes proteínas em pequenos peptídeos e aminoácidos para nutrição celular (Graminho et al. 2013). A cada dia mais e mais processos industriais criam necessidade de hidrolisados proteicos contendo peptídeos específicos e preservados, que, portanto necessitam da aplicação de uma protease melhores características, porém para atingir essas características requer escolher uma protease adequada e frequentemente é necessário processá-la antes do uso (Tavano et al. 2013).

A utilização de enzimas imobilizadas pode trazer muitas vantagens em relação ao uso da enzima nativa, como isolamento, possibilidade de reuso, e aumento de estabilidade (Werner et al. 1982). A utilização de enzimas imobilizadas em processos industriais pode reduzir significativamente os custos operacionais por permitir a recuperação da enzima ao final do processo e possibilitar a reutilização desta por vários ciclos, reduzindo a quantidade necessária de enzima. Diferentes suportes podem ser utilizados para imobilização de uma enzima via diferentes processos físicos ou químicos, envolver diferentes partes e atingir diferentes orientações da enzima (Tavano et al. 2013).

A utilização de suportes magnéticos para imobilização de enzimas trás uma nova característica que elimina indesejáveis etapas, como a necessidade de centrifugação, diluição da amostra, e perda de suporte durante as lavagens. Sendo a separação magnética rápida e eficiente, há ainda a economia de tempo (Tüzmen et al. 2012). A utilização de partículas em escala nano amplia a gama de aplicações possíveis e, devido ao seu tamanho reduzido, amplia a área superficial, diminui a toxicidade, além da

barreira de transferência, e aprimorando a eficiência catalítica (Neri et al. 2008; Jordan et al. 2011). Dentre os revestimentos utilizados em imobilização, a polianilina (PANI) vem sendo bastante estudada e representa uma classe de polímeros promissores (Neri et al. 2008; Caramori, 2007).

Peptídeos são normalmente gerados por hidrólise de uma proteína precursora e aqueles que apresentam atividade em processos biológicos são chamados peptídeos bioativos. Estas importantes moléculas, dentre outras funções, podem agir como imunomoduladores, possuir atividade em sistemas de interação protéica, promover inibição ou ativação enzimática, mediar transporte de substâncias e promover rompimento de membranas celulares microbianas. Vários benefícios para a saúde têm sido associados com peptídeos bioativos, especialmente aqueles derivados de fontes alimentares, como leite bovino (Mooney et al. 2013). Por causa dos seus efeitos benéficos, peptídeos bioativos podem ser utilizados na formulação de alimentos funcionais, nutracêuticos e drogas naturais, envolvendo vários segmentos industriais (Haque et al. 2009).

Nos últimos anos, houve um aumento crescente no interesse comercial da produção de peptídeos bioativos a partir de várias fontes para utilizá-los como ingredientes em alimentos funcionais (Kunda et al. 2012). Dentre estes, os peptídeos com atividade antioxidante, produzidos a partir da caseína, têm se destacado devido ao fato de interferirem em processos oxidativos e a possibilidade de serem utilizados em seres humanos (Muro Urista et al. 2011; Ao and Li, 2013). Além disto, podem ser utilizados como conservante de alimentos o que é de extremo interesse da indústria alimentícia (Sakanaka, 2005).

Como os microrganismos podem ser cultivados através de métodos bem estabelecidos, produzindo elevadas quantidades de enzimas, as proteases microbianas têm sido utilizadas com sucesso na produção de peptídeos bioativos em uma escala comercial (Corrêa et al., 2011, Kunda et al. 2012). Neste contexto, a protease produzida pelo *Penicillium aurantiogriseum* URM 4622 representa uma fonte alternativa desta enzima para produção de peptídeos bioativos, considerando-se a sua obtenção com alto rendimento e da sua imobilização em nanopartículas de magnetita revestida com PANI que permite a separação desta enzima do produto com elevada eficiência e baixo custo.

2. Revisão de Literatura

2.1. Proteases

Enzimas com capacidade de hidrolisar ligações peptídicas são chamadas de proteases, estas existem em todas as ordens de vida e constituem a maior família de enzimas presentes no ser humano (Lindsay et al. 2013). As proteases são responsáveis por inúmeras funções fisiológicas complexas. Sua importância nas vias regulatórias e metabólicas fica evidente pela sua constante presença em todos os organismos vivos (Graminho et al. 2013). A proteólise caracteriza um processo em que ocorre a quebra de proteínas em peptídeos menores ou aminoácidos através da hidrólise de suas ligações peptídicas (Lindsay et al. 2013). Essa quebra pode ser ocasionada por processos químicos ou enzimáticos, porém a hidrólise química seja mais difícil de controlar. Através da hidrólise enzimática, é possível realizar a proteólise em condições mais amenas e evitar condições extremas requeridas para hidrólises químicas, além disso, enzimas possuem alta especificidade ao substrato, o que permite maior controle das características do produto da hidrólise (Tavano, 2013).

Porém para entender completamente as funções de uma protease, é preciso compreender quais proteínas são processadas por essa protease, as funções desses substratos e as etapas do seu processamento (Lindsay et al. 2013). O papel de uma protease extracelular está diretamente relacionado ao catabolismo de grandes proteínas em pequenos peptídeos e aminoácidos para absorção celular (Graminho et al. 2013). Sendo assim, processos que necessitem de hidrolisados proteicos contendo peptídeos específicos e preservados podem requerer a aplicação de protease. Para tal propósito é necessário escolher a protease com melhores características e frequentemente modificar esta enzima (Tavano, 2013).

O mercado mundial de enzimas industriais foi avaliado em 3,6 bilhões de dólares em 2010, com crescimento anual de 9,1%, e irá alcançar o valor de 6 bilhões de dólares no ano de 2016. A indústria alimentícia responde pelo maior segmento industrial de uso dessas enzimas, faturando quase 1,2 bilhões de dólares em 2010. Espera-se que o mercado atinja a marca dos 2,1 bilhões em 2016 (Shalini and Dewan, 2012). As enzimas proteolíticas lideram esse mercado e respondem por aproximadamente 60% do mesmo. Suas principais aplicações são no setor alimentício, produção de detergentes e processamento de couros (Anitha et al, 2013).

2.2. Imobilização de enzimas

Enzimas imobilizadas são aquelas que estão fisicamente confinadas ou localizadas em certa região definida do espaço, com retenção das suas atividades catalíticas, e que podem ser usadas repetidamente e continuamente (Chibata et al. 1978). A utilização de enzimas imobilizadas trás muitas vantagens sobre a enzima nativa, como isolamento, possibilidade de reuso, e aumento de estabilidade (Werner et al. 1982).

A imobilização de enzimas em suportes não solúveis tem sido um tópico ativo de pesquisas há décadas e possibilita não somente a reutilização da enzima como também a modulação das suas propriedades catalíticas. Através da imobilização covalente de cada molécula da enzima aos variados grupos ativados do suporte, é possível atingir um alto grau de estabilização enzimática e interferir nas propriedades catalíticas da enzima, como pH ótimo, temperatura ótima, ação de inibidores e cinética (Liu et al. 2013). Tal processo também mostrou ser capaz de aumentar o tempo de armazenamento e possibilitar a reutilização dessas enzimas (Werner et al. 1982). Além disso, a autólise pode ser reduzida e a concentração efetiva de enzima é maior quando comparada as hidrólises realizadas com a enzima livre, o que pode resultar em tempos de hidrólise significativamente menores (Sun et al. 2012). Todas essas modificações agregam características de interesse industrial ao uso de enzimas.

A partir dos anos 1960 houve um grande avanço nas técnicas de imobilização. A possibilidade de uso contínuo e repetido das enzimas a partir dessas técnicas proporcionou grades avanços dos processos industriais, no sentido de diminuir os custos e aumentar a produção (Vitolo, 2001). O uso de enzimas imobilizadas na indústria pode reduzir os custos operacionais por reduzir a quantidade de enzima necessária, caso a enzima imobilizada possa ser recuperada ao final do processo e reutilizada por vários ciclos, enquanto mantiver sua atividade. Suportes diferentes podem ser utilizados para imobilização de uma enzima via diferentes processos físicos ou químicos, envolver diferentes partes e atingir diferentes orientações da enzima (Tavano et al. 2013).

Os novos avanços no campo da biocatálise por enzimas imobilizadas vêm oferecendo a possibilidade de uma aplicação mais ampla e mais econômica das enzimas no setor industrial, no tratamento de efluentes, na medicina e no desenvolvimento de biossensores. Os métodos de imobilização de enzimas basicamente incluem adsorção a

materiais insolúveis, aprisionamento em matriz polimérica, encapsulamento, reticulação com um reagente bifuncional, ou ligação covalente a um suporte insolúvel. Os diversos materiais utilizados como suportes na imobilização de enzimas são classificados entre suportes inorgânicos, polímeros sintéticos ou macromoléculas naturais. Os materiais poliméricos tornam-se bons candidatos devido a seus grupos funcionais reativos, boas propriedades mecânicas, simples método de preparação e capacidade para acomodar componentes atóxicos para aumentar a biocompatibilidade (Tüzmen et al. 2012). Além disso, características adicionais de interesse industrial podem ser adicionadas aos derivados, através do suporte utilizado, a exemplo da magnetização.

2.2.1. Suportes Magnéticos

Magnetização é uma possível solução para materiais que são de difícil remoção do meio de reação (Barbosa et al. 2012). O uso de suportes magnéticos para imobilização de enzimas é principalmente baseado na propriedade magnética da fase sólida que possibilita atingir uma rápida separação por campo magnético, bem como a diminuição do custo operacional. A imobilização de enzimas ou biomoléculas em micropartículas magnéticas normalmente é alcançada através dos grupos reativos existentes na sua superfície. Hoje em dia esses compostos funcionais podem ser produzidos de diversas formas, porém normalmente envolve um revestimento da partícula suscetível magneticamente com um polímero ou biomacromoléculas. O uso de partículas magnéticas em tecnologia enzimática está crescendo constantemente (Neri et al. 2008).

O uso de suportes com núcleo magnético diminui a necessidade de centrifugação, indesejável diluição da amostra e perda do suporte durante as lavagens, eventos que muitas vezes complicam o uso de reatores de enzimas não-magnéticas. As separações magnéticas são relativamente rápidas, fáceis e requerem aparelhagem simples. Portanto, técnicas de separação magnética têm atualmente encontrado muitas aplicações em áreas diferentes das ciências biológicas, especialmente em escala de laboratório. A imobilização enzimática em suportes magnéticos vem tornando-se preferível à enzima nativa para fins industriais (Tüzmen et al. 2012).

Nos últimos anos, as partículas magnéticas em escala nano vem recebendo atenção aumentada em vários campos da biologia, incluindo aplicações biomédicas e ambientais, devido ao seu tamanho reduzido, maior área superficial e baixa toxicidade,

além de diminuir a barreira de transferência, e então melhorar a eficiência catalítica (Neri et al. 2008; Jordan et al. 2011).

Mais especificamente, nanoparticulas de magnetita são um dos mais prevalentes materiais magnéticos em uso comum. Elas são um material biocompatível super-paramagnético com baixa toxicidade e fortes propriedades magnéticas, encontrando aplicações na imobilização e purificação de enzimas e proteínas. Alguns outros campos que se beneficiam da utilização de nanopartículas magnéticas e nanopartículas de magnetita, em particular, incluem absorção e separação intracelular, carreamento de drogas, hipertermia magnética, melhoramento de contraste em ressonância magnética, tratamento de efluentes e processamento têxtil (Jordan et al. 2011).

Dentre os revestimentos utilizados sobre essas partículas magnéticas a polianilina (PANI) representa uma classe de polímeros altamente promissores, em função de seu baixo custo de síntese, facilidade operacional e por apresentar propriedade condutora de elétrons, pois, dependendo das condições de síntese, o polímero pode atuar como semicondutor (Caramori, 2007). A polianilina vem sendo estudada como suporte para imobilização de diversas enzimas, e vários estudos de caracterização deste suporte em imobilização já foram realizados, Figura 1 (Mooney et al. 2007).

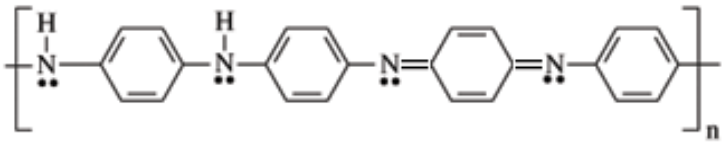

Figura 1. Estrutura da polianilina no formato de base de esmeraldina (Galiani, et al. 2007).

Utilizando enzimas proteolíticas é possível hidrolisar proteínas precursoras a fim de liberar seus peptídeos. Esses peptídeos podem ser atraentes, industrialmente, pela sua importância fisiológica e pelas diversas funções que podem desempenhar. Estes peptídeos podem ser utilizados na indústria alimentícia para agregar valor nutracêutico a produtos além de poder serem utilizados pra produção de medicamentos (Mooney et al, 2013).

2.3. Peptídeos Bioativos

Peptídeos, normalmente gerados por hidrólise de uma proteína precursora, são importantes moléculas encontradas em todos os organismos e estão envolvidos em muitos processos biológicos. Eles podem funcionar como hormônios e agentes imunomoduladores, através de interações com citocinas, receptores e outras proteínas sinalizadoras, e tem atraído atenção significante com a recente descoberta de sua atividade inibidora ou antagonista de sistemas de interação proteica. Outras funções incluem sinalizadores de *quorum* ou *"quorum sensing*" na regulação de populações bacterianas intestinais, inibição e ativação enzimática, canais de proteínas ligadas à membrana, peptídeos transportadores, receptores de toxinas e venenos e rompimento de membranas celulares nos casos dos peptídeos antimicrobianos (Mooney et al. 2013).

Peptídeos bioativos foram definidos como fragmentos de proteínas específicos que têm um impacto positivo sobre as funções ou condições corporais e pode vir a influenciar a saúde (Kittis e Weiler, 2003). Esses peptídeos encontram-se encriptados dentro de muitas sequências de proteínas da dieta e, quando liberados por várias proteases, podem exercer as funções fisiológicas em diversos locais no corpo humano, como no coração, nos ossos e dentes. Modulam eventos fisiológicos como a saúde digestiva, imunomodulação, alterações de humor, memória e controle do estresse. Peptídeos bioativos descritos na literatura foram liberados a partir de proteínas dietéticas pelas enzimas do trato digestivo, como tripsina, ou pelo sistema proteolítico de microorganismos durante a fabricação de alimentos. Numerosos estudos abordam a capacidade de lactococci ou lactobacili de gerar peptídeos bioativos a partir de proteínas do leite durante a fermentação, por exemplo: Miclo et al. 2012.

Vários benefícios para a saúde têm sido associados com peptídeos bioativos, especialmente aqueles derivados de fontes alimentares: como plantas e leite bovino. Dipeptídeos simples originados da caseína do leite são capazes de inibir a enzima conversora de angiotensina (ECA), enquanto peptídeos maiores obtidos da mesma fonte proteica apresentaram ações importantes na imunogenicidade e nutrição no início do desenvolvimento (Mooney et al. 2013).

Os meios para a produção de peptídeos bioativos incluem hidrólise da proteína por protease, fermentação por bactérias, e síntese química. Muitos peptídeos bioativos de origem vegetal ou animal com potencial relevante já foram descobertos. Proteínas provedoras contendo estas atividades biológicas latentes são encontradas no leite, nos

ovos, nas carnes, nos peixe, bem como diferentes fontes de proteína vegetal: como soja, trigo e brócolis (Abidi et al. 2013).

Por causa dos seus efeitos benéficos, peptídeos bioativos podem ser utilizados na formulação de alimentos funcionais, nutracêuticos e drogas naturais. Tais efeitos podem vir a serem utilizados no tratamento da diarreia, hipertensão, trombose, cáries dentárias, estresse oxidativo, absorção de minerais e imunodeficiência, dentre outros (Haque et al. 2009). Nos últimos anos, houve um aumento crescente no interesse comercial da produção de peptídeos bioativos a partir de várias fontes para utilizá-los como ingredientes em alimentos funcionais, cosméticos ou medicamentos; com promoção da saúde ou prevenção de doenças. Imunomoduladora, antimicrobiana, opióide, inibição da enzima conversora de angiotensina (ECA), ligação de minerais, antitrombótica, osteoprotetora, antilipidêmica, hipocolesterolêmica, regulação hormonal e antialérgica são algumas das bioatividades descritas (Kunda et al. 2012; Muro et al. 2011; Pihlanto, 2013) Figura 2.

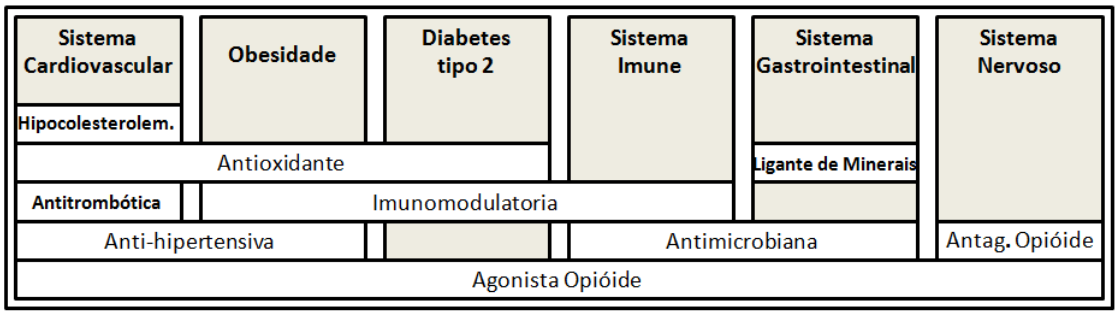

Figura 2. Atividades biológicas de peptídeos derivados de alimentos (Hartmann, R. e Meisel, H. 2007).

Enzimas extraídas de microrganismos têm sido utilizadas com sucesso no processo proteolítico para libertar os peptídeos a partir de proteínas do leite. Enzimas purificadas a partir de espécies de bactérias ácido-laticas e *Aspergillus oryzae*, tais como a Alcalase, Termolisina e Proteinase K, são proteases comercialmente disponíveis para a produção de pequenos peptídeos com um resíduo de prolina na extremidade carboxi-terminal. Muitos estudos têm demonstrado atividade de inibição da ECA *in vitro* e efeito anti-hipertensivo de pequenos peptídeos de hidrolisados de caseína e soro de leite em ratos espontaneamente hipertensos (Muro et al. 2011).

A combinação correta da fonte protéica e de enzimas específicas tem apresentado grande importância no que concerne a produção de peptídeos bioativos. Propriedades funcionais e biológicas dos peptídeos vão depender da escolha da fonte

proteica, sua sequencia de aminoácidos e da ação das enzimas proteolíticas (Muro et al. 2011). Por exemplo, é conhecido que a prolina é um aminoácido resistente à degradação por enzimas digestivas e pode atravessar a parede do intestino delgado quando presente em pequenos peptídeos (Vermeirssen et al. 2004). Assim, enzimas que produzam peptídeos com resíduos de prolina são interessantes para a produção de alimentos funcionais.

2.4. Aplicação de peptídeos em alimentos

Para direcionar o *boom* do efeito que os peptídeos bioativos do leite podem produzir no metabolismo, os pesquisadores tem se focado em estudos sobre a produção de "alimentos funcionais" para a fabricação de muitos produtos de consumo ou ingredientes nutracêuticos (Kunda et al. 2012). Aditivos funcionais em alimentos é uma área cada vez mais interessante de desenvolvimento, dado o potencial para produzir melhorias na dieta, saúde e resistência à infecção. Peptídeos oferecem uma fonte natural significativa de tais alimentos funcionais e, como resultado, tem havido interesse na identificação de peptídeos bioativos em produtos alimentares, como, por exemplo, o leite. Vários peptídeos já foram comercializados como nutracêuticos, demonstrando a necessidade de mais métodos para a detecção e caracterização de novos peptídeos bioativos nesta área (Mooney et al. 2007).

Produtos lácteos fermentados têm naturalmente elevados valor nutritivo, e como benefícios extras, muitos efeitos na promoção da saúde, como: a melhoria do metabolismo da lactose, a redução dos níveis séricos de colesterol e redução do risco de câncer. Estes benefícios à saúde podem, em parte, ser atribuído à liberação de sequências de peptídeos bioativos durante a fermentação. Numerosos peptídeos e frações peptídicas, tendo propriedades bioativas, foram isolados de produtos lácteos fermentados. Muitos artigos e capítulos de livros recentes analisaram a liberação de vários peptídeos bioativos de proteínas do leite através de proteólise microbiana (Pihlanto, 2013).

Neste contexto, as propriedades multifuncionais de peptídeos do leite tem promovido o desenvolvimento de numerosas investigações sobre os peptídeos funcionais e os seus efeitos no sistema humano. Os benefícios potenciais de diferentes peptídeos bioativos do leite tem sido um tema de crescente interesse comercial e farmacêutico no contexto dos alimentos funcionais e tratamentos clínicos. Atualmente,

peptídeos bioativos derivados do leite são considerados candidatos importantes para vários alimentos funcionais de promoção da saúde voltadas para o coração, ossos e saúde do sistema digestivo, bem como para melhorar a defesa imunológica, e para trazer o controle do humor e do estresse (Lahrich et al. 2013).

Vários peptídeos provenientes de proteínas alimentares tem demonstrado capacidade antioxidante, despertando estudos extensivos desde a primeira publicação sobre a bioatividade. Em geral peptídeos antioxidantes apresentam de 5-16 resíduos de aminoácidos, são considerados componentes alimentares seguros, de baixo peso molecular, baixo custo, alta atividade e fácil absorção. Eles ainda possuem vantagens sobre o uso de antioxidantes enzimáticos por apresentarem estrutura mais simples que lhes confere maior estabilidade sem despertar reação danosa do sistema imunitário (Pepe et al. 2013).

A esta área de conhecimento se atribui o nome de “peptidomica alimentar”, que dispõe sobre a identificação e quantificação de peptídeos nutricionalmente relevantes geralmente chamados de "peptídeos bioativos". Nos últimos cinco anos, este campo foi amplamente expandido e várias revisões publicadas. Um parâmetro importante é que após a hidrólise do ingrediente, é preciso também haver a passagem através do sistema digestivo, a fim de entregar seu benefício. Considerando a variedade de enzimas disponíveis nos sistemas digestivo (por exemplo, a pepsina, tripsina, quimotripsina, elastase, carboxi-peptidases, amino-peptidases, di e tripeptidases), não é surpreendente que os pequenos peptídeos são a categoria com conhecidas atividades biológicas e, portanto, representam os candidatos mais promissores para qualquer aplicação de alimentos que vise oferecer benefícios à saúde (Lahrich et al. 2013).

O uso de culturas microbianas produtoras de peptídeos bioativos podem permitir o desenvolvimento de novos produtos lácteos fermentados. Os oligopeptídeos formados podem ser uma fonte direta de peptídeos bioativos após hidrólise por enzimas gastrointestinais. Porém o fato de que as atividades de peptidases são afetadas pelas condições de crescimento, faz com que a manipulação de peptídeos seja limitada. A produção de peptídeos com sequência de aminoácidos específica e funcional requer estudos sobre a seleção da proteína provedora e o melhor método catalítico (digestão, fermentação ou hidrólise enzimática) para obtenção de resultados promissores (Muro et al. 2011).

Por este motivo, a forma mais comum de gerar peptídeos bioativos é por hidrólise enzimática de proteínas inteiras. Enzimas proteolíticas de várias fontes (animal, vegetal ou microbiana) podem ser usadas com sucesso na produção de peptídeos bioativos. As proteases microbianas, porém, se destacam como mais atrativas à obtenção de hidrolisados e peptídeos bioativos em escala comercial, devido seus métodos de cultivo serem bem estabelecidos e apresentarem produtividade elevada (Corrêa, 2011).

2.5. Caseína

O leite bovino contém cerca de 32 g/L de proteínas, dos quais 20% são proteínas do soro de leite e 80% são as caseínas. Caseínas podem ser divididas em α, β e k-caseínas. Já a fração de soro de leite contém uma lactalbumina, β-lactoglobulina e outras proteínas, como imunoglobulinas e lactoferrinas. As atividades biológicas e fisiológicas de proteínas do leite são parcialmente atribuídas aos vários peptídeos codificados nas moléculas de proteínas nativas. Estudos identificaram um grande número de sequências peptídicas com bioatividades específicas em caseínas e proteínas de soro de leite. Além disso, grande número de peptídeos bioativos vem sendo encontrados em derivados de produtos fermentados a partir do leite (Muro et al. 2011). As proteínas provenientes do leite, principalmente as caseínas, são disponíveis comercialmente em grandes quantidades com elevado grau de pureza e por um baixo preço, o que faz delas atrativas para a busca de peptídeos bioativos (Corrêa, 2011).

Caseínas são proteínas insolúveis e, como tal, elas formam precipitados no leite. Sua estrutura aberta e flexível facilita a digestão e hidrólise para liberar uma quantidade considerável de peptídeos com diferentes sequências de aminoácidos. Muitas pesquisas foram realizadas mostrando bioatividade desses peptídeos derivados de caseína (Silva e Macalta, 2005). Os peptídeos de caseína têm potencial para exercer numerosos efeitos biológicos benéficos à saúde. Exemplos de peptídeos bioativos derivados de caseína incluem caseinofosfopeptídeos, capazes de desempenhar um papel no transporte e absorção de certos minerais, dentre outras propriedades, os glicomacropeptídeos que se ligam às toxinas, e que possuem propriedades de se comportarem como casoxinas, imunomoduladores, antagonistas opiáceos, enquanto casomorfinas podem se comportar como agonista de receptor opióide (Muro et al. 2011).

Recentemente, outros peptídeos bioativos multifuncionais específicos foram identificados em sequências de caseína com atividade, por exemplo, imunoestimulantes, opióides e inibidores da ECA. Peptídeos de caseína também agem no corpo como componentes de regulação. Os tri-peptídeos Ile-Pro-Pro e Val-Pro-Pro, por exemplo, apresentam propriedades anti-hipertensivas, mostram efeitos de modulação do sistema imunológico, além de uma capacidade para aumentar a proliferação, diferenciação e sinalização dos osteoblastos (Muro et al. 2011).

2.6. Ação antioxidante de peptídeos

Resultados recentes de peptídeos antioxidantes são revisados para estudar as possíveis aplicações em seres humanos, por três razões principais: inibição de espécies reativas de oxigênio (ROS), atividade antioxidante e modulação do sistema imunitário, que estão envolvidas na inflamação. A inflamação é, por sua vez, envolvida na iniciação e patogênese de diversas doenças crônicas humanas. O mecanismo exato através do qual os peptídeos desempenham atividade antioxidante ainda não foi completamente elucidado, estudos apontam inibição da peroxidação de lipídios, captura de radicais livres e quelação de metais de transição. A identificação de qualquer composto que possa interferir no processo de oxidação é altamente desejável (Muro et al. 2011; Sarmadi e Ismael 2010). Além disso, o uso de antioxidantes naturais em alimentos, para evitar deterioração de produtos estocados, é de extremo interesse da indústria alimentícia e possui demanda crescente (Sakanaka et al. 2005).

Também foi provado que alguns pequenos peptídeos são capazes de estimular expressão e genes cujas proteínas protegem células contra estresse oxidativo. Outros peptídeos foram relacionados com o estímulo de enzimas antioxidantes, como a glutationa peroxidase e superoxido dismutase. Atividade antioxidante de peptídeos está mais relacionada à sua composição estrutura e hidrofobicidade. Aminoácidos com resíduos aromáticos podem doar prótons para radicais deficientes em elétrons. A sequencia e arranjo destes aminoácidos também pode influenciar na capacidade antioxidante dos peptídeos (Sarmadi e Ismael 2010).

Porém, já foi provado que hidrolisados de proteínas exercem maior atividade antioxidante do que peptídeos purificados. E que em se tratando da área de ciências nutricionais, a utilização de hidrolisados de proteínas não purificados pode ter

determinados benefícios além dos peptídeos purificados, pois a absorção de oligopeptídeos pode ser aumentada na presença de açúcares e aminoácidos (Sarmadi e Ismail, 2010).

2.7. Penicillium

Fungos filamentosos são utilizados em muitos processos industriais para produção de enzimas e metabólitos. O gênero Penicillium e suas espécies relacionadas mesofílicas, termofílicas e ácido-toletantes, são capazes de produção de proteases extracelulares, uma característica de interesse industrial. Espécies de Penicillium são altamente disseminadas naturalmente, podendo ser encontradas em solos e frutas decaídas por causa da sua natureza nutricionalmente pouco exigente o que lhes permite crescer em uma gama de condições diversificada (Graminho et al. 2013).

Segundo o MycoBank – *International Mycological Association*, este gênero pertence ao filo *Ascomycota*, da classe dos *Eurotiomycetes*, ordem *Eurotiales* e família *Trichocomaceae*. O micélio tipicamente consiste numa hifa em rede multinucleada altamente ramificada, septada e normalmente sem cor. Conidióforos multi-ramificados brotam do micélio, trazendo conidiósporos constritos individualmente. Os conidiósporos são a principal forma de dispersão do fungo e muitas vezes são de coloração esverdeada. A reprodução sexuada envolve a produção de ascósporos, começa com a fusão de um arquegônio e um anterídio, com o compartilhamento de núcleos. Os asci distribuídos irregularmente contêm oito ascósporos unicelulares cada um (Encyclopedia of Life).

Em ágar, os fungos deste gênero são caracterizados por um micélio vegetativo, formam um tapete de tecido. Os conidióforos se elevam mais ou menos perpendiculares as hifas submersas ou aéreas, com a porção basal ou haste principal (estipe) septadas, relativamente estreita, tendo uma estrutura ramificada de frutificação no vértice, o último conidióforo pode consistir de células fiálides suportadas diretamente no ápice ou em um ou mais níveis de ramos; as células fiálides são geralmente em forma de frasco com uma base inchada estreitamento gradual ou abrupto a um tubo interno onde conídios são produzidos em cadeias não ramificadas basais, ou seja, com o mais velho na parte superior, o conídios são asseptados, hialinos, pequenos, de formas variando de globosa à cilíndrica, e suave a fortemente áspera (Onions, 1987).

3. Objetivos

3.1. Objetivo Geral

Imobilização da protease produzida pelo *P. aurantiogriseum* URM 4622 em nanopartículas magnéticas para obtenção de peptídeos com propriedades antioxidantes a partir da hidrólise da caseína.

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3.2. Objetivos Específicos

- Produzir protease em cultura líquida submersa utilizando o *P. aurantiogriseum* URM4622;
- Pré-purificar a protease utilizando o fracionamento salino;
- Imobilizar a protease em nanopartículas de magnetita revestida com PANI;
- Otimizar a imobilização da protease através de um planejamento fatorial completo 2^4, no qual foi avaliada a influência do tempo de ativação com glutaraldeído, concentração da proteína ofertada, pH,e tempo de imobilização. Seguido de um planejamento central composto 2^2, no qual foi avaliada a influência do pH e do tempo de ativação com glutaraldeído;
- Caracterizar a protease livre e imobilizada quanto aos aspectos físico-químicos, tais como: pH ótimo, temperatura ótima, Km e Vmáx.
- Hidrolisar a caseína utilizando a enzima livre e imobilizada nas nanopartículas magnéticas;
- Caracterizar o perfil dos peptídeos gerados através de espectrometria de massa;
- Avaliar a atividade antioxidante do pool de peptídeos.

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5. Artigo 1

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Optimization of *Penicillium aurantiogriseum* URM4622 protease immobilization on polyaniline-coated magnetic nanoparticles

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5.1. Abstract

The proteolysis process can activate, inactivate, or even completely change protein function. Therefore proteolytic enzymes can be found in all orders of life. Although, enzymes often may be fragile and easily denatured, increasing the operational costs. Enzyme immobilization offers a well-established method for archive economical viability of the process, especially when it comes to industry. In this study, protease from *Penicillium aurantiogriseum* URM4622 was immobilized on glutaraldehyde-activated magnetic nanoparticles coated with polyaniline (PANI) and the process was optimized using a full two-level factorial design (2^4) followed by an response surface methodology (RSM) to characterize the enzyme immobilized under the most favorable conditions. The protease was covalently bound to the nanoparticles via glutaraldehyde at 25 ºC. A model representing the relationship between the residual activity and the four immobilization parameters (immobilization time, pH, offered protein amount and activation time) could determine pH and activation time as the most significant variables in the protease immobilization process. A new RSM model, based on those significant variables, with the following equation $Y=1.0+0.006x_1+0.010x_1^2-0.055x_2-0.067x_2^2-0.010x_1x_2$, predicted the optimum conditions for immobilization, as an immobilization time of 2 hours, an offered protein amount of 200 µg/mL, an immobilization pH of 6.3 and an activation time of 7.3 hours. The caracterization of the immobilizated enzyme showed a slightely diferent behavior. Though immobilization we could be able to reuse the enzime up to 5 times keeping over 50% of its original activity. The results also proves the PANI-coated ferromagnetic nanoparticles as an efficient support for fungal protease immobilization.

Keywords

Glutaraldehyde, pH, Activation time, PANI, Polyaniline, Magnetite,

5.2. Introduction

Proteolytic enzymes are characterized as capable of hydrolyses the peptide bonds in a protein into smaller proteins, polypeptides or amino acids. The proteolysis process can activate, inactivate, or even completely change protein function. Its products may be involved in a vast array of biological processes in homeostasis (Barret et al. 1998). The global enzyme market was valued at $3.1 billion in 2010 and it's expected to reach 6% billion by 2016. The proteases represent ~66% of this industrial market, and are widely used for industrial and medical purposes (Shalini and Dewan, 2012; Rogers and Overall, 2013; Singh et al. 2012). Proteases are mostly used in food and leather processing, detergent making, and in pharmaceutical industry (Djamel et al. 2009).

In all orders of life, proteases can be found. Microbial proteases possess industrial interesting characteristics, as biochemical diversity, temperature and low water activity resistance (Agrawal et al. 2004; Takami et al. 1990). The *Penicillium* genus is known by protease production, are widely disseminated in nature and can grow under a big variety of substrates, thus they are not nutritionally exigent (Graminho et al. 2013). *P. charlesii, P. griesofulvin* and *P. waksmanii*, are examples of studied protease producing species (Abbas et al. 1989; Dixit and Verma, 1995).

Enzymes often may be fragile and easily denatured, increasing the operational costs. Enzyme immobilization offers a well-established method for archive economical viability of the process, especially when it comes to industry (Verma el al. 2013; Yücel, 2012). Trough immobilization, the process costs can be reduced by reusing the immobilized derivative, reducing the quantity of enzyme required. Besides, the immobilization process can improve enzyme viability in a bigger temperature spectrum, inhibitors action, autolysis and even enable the use in some denaturation reaction media (Yücel, 2012; Tavares, 2013).

Different supports can be used in immobilization. The use of nanomaterials as support has grown in popularity lately. It is described as interesting to enzyme support, due it nano-scale dimension that allow enzyme-bound nanomaterial to behave as a free enzyme. Some favorable phenomena appears when the support size approaches nanometers, as large surface area to volume ratio that enable high enzyme loading, better mobility and diffusivity, low mass transfer resistance and thus higher activity and stability. Moreover, the use of magnetic nanoparticles adds advantage of easy separation

from reaction medium by applying a magnetic field (Verma, 2013). Ferric and ferrous coprecipitation forms inexpensive magnetic particles that are being diversely used, ranging from ferrofluid technology to controlled drug delivery, due to their nanosize effects and superparamagnetic properties (Yücel, 2012).

The use of an appropriate support in suitable immobilization conditions can provide high stability and good activity values of the derivative. Usually, immobilization techniques involves physical adsorption and covalent bonding onto a solid support. Glutaraldehyde is one of the most used support activation actor, as it allows stronger and more stable bound (Yücel, 2012). The Polyaniline matrix is also being largely studied in various enzymes immobilization applications, due to it stability (Barbosa et al. 2012).

In this study, protease from *Penicillium aurantiogriseum* URM4622 was immobilized on glutaraldehyde-activated magnetic nanoparticles coated with polyaniline and the process was optimized using a full two-level factorial design (2^4) followed by an response surface methodology (RSM) to characterize the enzyme immobilized under the most favorable conditions.

5.3. Materials and Methods

Microorganism and culture medium

The *P. aurantiogriseum* dierchx (URM4622) strain was o obtained from the University Recife Mycologia (URM), inscribed in the Commonwealth Mycological Institute (CMI). The strain was maintained at 4 °C in a malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose and 1.5% (w/v) agar.

The soybean flour medium described by Lima et al. 2011, composed of 1.65% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 1.0% (v/v) mineral solution, pH 7.21, was used for protease production. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4 \cdot 7H2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$ and 100 mg $CaCl_2 \cdot H_2O$. This medium was sterilized in an autoclave at 121 °C for 20 min.

Protease production

Inoculum spores were produced in agar plates containing a cell culture grown for 5 days at 28 °C and then suspended in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution previously sterilized at 121 °C for 20 min. After inoculation with 10^6 spores/mL, fermentations were carried out at 24 °C and 200 rpm, in 250 mL Erlenmeyer flasks containing 50 mL of the culture medium. The broth obtained at the end of fermentation (72 h) was vacuum filtered through 0.45 µm pore diameter nitrocellulose membranes to remove the mycelia. Since the target protease was an extracellular one, the filtrate was analyzed to determine the final protein concentration and protease activity and used as an enzyme source (referred to as the crude extract).

Protein determination and protease activity assay

Protein concentration was determined according to Smith et al. 1985, using bovine serum albumin as standard.

Protease activity was determined according to Leighton et al. (1976), using 1% (w/v) azocasein as substrate, in 0.1 M Tris-HCl buffer (pH 7.5). One unit of protease activity was defined as the amount of enzyme producing an increase of one unit in the optical density at 440 nm in 1 h, and was expressed in U enzyme/ml.

Protease Partial Purification by Ammonium Sulphate

The crude extract was precipitated by 60-80% ammonium sulphate saturation at 4°C. The obtained precipitates (partial purified enzyme) were dissolved in 0.05M Tris-HCl buffer (pH 7.5), dialysed over water and used for immobilization experiments.

Magnetic nanoparticles coated with PANI preparation

In 50 mL of distilled water were added 5 mL of 1.1 M $FeCl_3 .6H_2O$ and of 0.6 M $FeCl_2 .4H_2O$. Then, under magnetic agitation, 5.0 M NaOH was added dropwise up to pH 10 when black particles precipitates were produced. The temperature was increased up to 50°C for 30 min with vigorous stirring. The magnetic particles were thoroughly washed with distilled water until pH 7. The material was dried up at 50 ºC, macerated and kept at 25°C.

The magnetic nanoparticles (0.5 g) were added to 50 mL of 0.1 M $KMnO_4$ solution at 25°C for 1 h to do an oxidative polymerization of aniline. After washed up with distilled water, the magnetic-$KMnO_4$ nanoparticles were immersed into 50 mL of 0.5 M aniline solution diluted in 1.0 M HNO_3. Polymerization occurred at 4°C for 1 h and, after that, the magnetic nanoparticles coated with polyaniline (mPANI) were successively washed with distilled water, 0.1 M citric acid and rewashed with distilled water, and finally the material was dried up at 50 °C and kept at 25°C.

Protease immobilization

The ferromagnetic nanoparticles were activated with a 2% (v/v) glutaraldehyde solution during different times (1-12 hours). After washed 10 times with distilled water and 3 times with 0,2 M Tris-HCl buffer (pH 5,5 – 9), the activated magnetic nanoparticles were mixed with the partially purified enzyme solution (100-300 µg/mL) for immobilization (1-3 hours). Then, the nanoparticles were washed over 30 min with 0,1M glycine solution, and other 2 times with distilled water. The washed up were used to measure non-binding proteins.

The residual activity was determined as the ratio of the activity in the partial purified protease to that present in immobilized protease, and expressed as percentage.

Screening of the most significant immobilization parameters

Protease immobilization on magnetic nanoparticles parameters screening was carried out with a 2^4 full factorial design (FFD). Nanoparticles activation time, offered protein amount, pH and immobilization time were selected as the four independent variables (factors), whereas the response was residual activity (RA), expressed by immobilized enzyme activity/free enzyme activity multiplied by 100.

For statistical analysis, the actual values of the independent variables (X_i) were coded according to the equation:

$$x_i = \frac{X_i - X_o}{\Delta X_i} \qquad (1)$$

where x_i represents the corresponding coded values, X_0 the actual values at the central point, and ΔX_i the step change value. The experimental design was composed of 16 runs and 4 repetitions at the central point, needed to calculate the pure error (Table 1). The goodness of fit was evaluated by the coefficient of determination (R^2) and the analysis

of variance (ANOVA); the first-order equation was determined by Fischer's test. The experimental and predicted values were compared and validated with *Statistica 8.0 (*Burns et al. 2006).

Optimization of protease immobilization using RSM

To optimize the protease immobilization, the offered protein amount and immobilization time were fixed at the best value found in the screening design (200 µg/mL and 2 h, respectively), whereas the levels of the other two factors were varied according to a 2^2 central composite design (CCD).

The levels of this second design were also chosen on the basis of the effect estimates obtained in the screening design. The CCD consists of a full 2^2 design augmented with four star (axial) points and a central point repeated six times (to provide an estimate, with five degrees of freedom, of the experimental error of a response). Since there are five levels for each factor, a full second-order model can be fitted to the new response values (Eq 2):

$$\hat{y}_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \qquad (2)$$

where represents the predicted value of the response variables, b_0 is the intercept coefficient, b_i are the linear coefficients, b_{ii} are the quadratic coefficients and b_{ij} are the interaction ones.

The CCD levels are given in Table 2. For simplicity, the star points were rounded off with respect to the theoretical values $\alpha=\pm\sqrt{2}$. This is a minor problem, which only makes the design slightly non-rotatable (Chen et al. 2012). The goodness of fit of the models was evaluated by the determination coefficient (R^2), the analysis of variance (ANOVA) and the Fischer's *t* test. Experimental and predicted values were compared by the Statistic 8.0 statistical program package.

The observed values of the response variable residual activity (RA) obtained by experiments in relation to the independent variable activation time were not optimized by the CCD model proposed. However, the value predicted by the model was calculated using the mathematical equation (Eq. 1) obtained for both independent variables, pH (optimized) and activation time (not optimized). So to actually confirm that the optimal conditions obtained by combination the pH optimized (6.3) and the activation time not optimized, but calculated by the equation (7.3) presented the highest residual activity

value, this assay was tested in triplicate (namely run A1) . Still, we compared the value of RA in the former test with two more tests of experimental design that presented high values of RA: 11 (C) and 5 runs, namely A2 and A3, respectively, replacing pH 6.5 for both by that optimized (6.3) and keeping the values of activation time of 4 and 8 h, respectively. All tests (namely A2 and A3) were done in triplicate.

pH and temperature characterization and reuse

To evaluate the effect of pH on free and immobilized enzyme activity, the pH of the reaction mixture containing 1.0% (w/v) of azocasein was varied over the range 3.0 - 11.0. The buffers used were 0.05 M citrate (pH 3 - 6), 0.05 M Tris-HCl (pH 7 - 9), and 0.05 M carbonate-bicarbonate (pH 10 - 11). An analogous study was done for temperature in the same azocasein-buffer solution. To determine the optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 20 to 65ºC). The immobilized enzyme was repeatedly used, and the first activity was compared with the subsequent ones.

5.4. Results and Discussion

Screening of the most significant immobilization parameters

To identify the most significant parameters for *P. aurantiogriseum* protease immobilization on magnetic nanoparticles, the influence of nanoparticles activation time, offered protein amount, pH and immobilization time was evaluated according to the design defined by the levels given in Table 1. Experimental runs were carried out at all possible combinations of these levels.

The matrix of the design's experimental results is presented in Table 3. Evaluating the twenty runs shown in Table 3, the best results for residual activity (93%) were obtained in run #17, but the run #18, #19 and 20#, which were performed in the same conditions, presented a lower residual activity (84-86%). Although, the run #3 presented residual activity almost as high as run #17 (92 %), indicating that its parameters must be considered (immobilization time of 1 h, activation time at the greater level of 2 h, 100 μg/mL of protein amount and pH 7). These parameters agree with our RSM results.

A full factorial model was fitted to the residual activity data. This model included four main effects, six two-factor, four three-factor and one four-factor interactions. The statistically significant estimates of the effects (at the 95% confidence level) are listed in Table 4. A simplified model based only on the effects of Table 4 showed no lack-of-fit, and had a coefficient of determination (R2) of 0.0018. The values of the significant effects indicate that, on average, higher protease immobilization were obtained when factors 2 (activation time) was selected at their highest level, and factor 3 (pH) at the lowest one. It should be noted that there was a significant interaction involving factors 3 (pH) and 4 (offered protein amount), even though the main effect of factor 4 (offered protein amount) was not significant. The negative interaction effect means that a decrease in pH with a simultaneous decrease in offered protein amount led to an increase in protease immobilization on magnetic nanoparticles.

The enzyme immobilization using glutaraldehyde occurs by covalent binding. The glutaraldehyde works as a spacer arm linking its aldehyde groups with the enzymes' amine groups. If the functional groups of the spacer arm are not occupied by an amine group it can randomly bind with another amine groups and inactivate the enzymes (Chae et al. 1998). The activation time of the particles by glutaraldehyde should be optimized, so there is enough room for maximum enzyme loading and lower inactivation rate, generating greater activity.

The pH of the buffer solution is a significant immobilization factor due to its influence on the conformation of the free enzyme and the amino group on the carriers (Peng, et al. 2012). In studies involving optimization of enzyme immobilization, pH currently is found as a highly significant factor (Kuo et al. 2012; Yücel, 2012; Li et al. 2013), to optimize the immobilization of proteases, assessed observed that the influence of pH on immobilization was highly significant factor and found an optimum pH near 7.5.

Optimization of Immobilization

According to the factorial model, a longer activation caused a higher rate of immobilization. Many research groups use higher than 12h activation times to activate nanomaterials using glutaraldehyde, indicating large activation time methods as more efficient (Zhu and Sun, 2012; López-Gallego et al. 2005; Yao et al. 2011).

The results of residual activity obtained from the *P. aurantiogriseum* URM4622 protease immobilization according to the 2^2 CCD used in this study are listed in Table 5. Overall, these values are much superior to the ones in Table 3, previously stated, and the highest RA was 105,72% (with an activation time of 4h and pH of 6,5). When full quadratic models are fitted to the data in Table 5, the following equations are obtained (Eq. 3):

$$Y = 1.0 + 0.006\, x_1 + 0.010\, x_1^2 - \mathbf{0.055}\, x_2 - \mathbf{0.067}\, x_2^2 - 0.010\, x_1 x_2 \qquad (3)$$

Were x_1 stands for activation time and x_2 for pH

Independent variables influence on the response was different, in that, according to Eq. (3), only linear and quadratic effects of pH are statistically significant at 95% confidence level (in bold). Indeed, the algebraic signs of both regression coefficients were negative, thereby indicating that any decrease in pH value resulted in corresponding residual activity increase. On the other hand, no main effect (linear or/and quadratic) of variable activation time was significant. In geometric terms, the response surface (Figure 1) shows a shape indicating that the variable pH optimal value is close to the values studied at central point runs (central region of the surface).

According regression analysis by Eq. (3), the maximum pH predicted value can be provided (pH=6.3). This predicted value is only 3.1 % lower than the experimental ones (6.5), thus demonstrating the validity of the model employed. The results of Kuo et al. 2012, immobilizing lipase on chitosan-coated Fe3O4 nanoparticles corroborate with the results of our work, extolling the significant pH influence on immobilization process and establishing a pH of 6,37 as the optimum condition. As the results presented by Yücel, Y. 2012, that emphasize the importance of the pH of the immobilization buffer.

When using glutaradehyde preactivated supports, the primary amino groups of the enzyme would react with the aldehyde groups that have been introduced on the support. The usual pH to perform the immobilization is near the neutral, given the low stability of glutaraldehyde activated supports at high pH values. Alkaline pH values interfere with ε-amino of Lys groups, lowering the reactivity and the intensity of the multipoint covalent attachment (López-Gallego et al. 2005).

Despite the independent variable activation time was not optimized by the model, the maximum predicted value (7.3 h) could be calculated by the Eq. (3) and an

additional experiments (A1, A2, and A3) were performed in triplicate using the combinations of value optimized for pH (6.3) with the activation time value calculated by the equation and the corresponding in runs 11 (C) and 5 of CCD. According to the results of those 3 additional experiments described in Table 6, the real optimal conditions were observed in run A1 composed of pH optimized value by the model and activation time calculated in the equation confirmed through the highest of residual activity. This means that despite not obtain the optimized model for the independent variable, activation time, it was possible to calculate by mathematical equation the predicted value by the model and test in triplicate, in the laboratory, joint with the pH optimized value so to confirm that this combination corresponds to the optimal conditions to obtain the highest protease residual activity.

The statistical significance of second-order model equations was confirmed by the F-test analysis of variance (ANOVA) and multiple regression (Table 7). In fact, the computed F-values (10.94 for x_2) was much greater than the tabulated ones [$F(5,8) = 3.69$]. Whereas the lack of fit was insignificant. In addition, the high values of the determination coefficients ($R2 = 0.87$) indicate that the models explained 87% of the variability data, and the regression was statistically significant ($p < 0.002$) at 95% confidence level.

Although it is relatively simple to activate supports using glutaraldehyde, the activation conditions can promote an increase in the amount of glutaraldehyde introduced in the support, suggesting a self-polymerization. Controlling the activation conditions, it is possible to activate the aminogroups in the support with fell glutaraldehyde molecules (Betancor et al. 2006). Besides, the activation happens over a layer of cationic groups (the amino groups), which can confer some ionic exchanger features to the support. Actually, on highly activated glutaraldehyde supports at low ionic strength a physical adsorption could be possible before the covalent reaction. Furthermore, if glutaraldehyde forms dimmers on support, a hydrophobic surface is formed over it, what may affect the immobilization of enzymes that do not have a high affinity for hydrophobic surfaces (Barbosa et al. 2012). Finding the optimum activation time can contribute to the maximum use of glutaraldehyde aminogroups activation to reach the best immobilization conditions. Considering the statistical data regarding the residual activity obtained, the 7,3 h activation time seems to reach the optimum glutaraldehyde aminogroups activation.

pH and temperature characterization and reuse

To find the optimal temperature for immobilized and free enzyme action, the protease activity was measured at various temperatures ranging between 20 and 65°C for 1 h. Figure 2A shows that both free and immobilized enzymes suffers a similar influence of temperature, with a slightly wane of the immobilized enzyme. It was shown to increase with temperature up to 37ºC, then decreased continuously. More than 85% (free enzyme) and 75% (immobilized enzyme) of the activity was found in the temperature range 20 - 45ºC.

Figure 2B shows the pH-dependence of free and immobilized enzyme activity on azocasein. The free and immobilized enzyme were very active at the pH range of 8-11 and showed the highest activity at pH 9.0 (free enzyme) and pH 10.0 (immobilized enzyme). These results demonstrated that with the immobilization process the enzyme shows highest activities values under alkaline conditions.

To understand the reusability of the immobilized nanoparticles, the protease activity was determined after repeated use (Figure 3). The protease was immobilized onto magnetic nanoparticles under optimum conditions. After 6 repeated uses the immobilized protease retained 51% of the original activity. The gradual decrease in enzyme activity could be partially attributed to the loss of particles during the washes. Although a slight decrease could be attributed to denaturation and/or leakage of immobilized protease from nanoparticles after repeated washing/dilutions (Kuo et al. 2012).

5.5. Conclusions

The PANI-coated ferromagnetic nanoparticles was successfully used for covalent immobilization of *Penicillium aurantiogriseum* protease. The protease was bound to the nanoparticles via glutaraldehyde at room temperature. A model representing the relationship between the residual activity and the four immobilization parameters (immobilization time, pH, offered protein amount and activation time) could determine the most significant variables that affects the immobilization of the protease, as pH and activation time. A new model based on those significant variables predicted the optimum conditions for immobilization, as an immobilization time of 2 hours, an

offered protein amount of 200 µg/mL, an immobilization pH of 6.3 and an activation time of 7.3 hours. Though immobilization we could be able to reuse the enzime for 5 times keeping over 50% of its original activity. The results also proves the PANI-coated ferromagnetic nanoparticles as an efficient support for fungal protease immobilization.

5.6. Acknowledgments

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5.8. List of Tables

Table 1. Real and Coded Factor Levels Used in the 2^4 Design to Investigate the *P. aurantiogriseum* URM 4622 protease immobilization on magnetic nanoparticles

Factors	Lower (-1)	Central (0)	Higher (+1)
X_1 - Immobilization time (hours)	1	2	3
X_2 - Activation time (hours)	1	1,5	2
X_3 – pH	7	8	9
X_4 - Offered protein amount (µg/mL)	100	200	300

Table 2. Real and Coded Levels of the Factors Used in the 2^2 Central Composite Design

Factors	Lower (-1)	Central (0)	Higher (+1)	Axial (-α)	Axial (+α)
X_1 - Activation time (hours)	6	8	10	4	12
X_2 – pH	6	6,5	7	5,5	7,5

Table 3. Matrix of the 2^4 Full Factorial Design Combinations (actual levels) and the Observed Response Values

Run	X_1 (hours)	X_2 (hours)	X_3	X_4 (μg/mL)	Residual Activity (%)
1	1	1	7	100	58
2	3	1	7	100	74
3	**1**	**2**	**7**	**100**	**92**
4	3	2	7	100	90
5	1	1	9	100	48
6	3	1	9	100	63
7	1	2	9	100	87
8	3	2	9	100	89
9	1	1	7	300	72
10	3	1	7	300	80
11	1	2	7	300	87
12	3	2	7	300	76
13	1	1	9	300	51
14	3	1	9	300	57
15	1	2	9	300	68
16	3	2	9	300	77
17	**2**	**1.5**	**8**	**200**	**93**
18	2	1.5	8	200	86
19	2	1.5	8	200	84
20	2	1.5	8	200	84

*the best results are shown in boldface.

Table 4. Statistically significant effect estimates ($p<0.05$) determined for the response given in Table 2. Single digits indicate main effects; digit combinations stand for two-factor interaction effects. Notation as in Table 1.

Factors	Effects on residual activity
X_1 - Immobilization time (hours)	0.53
X_2 - Activation time (hours)	**0.20**
X_3 – pH	**-0.11**
X_4 - Offered protein amount (µg/mL)	-0.04
1*2	-0.06
1*3	0.02
1*4	-0.02
2*3	0.05
2*4	-0.08
3*4	**-0.04**

*the significant effects are shown in boldface.

Table 5. Level Combinations of the Two Factors (activation time and pH) Used in the 2^2 Central Composite Design and Results of the Residual Activity

Run	Activation Time	pH	Residual Activity (%)
1	6	6,0	94,2953
2	6	7,0	86,1063
3	10	6,0	103,0201
4	10	7,0	90,7376
5	**4**	**6,5**	**105,7252**
6	12	6,5	100
7	8	5,5	95,7627
8	8	7,5	78,9384
9 (C)	8	6,5	95,8015
10 (C)	8	6,5	99,8092
11 (C)	8	6,5	102,2901
12 (C)	8	6,5	100,5725
13 (C)	8	6,5	100,9542
14 (C)	8	6,5	100,5725

*the best results are shown in boldface.

Table 6. Analysis of variance applied to the regression models calculated for residual activity variable response, according to the 2^2-Central Composite design

Residual activity	Degrees of Freedom	Sum of Squares	Mean of Square	F_{calc}	F_{tab}	R^2	$R^2_{adjusted}$	p_{value}
Regression	5	0.060	0.012	10.94	3.69	0.87	0.79	0.002
Residues	8	0.009	0.001					
Total	13	0.069	0.013					

Table 7. Results Obtained at the Level Combination for which a Maximum is Predicted by Eq. 3.

Tests	A1	A2	A3
	Activation time: 7,3 h	Activation time: 4 h	Activation time: 8 h
Residual activity	**110,5005 %**	95,8274 %	105,0535 %
Standard deviation	**0,04506**	0,05001	0,02665

*the best results are shown in boldface.

5.9. List of Figures

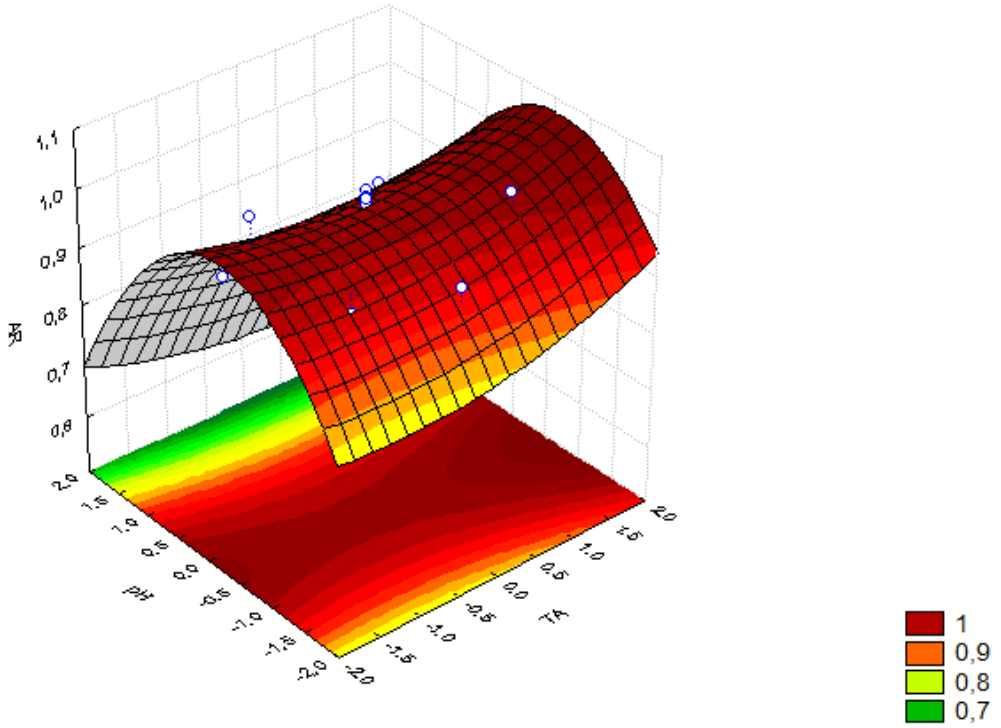


Fig 1. Three-dimensional response surface plot of the residual activity (AR) showing the interactive effects of pH and activation time (TA).

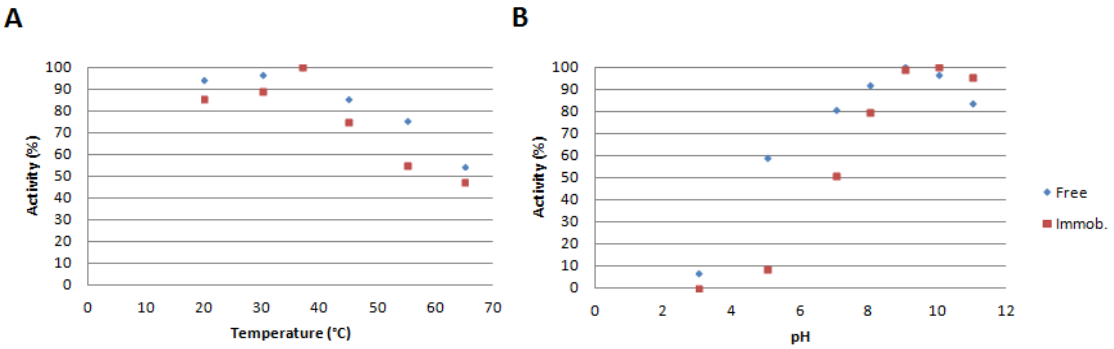


Fig 2. Effects of (A) temperature and (B) pH on the relative activity of free and immobilized protease from *P. aurantiogriseum* on magnetic nanoparticles.

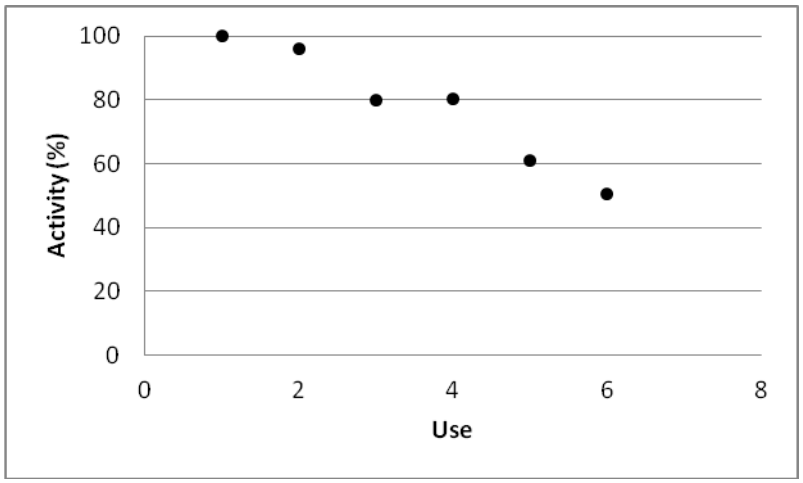
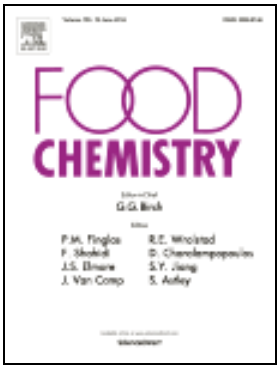


Fig 3. Reusability of immobilized protease from *P. aurantiogriseum* on azocasein hydrolysis.

Artigo 2



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MS Analysis of antioxidant bioactive peptides from casein produced by free and immobilized *Penicillium aurantiogriseum* protease on magnetic nanoparticles

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6.1. Abstract

The aim of this study was to use a protease produced by *Penicillium aurantiogriseum*, free and immobilized on polyaniline-coated magnetic nanoparticles, for bovin casein hydrolysis. Also it was to evaluate the hydrolysates antioxidant properties and Peptides Mass Fingerprints (PMFs) by mass spectrometer. The peptides present in higher amount were, then, sequenced. The 60-80% saline fraction of crude extract was used for immobilization. The casein hydrolysates were characterized by SDS-PAGE and Mass Spectrometer. Thereafter, were used in ABTS radical scavenging activity and Hydrogen peroxide scavenging activity assays. Although some differences in peptides mass peaks were found between the free and immobilized enzymes casein hydrolisates, they both presented similar PMF and those peptides could be sequenced: D-V-P-S-E-R-Y-L-G-Y; G-L-P-Q-E-V-L-N-E-N-L-L-R-F; L-S-L-S-Q-S-K-V, Y-Q-E-P-V-L-G-P-V-R-G-P-F and L-L-Y-Q-E-P-V-L-G-P-V-R. The hydrolysates presented an excellent ROS scavenging activities, which validates the protease capacity of developing casein derived natural ingredients with potential for nutraceutical and functional foods.

6.1. Introduction

Reactive oxygen species (ROS), normally produced in living organisms during metabolism, is a important component of our defense system, helping the host against microbial infection, but when overexpressed and accumulated beyond the cell antioxidant capacity, ROS can affect energy production, survival, cell growth and numerous signaling pathways (Duan et al. 2014; Kim et al. 2013; Guo et al. 2014). ROS include a variety of free radicals, the most common are superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), peroxyl radical ($RO^{\bullet-}$), and some non-free radical species such as hydrogen peroxide (H_2O_2) (Ko et al. 2013). Under redox homeostasis, the excess ROS can be scavenged by the cellular antioxidant system, that involves non-enzymatic antioxidants (as glutathione and vitamin C) and antioxidant enzymes (as catalase, glutathione reductase, glutathione peroxidase and superoxide dismutase). The excessive ROS are involved in different disorders, such as diabetes, chronic inflammation, hypertension, atherosclerotic cardiovascular disease, aging and cancer (Duan et al. 2014; Guo et al. 2014).

Moreover, in food system, oxidative products as well as free radicals can be produced by lipids oxidation, especially in polyunsaturated fatty acids rich foods. It ends up compromising the taste of food, decreasing the l shelf-life of the product (Intarasirisawat et al. 2013). A variety of synthetic antioxidants are commonly used in the food industry due to its stronger antioxidant activity, such as butylated hydroxylanisole and propyl gallate. However have been proven to have potential health risks, so its use should be kept under strict control. Since natural antioxidants, such as tocopherols, can have higher production costs and lower efficiency, together with the fact of the growing concern of the consumed food additives, there is a growing demand for the identification of new natural sources of food antioxidants. This scenario, along with the well-known safety of natural peptide sequences, has led to an increasing interest in food-derived antioxidant peptides (Girgih et al. 2013).

Bioactive peptides are found within the precursor proteins and may be released during food processing or digestion. After digestion they can be absorbed by the intestines and manifest their activity in several other body tissues (Puchalska et al. 2014). Several bioactivities have been associated to the action of bioactive peptides from foods, especially those derived from bovine milk. The functionality of these

bioactive peptides has been associated with both small peptides, such as dipeptides with angiotensin-converting enzyme (ACE) inhibitory activity, as larger peptides with immunomodulatory activity. Cytomodulatory, immunomodulatory, mineral binding, opioid, antimicrobial, blood pressure reducing and antioxidant, are some of the known health promoting activities of bioactive peptides from food, with some of these exhibiting multifunctionality (Mooney, et al, 2013). Different parameters can affect the bioactivity of these compounds, such as source of protein, degree of hydrolysis, peptide structure, amino acid composition and type of protease used. Among these, antioxidant peptides have received more attention due to its health promoting capacity in several pathological scenarios (Memarpoor-Yazdi et al. 2013).

Although, it has been proven that hydrolysate exerts higher antioxidant activity than purified peptides. When it comes to the area of nutritional sciences application of non-purified protein hydrolysate can have certain benefits over those of purified peptides since the absorption of oligopeptides can be increased in the presence of sugar and amino acids (Sarmadi and Ismail, 2010).

The casein peptides have the potential to exert numerous health improving effects. Examples of bioactive peptides derived from casein include casein phosphopeptides, can play a role in the transport and absorption of certain minerals, among other properties, glycomacropeptides that bind toxins, which have properties to behave as casoxins, immunomodulators, opioid antagonists, while casomorphins can behave as agonist opioid receptor (Muro Urista et al. 2011). Caseins are available in large amounts at a high degree of purity and at low price in market which, make them attractive in the search for bioactive peptides (Corrêa et al. 2011). Several plants, animals, and microbial proteases are employed for peptides production. Microorganisms serve as the preferred source of proteases because of their well-known methods of rapid growth, the limited cultivation space required and the easy genetic manipulation to generate new enzymes with improved properties (Abidi et al. 2013).

The Penicillium genus and its related mesophilic, thermophilic and acid-toletants species, are capable of producing extracellular proteases and due to its nutritionally undemanding nature they can grow in a range of diverse conditions, characteristics of industrial interest (Graminho et al. 2013). The *Penicillium aurantiogriseum* was described as a large amount producer of extracellular collagenolytic protease (Lima et al. 2011).

In the usage of free enzymes some disadvantages can occur, such as difficult enzyme recovery after use and they tend to be more sensitive to denaturing agents, for example. On the other hand, immobilized enzymes proved to be more practical and advantageous for catalysis. Immobilized enzymes can be easily separated from the products of hydrolysis, allowing continuous processes and reuse of the enzyme. The different methods of immobilization may influence the biocatalytic properties of enzymes, it is important to find a strategy to improve the immobilization of the enzyme properties such as activity, reduced inhibition, cost, among others (Tavares et al. 2013). The immobilization on magnetic particles is becoming preferable to other supports for industrial use due to easier recovery of the immobilized enzyme (Neri et al. 2008).

The objectives of the present study were to use the protease produced by *Penicillium aurantiogriseum*, free and immobilized on magnetic nanoparticles, to hydrolyses commercial bovine casein to evaluate its antioxidant properties and to do a peptide characterization using time of flight mass spectrometer, aiming the potential use of this enzyme in the production of antioxidant functional food ingredients derived from casein.

6.2. Material and Methods

Microorganism and culture medium

The filamentous fungi *Penicillium aurantiogriseum* dierchx (URM4622) strain was obtained from the University Recife Mycologia (URM), inscribed in the Commonwealth Mycological Institute (CMI). The maintenance medium used was malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose and 1.5% (w/v) agar, and the strain was kept under 4 °C until use.

The production medium used was the soybean flour medium described by Lima et al. 2011, composed of 1.65% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 1.0% (v/v) mineral solution, pH 7.2. The mineral solution content was 100 mg $FeSO_4 \cdot 7H2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$ and 100 mg $CaCl_2 \cdot H_2O$, per 100 mL of distilled water. Both mediums were sterilized in an autoclave at 121 °C for 20 min.

Protease production

The *P. aurantiogriseum* was incubated in the maintenance medium during 120 h at 28 °C to spores production. The inoculum was prepared suspending the spores in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution previously sterilized at 121 °C for 20 min, with 10^6 spores/mL, counted in Neubauer chamber. After inoculation, production were set up in 250 mL Erlenmeyer flasks containing 50 mL of the production medium and the fermentation occurred at 24 °C and 200 rpm over 72 h. At the end of fermentation, the broth obtained was vacuum filtered through 0.45 µm pore diameter nitrocellulose membranes to remove the mycelia. The filtrate was used as an enzyme source (referred to as the crude extract), as the target protease is an extracellular one.

The crude extract was precipitated by 60-80% ammonium sulphate saturation at 4°C. The obtained precipitates (partial purified enzyme) were dissolved in 0.05M Tris-HCl buffer (pH 9), dialysed over water and used on the hydrolisys and immobilization process.

Protein determination and protease activity assay

Protein concentration was determined according to Smith et al. 1985, using bovine serum albumin as standard. Protease activity was determined according to Leighton et al. (1976), using 1% (w/v) azocasein as substrate, in 0.1 M Tris-HCl buffer (pH 9). One unit of protease activity was defined as the amount of enzyme producing an increase of one unit in the optical density at 440 nm in 1 h, and was expressed in U enzyme/ml.

Protease immobilization

To produce the magnetite core nanoparticles, 5 mL of 1.1 M $FeCl_3 .6H_2O$ and of 0.6 M $FeCl_2 .4H_2O$ was added in 50 mL of distilled, under vigorous stirring, 5.0 M NaOH was added dropwise up to pH 10 and the temperature was increased up to 50°C for 30 min. The particles were thoroughly washed with distilled water until pH 7 was reached. After dried up (at 50 ºC) and macerated. Then 0.5 g of nanoparticles were added to 50 mL of 0.1 M $KMnO_4$ solution at 25°C for 1 h. After washed up, the magnetic-$KMnO_4$ nanoparticles were immersed into 50 mL of 0.5 M aniline solution diluted in 1.0 M HNO_3. Polymerization occurred at 4°C for 1 h. After that, the PANI

coated nanoparticles were successively washed with distilled water, 0.1 M citric acid and rewashed with distilled water, and finally dried up at 50 °C and kept at 25°C.

For immobilization, the magnetic nanoparticles coated with polyaniline (PANI), was activated with a 2% (v/v) glutaraldehyde solution for 7.3 h. After washed 10 times with distilled water and 3 times with 0,2 M Tris-HCl buffer pH 6.3, the activated magnetic nanoparticles were mixed with the partially purified enzyme solution, at a concentration of 200 µg/mL for 2h, to immobilize. Then, the nanoparticles were washed over 30 min with 0.1M glycine solution, under gentil rotation, and other 2 times with distilled water. The washed up were used to measure non-binding proteins and estimate the immobilized amount.

Preparation of casein hydrolysates

Commercial casein from bovine milk, Sigma-Aldrich, was dissolved into 0.05M Tris-HCl buffer (pH 9) in a concentration of 1% (w/v). 100 µL of protease, in a concentration of 1.5 mg/mL, was added in a 20 mL of casein solution, in a 50 mL Erlenmeyer flask in water bath under 37 ºC. The hydrolisys occurred for 2.3h, 2mL aliquots were collected at 5, 15, 25, 35, 45, 65, 95, 125 and 155 minutes. A control was aliquotted just after adding the enzyme. All aliquots were heated at 80 ºC for 20 min, to stop the hydrolysis. Finally, the hydrolysates were lyophilized and stored at -18 ºC for further use. The same process was done using approximately the same amount of immobilized protease.

SDS-PAGE Analysis

All of the hydrolysis aliquots were used in the SDS-PAGE, to show the hydrolysis pattern. The SDS-PAGE was carried out as described by Laemmli, 1970, using a separation faze of 15% acrilamide/bis-acrilamide. Samples were mixed with Laemmli buffer without using β-mercaptoethanol. The molecular weight was estimated using a Low Molecular Weight Calibration Kit (Amersham), containing: Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30,1 kDa), Trypsin inhibitor (20,1 kDa) and α-Lactalbumin (14,4 kDa). The protein visualization was done by the Coomassie blue staining method. Gels were stained overnight with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (45:10:45) and destained in the same solution without dye.

Peptides mass fingerprint (PMF)

The PMF was obtained by mass spectrometry, using a MALDI-TOF (*Matrix Assisted Laser Desorption Ionization - Time of Flight*) mass spectrometer Autoflex III (Bruker Daltonics, Billerica, MA, USA), equipped with a Nd:YAG laser (355 nm). The hydrolysates were mixed with alpha-cyano-4-hydroxycinnamic Acid – HCCA (10 mg/mL) on 50% acetonitrile and 0.3% trifluoroacetic acid. The mass spectra were obtained in positive reflective mode with acceleration voltage of 19 kV and laser at 100 Hz. The detection range was set to m/z 700 - 4480. The fragmentation of parental ions was done in LIFT mode and the external calibration with standard mix of peptides (Bruker Daltonics). The data was analyzed using FlexControl 3.0 (Bruker Daltonics), and the spectra were processed using the FlexAnalysis 3.0 (Bruker Daltonics). The sequences found were compared with the sequences of NCBI protein data bank (www.ncbi.nlm.nih.gov).

ABTS radical scavenging activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was measured using the method described by (Re et al. 1999). Using 7 mM of ABTS solution and 140 mM of potassium persulphate solution. The working solution was prepared by mixing 5 mL of ABTS solution with 88 µL of potassium persulphate solution and allowing them to react for 10 h at room temperature in dark. The solution was then diluted by mixing 1mL of ABTS working solution with approximately 50 mL of ethanol in order to obtain an absorbance of 0.7 units at 734 nm using a Ultrospec® 3000 spectrophotometer (Amersham). Fresh ABTS solution was prepared daily. Sample (30 µL) with different concentrations (1%, 0,5% and 0,1% w/v) was mixed with 3 mL of ABTS working solution and the mixture was left for 5 min at 25 ºC in dark, the same ABTS working solution with distilled water was used as blank sample and the non-hydrolyzed casein was used as reference sample. The absorbance was then measured at 734 nm. A standard curve of Trolox ranging from 100 to 1500 mM was prepared.

Hydrogen peroxide scavenging activity

The Hydrogen peroxide scavenging activity was assayed according to the method of Kittiphattanabawon et al. 2012. 850 µL from sample was mixed with 150 µL of 43 mM hydrogen peroxide in 0.1 M phosphate buffer, pH 7.4. After 5 min of reaction at 25 ºC, the absorbance was recorded at 230 nm. In the blank sample we used the same phosphate buffer without hydrogen peroxide and also used non-hydrolyzed casein as reference sample. Trolox (0–10 mM) was used as standard. The activity was expressed as µmol Trolox equivalents (TE)/mg sample.

6.3. Results and Discussion

SDS-PAGE

The SDS-PAGE gels of casein hydrolysis over time is presented in Figure 1. SDS-PAGE gel showed the pattern of hydrolysis of bovine casein by *P. aurantiogriseum* protease over the time. After the molecular marker, the first column shows the not hydrolyzed bovine casein (column #1), showing different bands for each casein protein, as α, β and κ (19 – 25 kDa). After the first five minutes of hydrolysis (column #2) the casein bands almost completely disappears. Just in the fifth column (45 minutes of hydrolysis) the casein bands are no longer visualized and just a group of bands between 14-20 kDa can be seen. After the fifth column, no visible difference can be seen to the next columns. No perceptive different can be observed when compared the free enzyme and immobilized gels.

Peptides mass fingerprint (PMF)

The PMF of both immobilized (A) and free (B) protease are presented in figure Y. Apparently the two PMFs shows the same peaks. All of the grater intensity peaks appear in both of the PMFs. Although, the free enzyme hydrolysate PMF presented 4 intense peaks (Intensity ranging from 225 to 667) that doesn't appear in the immobilized enzyme hydrolysate one (2014,2; 2040,3; 2129,4; 2150,3 m/z). Although different results in peptides mass between free and immobilized enzymes is seen in other works (Nicoli et al. 2008), in this work the free and immobilized enzyme hydrolysate peptides mass presented almost the same pattern.

Sequenced Peptides

For some peptides present in greater amount, it was possible to have their amino acid sequence elucidated. Four peptides were successfully sequenced, α-S1-casein fragments: D-V-P-S-E-R-Y-L-G-Y (f85-94) and G-L-P-Q-E-V-L-N-E-N-L-L-R-F (f10-23), β-casein fragments Y-Q-E-P-V-L-G-P-V-R-G-P-F (f193-205) and L-L-Y-Q-E-P-V-L-G-P-V-R (f191-202), presented in Figure 2. The amino acid proline can be seen in 4 of the 5 sequenced peptides. Proline and hydroxyproline containing peptides are usually resistant to degradation by digestive enzymes (Savoie et al. 2005), what is interesting fact for food peptides, as it promotes digestion resistance and facilitates absorption. Moreover peptides containing P, A, L, F and Y amino acids in their sequence can contribute to strong scavenging effect of ROS and the L-G and G-P sequence seems to play an important role in radical scavenging (Byun et al. 2009), both those amino acid residues as L-G and G-P sequences can be evidenced in the sequenced peptides, suggesting that its sequence provides a ROS scavenging capacity. Besides, the presence of Y residue, as in f193-205 sequence, at peptide C-terminus end was associated with strong hydrogen peroxide scavenging activity (Guo et al. 2009), also suggesting that this peptide sequence can promote a ROS scavenging capacity.

The antioxidative properties of the peptides are more related to their composition, structure, and hydrophobicity. As example of composition influence, amino acids with aromatic residues (Y, H, W, F) can donate protons to electron deficient radicals, improving the radical-scavenging properties of peptides. Peptide linkage and/or specific structural features of the peptides have been claimed to influence antioxidant capacity. Amino acid correct positioning in peptide sequence plays an important role in antioxidant activity of peptides. However, peptide bond or its structural conformation can reduce the antioxidant activity of the constituent amino acids. Therefore, apparently peptide conformation shows both synergistic and antagonistic effects, concerning to antioxidant activity. Moreover, the configuration of peptides can also affect antioxidant activity. The correct positioning of imidazole group is a influencing factor in antioxidant activity (Sarmadi et al. 2010).

The **Y-Q-E-P-V-L-G-P-V-R-G-P-F**-P-I-I-V (f193-209), also known as casedecin-17, that contains one of the peptides found in our work (f193-205), have been showed to have strong antioxidant capacity (Eisele et al. 2013) and also Angiotensin-

converting enzyme inhibitory activity (Gómez-Ruiz et al. 2002). Amato, A. et al. 2003, indicates the L-L-Y-Q-E-P-V-L-G-P-V-R (f191-202) fragment as a highly conserved stretch in mammalian β-caseins (Amato et al. 2003). The β-casein fragments found seems to be in the same preferential cleavage sites as found by Lozo et al. 2011 using *Lactobacillus paracasei paracasei* for casein hydrolysis, in the C-terminal portion, what follows the same process described by Schmelzer et al. 2007, that says that the C-terminal portion is the first to be hydrolysed. Besides, the G-L-P-Q-E-V-L-N-E-N-L-L-R-F (f10-23) αS1-casein fragment, was detected in casein hydrolysates by *Bifidobacterium longum* (Chang et al. 2013) and in low-salt Cheddar cheese (Møller et al. 2013).

Antioxidative activity

Casein hydrolysates were reported to have higher concentration of histidine, lysine, proline and tyrosine, and all these amino acids have been previously found to act as free radical scavengers (Pihlanto, 2006). Scavenging activities of bovine casein hydrolysates were determined using two radicals: ABTS and H_2O_2, results presented in Table 1. The radical ABTS is reduced with concomitant conversion to a colourless product in the presence of antioxidants with hydrogen-donating or chain-breaking properties. The hydrolysis of bovine casein with *P. aurantiogriseum* protease tended to enhance the antioxidant activity of hydrolysates measured by the ABTS assay. The antioxidant activity of the milk proteins hydrolysates seems to be inherent to the characteristic amino acid sequences of peptides derived, depending on the used protease specificity, although the relationship between peptide structure and activity or the antioxidant mechanism is yet not fully understood (Pihlanto, 2006). The increased antioxidant activity through hydrolysis suggests that this process contributed to antioxidant activity by releasing previously inactive peptides encrypted in the sequence of bovine casein. The 1% w/v hydrolysate demonstrated an equivalent activity to 1341 µmol of Trolox, and reaching 44,35% of ABTS inhibition. Intact bovine casein also shown to possess antioxidant properties, as measured by the ABTS method, although, hydrolysates demonstrated a 2,72 times higher activity than the non-hydrolyzed casein, as observed in Table 1.

The Hydrogen peroxide, as reactive oxygen species (ROS), can cause oxidative stress and damage of biomolecule in the cell, leading to cell death and serious chronic

diseases. Hydrogen peroxide, a weak oxidizing agent, is implicated indirectly in lipid oxidation. Hydrogen peroxide is a reactive non radical, which can permeate biological membranes and be converted to more reactive species as hydroxyl radical and singlet oxygen (Intarasirisawat et al. 2013). The casein hydrolysate exhibited strong hydrogen peroxide scavenging activity. 0,1 % w/v of the hydrolysate showed to be equivalent to 547,82 µmol of Trolox, indicating an H_2O_2 scavenging activity of 644,5 µmol of trolox/mg of sample. Although the non-hydrolyzed casein also presented a scavenging activity, hydrolysate sample showed a 3,44 times higher activity.

Furthermore, many factors can influence antioxidant activity of bioactive peptides as well. The antioxidant and biological activities can be affected by the operational conditions applied to isolate proteins, degree of hydrolysis, type of protease and peptide concentration. However, it has been postulated that the overall antioxidative activity must be ascribed to the integrative effects of these actions rather than to the individual actions of peptides (Sarmadi and Ismail, 2010).

6.4. Conclusion

Commercial bovine casein hydrolysis using *Penicillium aurantiogriseum* free and immobilized, on magnetic nanoparticles coated with PANI, protease, was successfully done. The PMFs from casein hydrolysis reveled slightly different pattern concerning the free and immobilized enzyme used. The most present peptides were sequenced, α_{S1}-casein peptides: D-V-P-S-E-R-Y-L-G-Y; G-L-P-Q-E-V-L-N-E-N-L-L-R-F; β-casein fragments: L-S-L-S-Q-S-K-V, Y-Q-E-P-V-L-G-P-V-R-G-P-F and L-L-Y-Q-E-P-V-L-G-P-V-R. The hydrolysates had their ROS scavenging properties tested, revealing a 1341 µmol of Trolox equivalent ABTS scavenging activity using 1% w/v hydrolysate concentration and 547,82 µmol of Trolox equivalent hydrogen peroxide scavenging activity using 0,1% w/v hydrolysate concentration. In both assays the hydrolysates presented a more than 2,5 times greater activity than non-hydrolyzed casein, confirming the potential use of *Penicillium aurantiogriseum* protease in antioxidant casein-derived functional foods production.

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6.6. List of Tables

Table 1. ABTS and H_2O_2 radical scavenging activities of casein hydrolysate.

Peptides (w/v)	**ABTS**		**H2O2**	
	Equivalent [Trolox] (µM)	(% inhibition)	Equivalent [Trolox] (µM)	µmol Trolox/mg sample
1%	1341	44,35		
0,5 %	771	23,65		
0,1%	297,67	6,47	547,82	644,5
0,05%			293,42	690,4
0,01%			47,42	557,88
Casein (w/v)				
0,1%	254,33	4,9	149,42	175,79

6.7. List of Figures

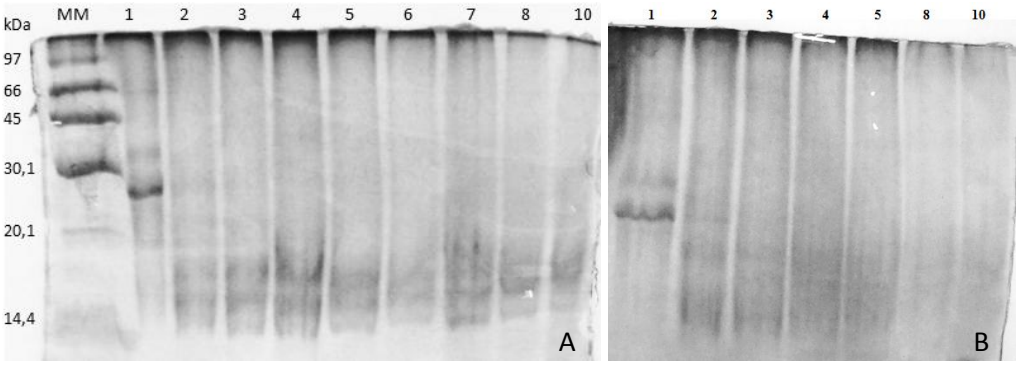

Fig 1. SDS-PAGE gel of casein hydrolysis over time. (A) Free enzyme casein hydrolysates, (B) Immobilized enzyme casein hydrolysates.

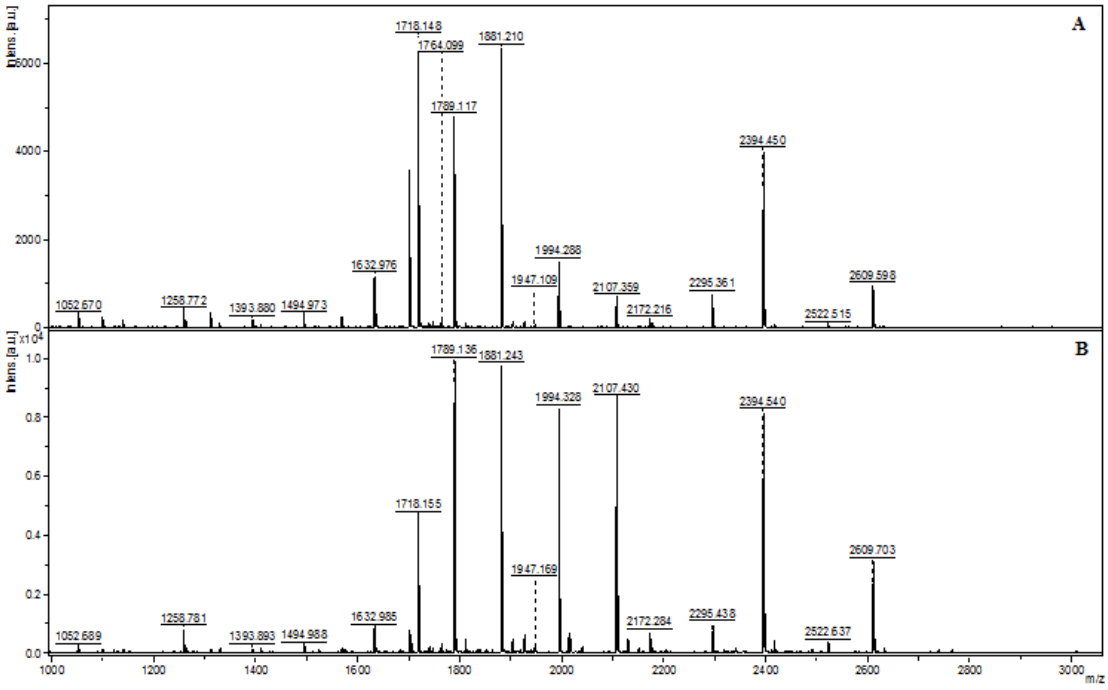

Fig 2. PMF of immobilized (A) and free enzyme (B) casein hydrolisates.

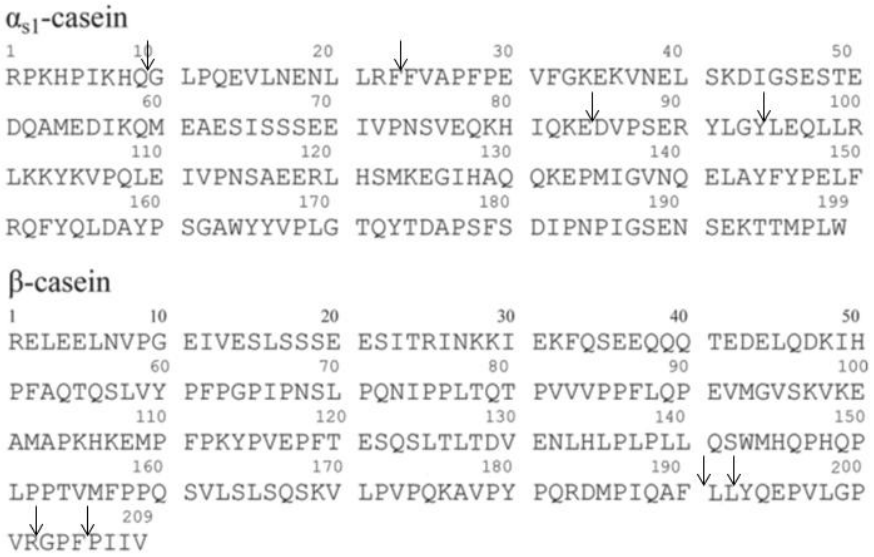

Fig 3. Cleavage region of peptide bonds on α_{S1}- and β-casein hydrolyzed with *P. aurantiogriseum* protease.

7. Anexos

7.1. Resumos apresentados em congressos

7.1.1. XII Congresso Latinoamericano de Microbiologia

Purificação parcial da protease caseinolítica produzida por *Penicillium aurantiogriseum* em meio de soja

Duarte Neto, J.M.W.; Lima, C.A.; Lima-Filho, J.L.; Porto, A.L.F.

Laboratório de Imunopatologia Keizo Asami, UFPE.

As enzimas proteolíticas são comumente associadas a várias aplicações em detergentes, processamento de peles, recuperação de prata, propósitos médicos, processamentos de alimentos, indústria química e tratamento de resíduos. As proteases microbianas se destacam na obtenção de peptídeos bioativos com aplicações potenciais na ciência dos alimentos e da nutrição. Assim, a pesquisa por novas proteases torna-se interessante, principalmente para produzir peptídeos bioativos com propriedades de interesse. Neste estudo objetiva-se a purificação parcial de protease caseinolítica produzida pelo *Penicillium aurantiogriseum* URM 4622 visando a obtenção de peptídeos bioativos. O *P. aurantiogriseum*, obtido na coleção da micoteca da UFPE, foi mantido em meio de cultura ágar extrato de malte e o meio utilizado para produção da enzima foi o meio de soja (MS). Após o inoculo padronizado (106 esporos/mL), a produção ocorreu em Erlenmayer contendo meio MS icubada em agitador orbital (200 rpm) a 24ºC durante 72h. Em seguida o extrato bruto foi tratado por precipitação salina com Sulfato de Amônio nas frações: 0/20%; 20/40%; 40/60%; 60/80%; 80/100% de saturação. Após diálise, foram determinadas a concentração proteica e atividade proteásica de cada uma das frações e do extrato bruto. Foi observado que a fração 60-80 possuiu atividade proteásica relativa muito superior às outras frações (1125 U/mL), porém uma atividade específica baixa se comparada com o extrato bruto (Fr60-80=579 U/mg; EB=866 U/mg). Isso se deve à alta quantidade de proteínas totais, inespecíficas, que precipitam nesta fração (1,94 mg/mL). Ao juntar as frações 40-60 e 60-80, obtivemos uma atividade específica de 971,7 U/mg, mostrando um fator de purificação 1,12, em relação ao extrato bruto. Essa fração será utilizada em ensaios futuros de purificação. Neste trabalho demonstrou-se que a protease caseinolítica produzida pelo *P. aurantiogriseum* em meio de soja, precipita numa saturação entre 40 e 80% de Sulfato de Amônio, com um fator de purificação de 1,12, Indicando que outros ensaios de purificação são necessários para isolamento desta enzima.

Palavras chave: proteases, *Penicillium aurantiogriseum*, pré-purificação, precipitação salina.

7.1.1. XII Congresso Latinoamericano de Microbiologia

Potencial proteolítico de linhagens de aspergillus terreus

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O processo de modificação de uma proteína normalmente se refere a tratamentos físicos, químicos ou enzimáticos que modifiquem a conformação ou estrutura e, consequentemente, suas propriedades funcionais. Dos muitos usos possíveis de tratamentos enzimáticos de proteínas, a hidrólise é o mais largamente utilizado. O processo de hidrólise enzimática envolve a ação proteolítica de uma enzima para separar ligações peptídicas específicas de uma proteína alvo. Enzimas microbianas proteolíticas são largamente utilizadas em vários processos industriais. As proteases apresentam muitas propriedades de interesse industrial, em especial atividade em baixas concentrações e ausência de toxicidade. Esta classe de enzimas pode ser isolada de muitas fontes, todavia proteases fúngicas sejam as principais responsáveis pela amplo uso de fungos na indústria de fermentação. Os fungos do gênero Aspergillus são sabidamente utilizados na produção de proteases. O objetivo deste trabalho é fazer uma triagem das linhagens de *Aspergillus terreus* com potencial para produção de protease em escala comercial. 19 linhagens de *A. tereus*, obtidos na micoteca da UFPE, foram inoculados 10^6 esporos/mL em meio de lactose, para fermentação. O meio foi mantido à 28ºC, por 7 dias, sob agitação de 150 rpm. Em seguida os extratos brutos foram utilizados para determinação da atividade proteásica, determinando o grau de hidrólise em espectrofotômetro, usando azocaseína como substrato. Então, foram determinadas as concentrações proteicas dos extratos, utilizando o kit comercial da "BCA Protein Assey" da Thermo®. A linhagem de maior atividade proteolítica foi a 3571, com atividade 1,47 vezes maior que a média das outras linhagens (116 U/mL). Esta linhagem mostrou muito alta concentrações de proteínas totais (3,72 mg/mL) o que contribuiu para demonstrar baixa atividade específica (31,18 U/mg), evidenciando que a enzima deve ser purificada para medição de sua atividade específica real. Demonstrou-se que dentre as 19 linhagens de *A. tereus* estudadas neste trabalho, a linhagem 3571 parece ser a de maior potencial para produção de protease, pela sua alta produtividade e maior atividade proteolítica, porém testes mais aprimorados devem ser realizados para atestar esses dados.

Palavras chave: proteases, atividade proteolítica, azocaseína, *Aspergillus tereus*.

7.1.2 XI Reunião Regional Nordeste da SBBq

Immobilization Of Protease From *Penicillium auratiogriseum* URM 4622 Fermentation On Magnetic Nanoparticles Coated With Polyaniline

Duarte Neto, J.M.W.; Maciel, J.C.; Carvalho Jr, L.B.; Lima-Filho, J.L.; Lima, C.A.; Porto, A.L.F.

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Bioactive peptides, with properties of interest, can be obtained from casein hydrolysis using fungal proteases. *Penicillium aurantiogriseum* fermentation, can produce protease capable of casein degradation. Therefore, the objective of this study was to immobilize this protease on magnetic nanoparticles for casein proteolytic hydrolysis. The *P. aurantiogriseum* was incubated in soybean flour medium at 200rpm, at 24ºC, over 72h, for enzyme production. The filtrated were treated by Ammonium Sulfate, and, after dialysed, the fractions had their proteolytic activity and protein concentration tested. The 40-60% and 60-80% fractions showed 660 µg/mL and 3680 µg/mL of protein concentration and 259 U/mL and 849 U/mL of proteolytic activity, respectively. Those enzyme fractions were diluted (~300 µg/mL) and immobilized via glutaraldehyde on magnetic nanoparticles coated with polyaniline. The amount of immobilized proteins from F40-60% and F60-80%, were 34% and 54%, respectively. The proteolytic activity shown by F40-60% was 66 ±5.11 U/mL and by F60-80% was 76 ±7.73 U/mL. The results have shown that the amount of immobilized proteins from F60-80% was bigger than F40-60%, although, we don't know the actual protease portion of this value. Also, the immobilized F60-80% showed bigger proteolytic activity than F40-60%, indicating that there is a higher protease amount per µg/mL. Those immobilized fractions were subjected to four reuse cycles. After that, showed more than 60% of the initial proteolytic activity. This results show that the F60-80% proven to be more effective and could be used on enzymatic derivatives production for bioactive peptides release from casein, without need of protease purification.

Word Keys: Casein, Enzyme, Fungus, Glutaraldehyde, PANI
Supported by: FACEPE

7.1.3. IV Simpósio Internacional em Diagnóstico e Terapêutica

Optimization Of *Penicillium Aurantiogriseum* URM 4622 Protease Immobilization On Magnetic Nanoparticles Coated With Polyaniline

Duarte Neto, J.M.W.; Maciel, J.C.; Carvalho Jr, L.B.; Lima-Filho, J.L.; Lima, C.A.; Porto, A.L.F.

Proteolytic enzymes are widely used commercially, as in medical purposes, chemical industry, waste treatment and food processing. Bioactive peptides, with properties of interest can be obtained through casein hydrolysis using fungal proteases, as the produced by *Penicillium aurantiogriseum*. Enzyme immobilization offers advantages as enhanced stability, protection against autolysis, the possibility of repeated use of the catalytic material and can reduce the capital and operation costs. The use of magnetic nanoparticles as support adds advantages to immobilization as easy separated from reaction medium by applying a magnetic field and higher surface area. In this work, we report the use of factorial designs and RSM to optimize *Penicillium aurantiogriseum* URM 4622 protease immobilization on magnetic nanoparticles coated with polyaniline. A full two-level design on four factors (magnetic nanoparticles activation time by glutaraldehyde, offered protein amount for immobilization, pH and immobilization time) was employed to identify the most significant immobilization parameters, and a subsequent central composite design (CCD) was used to find the optimal levels of the two most significant factors (pH and activation time). The design results indicated that pH had significant negative main effect, whereas the activation time had a positive effect on protease immobilization. The most favorable immobilization conditions were found to be 6 hours of activation time, pH 6,5, 200 µg/mL of offered protein amount and 2 h of immobilization time which led to a 1,3 residual activity. Through this process, our results indicate that we can make biocatalysis operation easier even improving protease activity, although more studies must be done to fully optimize the technique.
Keywords: factorial design; central composite design; glutaraldehyde.
Support: FACEPE.

7.1.4. VIII Encuentro Latinoamericano y Del Caribe de Biotecnologia

Optimization Of *Penicillium Aurantiogriseum* URM 4622 Protease Immobilization On Magnetic Nanoparticles

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Proteolytic enzymes represent a biotechnologically important enzymes class used in various industry segments and medical purposes. Enzyme immobilization offers advantages as enhanced stability, protection against autolysis, possibility of repeated use and can reduce the capital and operation costs. Magnetic nanoparticles used as support adds advantages as easy separation from reaction medium by applying a magnetic field and higher surface area. This work aimed to use magnetic nanoparticles coated with polyaniline as support to immobilize *Penicillium aurantiogriseum* URM 4622 protease and optimize this process through factorial designs and response surface methodology. We employed a full two-level design on four factors (nanoparticles activation time, offered protein amount for immobilization, pH and immobilization time) to identify the most significant parameters, and, thereon, used central composite design to find the optimal levels of the two most significant factors (pH and activation time). Results indicated that pH had negative main effect, whereas the activation time was positive. The most favorable immobilization conditions were found to be 4 hours of activation time, pH 6,5, 200µg/mL of offered protein amount and 2h of immobilization time, which led to a residual activity of 1,06. Our results indicate that we could make biocatalysis operation easier even improving protease activity.

7.1.4. VIII Encuentro Latinoamericano y Del Caribe de Biotecnologia

Production Of *Penicillium Aurantiogriseum* URM 4622 Fibrinolytic Protease On Submerged Fermentation

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About 17,3 million deaths from cardiovascular disease were reported in 2008. Intravascular thrombosis is one of the main causes of cardiovascular diseases, it happens when fibrin aggregates forming clots in unbalanced situations caused by certain disorders. The most common treatment is fibrinolytic therapy using plasminogen activators that releases plasmin (EC 3.4.21.7) hydrolyzing fibrin. Microbial fibrinolytic enzymes have been attracting medical attention, being cheaper and safer production sources. Some species of *Penicillium* genus have already being described as fibrinolytic producer. The objective of this study was to test fibrinolytic protease production by *P. aurantiogriseum* in soybean flour medium on submerged fermentation. The *P. aurantiogriseum* was inoculated (10^6 espores/mL) in soybean flour medium and incubated at 200 rpm, at 24 ºC, over 72h, for enzyme production. After fermentation, the medium was filtrated and stored at -20 ºC. To measure fibrinolytic activity a solution with fibrinogen and thrombin was incubated at 37 ºC for 10-15 min to form a clot and the *P. aurantiogriseum* extract was added. After centrifugation with trichloroacetic acid, the hydrolyzate was measured at spectrophotometer (275 nm). The extract showed 17,24 ± 0,34 fibrinolytic unities, showing that *P. aurantiogriseum* produces a stable fibrinolytic protease.

7.2. Patente

Pedido de concessão de patente frente ao INPI, processo Nº.: BR 10 2013 027182 9

RESUMO

PROCESSO DE IMOBILIZAÇÃO DE SUBSTRATO ENZIMÁTICO EM PARTÍCULAS MAGNÉTICAS E SUA UTILIZAÇÃO NA PURIFICAÇÃO, ISOLAMENTO, EXTRAÇÃO E/OU SEPARAÇÃO DE ENZIMAS.

O presente invento descreve o método de utilização de partículas magnéticas revestidas e ativadas como suporte para imobilização de substrato enzimático com a finalidade de uso na purificação, isolamento, extração e/ou separação de enzimas de uma amostra contendo, pelo menos, mais outra substância (contaminante), como amostras biológicas. Esse método trás benefícios como menor custo, menor tempo operacional e alta especificidade em relação aos outros métodos descritos por se tratar de um processo de única etapa de purificação envolvendo afinidade enzima-substrato.

7.3. Normas do periódicos *Applied Microbiology and Biotechnology*

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Book

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7.4. Normas do periódico *Food Chemistry*

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