RUANN JANSER SOARES DE CASTRO

PRODUCTION, BIOCHEMICAL CHARACTERIZATION OF A PROTEASE FROM *Aspergillus oryzae* AND ITS APPLICATION TO PROTEIN HYDROLYSIS FOR OBTAINING HYDROLYSATES WITH ANTIOXIDANT ACTIVITY

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CAMPINAS
2012

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ENGENHARIA DE ALIMENTOS

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Orientadora: Prof[a]. Dr[a]. Helia Harumi Sato

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MASTERS THESIS PRESENTED TO THE FACULTY OF FOOD ENGINEERING
OF THE UNICAMP TO OBTAIN THE MsC GRADE IN FOOD SCIENCE

DISSERTAÇÃO DE MESTRADO APRESENTADA À FACULDADE DE ENGENHARIA DE ALIMENTOS DA UNICAMP PARA OBTENÇÃO DO TÍTULO DE MESTRE NA ÁREA DE CIÊNCIA DE ALIMENTOS

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2012

FICHA CATALOGRÁFICA ELABORADA POR
LUCIANA P. MILLA – CRB8/8129- BIBLIOTECA DA FACULDADE DE ENGENHARIA DE ALIMENTOS – UNICAMP

C279p

Castro, Ruann Janser Soares de, 1987-
Produção, caracterização bioquímica de proteases de *Aspergillus oryzae* e aplicação na hidrólise de proteínas para obtenção de hidrolisados proteicos com atividade antioxidante / Ruann Janser Soares de Castro. -- Campinas, SP: [s.n], 2012.

Orientador: Helia Harumi Sato.
Dissertação (mestrado) – Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos.

1. Fermentação. 2. Proteases. 3. *Aspergillus oryzae*. 4. Hidrólise enzimática. 5. Antioxidante. I. Sato, Helia Harumi. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em inglês: Production, biochemical characterization of a protease from *Aspergillus oryzae* and its application to protein hydrolysis for obtaining hydrolysates with antioxidant activity.
Palavras-chave em inglês (Keywords):
Fermentation
Proteases
Aspergillus oryzae
Enzymatic hydrolysis
Antioxidant
Área de concentração: Ciência de Alimentos
Titulação: Mestre em Ciência de Alimentos
Banca examinadora:
Helia Harumi Sato
Luciana Ferracini dos Santos
Luciana Francisco Fleury
Data da defesa: 31/08/2012
Programa de Pós Graduação: Ciência de Alimentos

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Dedico este trabalho à minha família e aos meus queridos amigos que foram, são e sempre serão suportes indispensáveis na construção de alicerces sólidos na minha vida pessoal e profissional.

Agradecimentos

Ao meu querido e bom Deus que sempre abençoou e continua abençoando tudo o que eu faço: colocando as pessoas certas no meu caminho, aliviando os momentos de estresse, acalmando meu coração nas horas de angústia e dando-me sabedoria para saber que tudo tem seu tempo.

À mainha e ao "pain", Ivoneide e Antonio, por serem as pessoas que mais se alegram pelas minhas conquistas, por todo amor, compreensão e apoio!

À minha querida irmã, Ruanna, pelo companheirismo, apoio e pelos ótimos momentos compartilhados.

Aos meus tios, tias, primos e avós por serem pessoas tão maravilhosas e que me fazem tão bem.

À professora Helia, pelo cuidado, carinho, atenção, preocupação, paciência, dedicação e por todo conhecimento compartilhado ao longo desses dois anos de muito aprendizado e crescimento.

À delegação cearense da FEA: Aliciane, Cinthia, Talita, Bruna, Ana Laura, Mirela e Wellington pelo companheirismo e por trazerem um pouco do Ceará pra Campinas. Agradecimento especial à Aliciane, que me deu total suporte quando cheguei em Campinas, e à Cinthia, pelos abraços que sempre me fazem tão bem.

Aos meus amigos da Engenharia de Alimentos da UFC. À minha turma: Renata, Moara, Talita, Thiago, Monique, Millena, Delane, Tatiane, Karina, Niédila e Cinthia, que sempre apostaram e torceram por mim. Aos professores: Marcos, Elisabeth, Patrícia, Zapata, Herlene, Carminha, Alice, Cláudio Guimarães, Cláudio Ernani, Evânia, José Maria... Aos funcionários: Augusto, Pereira, Luiz Bitu e Rose, que foram de suma importância para minha formação.

Às professoras Suzana Cláudia e Claudia Martins da UFC, por terem me ensinado a base da pesquisa e pelo conhecimento imprescindível que tem me acompanhado ao longo desses anos.

À professora Maria do Carmo Passos Rodrigues da UFC, pelos ensinamentos, apoio, carinho, torcida e pelas longas horas de conversa. Por ser uma pessoa tão especial, que me incentiva a ir cada vez mais longe e se alegra por cada conquista alcançada.

Ao Dr. Gustavo Saavedra da Embrapa Agroindústria Tropical de Fortaleza pela oportunidade de estágio, incentivo e conhecimento compartilhado.

Aos meus amigos da Embrapa: Natália Lima, Virna, Helder, Janaína, Adriana, Genilton, Rakel, Luciana, Mariza, Carol, Carina, Leise, Manuella, Natália Moura, Kally, Andréa, Ana Paula, Cyntia, Millena e Myrella por sempre me receberem de portas abertas, pela amizade sincera e por todos os momentos de apoio, incentivo e força.

Aos meus amigos do Laboratório de Bioquímica de Alimentos da FEA: Paulinha, Fabíola, Marcela, Lívia Rosas, Joelise, Dani, Camilo, Fabiano, Leonardo, Zé, Haroldo, Thiago, Márcio, André, Lívia Dias, Giulia, Jéssika, Ketlyn, Carol, Val, Bia, Priscila Becker, Priscila Hoffman, Viviane, Evandro, Mel, Ricardo, Erica, Fernanda, Patrícia e Isabela pelos momentos de descontração, ajuda, discussões, acolhimento e pela saudável convivência.

Agradecimento especial à Paulinha por tornar meus dias mais alegres, pelos conselhos e confiança. À Fabíola pela demonstração diária de carinho, cuidado e companheirismo. À Marcela pela companhia nos cafés, momentos de descontração e conhecimento compartilhado. À Val e à Bia por todo o carinho e ajuda.

À Lívia Rosas por ser minha parceira oficial de dança nas festas da Bioquímica, por ter me auxiliado nas análises de atividade antioxidante, pelos momentos de esclarecimentos de dúvidas, discussões e por estar sempre disposta a ajudar.

À professora Gláucia Maria Pastore e ao Laboratório de Bioaromas pelo auxílio nas análises de atividade antioxidante.

Aos amigos adquiridos ao longo desses dois anos na república, disciplinas e outros laboratórios: David, Cyntia Cabral, Alaíde, Renata, Érica, Verônica, Juliana, Flávia, Angélica, Janclei e Fábio.

À Bianca Pelici pela amizade, parceria nas corridas e pelos ótimos momentos de estudo.

Aos alunos de graduação da FEA que tive o privilégio de ser PED nas disciplinas de Bioquímica e que foram tão importantes para o meu amadurecimento profissional.

A todos os professores que fazem parte da FEA pela competência e contribuição com os valiosos conhecimentos repassados durante as disciplinas.

Aos funcionários da FEA: Cosme, Marcos Sampaio, Marcos A. de Castro, Guiomar e Jardette pela competência e auxílio prestados.

À Bunge Alimentos S.A., Cooper Ovos e Alibra pela doação de material para execução deste trabalho.

Aos professores membros da banca examinadora: Gabriela Alves Macedo, Carlos Raimundo Ferreira Grosso, Luciana Francisco Fleuri e Luciana Ferracini dos Santos pela valorosa contribuição neste trabalho.

Agradecimento especial à professora Gabriela Alves Macedo pelo incentivo e valorização deste trabalho.

Ao CNPq pela concessão da bolsa de estudos.

À FAPESP pelo apoio financeiro necessário ao desenvolvimento deste trabalho.

Ao Departamento de Ciência de Alimentos, à Faculdade de Engenharia de Alimentos e à Unicamp pela grande oportunidade de desenvolvimento, aprimoramento dos conhecimentos científicos e formação profissional.

"Um homem que nunca muda de opinião, em vez de demonstrar a qualidade da sua opinião demonstra a pouca qualidade da sua mente."

(Marcel Achard)

Índice

Resumo

As proteases constituem um dos mais importantes grupos de enzimas produzidos comercialmente, apresentando diversas aplicações nas indústrias de alimentos e farmacêutica. A utilização de proteases na hidrólise enzimática de proteínas para obtenção de peptídeos com propriedades antioxidantes tem recebido grande notoriedade nas pesquisas científicas. Nesse contexto, o presente trabalho visou estudar a produção e caracterização bioquímica de protease de *Aspergillus oryzae* LBA 01 obtida por processo fermentativo em estado sólido e avaliar a aplicação desta protease e de preparações comerciais na hidrólise de proteínas para obtenção de hidrolisados com atividade antioxidante. A maior produção de protease por *A. oryzae* LBA 01 foi observada em meio de cultivo composto de farelo de trigo, peptona (2,0% p/p) e extrato de levedura (2,0% p/p) sob as seguintes condições: 50,0% de umidade inicial, inóculo de 10^7 esporos.g^{-1} e incubação a 23°C por 72h. A caracterização bioquímica, realizada por planejamento experimental, mostrou que a protease apresentou maior atividade na faixa de pH 5,0-5,5 e 55-60°C, e estabilidade no intervalo de pH 4,5-6,0 após 1h de tratamento na faixa de temperatura de 35-45°C. Proteína isolada de soja, soro de leite e clara de ovo apresentaram aumento expressivo nas suas propriedades antioxidantes quando hidrolisadas com diferentes proteases microbianas. A aplicação de protease comercial Flavourzyme® 500L, obtida de *A. oryzae*, para a hidrólise de proteína isolada de soja, resultou na obtenção de hidrolisados com maior atividade antioxidante quando comparados aos hidrolisados preparados com as proteases de *A. oryzae* LBA 01 e a protease comercial Alcalase® 2.4L de *Bacillus licheniformis*. As condições de hidrólise, definidas a partir de delineamento composto central rotacional (DCCR), foram: concentração de substrato de 90,0 mg.mL^{-1} e adição de 70,0 U de protease por mL de mistura reacional (U.mL^{-1}), resultando em 775,17 e 11,83 Trolox EQ µmol.g^{-1}, para os ensaios de ORAC e DPPH, respectivamente. Os hidrolisados de soro de leite com maior capacidade antioxidante foram obtidos com a protease de *A. oryzae* LBA 01. A adição de 70,0 U.mL^{-1} de protease a solução de soro de leite 80,0 mg.mL^{-1}, resultou em 424,32 e 16,39 Trolox EQ µmol.g^{-1}, para os ensaios de ORAC e DPPH, respectivamente. Na preparação de hidrolisados de proteínas de clara de ovo, a utilização de 30,0 mg.mL^{-1} de substrato e 20,0 U.mL^{-1} da protease comercial Flavourzyme® 500L de *A. oryzae*, resultou em 1.193,12 e 19,05 Trolox EQ µmol.g^{-1} para

os ensaios de ORAC e DPPH, respectivamente. Os maiores valores de atividade antioxidante, para os três substratos, foram detectados entre 30 e 180 minutos de incubação, onde o grau de hidrólise variou de 40,0 a 66,0%. Os resultados obtidos mostraram que a preparação de protease de *A. oryzae* LBA 01 obtida por fermentação em estado sólido e posterior concentração por precipitação com sulfato de amônio, diálise e liofilização, apresentou atividade enzimática semelhante às preparações comerciais avaliadas, tendo, portanto, potencial para aplicação na hidrólise proteica. A hidrólise enzimática, nas condições de estudo avaliadas, aumentou de 2 a 23 vezes a capacidade antioxidante de proteína isolada de soja, soro de leite e clara de ovo, mostrando-se um processo eficaz para obtenção de peptídeos com atividade antioxidante.

Palavras-chave: fermentação, protease, hidrólise, peptídeos bioativos, antioxidantes.

Summary

Proteases are one of the most important groups of enzymes produced commercially, with several applications in the food and pharmaceutical industries. The use of proteases in the enzymatic hydrolysis of proteins to obtain peptides with antioxidant properties has gained great notoriety in scientific research. In this context, the main objectives of the present study were to optimize the production of the protease from *Aspergillus oryzae* LBA 01 by solid state fermentation, and to determine its biochemical characteristics. The application of this protease and of commercial preparations to protein hydrolysis, and the study of the antioxidant properties of the hydrolysates obtained, was evaluated. The optimum fermentation medium was composed of wheat bran, 2.0% (w/w) peptone and 2.0% (w/w) yeast extract, and the conditions for maximum protease production were an initial moisture content of 50.0%, an inoculum level of 10^7 spores.g^{-1} and incubation at 23°C for 72h. The biochemical characterization, evaluated using an experimental design, showed that the enzyme was most active in the pH range 5.0-5.5 and 55-60°C. The enzyme was stable from pH 4.5 to 6.0 after 1h incubation at 35-45°C. Soy protein isolate, bovine whey protein and egg white protein exhibited increases in antioxidant activity when hydrolyzed with the different microbial proteases. For the hydrolysis of soy protein isolate, application of the commercial protease Flavourzyme® 500L from *A. oryzae* resulted in hydrolysates with greater antioxidant activity as compared to hydrolysates prepared with the protease from *A. oryzae* LBA 01 and the commercial protease Alcalase® 2.4L from *Bacillus licheniformis*. The hydrolysis conditions, as defined by a central composite rotational design (CCRD), were: 90.0 mg.mL^{-1} substrate concentration plus 70.0 U of protease per mL of reaction mixture (U.mL^{-1}), which resulted in 775.17 and 11.83 Trolox EQ μmol.g^{-1} as determined by the ORAC and DPPH assays, respectively. For the whey protein hydrolysates, the greatest antioxidant activity was obtained with the protease from *A. oryzae* LBA 01. According to the CCRD, the use of 80.0 mg.mL^{-1} of bovine whey protein and 70.0 U.mL^{-1} of protease resulted in 424.32 and 16.39 Trolox EQ μmol.g^{-1}, respectively, as determined by the ORAC and DPPH assays. For the egg white protein, hydrolysis with 20.0 U.mL^{-1} of Flavourzyme® 500L from *A. oryzae* with 30.0 mg.mL^{-1} substrate concentration, resulted in 19.05 and 1,193.12 Trolox EQ μmol.g^{-1}, respectively, as determined by the ORAC and DPPH assays. The maximum antioxidant activities were obtained in the range from 30 to

180 min of hydrolysis, with a degree of hydrolysis of about 40.0-66.0%. The results showed that the protease preparation from *A. oryzae* LBA 01 obtained by solid state fermentation produced enzymatic activity similar to that of the commercial preparations, and was an attractive enzyme to apply in protein hydrolysis. Under the conditions evaluated in this study, enzymatic hydrolysis resulted in a 2.0- to 23.0-fold increases in antioxidant activity for the soy protein isolate, bovine whey protein and egg white protein, being an effective process to obtain peptides with antioxidant activity.

Keywords: fermentation, protease, hydrolysis, bioactive peptides, antioxidants.

Introdução

As proteases constituem um dos mais importantes grupos de enzimas produzidos comercialmente (Uyar e Baysal, 2004), e têm aplicação em diferentes indústrias, como de alimentos, têxtil, farmacêutica e de detergentes (Horikoshi, 1999; Kanekar *et al*., 2002). Proteases respondem por mais de 60,0% de todo o mercado mundial de enzimas (Oskouie *et al*., 2008), sendo 40,0% desse total de proteases de origem microbiana (Rao *et al*., 1998), portanto, o estudo de processos que envolvam a produção e aplicações desse grupo de enzimas apresenta grande relevância em diversas áreas de pesquisa.

Os micro-organismos representam uma excelente fonte de proteases devido à sua ampla diversidade bioquímica e susceptibilidade a manipulação genética (Rao *et al*., 1998). *Aspergillus oryzae* é uma espécie de micro-organismo considerada não toxicogênica (Vishwanatha, Rao e Singh, 2009), sendo utilizada na indústria na produção de alimentos fermentados tradicionais japoneses, tais como shoyu e saquê.

As enzimas microbianas podem ser obtidas tanto em processo submerso, quanto em fermentação em estado sólido (FES). No caso de cultivo de fungos filamentosos, a FES apresenta diversas vantagens em relação à fermentação submersa. Uma delas é que as condições de cultivo são mais parecidas com o habitat natural, assim, os fungos estão mais adaptados para crescer e secretar maior quantidade de enzimas (Pandey, 2003). A concentração dos produtos após extração é bem maior que os obtidos no processo de fermentação submersa e gera menos resíduo líquido. Este processo desperta maior interesse econômico em regiões, como o Brasil, com abundância em biomassa e resíduos agroindustriais de baixo custo (Castilho, Medronho e Alves, 2000), como a soja, o trigo e o algodão que atingiram juntos no ano de 2010, uma produção mundial de aproximadamente 979,0 milhões de toneladas (FAO, 2012). O processamento destas culturas dá origem a subprodutos de baixo valor agregado, como farelos e tortas, mas de alto valor nutritivo, sendo grande parte destinada à alimentação animal. A utilização destes resíduos como substrato para o desenvolvimento de processos biotecnológicos, como a produção de enzimas por fermentação em estado sólido é um exemplo promissor da obtenção de biomoléculas de alto valor agregado a partir de substratos de baixo custo.

Além dos aspectos de produção, a caracterização de enzimas é importante para avaliar o seu potencial biotecnológico. O estudo de proteases com diferentes propriedades, tais como especificidade de substrato, termoestabilidade, atividade em amplas faixas de pH e temperatura, são importantes para o êxito da aplicação destas enzimas na indústria.

As proteases têm sido estudadas para a obtenção de peptídeos bioativos, que podem ser definidos como sequências de aminoácidos específicas que promovem um impacto positivo em várias funções biológicas, incluindo atividades: antioxidante, antihipertensiva, antitrombótica, antiadipogênica e antimicrobiana (Biziulevicius *et al*., 2006; Zhang, Li e Zhou, 2010; Tsou *et al*., 2010; Tavares *et al*., 2011). Essa enorme diversidade funcional coloca os peptídeos e as proteínas em posição de destaque no campo das aplicações biotecnológicas (Miranda e Liria, 2008), sendo apontados como potenciais substitutos de substâncias químicas utilizadas como fármacos ou conservadores de alimentos (Hong *et al*., 2008). Dentre as diversas atividades biológicas citadas, a atividade antioxidante de peptídeos bioativos tem atraído grande atenção por serem capazes de minimizar os efeitos de radicais livres sobre o organismo humano e em alimentos.

Antioxidantes são considerados importantes nutracêuticos apresentando diversos benefícios à saúde, e são definidos como qualquer substância que retarda ou inibe significativamente a oxidação de um substrato. A glutationa (γ-Glu-Cys-Gly) e a carnosina (β-alanil-L-histidina) são peptídeos antioxidantes naturalmente presentes em tecidos musculares e apresentam capacidade de doar elétrons, quelar metais e íons e inibir a peroxidação lipídica (Samaranayaka e Li-Chan, 2011). Além dos naturalmente presentes, peptídeos obtidos a partir de alimentos proteicos hidrolisados têm sido relatados por terem capacidade antioxidante similar ou superior a antioxidantes sintéticos, sendo assim uma fonte segura para aplicação em alimentos.

O objetivo deste trabalho foi estudar a produção de proteases por *A. oryzae* LBA 01 em fermentação em estado sólido, caracterizar bioquimicamente a enzima obtida e aplicá-la na hidrólise de proteínas para obtenção de peptídeos com atividade antioxidante. Uma avaliação comparativa com preparações comerciais de proteases assim como o estudo das variáveis de processo envolvidas na hidrólise enzimática foram utilizados na determinação das condições mais adequadas para obtenção de hidrolisados com atividade antioxidante.

O Capítulo I consiste em uma Revisão Bibliográfica que visou aliar o conhecimento da obtenção de peptídeos bioativos através de diferentes técnicas, substratos e enzimas, com foco nas atividades antimicrobiana, antioxidante, antiadipogênica e anti-hipertensiva e seus respectivos mecanismos de ação e potencial de aplicação.

O Capítulo II foi composto pelo estudo dos parâmetros de cultivo para produção de proteases por *A. oryzae* LBA 01 por fermentação em estado sólido e caracterização bioquímica da protease extracelular obtida. Neste capítulo também foi realizado um estudo preliminar das propriedades antioxidantes de hidrolisados proteicos de proteína isolada de soja, soro de leite e clara de ovo, obtidos após hidrólise com a protease de *A. oryzae* LBA 01, por meio dos ensaios de ORAC e DPPH.

Os Capítulos III, IV e V abordaram o estudo das condições de hidrólise de proteína isolada de soja, soro de leite e clara de ovo, respectivamente. Os parâmetros avaliados incluíram a combinação mais adequada de enzima/substrato, a influência da concentração de substrato e protease, e do tempo de hidrólise na atividade antioxidante dos hidrolisados, avaliada pelos ensaios de ORAC e DPPH.

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Capítulo I: Obtenção, propriedades multifuncionais e aplicação de peptídeos com atividade biológica

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Revista: Química Nova

Production, multifunctional properties and application of protein hydrolysates with biological activity

Abstract

Bioactive peptides can be defined as specific protein fractions with amino acid sequence that promotes a positive impact on several human systems, including the digestive, immune, endocrine and cardiovascular. Some bioactive peptides have been established for their antimicrobial, antioxidant, anti-adipogenic and antihypertensive functions. This functional diversity of peptides and proteins place them in a prominent position in biotechnology applications, thus have prospects of being incorporated as ingredients in foods or as substitutes of chemical compounds, where their biological activities may assist in the food preservation, control, and prevention of diseases. Fermentation and enzymatic hydrolysis are the most common methods to produce bioactive peptides from whole proteins, however scientific researches are being intensified to produce multifunctional peptides with different characteristics by combining different methods. In this context, this review aimed to show the advances involving the processes of production, biological activities and potential applications of these bioactive peptides.

Keywords: protein hydrolysis, proteases, bioactive peptides.

1. Introdução

As proteínas têm relevância fundamental como componentes dos alimentos. Nutricionalmente, são fontes de aminoácidos essenciais, indispensáveis para o crescimento, manutenção do organismo e também fonte de energia. Em alimentos proteicos, possuem a capacidade de afetar propriedades físico-químicas e sensoriais, como a solubilidade, viscosidade, gelificação e estabilidade da emulsão. Algumas proteínas da dieta possuem propriedades biológicas específicas, fazendo destas, ingredientes potenciais de alimentos funcionais.[1]

Estudos recentes têm relacionado a prevalência de doenças cardiovasculares, obesidade, hipertensão, diabetes e câncer à fatores alimentares. Em resposta ao aumento na percepção sobre a relação entre alimentos e saúde, o mercado de alimentos funcionais sofreu um grande impulso. Um alimento funcional pode ser definido como qualquer alimento, que além das funções nutritivas básicas, fornece benefícios adicionais à saúde, regulando uma ou mais funções no organismo.[2,3]

Processos envolvendo a hidrólise de proteínas têm sido estudados para a produção de peptídeos com atividade biológica. Peptídeos bioativos são definidos como frações específicas de proteínas, com sequência de aminoácidos que promovem um impacto positivo em várias funções biológicas, incluindo atividades: antioxidante, antihipertensiva, antitrombótica, antiadipogênica e antimicrobiana.[4-7] Estes peptídeos apresentam sequências de 2-20 aminoácidos e massas moleculares inferiores a 6000 Da. A bioatividade é definida principalmente pela composição e sequência de aminoácidos.[8] Essa enorme diversidade funcional coloca os peptídeos e as proteínas em posição de destaque no campo das aplicações biotecnológicas,[9] sendo apontados por alguns autores como possíveis substitutos de substâncias químicas utilizadas como fármacos ou conservadores de alimentos.[10]

Diferentes vias são utilizadas na obtenção de peptídeos bioativos, dentre as quais podemos citar: fermentação direta, hidrólise enzimática ou a associação dos dois processos (Figura 1).

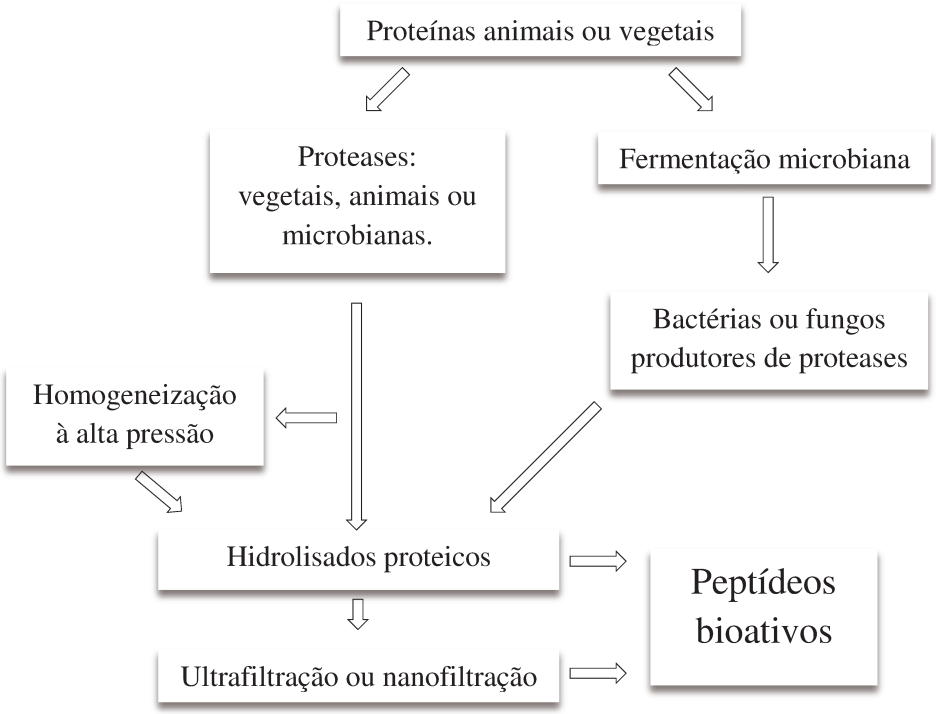

Figura 1 – Principais vias de obtenção de peptídeos bioativos.

No processo de fermentação direta, a aplicação de culturas de bactérias lácticas com atividade proteolítica, leva à formação de peptídeos bioativos, principalmente durante a fabricação de produtos lácteos. A hidrólise enzimática envolve a aplicação de enzimas proteolíticas digestivas, vegetais ou de origem microbiana em um processo de hidrólise limitada, levando a redução de fatores alergênicos, assim como melhoria da digestibilidade e formação de peptídeos com atividade biológica.[11]

Em adição aos processos convencionais citados anteriormente, a associação de diferentes tecnologias vem mostrando resultados eficazes na geração de peptídeos funcionais.[11] O uso de ultrafiltração e nanofiltração são exemplos de tecnologias que têm sido estudadas para refinar e fracionar peptídeos bioativos, permitindo uma separação em tamanhos selecionados e direcionando para aplicações específicas.[12,13]

Peptídeos bioativos podem ser obtidos a partir de fontes proteicas animais e vegetais, como o ovo, caseína, peixe, soja, etc.[5,6,14,15] Nesse contexto, o presente trabalho visou abordar alguns avanços da pesquisa científica envolvendo os processos de obtenção, atividades biológicas e potencial de aplicação de peptídeos bioativos.

2. Principais processos de obtenção de peptídeos bioativos

2.1. Fermentação

A aplicação de fermentação direta para obtenção de peptídeos bioativos está relacionada principalmente com a fabricação de produtos derivados de leite, o qual possui naturalmente proteínas precursoras de moléculas bioativas.[16] Durante o processo de fermentação, bactérias ácido lácticas (BAL) hidrolisam as proteínas do leite, principalmente as caseínas, em peptídeos e aminoácidos que são utilizados como fontes nutricionais para o seu crescimento. A capacidade destes micro-organismos em produzir enzimas proteolíticas faz delas potenciais produtoras de peptídeos bioativos, os quais podem ser liberados durante o processo de fabricação de produtos fermentados. Alguns micro-organismos são extensivamente relatados na literatura por possuírem um sistema proteolítico eficaz na hidrólise de proteínas e liberação de peptídeos com atividade biológica, dentro os quais merecem destaque: *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *diacetylactis*, *Lactococcus lactis* ssp. *cremoris* e *Streptococcus salivarius* ssp. *thermophylus*.[3] Além da utilização de micro-organismos vivos, as enzimas proteolíticas isoladas de BAL também têm sido utilizadas com sucesso em processos de hidrólise enzimática e produção de peptídeos bioativos.[17]

Embora, os produtos lácteos tenham destaque nas pesquisas científicas que envolvem a produção destes peptídeos por fermentação, foi demonstrado que produtos fermentados derivados de soja também apresentaram atividade biológica.[18,19] Inúmeros processos de fermentação para produção de peptídeos bioativos têm sido descritos na literatura (Tabela 1).

Tabela 1 - Obtenção de peptídeos com diferentes atividades biológicas por meio de processo fermentativo utilizando diversas fontes de proteína.

Micro-organismo	Fonte proteica	Condições do processo fermentativo	Peptídeos	Bioatividade	Referência
Lactobacillus delbrueckii subsp. *bulgaricus* IFO13953	Leite bovino (kappa-caseína)	Processo de fermentação submerso conduzido durante 48h a 37°C	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met (aa96-aa106)	Antioxidante	20
Lactobacillus helveticus	Leite bovino	Processo de fermentação submerso conduzido durante 24h a 42°C sob agitação de 200 rpm e pH 6,0	Peptídeos não caracterizados	Antihipertensiva	21
Bacillus subtilis ATCC 41332	Soja	Processo de fermentação em estado sólido conduzido durante 30h a 38°C	Peptídeos não caracterizados	Antihipertensiva Antitrombótica Antioxidante	18
Não especificado	Molho de mexilhões	Fermentação natural conduzida durante 6 meses	His-Phe-Gly-Asp-Pro-Phe-His	Antioxidante	22
Lactobacillus paracasei	Soro de leite caprino (α-lactoalbumina)	Processo de fermentação submerso conduzido durante 96h a 37°C sob agitação de 100 rpm	Trp–Leu–Ala–His–Lys (aa104-aa108)	Antihipertensiva	23
Cultura mista de bactérias ácido lácticas	Leite bovino (β-lactoglobulina e αS1-caseína)	Processo de fermentação submerso conduzido durante 30h a 42°C	Gly–Thr–Trp (aa17–aa19) Gly–Val–Trp (aa162-aa164)	Antihipertensiva	24
Lactobacillus helveticus LBK 16 H	Leite bovino	Processo de fermentação submerso conduzido durante 20h a 37°C	Peptídeos não caracterizados	Antihipertensiva	25
Aspergillus oryzae	Arroz, soja e caseína	Processo de fermentação em estado sólido conduzido durante 40h a 30°C	Val-Pro-Pro; Ile-Pro-Pro	Antihipertensiva	26
Lactobacillus kefiranofaciens	Soro de leite bovino	Processo de fermentação submerso conduzido durante 24h a 37°C	Peptídeos não caracterizados	Imunomoduladores	27
Aspergillus sojae	Soja e trigo	Processo de fermentação em estado sólido conduzido durante 192h a 20-45°C e umidade de 95%	Gly-Tyr; Ala-Phe; Val-Pro; Ala-Ile; Val-Gly	Antihipertensiva	19
Lactobacillus helveticus	Leite bovino	Processo de fermentação submerso conduzido durante 50h a 37°C	Peptídeos não caracterizados	Antimicrobiana	28

2.2. Hidrólise enzimática

Uma das formas mais comuns e rentáveis para produzir peptídeos bioativos é através da hidrólise enzimática de proteínas.[3] Esse processo oferece algumas vantagens, como: especificidade e emprego de enzimas em concentrações muito baixas, reações rápidas em condições suaves e obtenção de produto livre de resíduos químicos e com melhores propriedades funcionais e nutricionais.[29]

As proteases catalisam a reação de hidrólise das ligações peptídicas das proteínas e ainda podem apresentar atividade sobre ligações éster e amida. Todas as proteases apresentam certo grau de especificidade quanto ao substrato, em geral relacionado aos aminoácidos envolvidos na ligação peptídica a ser hidrolisada e àqueles adjacentes a eles.[30] Essa especificidade em adição às condições de hidrólise (pH, temperatura, tempo) afetam o tamanho e a sequência de aminoácidos na cadeia peptídica, além da quantidade de aminoácidos livres, que por sua vez influenciam a atividade biológica dos hidrolisados.[6,8] Alguns estudos demonstraram que a hidrólise de proteína de soja com enzimas proteolíticas de fontes diversas (animal, vegetal e microbiana) produziram hidrolisados com diferentes graus de hidrólise e poder antioxidante.[4, 31]

Um grande número de estudos demonstrou a liberação de peptídeos com atividade biológica após a hidrólise de proteínas (Tabela 2). As principais enzimas utilizadas nestes processos incluem enzimas gastrointestinais, tais como pepsina, tripsina, quimotripsina. No entanto, a perspectiva econômica do processo muitas vezes limita o tipo e a quantidade de enzimas que podem ser utilizadas na hidrólise de proteínas na indústria. Portanto, enzimas microbianas estão sendo amplamente aplicadas para a hidrólise em escala industrial.[32] A utilização de preparações comerciais de proteases microbianas de grau alimentício é vantajosa, uma vez que estas enzimas são de baixo custo e seguras, com alta rentabilidade de produtos.[33]

Tabela 2 - Aplicação de proteases para obtenção de peptídeos com atividade biológica a partir de diferentes fontes de proteína.

Protease	Condições de hidrólise	Fonte proteica	Bioatividade dos peptídeos	Referência
Orientase	pH 10,0; 50°C; 6h	Gema de ovo	Antioxidante	14
Alcalase®	pH 8,0; 55°C; 6h	Caseína	Anti-hipertensiva	33
Pepsina Pancreatina	pH 3,5; 37°C; 0,5h pH 7,0; 37°C; 1h	Leite humano	Anti-hipertensiva Antioxidante	34
Alcalase® Bromelina	pH 8,5; 50°C; 3h pH 5,5; 50°C; 3h	Carne de ostra	Antimicrobiana	35
Protease alcalina Tripsina Pepsina	pH 8,0; 40°C; 4h pH 8,0; 37°C; 4h pH 2,0; 37°C; 4h	Carne de tubarão	Antioxidante	36
Neutrase Papaína Quimotripsina Flavorase Alcalase®	pH 7,0; 37°C; 4h pH 6,5; 60°C; 4h pH 7,5; 50°C; 4h pH 6,0; 50°C; 4h pH 8,5; 60°C; 4h	Arroz	Antioxidante	37
Alcalase®	pH 8,0; 50°C; 3h	Soja	Antiadipogênese	38
Flavourzyme®	pH 7,0; 50°C; 2h	Proteína isolada de soja	Antiadipogênese	6
Neutrase	pH 6,0; 45°C; 4h	Proteína isolada de soja	Antiadipogênese	39
Pepsina	pH 5,5; 23°C	Hemoglobina bovina	Antimicrobiana Anti-hipertensiva	40
Protamex Tripsina Neutrase Savinase NS37005 Esperase Alcalase®	pH 6,5; 60 °C; 4h pH 8,0; 55°C; 4h pH 6,5, 55°C; 4h pH 9,5; 55°C; 4h pH 7,0; 55°C; 4h pH 8,5; 60°C; 4h pH 8,0; 50°C; 4h	Gelatina	Anti-hipertensiva Antioxidante Anticâncer	41
Protease alcalina de *Bacillus licheniformis* Protease neutra de *Bacillus subtilis*	pH 8,5; 55°C; 3h. pH 7,0; 45°C; 2h	Milho	Anti-hipertensiva	42

2.3. Métodos de ultrafiltração, nanofiltração e homogeneização à alta pressão hidrostática para obtenção, fracionamento e concentração de peptídeos

Métodos de ultrafiltração (UF) utilizando membranas de 0,001 a 0,1µm de diâmetro e pressões de 100 a 1000 kPa, e de nanofiltração (NF) empregando membranas com poros de diâmetros menores que 0,001µm e pressões de 500 a 3500 kPa[43] têm sido empregados para refinar, fracionar e obter peptídeos na forma concentrada e em tamanhos selecionados, no entanto há relatos que reportam aumento ou diminuição das atividades biológicas das frações obtidas.[12,44,45] Picot *et al*.[12] estudaram a aplicação de UF e NF com o intuito de verificar o impacto sobre a estabilidade e atividades biológicas de peptídeos bioativos obtidos por hidrólise enzimática de proteína de peixe e observaram que o fracionamento não resultou em aumento, ao contrário, diminuíram as atividades antioxidante e anti-hipertensiva. Hidrolisados de proteínas sarcoplasmáticas de carne bovina obtidos por hidrólise com papaína tiveram a capacidade de quelar Fe^{2+} (atividade antioxidante) aumentada após fracionamento por UF em membranas de 10 kDa.[44] Resultados semelhantes foram observados por Gómez-Guillén *et al*.[45], onde a atividade antioxidante de hidrolisados de gelatina aumentou até 62,0% quando fracionados em membranas de 1 kDa.

A homogeneização à alta pressão hidrostática é utilizada no processamento de alimentos visando principalmente à eliminação de micro-organismos e enzimas. Neste processo, o material de interesse é submetido a pressões entre 100 e 1000 MPa durante um tempo variável que pode durar de milisegundos a minutos, dependendo do objetivo que se deseja atingir.[46] Embora a técnica seja utilizada para fins de inativação enzimática, há relatos que a utilização de alta pressão hidrostática pode aumentar o efeito catalítico de proteases assim como sua estabilidade. Mozhaev *et al*.[47] estudaram os efeitos da aplicação de alta pressão hidrostática na atividade e estabilidade térmica de quimotripsina e verificaram que a técnica foi muito eficaz na estimulação da atividade catalítica e desaceleração da inativação térmica em temperaturas elevadas. É importante ressaltar, que os efeitos observados são aumentados em altas pressões até certo valor crítico, pois à pressões muito elevadas a desnaturação proteica pode causar perda da atividade das enzimas. A aplicação desta técnica como processo auxiliar na obtenção de peptídeos bioativos tem sido relatada como bastante eficiente, pois aumenta a hidrólise de proteínas, devido a alterações conformacionais que tornam a proteína mais passível de hidrólise pela

exposição de cadeias que antes eram inacessíveis pelas enzimas. Além disso, o aumento da proteólise sob pressão também tem sido atribuída aos efeitos físicos da pressão sobre a enzima e/ou efeitos sobre a interação enzima-substrato.[48] Quirós *et al*.[13] estudaram a aplicação de alta pressão hidrostática em conjunto com hidrólise enzimática para produção de peptídeos com atividade anti-hipertensiva. O tratamento de ovoalbumina foi realizado sob pressões elevadas, até 400 MPa, com quimotripsina, tripsina e pepsina. Os resultados obtidos mostraram que a aplicação de altas pressões melhorou a hidrólise e promoveu mudanças no perfil proteolítico. Apesar dos peptídeos obtidos a alta pressão e a pressão atmosférica não terem apresentado diferenças significativas na atividade anti-hipertensiva, a proteólise sob pressões de 200-400 MPa diminuíram o tempo de obtenção destes peptídeos.

3. Multifuncionalidade dos peptídeos bioativos

Peptídeos bioativos de proteínas alimentares têm sido estudados extensivamente ao longo da última década para elucidar seu potencial biológico e influência sobre os principais sistemas do corpo humano, como: digestivo, cardiovascular, nervoso e imunológico. Alguns peptídeos bioativos apresentaram atividades biológicas com impacto positivo sobre a saúde, dentre as quais podemos citar: atividade antimicrobiana,[40] antihipertensiva,[42] antioxidante,[37] anticancerígena,[41] antiadipogênica[6] e imunomoduladoras,[27] e, portanto têm perspectivas de serem incorporados como ingredientes em alimentos funcionais, nutracêuticos e medicamentos, onde essas bioatividades podem ser aliadas no controle e prevenção de doenças.[49]

A obtenção e características de peptídeos com atividade antimicrobiana, antioxidante, antiadipogênica e anti-hipertensiva estão descritas neste trabalho.

3.1. Peptídeos com atividade antimicrobiana

Peptídeos antimicrobianos estão amplamente distribuídos na natureza e representam um componente essencial do sistema imunológico. Eles são reconhecidamente, a primeira linha de defesa do organismo contra a colonização de micro-organismos exógenos, com papel fundamental na regulação de populações bacterianas em mucosas e outras superfícies epiteliais.[50,51] Mais de 800 peptídeos antimicrobianos já foram descritos em plantas e animais.[52] Apesar da diversidade na estrutura primária, a grande maioria dos peptídeos

antimicrobianos possui cadeias curtas de aminoácidos, que são caracterizadas pela predominância de aminoácidos catiônicos e hidrofóbicos. Embora haja diferenças significativas nas estruturas secundária e terciária, peptídeos antimicrobianos são geralmente compostos por uma superfície hidrofóbica e uma hidrofílica. O caráter anfipático destas moléculas é fundamental no mecanismo de ação antimicrobiana permitindo uma maior interação com a membrana bacteriana.[53] Em adição a característica anfipática, a reduzida massa molecular das frações peptídicas, com consequente maior exposição dos resíduos de aminoácidos e suas cargas, e a formação de pequenos canais na bicamada lipídica, foram relacionados com o poder antimicrobiano, pois causam modificações que aumentam a interação peptídeo-membrana.[45,54,55]

O mecanismo exato de ação de muitos peptídeos antimicrobianos ainda não está bem estabelecido, e devido ao grande número de peptídeos já conhecidos, acredita-se na probabilidade de existirem mecanismos distintos de ação.[53]

Além dos naturalmente presentes nos sistemas de defesa de plantas e animais, peptídeos com atividade antimicrobiana já foram identificados em diversos hidrolisados proteicos. Biziulevicius *et al*.[5] avaliaram o potencial antimicrobiano de hidrolisados protéicos obtidos a partir do tratamento enzimático de caseína, α-lactoalbumina, β-lactoglobulina, ovalbumina e albumina com proteases (tripsina, α-quimiotripsina, pepsina e pancreatina) e verificaram que todos os hidrolisados obtidos apresentaram atividade antimicrobiana contra as linhagens de bactérias e leveduras (*Escherichia coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Candida lambica* e *Saccharomyces cerevisiae*) testadas.

Três frações peptídicas obtidas de caseinato de sódio fermentado com *Lactobacillus acidophilus* DPC6026, estudadas por Hayes *et al*.[56], apresentaram atividade antibacteriana contra linhagens patogênicas de *Enterobacter sakazakii* ATCC 12868 e *Escherichia coli* DPC5063.

Liu *et al*.[35] descreveram a obtenção de peptídeo antimicrobiano após digestão de ostra (*Crassostrea gigas*) com Alcalase® 2.4L (Novo Nordisk, Denmark) e bromelina (Shanghai Co. Ltd., China). A fração denominada *CgPep33* purificada por ultrafiltração, cromatografia de troca iônica, filtração em gel e cromatografia líquida de alta eficiência (CLAE), apresentou amplo espectro de ação contra diversos micro-organismos, incluindo

bactérias Gram+, Gram- e fungos. O peptídeo *CgPep33* foi capaz de inibir o crescimento de todas as bactérias estudadas (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* e *Staphylococcus aureus*) e fungos (*Botrytis cinerea* e *Penicillium expansum*). Os valores de IC 50 (concentração necessária para inibir 50,0% do crescimento) variaram de 18,6-48,2 $\mu g.mL^{-1}$. As bactérias Gram+ foram as que apresentaram maior sensibilidade, com valores de CIM (concentração inibitória mínima) entre 40 e 60 $\mu g.mL^{-1}$.

Peptídeos com atividade antimicrobiana foram preparados a partir de gelatina hidrolisada com Alcalase® 2.4L (Sigma-Aldrich, Estados Unidos). As frações obtidas por ultrafiltração em membranas de 1 e 10kDa foram utilizadas para testes antimicrobianos contra 18 cepas bacterianas. As bactérias mais sensíveis na presença das frações testadas foram: *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Shewanella putrafaciens* e *Photobacterium phosphoreum*.[45] Hidrolisados de hemoglobina bovina tratada com pepsina foram purificados por CLAE e testados quanto ao poder antimicrobiano contra duas linhagens Gram- (*Escherichia coli*, *Salmonella enteritidis*) e três Gram+ (*Kocuria luteus* A270, *Staphylococcus aureus* e *Listeria innocua*). Os resultados obtidos mostraram que as frações peptídicas purificadas apresentaram amplo espectro de ação, agindo contra 4 das 5 bactérias testadas (*Kocuria luteus* A270, *Listeria innocua*, *Escherichia coli* e *Staphylococcus aureus*) com CIM variando entre 35,2 e 187,1 µM.[40] Tellez *et al*.[28] mostraram a eficiência de uma fração peptídica, isolada a partir de leite fermentado com *Lactobacillus helveticus*, contra uma infecção proposital com *Salmonella enteritidis* em ratos. A taxa de sobrevivência no grupo alimentado com a fração peptídica (0,02 µg por dia) foi superior ao grupo alimentado com metade da dose (0,01 µg por dia) e ao grupo controle.

3.2. Peptídeos com atividade antioxidante

A formação de radicais livres, tais como superóxido (O_2^-) e hidroxila (OH), é uma consequência inevitável em organismos aeróbios durante a respiração. Estes radicais são muito instáveis e reagem rapidamente com outros grupos ou substâncias no organismo, ocasionando lesões celulares ou nos tecidos.[37] Uma quantidade excessiva desses radicais no organismo foi associada ao desenvolvimento de várias doenças, como aterosclerose, artrite, diabetes e câncer.[57] Por serem espécies altamente reativas, os radicais livres podem causar

danos às proteínas e mutações no DNA, oxidação de fosfolipídeos de membrana e modificação em lipoproteínas de baixa densidade (LDL).[58] Em alimentos, a oxidação também afeta diretamente a qualidade, comprometendo caraterísticas como sabor, aroma e coloração. Nesse contexto, a presença de substâncias que inibem reações oxidativas que comprometem a qualidade dos alimentos é de suma importância.

Antioxidantes são considerados importantes nutracêuticos apresentando diversos benefícios à saúde, e são definidos como quaisquer substâncias que retardam ou inibem significativamente a oxidação de um substrato. Antioxidantes artificiais (BHA, BHT e n-propil-galato) exibem forte efeito protetor contra diversos sistemas de oxidação. No entanto, essas substâncias apresentam riscos potenciais *in vivo*, sendo seu uso restrito ou proibido em alguns países para gêneros alimentícios.[59] Alguns peptídeos com atividade antioxidante têm ocorrência natural em alimentos. A glutationa (γ-Glu-Cys-Gly) e a carnosina (β-alanil-L-histidina) são antioxidantes naturalmente presentes em tecidos musculares e apresentam capacidade de doar elétrons, quelar metais e íons e inibir a peroxidação lipídica.[60] Além dos naturalmente presentes, peptídeos obtidos a partir de alimentos proteicos hidrolisados têm sido relatados por terem capacidade antioxidante similar ou superior a antioxidantes sintéticos como o BHT, sendo assim uma fonte segura para aplicação em alimentos.[20]

Os mecanismos de ação que explicam a atividade antioxidante de peptídeos não são totalmente compreendidos, mas vários estudos mostraram a capacidade de peptídeos em inibir a peroxidação lipídica,[14] eliminar radicais livres,[45] quelar íons metálicos[41] e eliminar espécies reativas de oxigênio.[61] Assim como para outras atividades biológicas, as propriedades antioxidantes dos peptídeos estão relacionadas com sua composição, estrutura e hidrofobicidade.[62] A presença dos aminoácidos tirosina, triptofano, metionina, lisina e cisteína, foi relatada como importante fator para a ação antioxidante dos peptídeos, especialmente pela capacidade de redução do Fe^{3+} a Fe^{2+} e atividade quelante de íons Fe^{2+} e Cu^{2+}.[63,64,65] Deve-se ressaltar, que não só a presença, mas também a sequência destes aminoácidos na cadeia peptídica desempenha papel importante no poder antioxidante.[22] A capacidade antioxidante de peptídeos pode ser avaliada por diversos métodos, nos quais estão envolvidos diferentes mecanismos de ação e consequentemente medem atividades distintas (Tabela 3).

Tabela 3 – Principais métodos de determinação de atividade antioxidante de peptídeos e respectivos mecanismos de cada método.

Método	Mecanismo	Reação	Medida realizada	Referência
DPPH	Captura do radical DPPH	O radical DPPH (2,2-difenil-1-picril-hidrazil) reage com antioxidantes doadores de hidrogênio, com mudança de coloração violeta para amarela.	Redução da absorbância a 517nm	70
ORAC	Captura de radical peroxila	O radical peroxila, gerado pela decomposição do AAPH [dicloreto de 2,2'-azobis (2-amidinopropano)] na presença de oxigênio atmosférico, reage com um indicador fluorescente formando um produto não fluorescente. Na presença de antioxidantes, a fluorescência é preservada.	Redução de fluorescência (excitação a 485nm e emissão a 520nm)	71
FRAP	Poder de redução de ferro	Na presença de antioxidantes doadores de elétrons, o complexo Fe^{3+}-TPTZ [2,4,6-tri(2-piridil)-1,3,5-triazina] é reduzido a Fe^{2+}-TPTZ, com mudança de coloração azul clara para azul escura	Aumento da absorbância a 593nm	72
ABTS	Captura do radical ABTS	O radical ABTS (ácido 2,2'-azinobis-(3-etilbenzotiazolino-6-sulfônico) é estabilizado na presença de antioxidantes doadores de hidrogênio, com mudança de coloração verde escura para verde clara.	Redução da absorbância a 734nm	45
Habilidade em quelar metais de transição (Cu^{2+})	Quelação de Cu^{2+}	Reação de complexação de Cu^{2+} com violeta de pirocatecol gerando um produto colorido. A presença de antioxidantes diminui a formação do complexo Cu^{2+}-pirocatecol com consequente redução da intensidade de cor.	Redução da absorbância a 620nm	15
Habilidade em quelar metais de transição (Fe^{2+})	Quelação de Fe^{2+}	Reação de complexação de Fe^{2+} com ferrozina gerando um produto colorido. A presença de antioxidantes diminui a formação do complexo Fe^{2+}-ferrozina com consequente redução da intensidade de cor.	Redução da absorbância a 562nm	68
TBARS	Quantificação de produtos de peroxidação de lipídica	Reação do ácido tiobarbitúrico com produtos da decomposição dos hidroperóxidos, sendo o malonaldeído, o principal elemento quantificado. Absorbância e atividade antioxidante são inversamente proporcionais.	Aumento da absorbância a 532nm	73

Hernández-Ledesma *et al.*[66] investigaram as propriedades antioxidantes de hidrolisados proteicos de soro de leite obtidos após tratamento com proteases comerciais utilizando o método ORAC. Quarenta e duas frações peptídicas foram identificadas por CLAE acoplada a espectrômetro de massas, sendo que uma delas (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) apresentou atividade antioxidante maior que o BHA (hidroxianisol butilado), um potente antioxidante sintético. As propriedades antioxidantes de hidrolisados proteicos de amendoim foram comprovadas por Hwang *et al.*[67], utilizando ensaios de DPPH, capacidade de quelar metais e o método do tiocianato férrico. Hidrolisados proteicos de soja preparados com proteases microbianas comerciais e fracionados por ultrafiltração apresentaram atividade antioxidante quando testados pelos métodos de DPPH e ORAC.[4] Nazeer e Kulandai[68] avaliaram as propriedades antioxidantes de hidrolisados proteicos de peixe obtidos por tratamento enzimático utilizando diferentes proteases (papaína, pepsina, tripsina e quimotripsina). A atividade antioxidante foi avaliada pela redução do radical DPPH, poder de redução do ferro e habilidade em quelar metais. Todos os hidrolisados apresentaram atividade antioxidante, sendo que os obtidos com pepsina e tripsina mostraram maior atividade. Li *et al.*[69] verificaram que hidrolisados proteicos de carpa preparados com Alcalase® 2.4L e papaína apresentaram atividade antioxidante utilizando-se as metodologias ABTS, DPPH, poder de redução do Fe^{3+} e habilidade em quelar Fe^{2+}.

3.3. Peptídeos com atividade antiadipogênica

A obesidade é resultado de um desequilíbrio entre a ingestão e a real necessidade de energia, levando a um crescimento patológico de células adipócitas.[74] A quantidade de tecido adiposo pode ser controlada por inibição da adipogênese em células precursoras ou pré-adipócitas, como os pré-adipócitos 3T3-L1, que são os modelos mais bem caracterizados para o estudo de adipogênese. Muitos fatores de transcrição estão envolvidos na diferenciação de células pré-adipócitas em adipócitos, e a inibição ou regulação destes fatores pode levar a uma diminuição do acúmulo de gordura no organismo.[6] A glicerol-3-fosfato desidrogenase (GPDH) é uma enzima que ocupa uma posição chave no metabolismo da glicose, e está ligada à biossíntese de fosfolipídeos e triglicerídeos.[6,75] A supressão da atividade GPDH pode resultar na inibição da diferenciação bem como na

redução do acúmulo de lipídeos em células 3T3-L1, assim a determinação da atividade desta enzima pode ser empregada para avaliar o efeito antiadipogênico.[76] Outra enzima envolvida no processo de adipogênese é a ácido graxo sintetase (FAS), a qual participa da síntese endógena de ácidos graxos saturados de cadeia longa a partir dos precursores acetil-CoA e malonil-CoA.[77,78] Tem sido relatado que certas frações de proteínas hidrolisadas possuem a capacidade de inibir a ação destas enzimas, regulando assim o processo de diferenciação celular e o acúmulo relativo de lipídeos. De acordo com Kim *et al*.[79] estes hidrolisados apresentam grande potencial em tratamentos antiobesidade por diminuírem o acúmulo de gordura no organismo.

Tsou *et al*.[6] estudaram a aplicação da preparação comercial de proteases Flavourzyme® na hidrólise de proteína isolada de soja e avaliaram a capacidade antiadipogênica das frações dos hidrolisados obtidas por ultrafiltração. Os resultados revelaram que a hidrólise limitada de proteína isolada de soja permitiu a obtenção de hidrolisados com grande capacidade antiadipogênica, e que as frações obtidas por ultrafiltração inibiram mais eficientemente a atividade GPDH, sendo a fração obtida com membranas de 1kDa, a mais efetiva (59,0% de inibição). A atividade antiadipogênica de hidrolisados de proteína isolada de soja após tratamento enzimático com Neutrase e o efeito do fracionamento por ultrafiltração sobre a bioatividade foram estudados por Tsou *et al*.[39]. Assim como no estudo anterior, os resultados mostraram que peptídeos com baixa massa molecular (entre 1300 e 2200 Da) foram mais efetivos na inibição da atividade GPDH.

Mejia *et al*.[38] avaliaram o efeito de hidrolisados proteicos de soja enriquecidos com β-conglicinina (proteína naturalmente presente na soja) sobre a atividade da FAS e adipogênese em adipócitos humanos *in vitro*. Os resultados mostraram que alterações genotípicas nas subunidades da proteína de soja (enriquecimento com β-conglicinina) produziram perfis peptídicos que levaram à inibição da FAS e diminuição do acúmulo de lipídeos in vitro. A quantidade de hidrolisados de proteína de soja necessária para inibir 50,0% da atividade da FAS (IC_{50}) variou de 50-175μM. Um peptídeo com capacidade antiadipogênica foi isolado por ultrafiltração, filtração em gel e CLAE a partir de hidrolisados proteicos de soja. A capacidade antiadipogênica foi confirmada por meio da inibição da diferenciação de células pré-adipócitas 3T3-L1. O inibidor de adipogênese foi

identificado com um tripeptídeo (Ile-Gln-Asn), tendo um valor de IC_{50} de 0,014 mg de proteína.mL^{-1}.[79]

3.4. Peptídeos com atividade anti-hipertensiva

A hipertensão arterial é um problema de saúde comum em todo o mundo, atingindo cerca de 15-20% dos adultos.[42] Embora seja um distúrbio controlável, a hipertensão está associada ao desenvolvimento de doenças cardiovasculares, como arteriosclerose, infarto de miocárdio e acidente vascular cerebral.[80] A enzima conversora de angiotensina (ECA) desempenha um papel importante na regulação da pressão arterial porque catalisa a conversão da angiotensina I (forma inativa) em angiotensina-II (vasoconstritor), além de inativar a bradicinina (vasodilatador). Consequentemente inibidores sintéticos da ECA, tais como captopril e enalapril são muitas vezes utilizados para tratar a hipertensão e outras doenças relacionadas com o coração. No entanto, os inibidores sintéticos podem causar diversos efeitos colaterais, como tosse, alteração do paladar, erupções cutâneas e angioedema.[41]

É bem reconhecido, que proteínas alimentares contêm sequências primárias de peptídeos capazes de modular funções fisiológicas específicas.[10] Muitos tipos de peptídeos bioativos com atividade inibidora da ECA foram isolados de hidrolisados proteicos e produtos fermentados.

Peptídeos com atividade anti-hipertensiva foram isolados de hidrolisados proteicos de leite após fermentação com bactérias lácticas e hidrólise enzimática com a protease comercial Prozyme 6. Os peptídeos foram identificados como Gly-Thr-Trp e Gly-Val-Trp, e apresentaram atividade inibitória da ECA com valores de IC_{50} de 464,4 e 240,0 μM, respectivamente.[24]

Hernández-Ledesma *et al*.[34] hidrolisaram proteínas do leite humano com pepsina e pancreatina para estudo das propriedades anti-hipertensivas de peptídeos e verificaram que os hidrolisados derivados da β-caseína mostraram potente ação inibidora da ECA, com IC_{50} de 21 μM.

Frações peptídicas de proteína de soja hidrolisada com pepsina foram separadas por cromatografia de troca iônica, filtração em gel e CLAE e apresentaram atividade inibitória sobre a ECA. Quatro sequências de aminoácidos foram identificadas como potenciais

inibidoras da ECA: Ile-Ala (IC_{50} 153 μM), Tyr-Leu-Ala-Gly-Asn-Gln (IC_{50} 14 μM), Phe-Phe-Leu (IC_{50} 37 μM) e Ile-Tir-Leu-Leu (IC_{50} 42 μM). Quando administrados em uma dose de 2,0 g de peso corporal/kg em ratos hipertensos durante 15 semanas, as frações de peptídeos reduziram consideravelmente a pressão arterial.[81]

4. Conclusão

Peptídeos com atividade biológica podem ser definidos como sequências específicas de aminoácidos que promovem efeitos fisiológicos benéficos. As tecnologias para obtenção de peptídeos bioativos envolvem a hidrólise de proteínas por enzimas exógenas de origem microbiana, vegetal ou animal e processos fermentativos utilizando-se fungos ou bactérias. A ampla diversidade bioquímica das proteases, assim como a existência de fontes proteicas com composições variadas de aminoácidos, torna possível a obtenção de peptídeos com funções biológicas distintas e/ou até mesmo com multifuncionalidade, como atividades antioxidante, antimicrobiana, antiadipogênica e anti-hipertensiva. Outro fator que merece destaque é o estudo das condições de hidrólise (fonte e concentração de proteína, tipo e concentração de protease, temperatura, pH, tempo de reação) que influenciam diretamente na liberação de peptídeos com atividade biológica. Sendo assim, o estudo sobre os processos de obtenção assim como o entendimento da sua multifuncionalidade, tornam-se aliados na aplicação de peptídeos bioativos como potentes agentes biológicos naturais que podem ser utilizados em conjunto ou até mesmo em substituição a substâncias sintéticas em processos de conservação de alimentos, na administração de alimentos funcionais e na produção de fármacos.

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Capítulo II: The production, biochemical characterization of a protease from *Aspergillus oryzae* and its application to protein hydrolysis for study of the antioxidant power of the hydrolysates

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Revista: Process Biochemistry

Abstract

This study reports the production optimization, the biochemical characterization and the application to protein hydrolysis of a partially purified protease obtained from *Aspergillus oryzae* LBA 01 under solid state fermentation. The study of the antioxidant properties of the hydrolysates produced with the protease is reported. The optimum fermentation medium was composed of wheat bran, 2.0% (w/w) peptone and 2.0% (w/w) yeast extract, and the conditions for maximum protease production were an initial moisture content of 50.0%, an inoculum level of 10^7 spores.g^{-1} and an incubation at 23°C for 72h. The biochemical characterization using experimental design showed that the protease was most active over the pH range 5.0-5.5 and it was stable from pH 4.5 to 6.0, indicative of an acid protease. The protease showed optimum temperature range for activity from 55-60°C, and it was stable after 1h incubation at 35-45°C. The soy protein isolate, bovine whey protein and egg white protein exhibited a 2.0- to 10.0-fold increase in antioxidant activity when hydrolyzed with the protease from *A. oryzae* LBA 01. The results suggest that the protease from *A. oryzae* LBA 01 is an attractive enzyme to apply to protein hydrolysis to increase the antioxidant power of proteins from various sources.

Keywords: protease; production; *A. oryzae*; biochemical characterization; protein hydrolysis; antioxidant peptides.

1. Introduction

Proteases are multifunctional enzymes and are extremely important in the pharmaceutical, medical, food and biotechnology industries, accounting for nearly 60.0% of the whole enzyme market [1,2]. They can be isolated from plants, animals and microorganisms. Of these sources, the microorganisms show great potential for protease production due to their broad biochemical diversity and their susceptibility to genetic manipulation. It has been estimated that microbial proteases represent approximately 40.0% of the total worldwide enzyme sales [3].

Aspergillus oryzae (*A. oryzae*) is a filamentous fungus listed as a ‘‘Generally Recognized as Safe (GRAS)’’ organism by the US Food and Drug Administration. It has a long history of use in the food industry in the production of traditional fermented foods, due to its high proteolytic activity [4,5]. According to Machida et al. [6] the molecular history of the organism shows that *A. oryzae* has the largest expansion of hydrolytic genes (135 proteinase genes).

Proteolytic enzymes can be produced by submerged and solid state fermentation. For the growth of fungi, solid state fermentation is most appropriate method because it resembles the natural habitat of the fungi. Some characteristics make solid state fermentation more attractive than submerged fermentation: simplicity, low cost, high yields and concentrations of the enzymes and the use of inexpensive and widely available agricultural residues as substrates [7].

The biochemical characterization of enzymes is important to evaluate their biotechnological potential. The study of the protease properties, such as the influence of inhibitors or activators, the substrate specificity, the optimum catalytic pH conditions and the temperature and stability profiles, can be used to predict the successful application of the enzyme to particular industries or processes.

In the last decade, the application of proteases to the hydrolysis of animal and plant proteins to produce bioactive peptides has attracted much attention. The antioxidant activity of peptides is extensively reported in several studies. The mechanism of action of the antioxidant peptides is related to their capacity to inactivate ROS (reactive oxygen species), scavenge free radicals, chelate prooxidative transition metals and reduce hydroperoxides

[8]. Thus, studies of the production of new proteolytic enzyme sources are critical to advancing the knowledge concerning bioactive peptides.

In this context, the main objectives of the present study were to optimize the production of the protease from *A. oryzae* LBA 01 by solid state fermentation and to determine the biochemical characteristics of the protease, including the optimum pH and temperature for activity and stability, the effects of ions and inhibitors on the protease activity and the kinetic parameters *K*m and *V*max. After the biochemical characterization, the application of the protease to protein hydrolysis for the study of the antioxidant properties of bioactive peptides was evaluated.

2. Materials and Methods

2.1. Microorganism culture

The strain used in this study was *A. oryzae* LBA 01, previously selected as a proteolytic strain from the culture collection of the Laboratory of Food Biochemistry, Faculty of Food Engineering, State University of Campinas. The strain was periodically subcultured and maintained on potato dextrose agar slants. To produce fungal spores, the microorganism was inoculated into a medium composed of 10 g wheat bran and 5 mL of solution containing 1.7% (w/v) $NaHPO_4$ and 2.0% (w/v) $(NH_4)_2SO_4$ and incubated for 3 days at 30°C. The fungal spores were dispensed into sterile Tween 80 solution (0.3%) to prepare the inoculum for fermentation. The number of spores per milliliter in the suspension was determined with a Neubauer cell counting chamber.

2.2. Protease production

Wheat bran, soybean meal, and cottonseed meal were kindly provided by Bunge Foods S/A. These agricultural residues were used to select the most appropriate substrate for the protease production by *A. oryzae* LBA 01. The protease production was performed under solid state fermentation in 500 mL Erlenmeyer flasks containing 40 g medium. For substrate selection, the initial cultivation parameters were 50.0% moisture, temperature set at 30°C, and an inoculum level of 10^7 spores.g^{-1}. The protease activity was tested at 24 h intervals during 120 h fermentation. The crude extract was obtained by the addition of 150 mL acetate buffer (200 mM, pH 5.0) to the fermented medium. The mixture was homogenized and maintained at rest for 1 h. The mixture was filtered through a filter membrane to obtain an enzyme solution free of any solid material. The crude extract was concentrated by precipitation with ammonium sulfate (80.0%), dialysis and freeze drying. The partially purified preparation was biochemically characterized and used for protein hydrolysis.

2.3. Screening of the cultivation parameters: Plackett–Burman statistical design

A Plackett-Burman (PB) design [9] was used to determine the effects of the nutrients (glucose, starch, yeast extract and peptone), initial moisture, temperature and inoculum amounts on the protease production by *A. oryzae* LBA 01.

The variables were screened in 15 trials, with triplicates at the central point. The coded and real values of the variables are given in Table 1. The protease activity was measured after 48 and 72 h of fermentation.

Table 1 – The coded and real values for the variables evaluated in the PB design to study the effects on the production of protease by *A. oryzae* LBA 01.

Factors	Levels		
	-1	**0**	**+1**
Initial moisture (%) (x_1)	30.0	45.0	60.0
Temperature (°C) (x_2)	23	30	37
Inoculum level (spores.g^{-1}) (x_3)	10^5	10^6	10^{7*}
Glucose (% w/w) (x_4)	0	1.0	2.0
Starch (% w/w) (x_5)	0	2.5	5.0
Yeast extract (% w/w) (x_6)	0	1.0	2.0
Peptone (% w/w) (x_7)	0	1.0	2.0

*For statistical analysis, the coded value +1 of the inoculum level was substituted by +10 (equivalent to the real value of 10^7 spores.g^{-1}).

The main effects of the variables were determined using the Statistica® 8.0 software package from Statsoft Inc. (Tulsa, Oklahoma, USA). The variables with confidence levels greater than 95.0% were considered to significantly influence the protease production. After evaluating the effect of each variable, the conditions that had the greatest effects on the protease production were selected. The substrate moisture variable was further evaluated over the range 40.0 to 70.0 %.

2.4. Determination of protease activity

The protease activity was measured using azocasein as the substrate according to Charney and Tomarelli, with modifications [10]. The reaction mixture containing 0.5 mL 0.5% (w/v) azocasein (Sigma), pH 5.0, and 0.5 mL of the enzyme solution was incubated for 40 min. The reaction was stopped by adding 0.5 mL 10.0% TCA and the test tubes were centrifuged at 17,000 x *g* for 15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of enzyme activity (U) was defined as the amount of enzyme required to increase the absorbance at 428 nm by 0.01 under the assay conditions described.

The effect of the substrate concentration on the protease activity was also studied using casein and hemoglobin, as described below.

The protease activity using casein as the substrate was assayed as described by Iaderoza and Baldine, with modifications [11]. The reaction mixture containing 2.5 mL 1.2% (w/v) casein, pH 6.0 and 0.5 mL of the enzyme solution was incubated at 55°C for 10 min. The reaction was stopped by adding 2.5 mL 5.0% TCA, and the reaction mixture was incubated in an ice-bath for 20 min. The reaction mixture was filtered through filter paper (Whatman nº 1), and the absorbance of the filtrate was measured at 280 nm. One unit of enzyme activity was defined as the amount of protease required to increase the absorbance at 280 nm by 0.01 under the assay conditions described.

The protease activity using hemoglobin as the substrate was determined according to the Food Chemical Codex [12]. The reaction mixture containing 5.0 mL 2.0% (w/v) hemoglobin, pH 4.7, and 1.0 mL of the enzyme solution was incubated for 30 min at 55°C. The reaction was stopped by adding 5.0 mL 15.0% TCA; the reaction mixture was left to stand for 1 h, and it was filtered and its absorbance was measured at 275 nm. One unit of enzyme activity was defined as the amount of protease required to increase the absorbance at 275 nm by 0.01 under the assay conditions described.

2.5. Effects of pH and temperature on the activity and stability of the protease determined using an experimental design

To determine the central composite rotatable design (CCRD) levels, a univariate study was previously performed. The temperature was studied over the range from 30°C to 80°C, and the pH was studied over the range from 5.0 to 9.0.

The optimum pH and temperature for activity and stability were determined using a CCRD with three replicates at the central point and four axial points (a total of 11 runs). The coded and real values of these variables are given in Table 2. To study the protease stability, the enzyme was incubated for 1 h at various pH values and temperatures.

Table 2 – The independent variables and levels for the determination of the optimum pH and temperature for the activity and stability of the protease from *A. oryzae* LBA 01.

Optimum activity					
Independent variables	**Levels**				
	-α*	**-1**	**0**	**+1**	**+α**
pH	5.0	5.3	6.0	6.7	7.0
Temperature (°C)	35.0	39.4	50.0	60.6	65.0
Stability					
pH	4.0	4.4	5.5	6.6	7.0
Temperature (°C)	35.0	39.4	50.0	60.6	65.0

*α =1.41

The experiments were randomized to maximize the variability in the observed responses caused by extraneous factors. A second-order model equation was used for this model, represented by Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (1)$$

where Y is the estimated response, i and j equal values from 1 to the number of variables (n), β_0 is the intercept term, βi values are the linear coefficients, βij values are the quadratic coefficients, and xi and xj are the coded independent variables. The coefficient of determination R^2 and the F test (analysis of variance (ANOVA)) were used to verify the quality of the fit of the second-order model equation. The relationships between the responses and the variables were determined using the Statistica® 8.0 software package from Statsoft Inc. The protease activity was determined using the azocasein method.

2.6. Determination of the kinetic parameters *K*m and *V*max

Casein, azocasein and hemoglobin were used over the concentration ranges 4.0-20.0, 1.0-10.0 and 2.0-20.0 mg.mL^{-1}, respectively, to determine the kinetic parameters of the protease from *A. oryzae* LBA 01. The Michaelis-Menten constant (*K*m) and maximum velocity (*V*max) values were determined as the reciprocal absolute values of the intercepts on the x and y axes, respectively, of the linear regression curve [13].

The measurements were made at the optimum temperature as previously determined.

2.7. Effects of ions and inhibitors on the protease activity

The effects of metal ions and inhibitors on the enzyme activity were evaluated by the pre-incubation of the enzyme with different concentrations (0.05, 0.5 and 5.0 mM) of various salts and inhibitors for 1 h at room temperature. The residual protease activity was assayed using azocasein as the substrate at optimum conditions [10]. Each experiment was performed in triplicate.

2.8. Application of the protease to protein hydrolysis

Soy protein isolate (SPI), bovine whey proteins (BWP) and egg white protein (EWP) were used as the substrates of the enzymatic hydrolysis and as the sources of the peptides with antioxidant activity. Fifty-milliliter aliquots of 100.0 $mg.mL^{-1}$ (BWP and SPI) and 30.0 $mg.mL^{-1}$ (EWP) suspensions in 100 mM acetate buffer and 0 (control), 20.0 and 40.0 U of protease per mL of reaction mixture were incubated for 240 min at the optimum temperature and pH conditions of the enzyme. After the hydrolysis, the protease was inactivated by heating in boiling water for 20 min. The reaction mixtures were centrifuged at 17,000 x *g* for 20 min at 5°C. The supernatants were collected and freeze-dried until determination of the antioxidant activity.

2.9. Determination of antioxidant activities of the protein hydrolysates

2.9.1. ORAC assay

The ORAC method used herein, with fluorescein (FL) as the ‘‘fluorescent probe’’, was developed by Dávalos et al. [14] and described by Macedo et al. [15]. The automated ORAC assay was performed on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96-well plate. The reaction was performed at 37°C; the reaction was initiated by the thermal decomposition of AAPH in a 75 mM phosphate buffer (PBS, pH 7.4) due to the sensitivity of FL to pH. The solution of FL (0.4 $\mu g.mL^{-1}$) in PBS (75 mM, pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 µM Trolox solution, prepared daily in

distilled water and diluted to 1500-1.5 µmol.L^{-1} to prepare the Trolox standard curve. In each well, 120 µL of the FL solution was mixed with either 20 µL sample, blank (distilled water), or standard (Trolox solutions) before 60 µL AAPH (108 mg.mL^{-1}) was added. The fluorescence was measured immediately after the addition of AAPH, and measurements were then taken every 1 min for 75 min. The measurements were taken in triplicate. The ORAC values were calculated using the difference between the area under the FL decay curve and the blank (net AUC). The regression equations between the net AUC and the antioxidant concentration were calculated for all samples. The ORAC values were expressed as µmol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ µmol.g^{-1}) [16].

2.9.2. DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bougatef et al. [17]. A 500 µL aliquot of the protein hydrolysates (5 mg.mL^{-1}) was mixed with 500 µL 99.5% ethanol and 125 µL 0.02% DPPH in 99.5% ethanol. The mixture was then kept at room temperature in the dark for 60 min, and the reduction of the DPPH radical was measured at 517 nm using a UV-visible spectrophotometer. The DPPH radical-scavenging activity was calculated as follows (Equation 2):

$$Radical\ scavenging\ activity(\%) = \left[\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right] * 100 \quad (2)$$

The control reaction was performed in the same manner, except that distilled water was used instead of sample. Trolox was used as a standard. The tests were performed in triplicate. The results of the tests were expressed as µMol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ µmol.g^{-1}) and DPPH radical scavenging (%).

3.0. Calculations and statistics

The statistical analyzes were performed using the Minitab® 16.1.1 software package from Minitab Inc. (USA). Values are expressed as the arithmetic mean. The Tukey test was used to check the significant differences between the groups analyzed. When $p < 0.05$, the differences were considered significant.

4. Results and Discussion

4.1. The protease production by *A. oryzae* LBA 01 under solid state fermentation

The highest protease activity was observed between 48 and 72 h, with a decrease after 72 h of incubation. The protease production using wheat bran as a substrate was higher compared with that using cottonseed meal or soybean meal. Therefore, wheat bran was selected as the most appropriate substrate for the protease production by *A. oryzae* LBA 01, reaching 29.27 $U.g^{-1}$ after 48 h of fermentation (Table 3). Some studies describe the use of wheat bran as a potent substrate for the production of proteases by *A. oryzae* [18, 19].

Table 3 – The protease production by *A. oryzae* LBA 01 under solid state fermentation using agroindustrial substrates.

Fermentation period (h)	Protease activity ($U.g^{-1}$)[a]		
	Wheat bran	**Cottonseed meal**	**Soybean meal**
24	20.81 ± 0.74	7.04 ± 0.66	4.22 ± 0.66
48	29.27 ± 0.93	10.78 ± 3.31	8.20 ± 2.98
72	22.38 ± 1.27	12.66 ± 1.33	4.68 ± 0.66
96	20.68 ± 2.20	11.26 ± 1.33	4.92 ± 0.33
120	20.34 ± 1.62	5.86 ± 0.33	*Undetected*

[a]The protease activities were measured using azocasein as the substrate.

In the PB experimental design, the protease production ranged between 0 (undetected) and 41.00 $U.g^{-1}$ after 48 h of fermentation; after 72 h, the values ranged from 0.09 (run 9) to 35.85 (run 1) in the various trials, which reflected the importance of the variables evaluated for the optimization of the protease production (Table 4).

Table 4 – Plackett–Burman (PB) experimental design matrix with coded values and responses for the protease production of by *A. oryzae* LBA 01 under solid state fermentation using wheat bran as substrate.

Runs	Variables/Levels							Protease activity ($U.g^{-1}$)[a]	
	x_1	x_2	x_3[b]	x_4	x_5	x_6	x_7	48 h	72 h
1	+1	-1	+1	-1	-1	-1	+1	38.65	35.85
2	+1	+1	-1	+1	-1	-1	-1	15.25	16.40
3	-1	+1	+1	-1	+1	-1	-1	10.38	0.54
4	+1	-1	+1	+1	-1	+1	-1	41.00	32.35
5	+1	+1	-1	+1	+1	-1	+1	28.15	11.25
6	+1	+1	+1	-1	+1	+1	-1	25.20	27.40
7	-1	+1	+1	+1	-1	+1	+1	5.12	14.99
8	-1	-1	+1	+1	+1	-1	+1	*Undetected*	6.57
9	-1	-1	-1	+1	+1	+1	-1	1.52	0.09
10	+1	-1	-1	-1	+1	+1	+1	4.10	30.65
11	-1	+1	-1	-1	-1	+1	+1	7.26	5.43
12	-1	-1	-1	-1	-1	-1	-1	0.14	0.72
13 (C)	0	0	0	0	0	0	0	23.81	15.94
14 (C)	0	0	0	0	0	0	0	23.81	15.07
15 (C)	0	0	0	0	0	0	0	25.63	17.23

[a]The protease activities were measured using azocasein as the substrate. [b]For statistical analysis, the coded value +1 of the inoculum level (x_3) was substituted by +10 (equivalent to the real value of 10^7 spores.g^{-1}).

The effect estimates for 72 h of fermentation were used to select the variables because the coefficient of determination value (R^2 = 0.96) was better at that point than the coefficient of determination value obtained after 48 h of fermentation (R^2 = 0.62). The screening performed using the PB design revealed that the initial moisture, the inoculum level, the supplementation with yeast extract, and the use of peptone as a nitrogen source were the variables most relevant to the protease production; these variables had a positive effect (Table 5). In contrast, the increase in temperature and the addition of carbon sources (glucose and starch) negatively affected the protease production in the ranges studied.

The addition of the carbon sources can cause catabolic repression, a mechanism particularly important in the regulation of the extracellular enzymes that degrade complex substrates in organisms exposed to changing environments. The catabolic repression was

observed in the present study. The highest protease activity (35.85 $U.g^{-1}$) was detected in the run without added carbon sources; lower activity was detected in run 3 (0.54 $U.g^{-1}$), to which starch was added (5.0% w/w), and in run 9 (0.09 $U.g^{-1}$), to which glucose (2.0% w/w) and starch (5.0% w/w) were added. Sindhu, Suprabha and Shashidhar [20] observed an increase in protease production by *Penicillium godlewskii* SBSS 25 when various nitrogen sources (NH_4NO_3, KNO_3 and $NaNO_3$) were added to the fermentation medium, which also occurred in this study.

To perform the next step, the inoculum level and the additions of yeast extract and peptone were fixed at the +1 level of the PB design, and the temperature and the additions of starch and glucose were fixed at the -1 level, because they showed positive and negative effects, respectively (Table 5). The initial moisture level was the variable with the greatest effect on protease production; thus, it was selected to be studied in the next stage, over the range 40.0% to 70.0%.

Table 5 – The effect estimates for the response of the protease production ($U.g^{-1}$) by *A. oryzae LBA* 01 from the results of the PB design after 48 and 72 h of fermentation using wheat bran as substrate.

Factors	Effect		*p*-value	
	48 h[a]	72 h[b]	48 h	72 h
Initial moisture (x_1)	21.32	20.93	0.018	0.000
Temperature (x_2)	0.99	-5.04	0.890	0.032
Inoculum level (x_3)	6.60	7.56	0.345	0.004
Glucose (x_4)	0.89	-3.16	0.902	0.138
Starch (x_5)	-6.35	-4.87	0.391	0.036
Yeast extract (x_6)	-1.40	6.60	0.846	0.010
Peptone (x_7)	-1.70	4.54	0.813	0.047

[a]Standard error = 3.15, R^2 = 0.62.
[b]Standard error = 0.86, R^2 = 0.96.

The effect of the initial moisture level on protease production is shown in Fig. 1. Initial moisture levels of 40.0% and 50.0% allowed greater protease production by *A. oryzae* LBA 01, reaching 32.59 $U.g^{-1}$ and 33.52 $U.g^{-1}$ after 72 h of fermentation, respectively. However, at higher moisture levels, the enzyme production decreased (Fig. 1). It is known that the initial moisture is an important factor that influences the fermentation

process; at high levels, moisture can reduce the substrate porosity, causing particles to stick together and obstructing the oxygen transfer to the medium. In contrast, a low moisture level reduces water activity to levels that are not conducive to good fungal growth [7]. Uyar and Baysal [21] studying the production of protease using wheat bran as a substrate under solid state fermentation, observed that initial moisture levels between 30.0% and 40.0% increased the protease synthesis. Other authors also reported the use of a medium with high moisture (50.0-60.0%) as the optimum condition for protease production [7, 22].

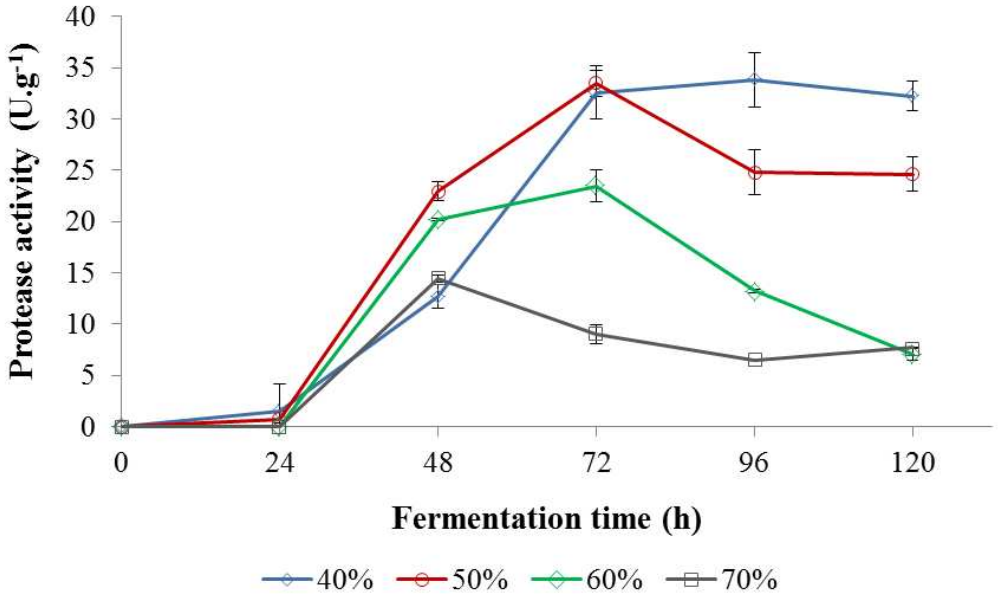

Fig. 1 - The effect of moisture content on protease production by *A. oryzae* LBA 01 under solid state fermentation using wheat bran as the substrate.

4.2. Biochemical characteristics of protease from *A. oryzae* LBA 01

The partially purified preparation was biochemically characterized and used to hydrolyze protein. The protease from *A. oryzae* LBA 01 exhibited optimum activity at pH 5.0 and in the temperature range 55°C to 60°C (Fig. 2). On the basis of the results of the univariate study, the levels for the experimental design were defined, and the CCRD was used to verify an interaction between the independent variables.

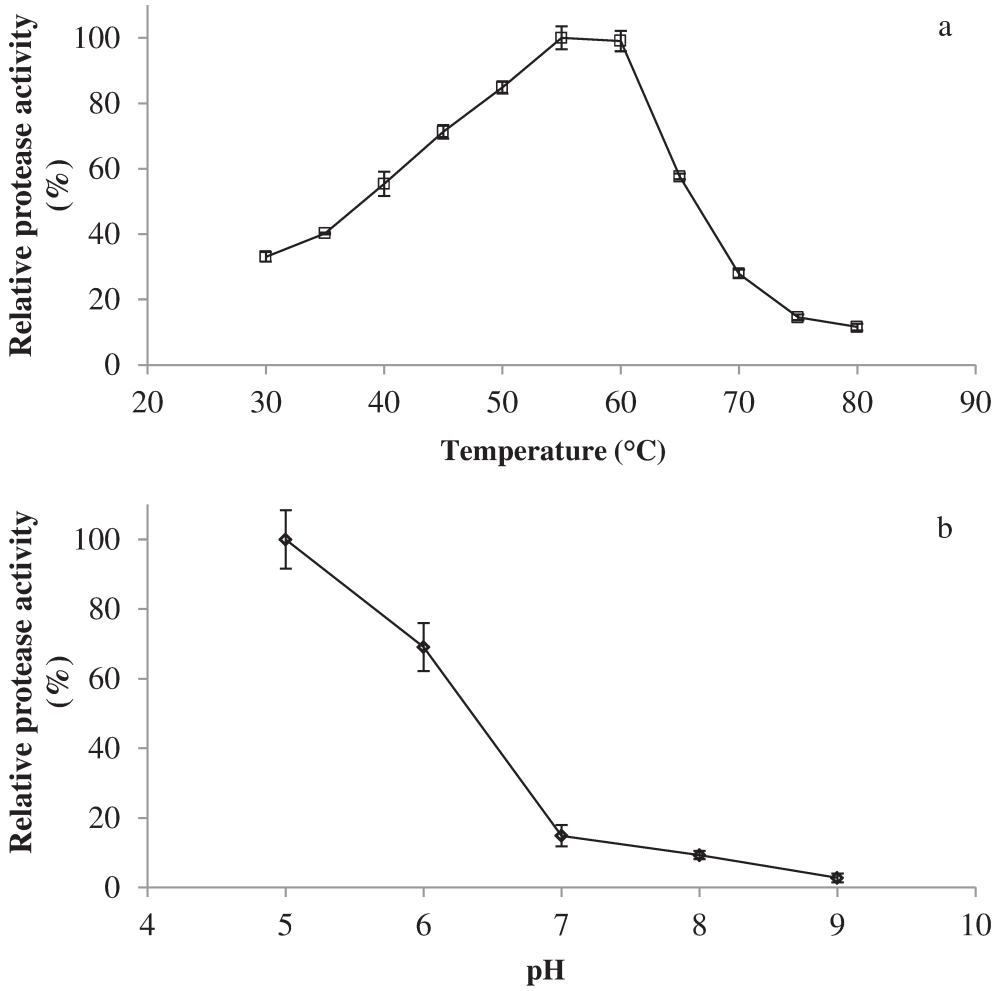

Fig. 2 - The effects of temperature (a) and pH (b) on the protease activity from *A. oryzae* LBA 01.

Table 6 shows the CCRD with its independent variables (pH and temperature) and the results for protease activity. The highest value obtained for the protease activity was observed in run 3 (3,961.30 $U.g^{-1}$), and the lowest was 880.76 $U.g^{-1}$ detected in run 6. The limited variability of the central points (runs 9-11) indicated good reproducibility of the experimental data.

Table 6 - The central composite rotatable design (CCRD) matrix for the determination of the optimum pH and temperature for the activity of the protease ($U.g^{-1}$) from *A. oryzae* LBA 01, with the coded and real values for the variables and responses.

Run	x_1/pH	x_2/Temperature (°C)	Protease activity ($U.g^{-1}$)[a]
1	-1 (5.3)	-1 (39.4)	2,151.34
2	+1 (6.7)	-1 (39.4)	921.91
3	-1(5.3)	+1 (60.6)	3,961.30
4	+1 (6.7)	+1 (60.6)	957.53
5	-1.41 (5.0)	0 (50.0)	2,703.55
6	+1.41 (7.0)	0 (50.0)	880.76
7	0 (6.0)	-1.41 (35.0)	1,361.51
8	0 (6.0)	+1.41 (65.0)	1,691.60
9	0 (6.0)	0 (50.0)	2,863.29
10	0 (6.0)	0 (50.0)	2,878.33
11	0 (6.0)	0 (50.0)	2,989.39

[a]The protease activities were measured using azocasein as the substrate.

The linear and quadratic terms for the pH and temperature as well as for the interaction demonstrated a significant effect on protease activity, with all *p*-values below 0.1 (Table 7).

Table 7 – Estimates for the coefficients obtained from the regression model in the CCRD for determination of the optimum pH and temperature for protease from *A. oryzae* LBA 01.

Factors	Coefficients	Standard error	*t*-value (5)	*p*-value
Intercept*	2,910.34	233.67	12.45	<0.001
pH (L)*	-851.38	143.09	-5.95	0.002
pH (Q)*	-474.43	170.31	-2.78	0.038
Temperature (L)*	289.05	143.09	2.02	0.099
Temperature (Q)*	-607.23	170.32	-3.56	0.016
pH x Temperature*	-443.59	202.36	-2.19	0.079

*Significant factors ($p < 0.1$)

An analysis of variance (ANOVA) showed that 92.0% of the total variation was explained by the model. The *F*-value computed (12.06) for the regression was greater than the tabulated *F*-value (3.45) (p value < 0.0001), reflecting the statistical significance of the model equation (Table 8).

Table 8 – ANOVA of the second-order polynomial model for the optimum activity ($U.g^{-1}$) of the protease from *A. oryzae* LBA 01.

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F* test
Regression	9,877,402.0	5	1,975,480.4	12.06
Residual	819,036.0	5	163,807.2	
Total	10,696,438.0	10		

$F_{0.1;\ 5,5}$ (*F* tabulated) = 3.45, R^2 = 0.92, *p*-value < 0.0001

Equation 3 (below) represents the second-order polynomial model for the experimental data:

$$\textit{Protease activity}\ (U.g^{-1}) = 2{,}910.34 - 851.38x_1 - 474.43x_1^2 + 289.05x_2 - 607.23x_2^2 - 443.59x_1.x_2 \quad (3)$$

The response surface and the contour plot were generated from the model. The protease from *A. oryzae* LBA 01 was more active in the pH range 5.0-5.5 and the temperature range 55-60°C. The protease activity increased as the temperature increased, indicating that the temperature had a positive effect. In contrast, the pH had a negative effect, with lower protease activity in the neutral region (Fig. 3).

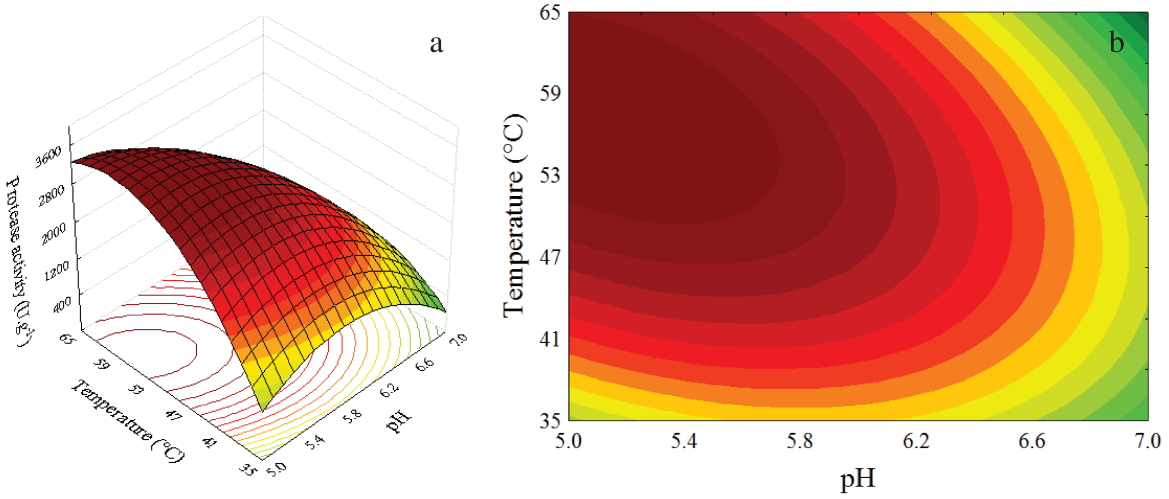

Fig. 3 - Response surface (a) and contour diagram (b) for the protease activity ($U.g^{-1}$) as a function of the pH and the temperature (°C).

The protease produced by *A. oryzae* LBA 01 demonstrated pH and temperature activity profiles similar to those of the acid proteases from *Rhizopus oryzae*, which showed optimum activity at pH 5.5 and 60°C, and from *A. oryzae* MTCC 5341, which showed more activity in the pH range 3.0-4.0 at 55°C [23,24]. The proteases from *Aspergillus parasiticus* MTCC 3558 showed optimum activity at pH 8.0 and 40°C, and the protease from *Aspergillus sojae* ATCC 42249 showed more activity at pH 9.0 and 70°C [25, 26].

Table 9 shows the experimental design used to determine the pH and temperature stability of the protease from *A. oryzae* LBA 01. The highest protease activity was 3,426.26 $U.g^{-1}$ in run 7, and the lowest value was 10.98 $U.g^{-1}$ in run 4. The central points (runs 9-11) showed little variation, reflecting good reproducibility of the experimental data.

Table 9 – The central composite rotatable design (CCRD) matrix used to determine the pH and temperature stability of the protease from *A. oryzae* LBA 01, with the coded and real values for the variables and responses.

Run	x_1 / pH	x_2 /Temperature (°C)	Protease activity ($U.g^{-1}$)[a]
1	-1 (4.4)	-1 (39.4)	3,135.09
2	+1 (6.6)	-1 (39.4)	2,464.69
3	-1 (4.4)	+1 (60.6)	1,363.33
4	+1 (6.6)	+1 (60.6)	10.98
5	-1.41 (4.0)	0 (50.0)	1,977.34
6	+1.41 (7.0)	0 (50.0)	616.06
7	0 (5.5)	-1.41 (35.0)	3,426.26
8	0 (5.5)	+1.41 (65.0)	20.31
9	0 (5.5)	0 (50.0)	2,753.73
10	0 (5.5)	0 (50.0)	2,757.18
11	0 (5.5)	0 (50.0)	2,753.95

[a]The protease activities were measured using azocasein as the substrate.

The estimated regression coefficients for the protease stability showed high statistical significance ($p < 0.05$). The pH and temperature showed negative effects on the protease stability, and the interaction was not significant (Table 10).

Table 10 – Estimated coefficients obtained from the regression model in the CCRD for stability of protease from *A. oryzae* LBA 01.

Factors	Coefficients	Standard error	t-value (5)	*p*-value
Intercept*	2,754.95	101.31	27.19	<0.001
pH (L)*	-493.49	62.04	-7.95	<0.001
pH (Q)*	-670.74	73.84	-9.08	<0.001
Temperature (L)*	-1,130.30	62.04	-18.22	<0.001
Temperature (Q)*	-457.45	73.84	-6.19	0.002
pH x Temperature	-170.49	87.74	-1.94	0.109

*Significant factors ($p < 0.05$)

The results of the analysis of variance (ANOVA) for the protease stability are shown in Table 11. The high value obtained for the coefficient of determination ($R^2 = 0.98$) indicated that the model could explain 98.0% of the variability in the experimental data. The value obtained in the *F* test (83.97) for the regression was 18.54 times higher than the critical value, indicating high significance ($p < 0.0001$).

Table 11 – ANOVA of the second-order polynomial model for the stability of the protease from *A. oryzae* LBA 01.

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F* test
Regression	15,127,478.0	4	3,781,869.5	83.97
Residual	270,238.0	6	45,039.67	
Total	15,397,716.0	10		

$F_{0.05;\ 4,6}$ (*F* tabulated) = 4.53, $R^2 = 0.98$, *p*-value < 0.0001

Equation 4 (below) represents the second-order polynomial model for the experimental data:

$$\textit{Protease activity}\ (U.g^{-1}) = 2{,}754.95 - 493.49x_1 - 670.74x_1^2 - 1{,}130.30x_2 - 457.45x_2^2 \quad (4)$$

The response surface and the contour plot of the second-order polynomial model were generated as a function of the independent variables (pH and temperature). An evaluation of the contour plot showed that the acid protease was more stable in the pH range 4.5-6.0 after 1h incubation at temperature range 35-45°C, under the assay conditions (Fig. 4).

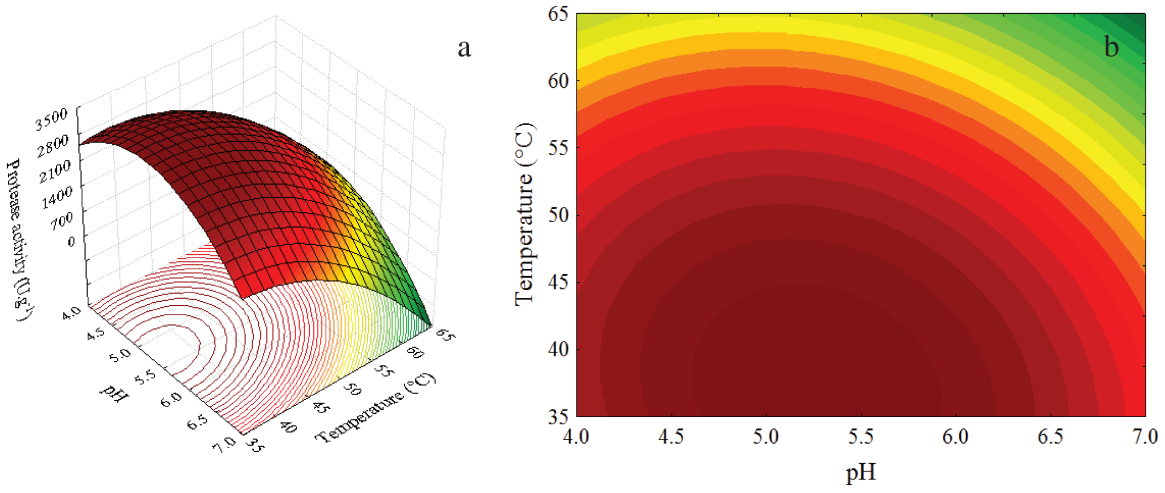

Fig. 4 – Response surface (a) and contour diagram (b) for the protease stability as a function of the pH and the temperature (°C).

In the enzyme kinetic studies, the protease from *A. oryzae* LBA 01 demonstrated the greatest affinity for the substrate hemoglobin, with *K*m and *V*max values estimated at 0.7 $mg.mL^{-1}$ and 2,764.5 $U.g^{-1}$, respectively. The *Km* and *V*max values of the protease for the substrates casein and azocasein were estimated at 4.9 and 5,446.3, and 2.5 $mg.mL^{-1}$ and 5,139.3, respectively (Table 12).

Table 12 - Kinetic parameters of the protease from *A. oryzae* LBA 01 using casein, azocasein and hemoglobin as substrates.

Substrate	*K*m ($mg.mL^{-1}$)	*V*max ($U.g^{-1}$)
Casein	4.9	5,446.3
Azocasein	2.5	5,139.3
Hemoglobin	0.7	2,764.5

The protease from *A. oryzae* LBA 01 retained more than 92.0% of its initial activity in the presence of the compounds $MgSO_4$, $CaCl_2$, NaCl, $(NH_4)_2SO_4$, glutathione, $Mg(NO_3)_2$, EDTA, p-chloromercuribenzoate, sodium azide and urea at concentrations from 0.05 to 5.0 mM. The addition of 5 mM $FeSO_4$ and $CoCl_2$ completely inhibited the protease activity. The addition of 5 mM $ZnSO_4$, $CuSO_4$, KCl, $CaCO_3$, cysteine, iodoacetamide and N-bromosuccinimide inhibited the protease with residual activities of 23.88%, 13.98%, 52.01%, 46.80%, 33.20% and 5.10%, respectively (Table 13). The enzyme was not

inhibited by the addition of EDTA, indicating that metal ions are not necessary for the activity of the protease from *A. oryzae* LBA 01. Morita et al. [27] reported that a protease from *A. oryzae* ATCC 42149 was activated in the presence of the metal ions Zn^{2+} and Co^{2+} at final concentrations of 1 mM. In the same study, the addition of 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+} inhibited the enzyme.

Table 13 - The effects of various salts and compounds on the protease activity.

Ions and inhibitors	**Residual activity (%)[a]**		
	0.05 mM	**0.5 mM**	**5.0 mM**
Control	100.00 ± 4.57		
$ZnSO_4$	96.59 ± 7.63	55.41 ± 3.82	23.88 ± 2.44
$MgSO_4$	97.10 ± 4.13	97.77 ± 4.10	97.21 ± 2.12
$FeSO_4$	90.45 ± 5.09	42.96 ± 3.44	*Undetected*
$MnSO_4$	105.99 ± 4.63	89.56 ± 4.67	80.87 ± 5.89
$CaCl_2$	95.03 ± 5.11	92.73 ± 5.31	93.96 ± 1.56
$CuSO_4$	82.68 ± 2.83	22.19 ± 0.61	13.98 ± 3.53
NaCl	99.39 ± 2.22	103.13 ± 2.01	100.29 ± 4.49
$CoCl_2$	88.71 ± 0.67	72.72 ± 4.51	*Undetected*
$(NH_4)_2SO_4$	100.66 ± 4.08	98.52 ± 3.34	95.73 ± 3.00
Glutathione	97.95 ± 3.97	97.29 ± 3.22	101.97 ± 3.36
Cysteine	98.26 ± 3.96	87.23 ± 2.76	46.80 ± 2.29
KCl	96.55 ± 4.93	91.62 ± 4.46	30.65 ± 4.77
$Mg(NO_3)_2$	98.01 ± 0.46	97.38 ± 3.16	94.31 ± 1.72
$MnCl_2$	101.74 ± 3.93	92.17 ± 8.80	73.83 ± 7.17
$CaCO_3$	101.65 ± 3.41	96.99 ± 1.36	52.01 ± 2.75
EDTA	101.52 ± 2.02	100.13 ± 3.47	99.25 ± 3.56
Iodoacetamide	79.98 ± 3.87	63.79 ± 2.51	33.20 ± 3.10
p-Chloromercuribenzoate	98.89 ± 4.55	97.52 ± 2.86	94.66 ± 2.47
Sodium azide	96.03 ± 0.26	97.20 ± 1.55	97.38 ± 5.20
N-bromosuccinimide	78.56 ± 3.76	10.56 ± 2.12	5.10 ± 1.40
Urea	96.73 ± 2.99	94.70 ± 3.43	92.58 ± 3.20

[a]Results are presented as the mean (n = 3) ± SD (standard deviation). The protease activities were measured using azocasein as the substrate.

4.3. Antioxidant activities of the protein hydrolysates

The antioxidant activity of the hydrolysates was evaluated using ORAC and DPPH assays. The ORAC assay has been broadly applied in academics and the food and supplement industry as a method of choice to quantify antioxidant capacity [28]. ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer. In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated easily by fluorescence [29]. In the study of the protease application to protein hydrolysis, the results showed that the antioxidant activity of the proteins increased after their enzymatic hydrolysis. For the ORAC assay, the enzymatic hydrolysis of SPI, BWP, and EWP by the protease from *A. oryzae* LBA 01 resulted in maximal antioxidant activity values of 645.20 ± 104.78, 172.11 ± 17.32 and 469.65 ± 47.80 Trolox EQ µmol.g^{-1}, respectively (Table 11). The best results were observed when 40.0 U protease per mL of reaction mixture was added to the protein solution. The antioxidant activity of the EWP hydrolysates prepared with the protease from *A. oryzae* LBA 01 increased the most compared with the control (Table 14).

Table 14 – The Trolox equivalents and linearity ranges for the ORAC assay (net AUC *vs.* concentration) performed on the control and protein hydrolysates.

Substrate	Protease (U.mL^{-1})	ORAC (Trolox EQ µmol.g^{-1})	Sample concentration range (mg.mL^{-1})	Slope	Intercept	R^2
SPI	Control	186.11 ± 48.45[a]	0.25-1.00	0.11	-0.07	0.94
	20.0	574.35 ± 46.15[b]	0.25-1.00	0.04	-0.08	0.99
	40.0	645.20 ± 104.78[b]	0.30-1.20	0.04	-0.22	0.99
BWP	Control	18.51 ± 7.20[a]	1.15-4.60	0.61	0.25	0.99
	20.0	132.61 ± 6.43[b]	1.00-4.00	0.14	-0.15	0.99
	40.0	172.11 ± 17.32[c]	1.20-4.80	0.13	-0.59	0.99
EWP	Control	47.18 ± 7.45[a]	1.05-4.20	0.34	-0.04	0.99
	20.0	423.43 ± 56.80[b]	0.50-2.00	0.06	-0.32	0.99
	40.0	469.65 ± 47.80[b]	0.50-2.00	0.05	-0.24	0.99

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. The comparisons were made between the values of each protein source (not between different protein sources). SPI: soy protein isolate; BWP: bovine whey protein; EWP: egg white protein.

Tavares et al. [30] investigated the antioxidant activity of bovine whey protein hydrolyzed by cardosins. The antioxidant activity of the whey protein hydrolysates was 0.96 ± 0.08 µmol Trolox EQ per mg hydrolyzed protein (ORAC values). Contreras et al. [31] tested the antioxidant activity of the whey protein concentrate hydrolyzed using Corolase PP® and thermolysin. The results showed that the higher enzyme:substrate ratio was best suited to produce the hydrolysates, resulting in antioxidant activity of 2.57 Trolox EQ µmol per mg protein (ORAC values).

DPPH radical is a stable free radical that shows maximal absorbance at 517 nm in ethanol and has been widely used to test the ability of natural compounds to act as free radical scavengers or hydrogen donors, as a means to evaluating their antioxidant potentials. When DPPH radical encounters a proton donating substance such as an antioxidant, the radicals would be scavenged and the absorbance is reduced [32, 33]. For the DPPH assay, the SPI and BWP hydrolysates prepared with the protease from *A. oryzae* LBA 01 showed higher antioxidant activity, with values of 4.61 ± 0.12 and 4.92 ± 0.09 Trolox EQ µmol.g^{-1}, respectively, compared with the EWP hydrolysates (Table 15). The greatest radical scavenging activity (73.62% at 5 mg.mL^{-1}) was observed in the BWP hydrolysates. For the SPI and BWP hydrolysates, the use of 20.0 U protease per mL of reaction mixture resulted in the maximum antioxidant activity. For the EWP hydrolysates, the hydrolysis had a negative effect, decreasing the antioxidant activity compared with the control (Table 15). The hydrolysis might be releasing the antioxidant peptides resulting in an increasing in antioxidant activity of the hydrolysates, while other treatments might product the peptides without antioxidant activities or hydrolysis the antioxidant peptides into amino acids and thus decrease the antioxidant activity of hydrolysate [34]. Zhang, Li and Zhou [35] investigated the production of antioxidant hydrolysates from soy protein using three commercial microbial proteases: neutral protease from *Bacillus subtilis*, validase from *Aspergillus oryzae* and alkaline protease from *Bacillus licheniformis*. The maximum ORAC value observed was 83.8 Trolox EQ µmol.g^{-1}, and the highest DPPH scavenging activity was 29.3% at 100 mg.mL^{-1}.

Table 15 – The protein hydrolysis by the protease from *A. oryzae* LBA 01 and the antioxidant activities of the hydrolysates in the DPPH assay.

Substrate	Protease ($U.mL^{-1}$)	DPPH (Trolox EQ $\mu mol.g^{-1}$)	DPPH radical scavenging (%)
SPI	Control	0.88 ± 0.14^{a}	30.77 ± 1.53^{a}
	20.0	4.61 ± 0.12^{b}	69.18 ± 1.54^{b}
	40.0	4.25 ± 0.08^{c}	66.43 ± 0.95^{c}
BWP	Control	2.44 ± 0.11^{a}	40.67 ± 0.28^{a}
	20.0	4.92 ± 0.09^{b}	73.62 ± 0.07^{b}
	40.0	4.53 ± 0.01^{c}	69.53 ± 0.07^{c}
EWP	Control	4.42 ± 0.11^{a}	63.03 ± 1.56^{a}
	20.0	1.89 ± 0.30^{b}	45.93 ± 3.17^{b}
	40.0	*Undetected*	33.99 ± 1.41^{c}

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. The comparisons were made between the values of each protein source (not between different protein sources). The DPPH assay was performed at 5 $mg.mL^{-1}$. SPI: soy protein isolate; BWP: bovine whey protein; EWP: egg white protein.

5. Conclusion

According to the optimization study, the best conditions for protease production by the strain *A. oryzae* LBA 01 were an initial moisture content of 50.0%, an inoculum level of 10^7 spores.g^{-1}, a temperature of 23°C, a medium that included 2.0% (w/w) peptone and 2.0% yeast extract (w/w), and a 72 h incubation using wheat bran as the substrate. The biochemical characterization using the CCRD showed that the protease was most active in the pH range 5.0-5.5 and stable from pH 4.5 to 6.0, indicative of an acid protease. The protease showed optimum temperature range for activity from 55-60°C, and it was stable after 1h incubation at 35-45°C. Enzyme kinetic studies resulted in *K*m ($mg.mL^{-1}$) and *V*max ($U.g^{-1}$) values of 2.5 and 5,139.3 for azocasein, 4.9 and 5,446.3 for casein and 0.7 and 2,764.5 for hemoglobin, respectively. The protease was not inhibited by 5 mM EDTA, indicating that metal ions were not necessary for enzyme activity. The enzyme was inhibited by 5.0 mM $ZnSO_4$, $CuSO_4$, KCl, $CaCO_3$, cysteine, iodoacetamide and N-bromosuccinimide, retaining 23.88%, 13.98%, 52.01%, 46.80%, 33.20% and 5.10% of its initial activity, respectively. The protease from *Aspergillus oryzae* LBA 01 can potentially

be applied to protein hydrolysis because it increased the natural antioxidant capacity of soy protein isolate, bovine whey protein and egg white protein.

Acknowledgements

The work described in this paper was substantially supported by grants from São Paulo Research Foundation – FAPESP (Project No. 2011/10429-9), which is gratefully acknowledged. Acknowledgements to the National Counsel of Technological and Scientific Development – CNPq by the granting of scholarship.

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Capítulo III: Antioxidant activities of soy protein isolate hydrolysates obtained using microbial proteases: optimization by response surface methodology

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Revista: LWT- Food Science and Technology

Abstract

The aim of this study was to evaluate the enzymatic hydrolysis of soy protein isolate (SPI) using different proteases to produce peptides with antioxidant activity. The hydrolysates were obtained using protease from *Aspergillus oryzae* LBA 01 (AO) produced by solid state fermentation and two commercial preparations: Flavourzyme® 500L from *Aspergillus oryzae* (CAO), and Alcalase® 2.4L from *Bacillus licheniformis* (CBL). A central composite rotatable design (CCRD) was used to assess the effect of the substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$ of reaction) concentrations on the antioxidant activity of the protein hydrolysates after 240 min of hydrolysis. Soy protein hydrolysates with higher antioxidant activities were obtained with the commercial protease CAO. The hydrolysis parameters, defined by the CCRD analysis, of a substrate concentration of 90.0 $mg.mL^{-1}$ and the addition of 70.0 U of protease per mL of reaction resulted in 775.17 ± 115.10 and 11.83 ± 0.31 Trolox EQ $\mu mol.g^{-1}$ for the ORAC and DPPH assays, respectively. The maximum antioxidant activities were observed between 120 and 180 min of hydrolysis, where the degree of hydrolysis and the residual protease activity were approximately 50.0%. The results suggest that the soy protein hydrolysates have potential for utilization as a natural source of antioxidants.

Keywords: Soy Protein Isolate; Response surface methodology; Enzymatic hydrolysis; Protease; Antioxidant activity.

1. Introduction

Proteins have a fundamental relevance as food components, both nutritionally and functionally. Nutritionally, they are a source of energy and amino acids, which are essential for growth and maintenance. In proteinaceous foods, they affect physicochemical and sensory properties, such as solubility, viscosity, gelation and emulsion stability. The presence of dietary proteins with specific biological properties make these components potential ingredients for functional food (Venugopal, 2009).

Processes involving protein hydrolysis have been studied for bioactive peptide production. Bioactive peptides can be defined as specific amino acid sequences that promote beneficial biological activities.

Bioactive peptides can be produced by enzymatic hydrolysis using digestive, microbial and plants enzymes, as well as by fermentation processes using proteolytic microorganisms. The limited and controlled proteolysis unfolds the protein chains, reducing the incidence of allergenic factors, thus resulting in the formation of small peptides with biological activities. The application of starter cultures with proteolytic activities improves the formation of bioactive peptides during the manufacture of fermented dairy products (Korhonen, 2009).

In the last decade, the enzymatic hydrolysis of proteins from animal and plant sources for the production of bioactive peptides has attracted much attention. The antioxidant activities of peptides are extensively reported in several studies. The mechanism of action of peptides with antioxidant properties is related to the inactivation of reactive oxygen species (ROS), scavenging of free radicals, chelation of prooxidative transition metals and reduction of hydroperoxides (Zhou et al., 2012).

According to Teng, Fang, Song, & Gao (2011), there is considerable evidence that free radicals play a critical role in causing some serious health disorders, such as cancer and cardiovascular diseases. Under normal conditions, free radicals can be effectively eliminated by antioxidant compounds and enzymes, such as superoxide dismutase and catalase. However, under pathological conditions, the balance between free radicals and antioxidant substances is upset, contributing to cell or tissue injury. In this context, peptides

can be used as sources of antioxidants to reduce the negative effects of free radicals in the human body.

It has been demonstrated that the hydrolysis of proteins from egg (Sakanaka, Tachibana, Ishihara, & Juneja, 2004), milk (Pihlanto, 2006), fish (Theodore, Raghavan, & Kristinsson , 2008), rice (Zhang et al., 2009), peanuts (Hwang, Shyu, Wang, & Hsu, 2010), and soybeans (Zhang, Li, & Zhou, 2010) resulted in peptides with antioxidant activities. It is important to note that peptides with distinct antioxidant activities are obtained when different protein sources are used. In addition, the antioxidant properties of peptides can be affected by the hydrolysis conditions: enzyme type, enzyme concentration, pH, temperature and hydrolysis time (Vastag et al., 2010).

In producing hydrolysates with antioxidant properties, the influence of the hydrolysis parameters on the antioxidant activities of the hydrolysates has been measured through the application of statistical experimental design techniques (Ren et al., 2008; Vastag et al., 2010; Sun, Luo, Shen, & Hu, 2011; Contreras et al., 2011; Zhou et al., 2012). Central composite rotatable design (CCRD) can be used as a valuable statistical and mathematical tool for the investigation of individual and interactive effect of the hydrolysis parameters on the progress of the enzymatic hydrolysis of different proteins (Vastag et al., 2010).

Information on the utilization of microbial enzymes for producing functional peptides from soy protein is scarce despite their abundance and commercial availability (Zhang, Li, & Zhou, 2010). In this study, we selected three microbial proteases: acid protease from *Aspergillus oryzae* LBA 01 produced under solid state fermentation, Flavourzyme® 500L from *Aspergillus oryzae*, and Alcalase® 2.4L from *Bacillus licheniformis*. Proteases from *Aspergillus oryzae* and *Bacillus licheniformis* are commonly used for protein hydrolysis, and they have considerably different substrate specialties and reaction characteristics. The main objective of the present study was to prepare hydrolysates by the enzymatic hydrolysis of soy protein isolate with different proteases and to investigate the antioxidant properties of the hydrolysates using methods such as ORAC- and DPPH-scavenging. The influence of the hydrolysis parameters, including substrate and protease concentrations, was evaluated by CCRD. In addition, the hydrolysis time was investigated in a univariate study under the optimal conditions determined from CCRD.

2. Materials and Methods

2.1. Reagents

Azocasein, trichloroacetic acid (TCA), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (97.0%) (AAPH), fluorescein, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available.

2.2. Enzymes

Three proteases were used in this study. Protease from *Aspergillus oryzae* LBA 01 (AO) (strain of culture collection of the Laboratory of Food Biochemistry, Faculty of Food Engineering, State University of Campinas) was obtained by solid state fermentation using wheat bran as a substrate in 500 mL Erlenmeyer flasks containing 40 g of cultivation medium. The cultivation parameters of moisture (50.0%), temperature (23°C), inoculum (10^7 spores.g^{-1}), peptone (2.0% w/w), and yeast extract (2.0% w/w) were previously determined. After 72 h of incubation, the fermented medium was homogenized with 150 mL of acetate buffer (0.2 M, pH 5.0), maintained at rest for 1 h and then filtered. The crude extract was concentrated by precipitation with ammonium sulfate (80.0%), dialysis and freeze-drying. The partial purified preparation was used for protein hydrolysis.

Commercial proteases Flavourzyme® 500L from *Aspergillus oryzae* (CAO) and Alcalase® 2.4L from *Bacillus licheniformis* (CBL) were purchased from Sigma Aldrich.

2.3. Determination of protease activity

The protease activity was measured using azocasein as a substrate, according to Charney, & Tomarelli (1948), with modifications. The reaction mixture was made up of 0.5 mL of azocasein (Sigma) (5 mg.mL^{-1}) in 0.05 M acetate buffer, pH 5.0), to which 0.5 mL of enzyme solution was added. The reaction was carried out under the optimum temperature and pH of each enzyme (AO: 55°C and pH 5.0; CAO: 50°C and pH 5.0 and CBL: 60°C and pH 7.0) and stopped after 40 min with the addition of 0.5 mL of TCA (0.6 mol.L^{-1}). Test tubes were centrifuged at 17,000 x *g* for 15 min at 25°C. A 1.0-mL aliquot of the supernatant was neutralized with 1.0 mL of 5N KOH and agitated before the

absorbance was measured at 428 nm. The control was prepared by adding 0.5 mL TCA (0.6 $mol.L^{-1}$) before the enzymatic solution. One unit of enzyme activity (U) was defined as the amount of enzyme required to cause an increase of 0.01 in absorbance at 428 nm under the assay conditions.

2.4. Preparation of protein hydrolysates: enzyme selection

Soy protein isolate (SPI) was used as a substrate for enzymatic hydrolysis. For the selection of the most appropriate enzyme to produce hydrolysates, the enzyme concentrations were adjusted to 0 (control), 20.0 and 40.0 U per mL of reaction, according to the activity of each protease, as previously determined. The proteins were suspended in a buffer to a final concentration of 100.0 $mg.mL^{-1}$. Fifty-milliliter aliquots of the mixtures were distributed in 125 mL Erlenmeyer flasks and incubated. The hydrolysis was carried out under the optimum temperature and pH of each enzyme for 240 min. After hydrolysis, the proteases were inactivated in a water bath at 100°C for 20 min. The peptide solution was separated from the residue by centrifugation at 17,000 x *g* at 5°C for 20 min. The supernatants were collected and freeze-dried for the determination of antioxidant activity.

2.5. Optimization of hydrolysis by response surface methodology

The Statistica® 8.0 software from Statsoft Inc. (Tulsa, Oklahoma, USA) was employed for experimental design, data analysis, and model building. A central composite rotatable design (CCRD) with three replicates at the central point and four axial points (with 11 runs) was used to determine the response pattern and establish a model. The 2 variables used in this study were the substrate ($mg.mL^{-1}$) (x_1) and protease ($U.mL^{-1}$ of reaction) (x_2) concentrations, while the dependent variables were the DPPH radical-scavenging activity and ORAC activity. The coded and real values of these variables are given in Table 1.

Table 1 – Independent variables and levels for the hydrolysis of SPI with proteases to increase antioxidant activity.

Independent variables	Levels				
	-α*	**-1**	**0**	**+1**	**+α**
[ ] Substrate (mg.mL^{-1})	33.6	50.0	90.0	130.0	146.4
[ ] Protease (U.mL^{-1})	9.8	20.0	45.0	70.0	80.2

*α = 1.41

The experiments were randomized to maximize the effects of unexplained variability in the observed responses because of extraneous factors. A second-order model equation was used for this model, represented by Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (1)$$

where Y is the estimated response, n is the number of variables, β_0 is the intercept term, βi is the linear coefficient, βij is the quadratic coefficient, and xi and xj are the coded independent variables. The coefficient of determination R^2 and the F test (analysis of variance-ANOVA) were used to verify the quality of fit of the second-order model equation. This step was carried out with the enzyme most appropriate for soy protein hydrolysis.

2.6. Determination of hydrolysis time

A kinetic study was used to determine the hydrolysis time for obtaining maximum antioxidant activity. The hydrolysis was carried out for 360 min, and samples were collected at different hydrolysis time points. The reactions were conducted similarly to that described above. During the hydrolysis, aliquots of the reaction mixture were collected for the determination of residual protease activity and the degree of hydrolysis.

2.7. Determination of the degree of hydrolysis (DH)

The DH of the SPI hydrolysates was determined with a modified version of the method described by Pericˇin et al., (2009). A 1.0 mL aliquot of the hydrolysates was added to an equal volume of 0.44 mol.L^{-1} trichloroacetic acid (TCA). The mixture was incubated

for 30 min at room temperature. Then, the mixture was centrifuged at 17,000 x *g* for 15 min. The obtained 0.22 mol.L^{-1} TCA-soluble protein fraction and the supernatant of the hydrolysate mixture (without the addition of TCA) were each analyzed to determine the protein content using the Lowry method (1951), which uses bovine serum albumin as the standard protein. The DH value, expressed as a percentage, was calculated as the ratio of 0.22 mol.L^{-1} TCA-soluble protein to total protein in the supernatant of hydrolysate mixture.

2.8. Determination of antioxidant activities

2.8.1. ORAC assay

The ORAC method, which uses fluorescein (FL) as the ‘‘fluorescent probe’’, was developed by Dávalos, Gómez-Cordovés, & Bartolomé (2004) and described by Macedo, Battestin, Ribeiro, & Macedo (2011). The automated ORAC assay was carried out on a Novo Star Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37°C; the reaction was started by the thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. A solution of FL (0.4 µg.mL^{-1}) in phosphate buffer (PBS) (75 mM, pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 µM Trolox solution, prepared daily in distilled water and diluted to 1500–1.5 µmol.L^{-1} solutions for the preparation of the Trolox standard curve. In each well, 120 µL of FL solution was mixed with either 20 µL of sample, blank (distilled water), or standard (Trolox solutions) before 60 µL of AAPH (108 mg.mL^{-1}) was added. The fluorescence was measured immediately after the addition of AAPH, and measurements were then taken every 1 min for 75 min. The measurements were taken in triplicate. ORAC values were calculated using the difference between the area under the FL decay curve and the blank (net AUC). Regression equations for the net AUC and antioxidant concentration were calculated for all samples. ORAC values were expressed as µmol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ µmol.g^{-1}) (Cao, Sofic, & Prior, 1996).

2.8.2. DPPH radical-scavenging activity

DPPH radical-scavenging activity of the hydrolysates was determined as described by Bougatef et al., (2009). An aliquot of 500 µL of each sample (5 $mg.mL^{-1}$) was mixed with 500 µL of 99.5% ethanol and 125 µL of DPPH (0.2 $mg.mL^{-1}$) in 99.5% ethanol. The mixture was then kept at room temperature in the dark for 60 min, and the reduction of the DPPH radical was measured at 517 nm using a UV-Visible spectrophotometer. The DPPH radical-scavenging activity was calculated as follows (Equation 2):

$$Radical\ scavenging\ activity(\%) = \left[\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right] * 100 \quad (2)$$

The control experiment was conducted in the same manner, except that distilled water was used instead of a sample. Trolox was used as a standard. The test was carried out in triplicate. The results of the tests were expressed as µmol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ µmol.g^{-1}).

2.9. Calculations and statistics

The statistical analyzes were performed using the Minitab® 16.1.1 software package from Minitab Inc. (USA). Values are expressed as the arithmetic mean. The Tukey test was used to verify for significant differences between the groups analyzed. The differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Selection of the most appropriate enzyme to produce hydrolysates

The enzyme preparations CAO, CBL and AO showed 4,295.83 $U.mL^{-1}$, 4,293.58 $U.mL^{-1}$ and 4,640.23 $U.g^{-1}$ of protease activity, respectively.

This study demonstrated that differences in the enzyme-substrate combination are responsible for different antioxidant activities of the hydrolyzed proteins. These differences could be attributable to the specificity of these enzymes on proteins, releasing peptides with different sizes, amino acid sequences and antioxidant activities (Contreras et al., 2011).

The results of the antioxidant assays (ORAC and DPPH) indicated an increased radical-scavenging capacity after enzymatic hydrolysis, suggesting the scavenging potential of the SPI hydrolysates. For the ORAC assay, the enzymatic hydrolysis of SPI with the protease CAO resulted in a maximum value of antioxidant activity (1,026.81 ± 181.57 Trolox EQ $\mu mol.g^{-1}$). The best results were observed when 40 U of proteases per mL of reaction were added to the protein solutions (Table 2). In contrast, for the DPPH assay, the hydrolysates obtained with 20 U of protease AO per mL of reaction showed the highest antioxidant activity (4.61 ± 0.12 Trolox EQ $\mu mol.g^{-1}$) (Table 3).

Zhang, Li, & Zhou (2010), investigated the production of antioxidant hydrolysates from soy protein using three commercial microbial proteases, namely neutral protease from *Bacillus subtilis*, Validase from *Aspergillus oryzae*, and alkaline protease from *Bacillus licheniformis*. The maximum ORAC value (83.8 Trolox EQ $\mu mol.g^{-1}$) was observed when the alkaline protease from *Bacillus licheniformis* was applied.

Table 2 – Trolox equivalents and linearity ranges for the ORAC assay (net AUC *vs*. concentration) performed on the control and SPI hydrolysate samples.

Enzyme	Protease ($U.mL^{-1}$)	ORAC (Trolox EQ $\mu mol.g^{-1}$)	Sample concentration range ($mg.mL^{-1}$)	Slope	Intercept	R^2
CAO	Control	174.42 ± 39.63[a]	1.30-5.20	0.17	-1.43	0.99
	20.0	790.64 ± 71.95[b]	0.25-1.00	0.03	-0.11	0.99
	40.0	1026.81 ± 181.57[b]	0.15-0.60	0.03	-0.13	0.98
CBL	Control	518.29 ± 116.84[a]	1.50-6.00	0.06	-0.19	0.99
	20.0	1023.01 ± 199.29[b]	0.15-0.60	0.03	-0.13	0.99
	40.0	904.98 ± 73.85[b]	0.13-0.52	0.03	-0.05	0.99
AO	Control	186.11 ± 48.45[a]	0.25-1.00	0.11	-0.07	0.94
	20.0	574.35 ± 46.15[b]	0.25-1.00	0.04	-0.08	0.99
	40.0	645.20 ± 104.78[b]	0.30-1.20	0.04	-0.22	0.99

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the values of each enzyme treatment (not between different enzymes). CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

Table 3 – Hydrolysis of SPI with proteases and the resulting antioxidant activities from the DPPH assay.

Enzyme	Protease ($U.mL^{-1}$)	DPPH (Trolox EQ $\mu mol.g^{-1}$)
CAO	Control	*Undetected*
	20.0	2.17 ± 0.03[a]
	40.0	1.07 ± 0.02[b]
CBL	Control	*Undetected*
	20.0	1.61 ± 0.11[a]
	40.0	1.92 ± 0.08[b]
AO	Control	0.88 ± 0.14[a]
	20.0	4.61 ± 0.12[b]
	40.0	4.25 ± 0.08[c]

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the values of each enzyme treatment (not between different enzymes). The DPPH assay was carried out at 5 $mg.mL^{-1}$. CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

When comparing the ORAC and DPPH values, the results showed no significant correlation (data not shown). This may be because the ORAC- and DPPH-scavenging assays have different reaction mechanisms. The DPPH compound is a stable free radical that has an unpaired valence electron at one atom of the nitrogen bridge and shows a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance reduced. Scavenging of the DPPH radical is the basis of the popular DPPH antioxidant assay (Guerard et al., 2007; Sharma, & Bath, 2009). The ORAC assay measures the antioxidant-scavenging activity against the peroxyl radical induced by AAPH at 37°C (Ou et al., 2001). The free radical causes damage to a fluorescent probe, decreasing the fluorescence intensity. The capacity of antioxidants to inhibit free radical damage is measured as the degree of protection against the change of probe fluorescence in the ORAC assay (Huang, Ou, & Hampsch-Woodi, 2002; Macedo, Battestin, Ribeiro, & Macedo, 2011). As such, a report of higher ORAC activity does not necessarily suggest a stronger DPPH-scavenging ability. In this context, the response for the ORAC assay was used to select the most appropriate enzyme. The protease CAO was selected for the optimization of the hydrolysis parameters by CCRD.

3.2. Hydrolysis optimization by response surface methodology

The CCRD with the independent variables (substrate and protease concentrations) and the results for antioxidant activity are presented in Table 4. For the ORAC assay, the highest value obtained for antioxidant activity was observed in run 2 (934.92 Trolox EQ $\mu mol.g^{-1}$), while the lowest value was 457.66 Trolox EQ $\mu mol.g^{-1}$, detected in run 1. For the DPPH assay, the highest value was 12.10 Trolox EQ $\mu mol.g^{-1}$, detected in run 8, while the lowest antioxidant activity was observed in run 1 (9.18 Trolox EQ $\mu mol.g^{-1}$). The little variation in the central points (runs 9-11), indicated good reproducibility of the experimental data.

Table 4 - CCRD matrix with coded and real values for the variables and responses for antioxidant activity of the SPI hydrolysates obtained with the protease CAO.

Runs	x_1 Substrate (mg.mL^{-1})	x_2 Protease (U.mL^{-1})	Antioxidant activity (Trolox EQ µmol.g^{-1})	
			ORAC	DPPH
1	-1 (50.0)	-1 (20.0)	457.66	9.18
2	+1 (130.0)	-1 (20.0)	934.92	9.87
3	-1 (50.0)	+1 (70.0)	836.30	11.29
4	+1 (130.0)	+1 (70.0)	864.02	11.00
5	-1.41 (33.6)	0 (45.0)	567.31	11.08
6	+1.41 (146.4)	0 (45.0)	823.75	10.86
7	0 (90.0)	-1.41 (9.8)	536.55	9.51
8	0 (90.0)	+1.41 (80.2)	811.86	12.10
9	0 (90.0)	0 (45.0)	627.35	11.79
10	0 (90.0)	0 (45.0)	689.74	11.38
11	0 (90.0)	0 (45.0)	667.63	11.17

In the ORAC assay, the linear terms of substrate (mg.mL^{-1}) and protease (U.mL^{-1}) as well as interaction, revealed significant effects on the protease activity with *p*-values less than 0.05. For the DPPH assay, the quadratic term of substrate concentration (mg.mL^{-1}) and the linear and quadratic terms of protease concentration (U.mL^{-1}) were statistically significant ($p < 0.1$) (Table 5).

Table 5 – Coefficient estimates for antioxidant activity by the regression model in CCRD.

Factors	Coefficients		Standard error		*t*-value (5)		*p*-value	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Intercept	661.58	11.45	37.39	0.245	17.68	46.756	0.000	0.000
x_1 (L)	108.45	0.012	22.90	0.149	4.73	0.076	0.005	0.942
x_1 (Q)	39.06	-0.38	27.25	0.178	1.43	-2.107	0.211	0.089
x_2 (L)	87.14	0.86	22.90	0.149	3.80	5.752	0.012	0.002
x_2 (Q)	28.40	-0.46	27.26	0.178	1.04	-2.573	0.345	0.049
$x_1 \times x_2$	-112.38	-0.24	32.39	0.212	-3.47	-1.147	0.018	0.303

The results of the analysis of variance (ANOVA) for the antioxidant activities are listed in Table 6. For the ORAC and DPPH assays, the coefficients of determination value

(R^2 = 0.87) indicated that the model can explain 87.0% of the experimental data's variability. The computed *F*-values for regressions were greater than the tabulated *F*-values, reflecting the statistical significance of the models (*p*-values < 0.05). Most of the residual can be explained by the lack of fit of the models, however, for both assays, the lack of fit was not significant at a confidence level of 95.0% (Table 6).

Table 6 – ANOVA of the regression model for antioxidant activity (Trolox EQ μmol.g^{-1}) of SPI hydrolysates.

Source of variation	Sum of squares		Degrees of freedom		Mean of squares		*F* test	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Regression	205,362.3	7.50	3	3	68,454.1	2.50	15.28	15.62
Residual	31,367.2	1.14	7	7	4,481.03	0.16		
Lack of fit	29,365.8	0.94	5	5	5,873.8	0.19	5.87	1.90
Pure error	2,001.4	0.20	2	2	1,000.7	0.10		
Total	236,729.5	8.64	10	10				

ORAC - Regression: $F_{0.05;3,7}$ (*F* tabulated) = 4.35, R^2 = 0.87, *p*-value = 0.002 / Residual: $F_{0.05;5,2}$ (*F* tabulated) = 19.30, *p*-value = 0.15.

DPPH - Regression: $F_{0,1;3,7}$ (*F* tabulated) = 3.07, R^2 = 0.87, *p*-value = 0.002 / Residual: $F_{0.1;5,2}$ (*F* tabulated) = 9.29, *p*-value = 0.38.

Equations 3 (ORAC) and 4 (DPPH) represent the models with the significant factors for the experimental data:

$$\textit{Antioxidant activity} = 710.64 + 108.45x_1 + 87.14x_2 - 112.38x_1x_2 \quad (3)$$

$$\textit{Antioxidant activity} = 11.45 - 0.38x_1^2 + 0.86x_2 - 0.46x_2^2 \quad (4)$$

where x_1 and x_2 are the coded values for the substrate and protease concentrations, respectively.

The surface response and contour plots of the models were generated as functions of the independent variables. For the ORAC assay, the maximum antioxidant activity was observed under two conditions: 1) substrate concentrations above 130.0 mg.mL^{-1} with enzyme concentrations in the range of 10.0 to 30.0 U.mL^{-1} of reaction and 2) substrate concentrations in the range of 30.0 to 70.0 mg.mL^{-1} with enzyme concentrations above 70.0 U.mL^{-1} of reaction (Fig. 1).

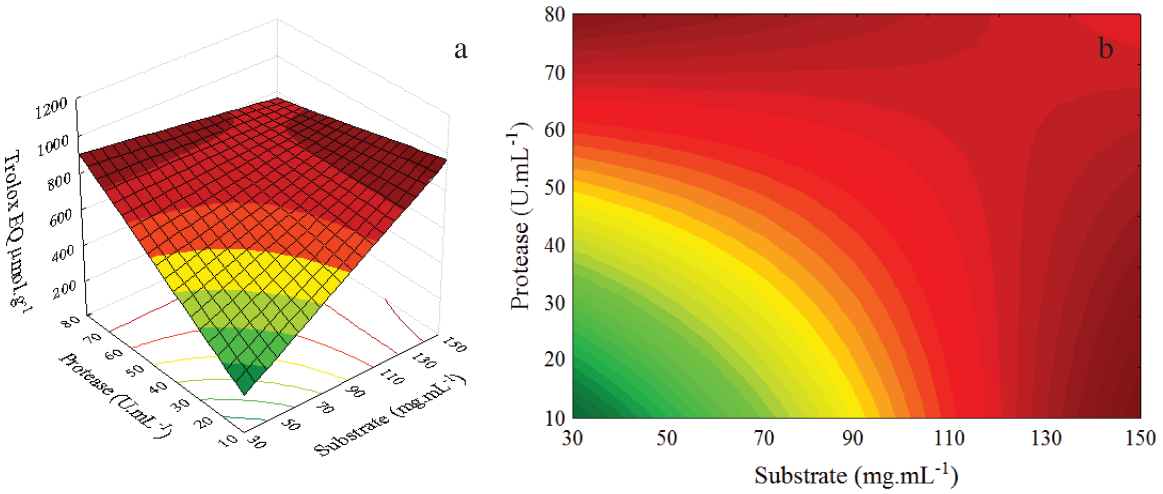

Fig. 1. Response surface (a) and contour diagram (b) for antioxidant activity (ORAC) as a function of the substrate (mg.mL^{-1}) and protease (U.mL^{-1}) concentrations.

The contour plot evaluation of the DPPH assay showed that the maximum antioxidant activity was obtained with substrate concentrations in the range of 70.0 to 110.0 mg.mL^{-1} and enzyme concentrations above 60.0 U.mL^{-1} of reaction mixture (Fig. 2).

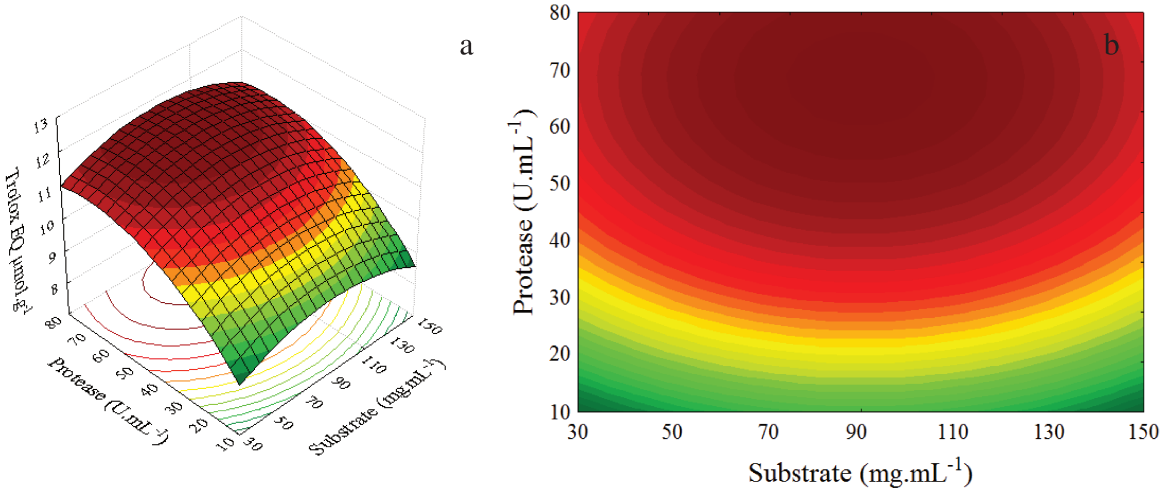

Fig. 2. Response surface (a) and contour diagram (b) for antioxidant activity (DPPH) as a function of the substrate (mg.mL^{-1}) and protease (U.mL^{-1}) concentrations.

The optimum conditions in coded and real values are presented in Table 7. The accuracy of the model was further tested by conducting hydrolysis experiments using intermediate values for increased antioxidant activity for the two responses, ORAC and DPPH, according to the superposition of contour diagrams (Fig. 3).

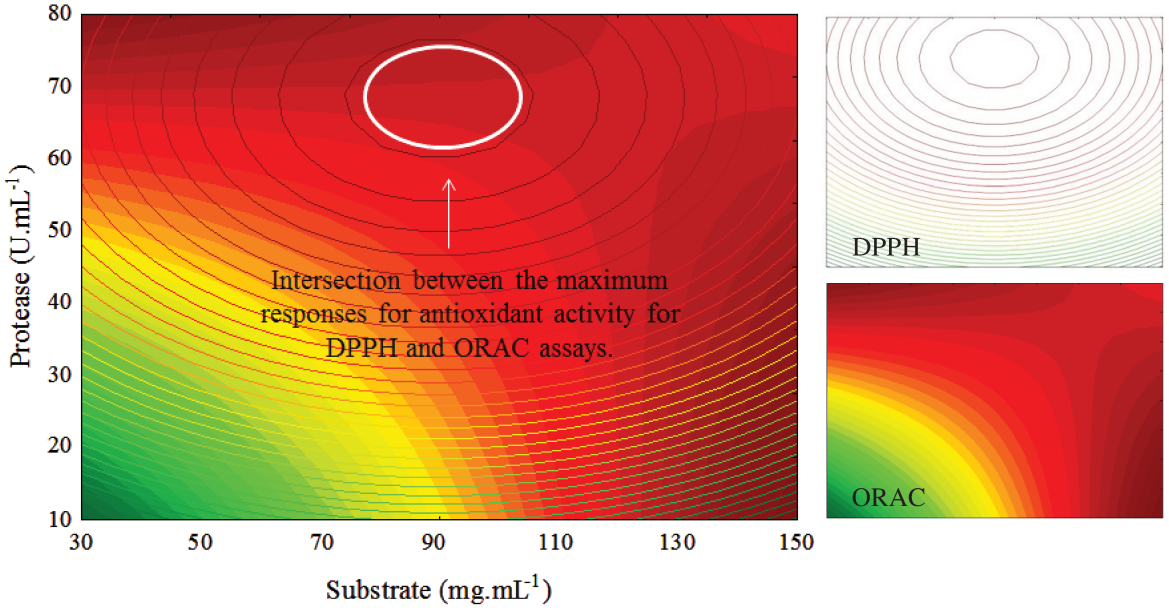

Fig. 3. Superposition of the contour diagrams for antioxidant activity (DPPH and ORAC assays) for the determination of the optimum conditions of hydrolysis.

The selected parameters were a substrate concentration of 90.0 $mg.mL^{-1}$ and the addition of 70.0 U of protease per mL of mixture reaction. According to the regression models (Equations 3 and 4), the predicted values for ORAC and DPPH in these conditions are 797.78 and 11.85 Trolox EQ $\mu mol.g^{-1}$, respectively. To confirm the validity of the model, three assays were performed under the optimal conditions given above. The experimental ORAC and DPPH values agreed with the values predicted by the model within a 95.0% confidence interval (Table 7).

Table 7 - Maximum antioxidant activity of the SPI hydrolysates and the corresponding values of the two processing parameters, substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$), obtained using CCRD.

Optimum processing conditions				
	ORAC		**DPPH**	
Independent variables	Substrate ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)	Substrate ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)
Experimental coded value	0	+1	0	+1
Experimental real value	90.0	70.0	90.0	70.0
Predicted response	797.78^{a}		11.85^{b}	
Experimental response*	775.17 ± 115.10^{a}		11.83 ± 0.31^{b}	

*Values are expressed as the mean ± standard deviation (n = 3), and those with different letters are significantly different, with $p < 0.05$.

3.3. Influence of the hydrolysis time in the antioxidant activity of the SPI hydrolysates

For the ORAC assay, the hydrolysis of the SPI was characterized by a high antioxidant activity within the first hour, when compared to the control sample. The highest value was observed at 120 min of hydrolysis (1,735.44 Trolox EQ $\mu mol.g^{-1}$). The antioxidant activity subsequently decreased after 120 min, but there was no significant difference ($p < 0.05$) between the results after this time (Fig. 4). The highest value observed in the DPPH assay was 18.09 Trolox EQ $\mu mol.g^{-1}$ for 300 min of hydrolysis. The statistical analysis showed there was no significant difference ($p < 0.05$) between the SPI hydrolysates obtained in ranges of 10 to 30, 60 to 240, and 180 to 360 min (Fig. 5).

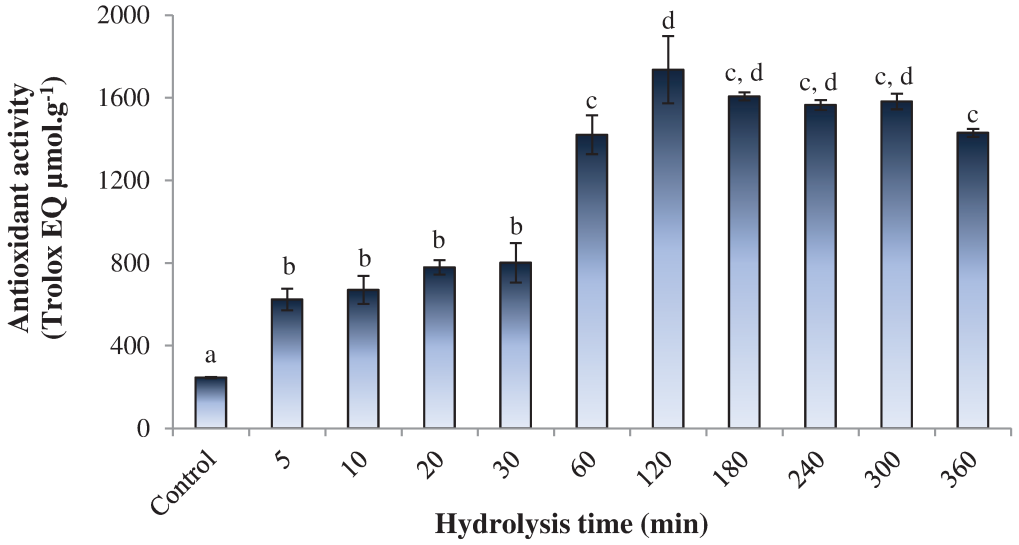

Fig. 4. Antioxidant activity (ORAC values) of the SPI hydrolysates for 360 min of hydrolysis. The results are presented as the mean (n =3) and those with different letters are significantly different ($p < 0.05$).

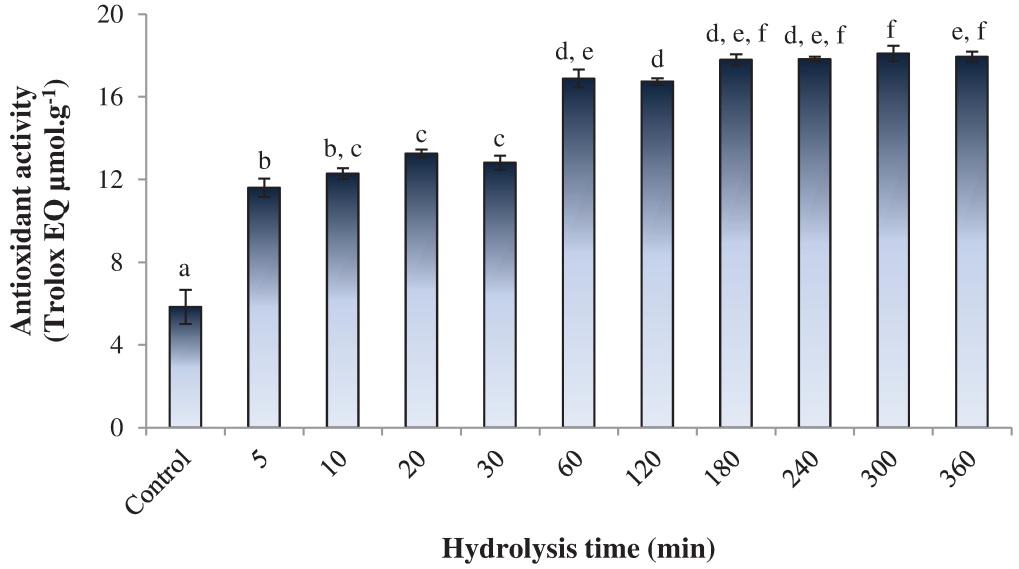

Fig. 5. Antioxidant activity (DPPH values) of the SPI hydrolysates for 360 min of hydrolysis. The results are presented as the mean (n = 3) and those with different letters are significantly different ($p < 0.05$).

The SPI hydrolysates were evaluated for their ability to scavenge DPPH radicals. The percent inhibition of DPPH radicals was measured by a decrease in absorbance at 517 nm. Inhibition of the DPPH radicals was more intense after 60 min of hydrolysis, reaching 66.18% at 300 min (Fig. 6). The results of antioxidant activity in the DPPH assay, expressed in Trolox EQ μmol.g^{-1}, and DPPH radical-scavenging (%) showed high correlation ($p < 0.05$) (data not shown).

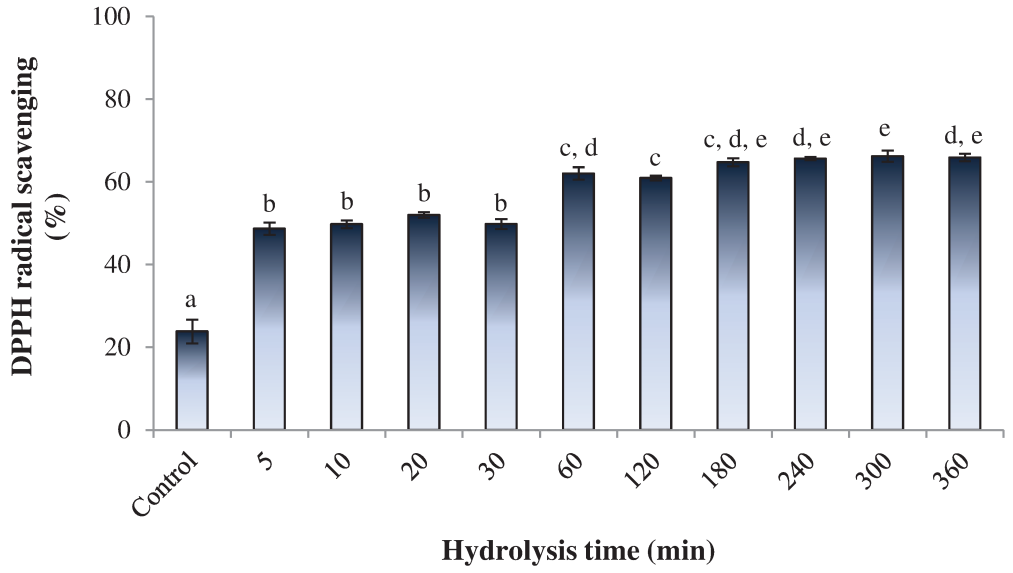

Fig. 6 – DPPH radical-scavenging (%) of the SPI hydrolysates for 360 min of hydrolysis. The results are presented as the mean (n = 3), and those with different letters are significantly different ($p < 0.05$).

Past research has shown a correlation between antioxidant activity and the DH of the protein substrate. In some studies, an increase in the DH of the protein hydrolysates increased antioxidant activity. However, other studies reported a decrease in antioxidant activity with an increase in DH. The maximum DH was observed after 60 min of reaction (54.01%) (Fig. 7). During hydrolysis, peptides with antioxidant properties could be continuously formed and degraded, depending on their molecular structure, which is primarily affected by the hydrolysis conditions (Vastag et al., 2010). The statistical analysis showed that there was no significant difference ($p < 0.05$) between the DH of the SPI hydrolysates obtained in range of 120 to 300 min (Fig. 7). The antioxidant values showed similar profiles; in the ORAC and DPPH assays, there were no significant differences ($p < 0.05$) between the SPI hydrolysates obtained in ranges of 120 to 300 min (Fig. 4) and 120

to 240 min (Fig. 5), respectively. Bougatef et al., (2009) used gastrointestinal proteases to hydrolyze smooth hound muscle protein for 4 h; they observed a high rate of hydrolysis for the first hour, which subsequently decreased, reaching a steady-state phase after 2 h. Theodore, Raghavan, & Kristinsson (2008) prepared protein hydrolysates from catfish protein isolates with a commercial protease, achieving 5.0, 15.0, and 30.0% degrees of hydrolysis. In these studies, the protein hydrolysates showed a decrease in DPPH radical-scavenging ability with increasing degrees of hydrolysis. Opposite results were observed in the ORAC assay; antioxidant activity increased with increasing DH. Raghavan, & Kristinsson (2008) studied the antioxidant activity of alkali-treated tilapia protein hydrolyzed with five different enzymes and observed that the ability of hydrolysates to inhibit the DPPH radical increased with increasing DH (7.5 < 15.0 < 25.0%).

The residual protease activity was measured during the enzymatic hydrolysis. The protease CAO retained approximately 99.8 ± 2.56% of the initial activity after 60 min of hydrolysis at 50°C. After this time, the protease activity showed a subsequent decrease, retaining only 24.40 ± 0.30% activity at the end of the process (Fig. 7). Parameters of the process, such as temperature and time, should be considered to justify the decrease in protease activity. As the enzymatic hydrolysis was carried out at 50°C for a longer time, the protease inactivation could have been caused by enzyme denaturation. Several studies reported a lower stability of proteases at temperatures around 50°C. Chien et al., (2002) studied the stability of protease from *Aspergillus sojae* and observed that 50.0% activity was retained after 1 h incubation at 50°C. García-Gómez, Huerta-Ochoa, Loera-Corral, & Prado-Barragán (2009) performed a comparative study between a protease produced by *Aspergillus oryzae* 2095 by solid-state fermentation and a commercial protease (Flavourzyme 500 MG®). The enzymes were subjected to a thermic treatment at 50°C for 2 h; the residual activity was 22.0% for the protease from *Aspergillus oryzae* 2095, while Flavourzyme 500 MG® was almost inactivated.

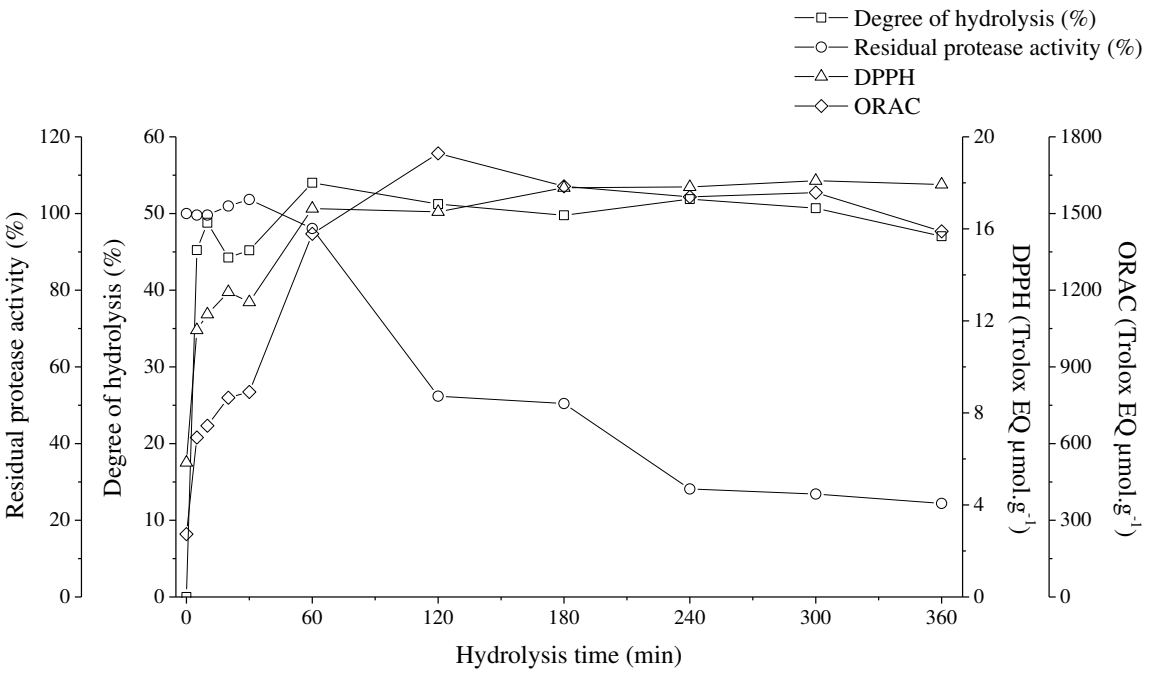

Fig. 7. Degree of hydrolysis (%), ORAC and DPPH values of SPI hydrolysates and residual protease CAO for 360 min of hydrolysis.

4. Conclusion

The results from this research demonstrated the viability of the applications of specific microbial proteases from *Aspergillus oryzae* LBA 01 and two commercial preparations for protein hydrolysis to increase the antioxidant capacity of SPI. The commercial protease Flavourzyme® 500L from *A. oryzae* (CAO) was selected as the most appropriate enzyme for SPI hydrolysate production. The hydrolysis parameters defined by the CCRD analysis were: a substrate concentration of 90.0 $mg.mL^{-1}$ and the addition of 70.0 U of protease CAO per mL of reaction resulted in 775.17 ± 115.10 and 11.83 ± 0.31 Trolox EQ $\mu mol.g^{-1}$, as measured by the ORAC and DPPH assays, respectively. The maximum antioxidant activities were observed in the range of 120 to 180 min of hydrolysis, where the degree of hydrolysis and the residual protease activity were approximately 50.0%. These results suggest that the soy protein hydrolysates have potential for utilization as a natural source of antioxidants.

Acknowledgements

The work described in this paper was substantially supported by grants from São Paulo Research Foundation – FAPESP (Project No. 2011/10429-9), which is gratefully acknowledged. Acknowledgements to the National Counsel of Technological and Scientific Development – CNPq by the granting of scholarship.

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Capítulo IV: Antioxidant activities of whey protein hydrolysates obtained by enzymatic hydrolysis

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Revista: International Dairy Journal

Abstract

Whey protein is a byproduct of the dairy industry that has high nutritional, functional and biological value. Enzymatic hydrolysis, which is a novel application of whey protein that is used to generate bioactive peptides with antioxidant properties, has attracted a great deal of attention. The aim of this study was to evaluate the effects of the enzymatic hydrolysis of bovine whey protein on its antioxidant properties. The hydrolysates were prepared with a protease from *Aspergillus oryzae* LBA 01 (AO) that was produced by solid-state fermentation and two commercial proteases: Flavourzyme® 500L from *Aspergillus oryzae*, and Alcalase® 2.4L from *Bacillus licheniformis* (CBL). A central composite rotatable design (CCRD) was used to assess the influence of the substrate ($mg.mL^{-1}$), and protease ($U.mL^{-1}$ of reaction) concentrations on the antioxidant activities of the protein hydrolysates after 240 min of hydrolysis. The results of this study demonstrate the viability of using the protease from *Aspergillus oryzae* LBA 01 (AO) to increase the antioxidant capacity of whey protein compared to two commercial protease preparations. The hydrolysis parameters were defined according to the CCRD analysis: substrate concentration of 80.0 $mg.mL^{-1}$ and addition of 70.0 U of protease per mL of reaction, resulting in 424.32 and 16.39 Trolox EQ $\mu mol.g^{-1}$ for the ORAC and DPPH assays, respectively. The maximum antioxidant activities were observed from 60 to 240 min of hydrolysis, where the degree of hydrolysis and residual protease activity were approximately 44.0% and 50.0%, respectively.

Keywords: antioxidant activity, bioactive peptides, hydrolysis, optimization**,** whey protein

1. Introduction

Whey protein is an abundant, low-cost byproduct of the dairy industry that has high nutritional, functional and biological value (Contreras et al., 2011). As a result of the expansion of whey production worldwide, there is a growing interest in novel applications of this byproduct (Pihlanto, 2006). Among these applications, the enzymatic hydrolysis of whey protein, which is used to generate bioactive peptides with antioxidant properties, has been reported (Tavares et al., 2011, Contreras et al., 2011). It is postulated that the antioxidant characteristics of peptides comes from their abilities to inactivate reactive oxygen species (ROS), scavenge free radicals, chelate pro-oxidative transition metals, and reduce hydroperoxides. These mechanisms are based on their inherent amino acid compositions and peptide sequences; they usually consist of 5–11 amino acids, including hydrophobic amino acids, proline, histidine, tyrosine and tryptophan (Korhonen & Pihlanto, 2006; Pihlanto, 2006; Elias, Kellerby, & Decker, 2008; Zhou et al., 2011).

Currently, the use of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) is restricted because of their toxic effects on human enzyme systems. On the other hand, bioactive peptides are considered natural antioxidants and have attracted a great deal of interest because of their safety and wide distribution properties (Zhang et al., 2009). Therefore, processes involving the production of bioactive peptides by enzymatic hydrolysis are very relevant to current scientific studies.

When attempting to produce hydrolysates with antioxidant properties, the influence of hydrolysis parameters such as the type and enzyme concentration, substrate concentration, and hydrolysis time on the antioxidant activities of hydrolysates must be determined (Vastag et al., 2010). The response surface methodology (RSM) also known as CCRD (central composite rotatable design) is a statistical tool that is used for multiple regression analyses using quantitative data that are obtained from properly designed experiments to solve multivariate equations simultaneously (Box, Hunter & Hunter, 1978; Khuri & Cornell, 1987; Colla et al., 2010). This methodology is a valuable tool for investigating the individual and interactive effects of various hydrolysis parameters on the enzymatic hydrolysis of different proteins, allowing the optimization of process parameters

to maximize the antioxidant activities of protein hydrolysates (Vastag et al., 2010; Contreras et al., 2011).

The main objective of the present study was to prepare hydrolysates by the enzymatic hydrolysis of bovine whey protein with a protease from *Aspergillus oryzae* LBA 01 and to make a comparative study with the commercial proteases Flavourzyme® 500L from *Aspergillus oryzae* and Alcalase® 2.4L from *Bacillus licheniformis* to investigate the antioxidant properties of hydrolysates by the ORAC and DPPH scavenging assays. The influence of hydrolysis parameters, including substrate and protease concentrations, was evaluated by the CCRD. In addition, the hydrolysis time and residual protease activity were investigated in a univariate study under optimal conditions, as determined in the CCRD.

2. Materials and Methods

2.1. Reagents

Azocasein, trichloroacetic acid (TCA), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (97.0%) (AAPH), fluorescein, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in commercially available grades.

2.2. Enzymes

A protease from *Aspergillus oryzae* LBA 01 (AO) (strain obtained from the culture collection of the Laboratory of Food Biochemistry, Faculty of Food Engineering, State University of Campinas) was obtained by solid state fermentation using wheat bran as a substrate in 500-mL Erlenmeyer flasks containing 40 g of cultivation medium. The following predefined cultivation parameters were used: 50.0% moisture, 23°C temperature, inoculum of 10^7 spores.g^{-1}, 2.0% (w/w) peptone, and 2.0% (w/w) yeast extract. After 72 h of incubation, the fermented medium was homogenized with 150 mL of acetate buffer (0.2M, pH 5.0), maintained at rest for 1 h and then filtered. The crude extract was concentrated by ammonium sulfate (80.0%) precipitation, dialysis and freeze-drying. The partial purified preparation was used for protein hydrolysis experiments.

The commercial proteases Flavourzyme® 500L from *Aspergillus oryzae* (CAO) and Alcalase® 2.4L from *Bacillus licheniformis* (CBL) were purchased from Sigma Aldrich.

2.3. Determination of protease activity

Protease activity was measured using azocasein as a substrate, according to Charney & Tomarelli (1948) with some modifications. The reaction mixture was composed of 0.5 mL of azocasein (Sigma) (0.5% w/v) in 0.05 M acetate buffer (pH 5.0), to which 0.5 mL of enzyme solution was added. The reaction was carried out in the optimal pH and temperature conditions for each enzyme (AO: 55°C, pH 5.0; CAO: 50°C, pH 5.0; and CBL: 60°C, pH 7.0) and stopped after 40 min with the addition of 0.5 mL of 10.0% TCA. Test tubes were centrifuged at 17,000 x *g* for 15 min at 25°C. An aliquot of 1.0 mL of each supernatant was neutralized with 1.0 mL of 5 N KOH. After agitation, the absorbance was

measured at 428 nm. The control sample was prepared by adding 0.5 mL TCA (10.0%) before the enzymatic solution. One unit of enzyme activity (U) was defined as the amount of enzyme required to cause an increase of 0.01 in the absorbance at 428 nm under the assay conditions.

2.4. Preparation of protein hydrolysates: enzyme selection

Bovine whey protein was donated by Alibra Ingredients, Ltd. To select for the most appropriate enzyme to produce the hydrolysates, the enzyme concentrations were adjusted to 0 (control), 20.0 and 40.0 U per mL of reaction, according to the activity of each protease, which was determined previously. The proteins were suspended in buffer to a final concentration of 100.0 $mg.mL^{-1}$. A total of 50 mL of each mixture was distributed in 125-mL Erlenmeyer flasks and incubated. The hydrolysis was carried out at the optimal temperature and pH conditions for each enzyme for 240 min. After hydrolysis, the proteases were inactivated in a water bath at 100°C for 20 min. The soluble peptides were separated from the residue by centrifugation at 17,000 x *g* at 5°C for 20 min. The supernatants were collected and freeze-dried to determine the antioxidant activities.

2.5. Optimization of hydrolysis by response surface methodology

The software Statistica® 8.0 from Statsoft, Inc. (Tulsa, Oklahoma, USA) was used for the experimental design, data analysis, and model building. A CCRD with three replicates at the central point and four axial points (with 11 runs) was used to determine the response pattern and establish a model. The 2 variables used in this study were substrate ($mg.mL^{-1}$) (x_1) and protease ($U.mL^{-1}$ of reaction) (x_2) concentrations, while the dependent variables were the DPPH radical scavenging activity and ORAC activity, respectively. The coded and real values of these variables are given in Table 1.

Table 1 – Independent variables and hydrolysis levels of whey protein with various proteases to increase antioxidant activity.

Independent variables	**Levels**				
	-α*	**-1**	**0**	**+1**	**+α**
[ ] Substrate ($mg.mL^{-1}$)	51.3	80.0	150.0	220.0	248.7
[ ] Protease ($U.mL^{-1}$)	9.8	20.0	45.0	70.0	80.2

*α =1.41

The experiments were randomized to maximize the effects of unexplained variability in the observed responses due to extraneous factors. A second-order model equation was used for this model, as represented by Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (1)$$

where Y is the estimated response, i and j have values from 1 to the number of variables (n), β_0 is the intercept term, βi is the linear coefficient, βij is the quadratic coefficient, and xi and xj are the coded independent variables. The coefficient of determination R^2 and F test (analysis of variance-ANOVA) were used to verify the quality of fit of the second-order model equation. This step was carried out with the enzyme that was most appropriate for obtaining whey protein hydrolysates with higher antioxidant activities.

2.6. Effect of incubation time and obtaining hydrolysates with maximum antioxidant activity

A kinetic study was used to determine the hydrolysis time at the point of maximum antioxidant activity. The hydrolysis was carried out for 360 min, and samples were collected at different incubation times. The reactions of enzymatic hydrolysis proceeded in a manner similar to the experiments described above.

2.7. Determination of the degree of hydrolysis (DH)

The DH of the whey protein hydrolysates was determined according to Peric˘in et al. (2009) with modifications. Mixtures of 1.0 mL of whey protein hydrolysates and 1.0 mL of 0.44 mol.L^{-1} trichloroacetic acid (TCA) were incubated for 30 min at room temperature. Then, each mixture was centrifuged at 17,000 x *g* for 15 min. The 0.22 mol.L^{-1} TCA-soluble protein fraction and the supernatant of the hydrolysate mixture (in the absence of TCA) were analyzed to determine the protein content by the method described by Lowry (1951) using bovine serum albumin as a protein standard. The DH value was calculated as the ratio of 0.22 mol.L^{-1} TCA-soluble protein to the total protein in the supernatant of the hydrolysate mixture, expressed as a percentage.

2.8. Determination of antioxidant activities

2.8.1. ORAC assay

The ORAC method used in this study, with fluorescein (FL) as the "fluorescent probe," was determined according Dávalos, Gómez-Cordovés, & Bartolomé (2004) and described by Macedo et al. (2011). The automated ORAC assay was carried out on a Novo Star Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96-well plate. The reaction was performed at 37°C and was started by the thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. The FL solution (0.4 $\mu g.mL^{-1}$) in phosphate buffer (75 mM PBS, pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 µM Trolox solution that was prepared daily in distilled water and diluted to 1,500–1.5 $\mu mol.L^{-1}$ solutions to prepare the Trolox standard curve. In each well, 120 µL of FL solution was mixed with either 20 µL of sample, blank (distilled water) or standard (Trolox solutions) before 60 µL of AAPH (108 $mg.mL^{-1}$) was added. The fluorescence was measured immediately after the addition of AAPH, and measurements were taken every minute for 75 min. The measurements were made in triplicate. The ORAC values were calculated using the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between the net AUC and antioxidant concentration were calculated for all of the samples. The ORAC values were expressed as µmol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ $\mu mol.g^{-1}$) (Cao, Sofic, & Prior, 1996).

2.8.2. DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bougatef et al. (2009). An aliquot of 500 µL of each sample at 5 $mg.mL^{-1}$ was mixed with 500 µL of 99.5% ethanol and 125 µL of 0.02% DPPH in 99.5% ethanol. The mixture was kept at room temperature in the dark for 60 min, and the reduction of the DPPH radical was measured at 517 nm using a UV-visible spectrophotometer. The DPPH radical-scavenging activity was calculated as follows (Equation 2):

$$Radical\ scavenging\ activity(\%) = \left[\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right] * 100 \quad (2)$$

The control was conducted in the same manner, except that distilled water was used instead of a sample. Trolox was used as a standard. The test was carried out in triplicate. The results of the tests were expressed as µmol of Trolox equivalent.g^{-1} of protein hydrolysates (Trolox EQ µmol.g^{-1}).

2.9. Calculations and statistics

The statistical analyzes were performed using the Minitab® 16.1.1 software package from Minitab Inc. (USA). Values are expressed as arithmetic means. Tukey's test was used to assess the significant differences among the analyzed groups. The differences were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Selection of the most appropriate enzyme to produce the whey protein hydrolysates

The enzyme preparations of CAO, CBL and AO had 4,295.83 $U.mL^{-1}$, 4,293.58 $U.mL^{-1}$ and 4,640.23 $U.g^{-1}$ of protease activity, respectively.

The ORAC assay has been broadly applied in academic and the food and supplement industries as the method of choice to quantify antioxidant capacity (Huang, Ou, & Prior, 2005). ORAC measures the antioxidant inhibition of peroxyl radical-induced oxidations, thus reflecting classical radical chain-breaking antioxidant activity by H atom transfer. In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated by fluorescence measurements (Prior, Wu, & Schaich, 2005). The antioxidant capacity (ORAC values) of whey protein increased between 2.0-fold and 9.3-fold after hydrolysis with the different proteases. The results obtained in our study are consistent with the results of Ren et al. (2008), who found that the antioxidant activities of grass carp sarcoplasmic hydrolysates that were prepared with five proteases showed different antioxidant properties. Contreras et al. (2011) hydrolyzed a whey protein concentrate using Corolase PP and Thermolysin to produce hydrolysates with antioxidant activities. The ORAC values in the hydrolysates obtained by Thermolysin were 2-fold greater than those obtained by Corolase PP under the same hydrolysis conditions. The differences observed in the antioxidant activities of hydrolysates when hydrolysis was carried out with different proteases could be attributed to the specificity of these enzymes on the protein substrate and the release of peptides of different sizes, amino acid sequences and antioxidant activities (Contreras et al., 2011). The largest increase was observed for the protease AO, reaching 172.11 Trolox EQ $\mu mol.g^{-1}$, a value that was 9.3-fold higher than the control reaction. The coefficients of determination (R^2) in the ORAC assay showed a good linearity between the net AUC and the sample concentrations in the ranges evaluated (Table 2).

Table 2 – Trolox equivalents and linearity ranges for the ORAC assay (net AUC *vs*. concentration) for control and whey protein hydrolysate samples.

Enzyme	Protease ($U.mL^{-1}$)	ORAC (Trolox EQ $\mu mol.g^{-1}$)	Sample concentration range ($mg.mL^{-1}$)	Slope	Intercept	R^2
CAO	Control	32.59 ± 1.61^{a}	6.00-12.00	0.67	-0.41	0.97
	20.0	133.79 ± 6.23^{b}	1.30-5.20	0.17	-0.63	0.99
	40.0	137.14 ± 19.40^{b}	1.30-5.20	0.17	-0.77	0.99
CBL	Control	70.95 ± 16.96^{a}	1.25-5.00	0.20	0.13	0.99
	20.0	136.74 ± 9.50^{b}	1.25-5.00	0.17	-0.57	0.99
	40.0	145.04 ± 19.09^{b}	1.30-5.20	0.19	-1.03	0.99
AO	Control	18.51 ± 7.20^{a}	1.15-4.60	0.61	0.25	0.99
	20.0	132.61 ± 6.43^{b}	1.00-4.00	0.14	-0.15	0.99
	40.0	172.11 ± 17.32^{c}	1.20-4.80	0.13	-0.59	0.99

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different ($p < 0.05$). Comparisons were made between the values of each enzyme treatment (not between different enzymes). CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

DPPH is a relatively stable organic radical that is characterized by a deep purple color and a maximum absorbance at 515–520 nm. When DPPH encounters a proton-donating substance, the radical is scavenged, and the absorbance is reduced. Therefore, DPPH is widely used as a substrate to evaluate the efficacy of antioxidants (Gao, Cao, & Li, 2010). The results in Table 3 indicate that the antioxidant capacity of whey protein increased after enzymatic hydrolysis, as observed in the ORAC assay. For the microbial enzymes that were evaluated, the antioxidant activity increased approximately 2-fold, and the highest level of DPPH radical scavenging (73.62%) was observed in whey protein hydrolysates that were prepared with AO (Table 3).

Table 3 – Hydrolysis of whey protein with proteases and antioxidant activities for the DPPH assay.

Enzyme	Protease ($U.mL^{-1}$)	DPPH (Trolox EQ $\mu mol.g^{-1}$)	DPPH radical scavenging (%)
CAO	Control	0.79 ± 0.05^{a}	39.40 ± 1.77^{a}
	20.0	1.20 ± 0.05^{b}	56.07 ± 2.93^{b}
	40.0	1.68 ± 0.07^{c}	61.24 ± 1.94^{c}
CBL	Control	0.61 ± 0.01^{a}	36.43 ± 0.29^{a}
	20.0	1.35 ± 0.05^{b}	55.01 ± 1.15^{b}
	40.0	1.25 ± 0.04^{c}	57.22 ± 1.18^{b}
AO	Control	2.44 ± 0.11^{a}	40.67 ± 0.28^{a}
	20.0	4.92 ± 0.09^{b}	73.62 ± 0.07^{b}
	40.0	4.53 ± 0.01^{c}	69.53 ± 0.07^{c}

[a, b, c] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different ($p < 0.05$). Comparisons were made between the values of each enzyme treatment (not between different enzymes). The DPPH assay was carried out at 5 $mg.mL^{-1}$. CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

Based on these results, it can be concluded that the protease from *A. oryzae* LBA 01 (AO) can be used to obtain a whey protein hydrolysate with maximum antioxidant activity; therefore, this enzyme was selected to perform the remaining experimental reactions.

3.2. Optimization of whey protein hydrolysis by response surface methodology

To optimize the process of hydrolysis, it is necessary determine the conditions and variables that significantly affect the antioxidant activities of a protein hydrolysate. In this study, all 11 experimental points were evaluated, and the mean results for the ORAC and DPPH assays were determined for each point (Table 4). For the ORAC assay, the highest antioxidant activity value was observed in run 8 (434.82 Trolox EQ $\mu mol.g^{-1}$), and the lowest was detected in run 7 (60.84 Trolox EQ $\mu mol.g^{-1}$). In the DPPH assay, the highest value was detected in run 3 (16.52 Trolox EQ $\mu mol.g^{-1}$), and the lowest was observed in run 7 (5.06 Trolox EQ $\mu mol.g^{-1}$). The low level of variation in the central points (runs 9-11) indicated good reproducibility of the experimental data.

Table 4 - Central composite rotatable design (CCRD) matrix with coded and real values for the independent variables and responses for the antioxidant activities of whey protein hydrolysates obtained with the protease AO.

Runs	x_1 Substrate (mg.mL^{-1})	x_2 Protease (U.mL^{-1})	Antioxidant activity (Trolox EQ µmol.g^{-1})	
			ORAC	DPPH
1	-1 (80.0)	-1 (20.0)	197.27	8.64
2	+1 (220.0)	-1 (20.0)	152.62	6.81
3	-1 (80.0)	+1 (70.0)	394.45	16.52
4	+1 (220.0)	+1 (70.0)	330.58	12.63
5	-1.41 (51.3)	0 (45.0)	341.25	17.07
6	+1.41 (248.7)	0 (45.0)	206.13	9.95
7	0 (150.0)	-1.41 (9.8)	60.84	5.06
8	0 (150.0)	+ 1.41 (80.2)	434.82	15.48
9	0 (150.0)	0 (45.0)	259.28	11.14
10	0 (150.0)	0 (45.0)	282.03	11.85
11	0 (150.0)	0 (45.0)	222.82	11.23

The results and significance of the linear and quadratic equations and the interaction of the independent variables are shown in Table 5. In the ORAC assay, only the linear terms were significant at the 95.0% confidence level. By contrast, for the DPPH assay, the linear and quadratic terms were significant at confidence levels of 99.0% and 95.0% ($p < 0.01$, and $p < 0.05$, respectively). Different effects of the independent variables on protein hydrolysis and antioxidant activity were expected because the ORAC and DPPH scavenging assays are involved in different reaction mechanisms, as described previously.

Table 5 – Coefficient estimates for the antioxidant activities of whey protein hydrolysates by the regression model in the CCRD.

Factors	Coefficients		Standard error		*t*-value (5)		*p*-value	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Intercept	254.68	11.41	19.62	0.51	12.97	22.30	0.000	0.000
x_1 (L)	-37.49	-1.98	12.03	0.31	-3.11	-6.29	0.026	0.001
x_1 (Q)	11.56	0.87	14.36	0.37	0.80	2.32	0.458	0.068
x_2 (L)	113.14	3.56	12.03	0.31	9.40	11.34	0.000	0.000
x_2 (Q)	-1.45	-0.76	14.36	0.37	-0.10	-2.03	0.924	0.098
$x_1 \times x_2$	-4.81	-0.51	16.99	0.44	-0.28	-1.16	0.789	0.298

The ANOVA indicated that the *p*-values for the antioxidant activities in the ORAC and DPPH assays were less than 0.001, demonstrating that the proposed models showed high significance at a confidence level of 99.9%. The coefficient of determination value (R^2) of the model could be used to check the experimental data variability. For the two tests, the R^2 values indicated that the models were able to explain 94.0% (ORAC assay) and 97.0% (DPPH assay) of the variability in the experimental data. The computed *F*-values for the regressions were greater than the tabulated *F*-values, reflecting the statistical significance of the models. The lack of fit of the models, for both assays, was not significant at a confidence level of 95.0% (Table 6).

Table 6 – ANOVA of the regression model for the antioxidant activities (Trolox EQ $\mu mol.g^{-1}$) of whey protein hydrolysates.

Source of variation	Sum of squares		Degrees of freedom		Mean of squares		*F* test	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Regression	113,316.5	142.77	2	4	56,658.25	35.69	67.05	43.00
Residual	6,759.9	4.98	8	6	844.98	0.83		
Lack of fit	4,976.0	4.68	6	4	829.33	1.17	0.93	7.80
Pure error	1,783.9	0.30	2	2	891.95	0.15		
Total	120,076.4	147.75	10	10				

ORAC – Regression: $F_{0.05;2,8}$ (*F* tabulated) = 4.46, R^2 = 0.94, *p*-value < 0.0001/Residual: $F_{0.05;6,2}$ (*F* tabulated) = 19.33, *p*-value = 0.601.

DPPH – Regression: $F_{0,1;4,6}$ (*F* tabulated) = 3.18, R^2 = 0.97, *p*-value = 0.00015/Residual: $F_{0.05;4,2}$ (*F* tabulated) = 19.25, *p*-value = 0.117.

Equations 3 (ORAC) and 4 (DPPH) represented the models with significant factors for the experimental data:

$$\textit{Antioxidant activity} = 262.00 - 37.49x_1 + 113.14x_2 \quad (3)$$

$$\textit{Antioxidant activity} = 11.41 - 1.98x_1 + 0.87\ x_1^2 + 3.56x_2 - 0.76\ x_2^2 \quad (4)$$

where x_1 and x_2 are the coded values for the substrate and protease concentrations, respectively.

The effects of the independent variables (protease and substrate concentrations) are illustrated in the surfaces response and contour plots (Figure 1). The CCRD analysis shows that the maximum antioxidant activity in the ORAC assay was observed using 50.0-100.0 $mg.mL^{-1}$ of the substrate and protease concentrations above 60.0 U per mL of reaction. Similar results were detected in the DPPH assay, where the antioxidant activity increased with increasing protease levels ($U.mL^{-1}$) and decreased at higher substrate concentrations.

Guerard et al. (2007) studied the optimization of the hydrolysis conditions (temperature, pH and Alcalase® 2.4L concentration) by a response surface methodology to obtain shrimp discard hydrolysates with antioxidant activity using the DPPH assay. The best results were observed when high levels of Alcalase® (110 to 130 AU/kg protein) were used.

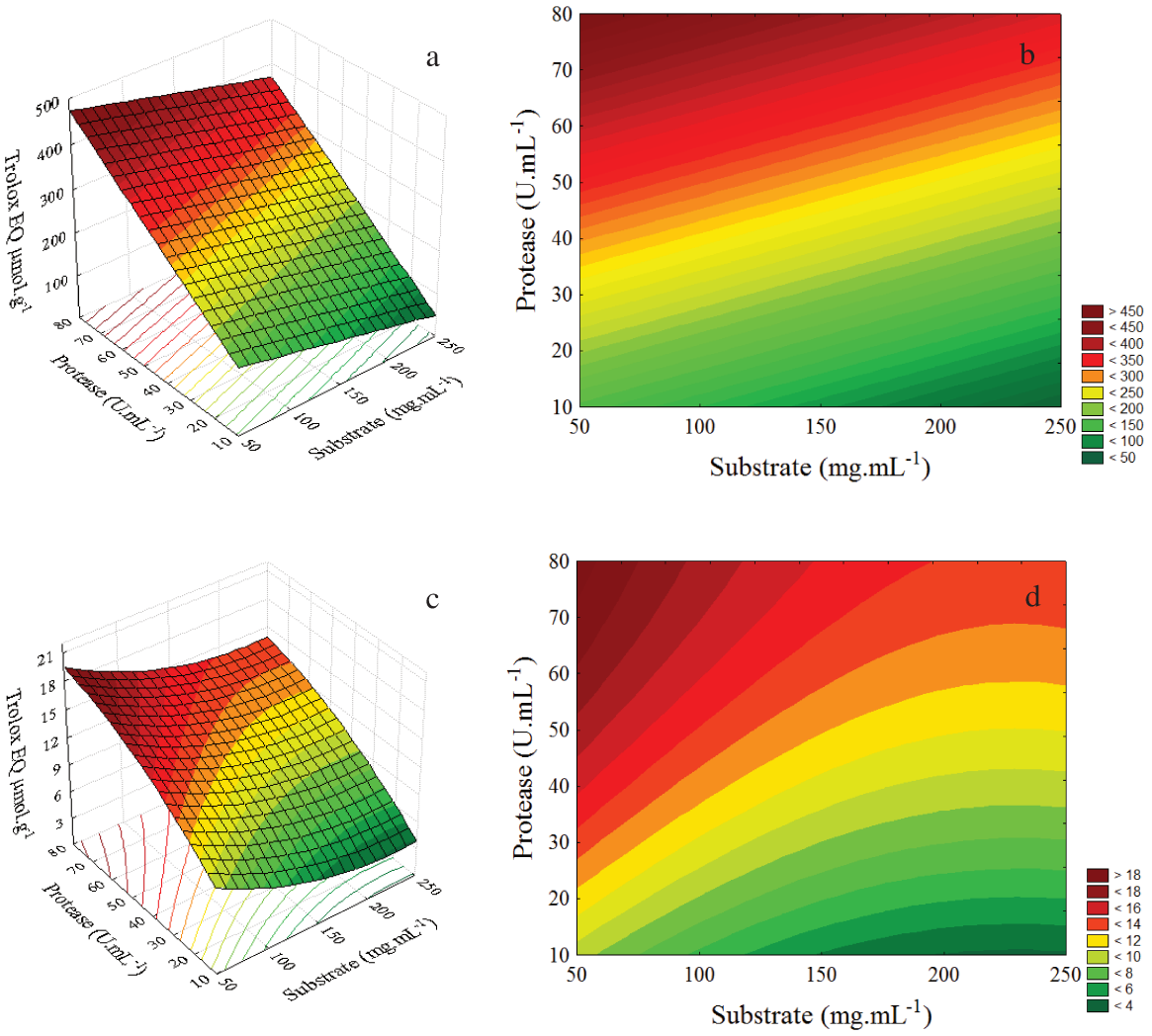

Figure 1 - Response surfaces (a and c) and contour diagrams (b and d) for antioxidant activity in the ORAC and DPPH assays, respectively, as a function of the substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$) concentrations.

The accuracy of the models was further tested by conducting hydrolysis experiments using intermediate values for increased antioxidant activity for the ORAC and DPPH responses according to the superposition of the contour diagrams (Figure 2).

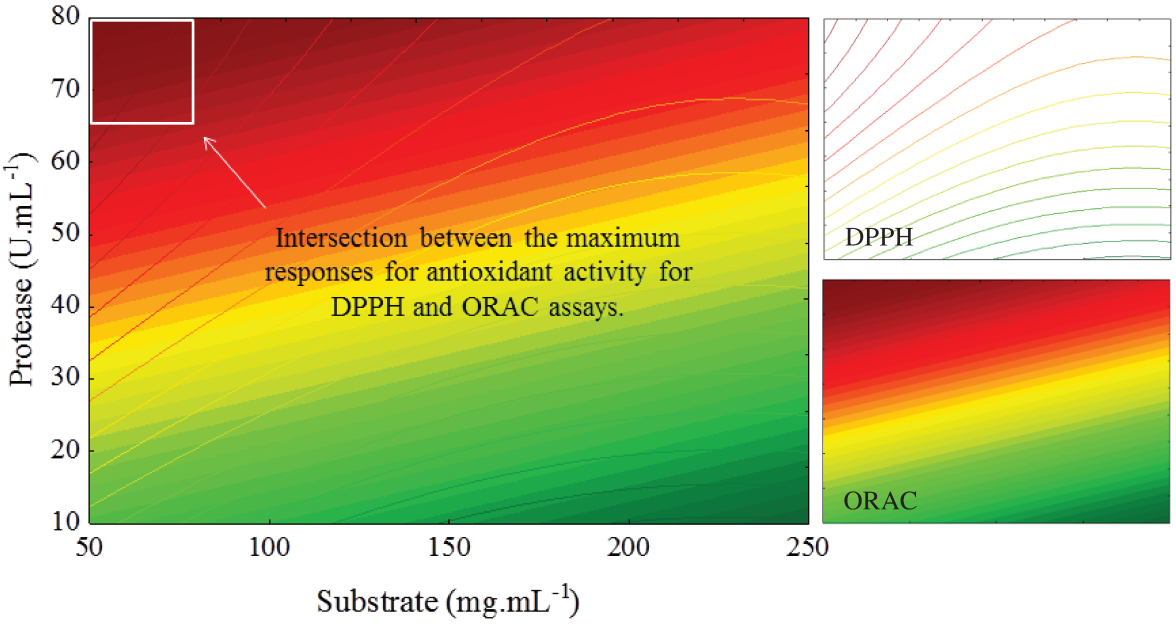

Figure 2 – Superposition of contour diagrams for antioxidant activity (DPPH and ORAC assays) for the determination of the optimal conditions for whey protein hydrolysis.

The selected parameters were a substrate concentration of 80.0 $mg.mL^{-1}$ and the addition of 70.0 U of protease per mL of reaction mixture. According to the regression models (Equations 3 and 4), the predicted values for ORAC and DPPH in these conditions are 412.63 ± 18.82 and 17.06 ± 0.56 Trolox EQ $\mu mol.g^{-1}$, respectively. To confirm the validity of the model, three assays were performed under the optimal conditions described above. The experimental ORAC and DPPH values agreed with the values predicted by the model within a 95.0% confidence interval (Table 7).

Table 7 - Maximum antioxidant activity of whey protein hydrolysates and the corresponding values of two processing parameters, substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$), that were obtained using CCRD.

Optimum processing conditions				
	ORAC		DPPH	
Independent variables	Substrate ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)	Substrate ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)
Experimental coded value	-1	+1	-1	+1
Experimental real value	80.0	70.0	80.0	70.0
Predicted response	412.63[a]		17.06[b]	
Experimental response[1]	424.32 ± 23.72[a]		16.95 ± 0.25[b]	

[1]Values are expressed as the mean ± standard deviation (n = 3) and those with different letters are significantly different ($p < 0.05$).

3.3. Effect of incubation time in antioxidant activity of the whey protein hydrolysates

Figure 3 shows the antioxidant profile as evaluated by the ORAC assay of whey protein hydrolysates during 360 min of hydrolysis. The antioxidant activity increased with increasing hydrolysis times, and a maximum value was observed at 120 min. The statistical analysis showed there was no significant difference ($p < 0.05$) between the hydrolysates obtained in range 60 to 360 min.

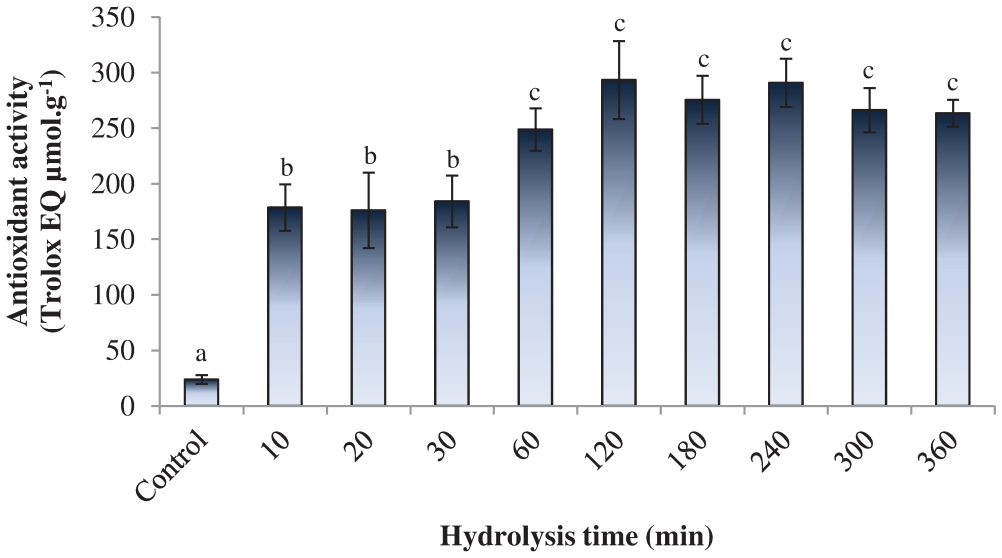

Figure 3 – Antioxidant activity (ORAC values) of whey protein hydrolysates for 360 min of hydrolysis with the protease AO. The results are presented as the mean (n = 3), and those with different letters are significantly different ($p < 0.05$).

The antioxidant profiles in the DPPH assay were characterized by high values during the initial hydrolysis times. At 10 min of hydrolysis, the antioxidant activity, expressed in Trolox EQ µmol.g^{-1}, increased 23.4-fold, and the DPPH radical scavenging (%) increased 5.9-fold compared to the control sample (Figure 4).

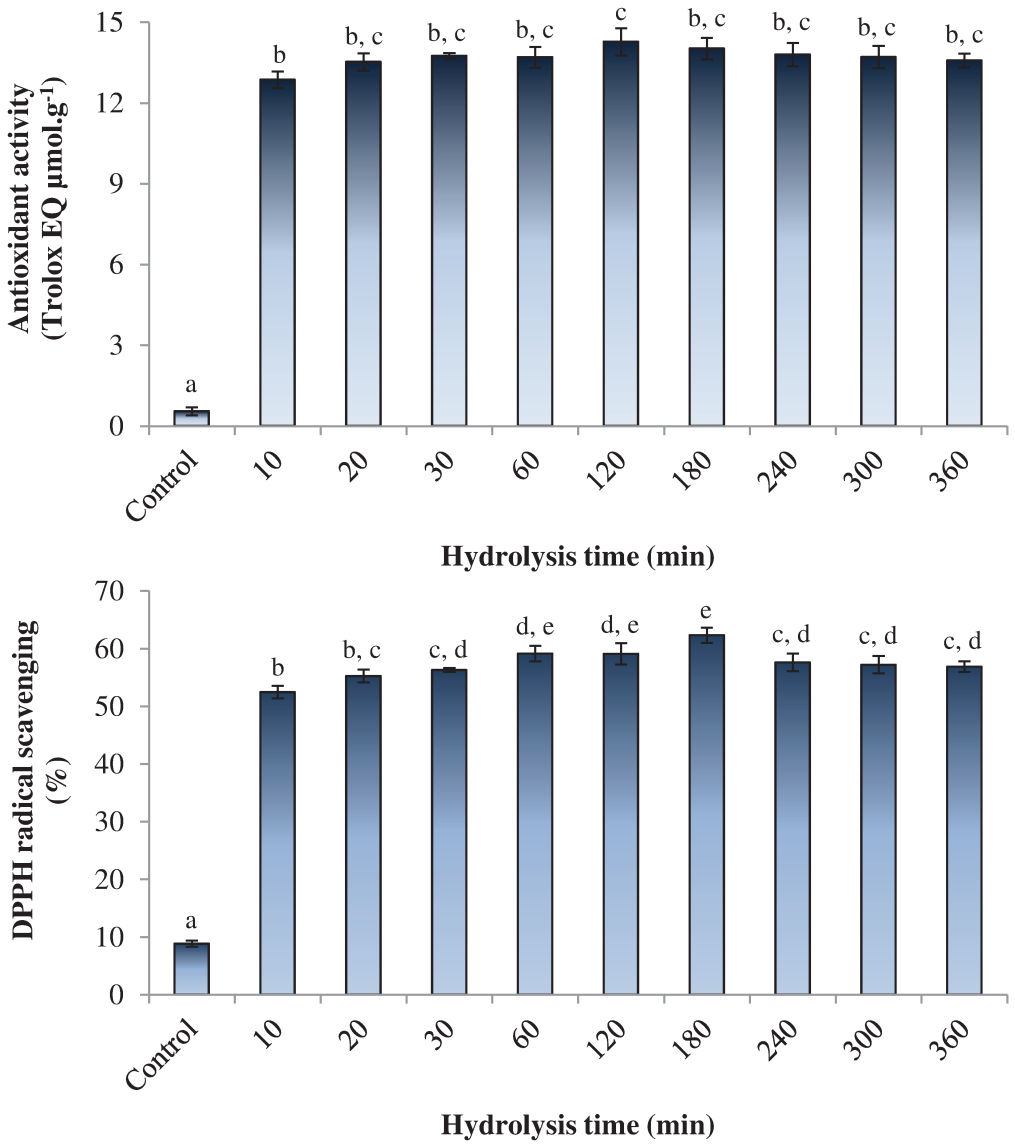

Figure 4 – Antioxidant activity, expressed in Trolox EQ µmol.g^{-1}, and DPPH radical scavenging of whey protein hydrolysates for 360 min of hydrolysis with the protease AO. The results are presented as the mean (n = 3), and those with different letters are significantly different ($p < 0.05$).

As was observed in the ORAC assay, the highest values were detected at 120 min of hydrolysis, and the whey protein hydrolysates exhibited a 62.3% inhibition of the DPPH radical at 5 $mg.mL^{-1}$ (Figure 4). The results for antioxidant activity in the DPPH assay, expressed in Trolox EQ $\mu mol.g^{-1}$, and DPPH radical scavenging (%) showed a high correlation ($p < 0.05$) (data not shown).

A standard assay is essential for comparing the results of different studies. In several studies, research groups used different methodologies to determine the antioxidant activity of protein hydrolysates, which may result in differences in the concentrations of radicals, incubation times, reaction solvents, pH and temperatures of the reaction mixtures. In addition, the inherent variables of the process, including substrate, enzyme, pH, temperature and hydrolysis time, can produce many types of hydrolysates, each with different antioxidant properties. The experimental data variability complicates the comparisons among different studies. Hsu (2010) studied the hydrolysis of tuna dark muscle with the commercial proteases Orientase and protease XXIII and obtained higher antioxidant activities, evaluated by the DPPH assay, at 2 h of hydrolysis. Yokomizo Takenaka, & Takenaka (2002) studied the antioxidant activity of okara protein hydrolysates that were obtained after treatment with seven different proteases. The maximum antioxidant activity, which was evaluated by the ferric thiocyanate method, was obtained after 3-6 h of hydrolysis and decreased after 8 h of hydrolysis. Related results in the literature suggest that the antioxidant properties of hydrolysates show an increase with increasing hydrolysis times, maintain a similar profile during a range of time, and then slow down, in agreement with the results of this study.

Another aspect that can be used to evaluate the catalytic action of proteases is the determination of the degree of hydrolysis (DH). The specific properties of the hydrolysates are dependent on the DH, which is influenced by the specific activity of the protease, the physical and chemical characteristics of the protein substrate and the reaction conditions (Peric˘in et al., 2009). The number of peptide bonds that are cleaved during the reaction is the parameter that most closely reflects the catalytic action of a protease. The DH is generally used as a parameter for monitoring proteolysis and is the most widely used indicator for comparing different protein hydrolysates (Hsu, 2010). In our study, the whey protein hydrolysis was characterized by high DH values during the initial hydrolysis times.

The DH ranged from 40.9% to 44.7%, but the results showed no significant difference ($p < 0.05$) between the whey protein hydrolysates obtained from 10 to 360 min of hydrolysis (Figure 6). The residual protease activity was measured during enzymatic hydrolysis. The protease activity decreased with increasing hydrolysis times, retaining approximately 94.20% and 26.12% of the initial activity after 20 and 360 min of hydrolysis, respectively (Figure 6). The highest antioxidant activity values, evaluated by the ORAC and DPPH assays, were detected in whey protein hydrolysates from 60 to 240 min of hydrolysis. The results were similar to those reported for the enzymatic hydrolysis of different protein sources. Sun et al. (2011) performed the DPPH assay to evaluate the antioxidant activity of porcine hemoglobin that was hydrolyzed with pepsin for 240 min. The results showed maximum antioxidant activity at 60 min of hydrolysis and observed that enzymatic hydrolysis for an extended period of time resulted in an increase in the DH and a decrease in the antioxidant activity. According to these authors, the release of hydrophobic amino acid residues in peptides, including Leu, Ile, Val, Phe, Tyr and Trp, are responsible for the antioxidant activity. Therefore, the release of these peptides may not be a direct function of the DH. Teng et al. (2011) prepared protein hydrolysates from goat placenta protein that was hydrolyzed by pepsin and studied the effects of reaction temperature, pH and enzyme-to-substrate ratio on the antioxidant activity of the peptides and the DH. The results showed that the maximum antioxidant activity values were detected in hydrolysis conditions where the DH had maximum values.

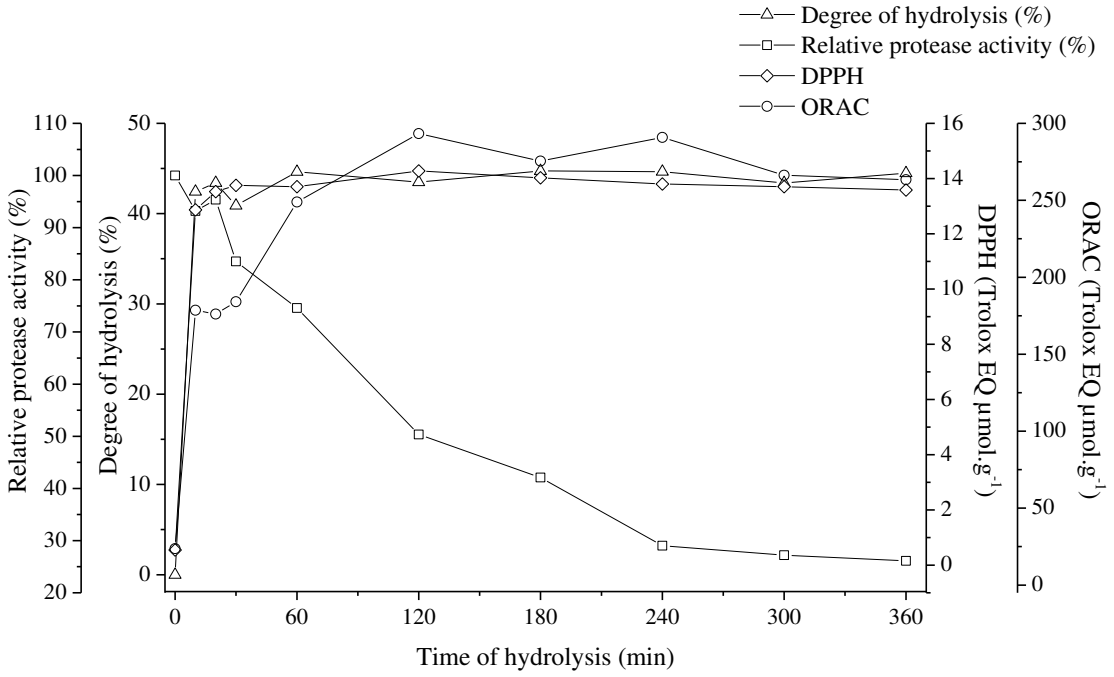

Figure 6 – Degree of hydrolysis (%), ORAC and DPPH values of whey protein hydrolysates and residual protease AO activity for 360 min of hydrolysis.

4. Conclusion

The protease preparation obtained from *Aspergillus oryzae* LBA 01 (AO) was superior to the commercial proteases Flavourzyme® 500 L from *Aspergillus oryzae* (CAO) and Alcalase® 2.4L from *Bacillus licheniformis* (CBL) for obtaining bovine whey protein hydrolysates with increased antioxidant activity. The hydrolysis parameters that were defined according to the CCRD analysis were a substrate concentration of 80.0 $mg.mL^{-1}$ and the addition of 70.0 U of protease per mL of reaction, which resulted in 424.32 and 16.39 Trolox EQ $\mu mol.g^{-1}$, for the ORAC and DPPH assays, respectively. The maximum antioxidant activities were observed between 60 and 240 min of hydrolysis, where the degree of hydrolysis and residual protease activity were approximately 44.0% and 50.0%, respectively.

Acknowledgments

The work described in this paper was substantially supported by grants from São Paulo Research Foundation – FAPESP (Project No. 2011/10429-9), which is gratefully acknowledged. Acknowledgements to the National Counsel of Technological and Scientific Development – CNPq by the granting of scholarship.

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Capítulo V: Optimization of protein hydrolysis parameters for the production of egg white hydrolysates with antioxidant properties

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Revista: Food Research International

Abstract

Bioactive peptides can be defined as specific protein fractions with an amino acid sequence that promotes a positive impact on several biologic activities. The aim of this study was to evaluate the effects of the enzymatic hydrolysis of egg white protein on their antioxidant properties. The hydrolysates were prepared with a protease from *Aspergillus oryzae* LBA 01 (AO), which was produced under solid-state fermentation, and two commercial proteases: Flavourzyme® 500L, from *Aspergillus oryzae* (CAO), and Alcalase® 2.4L, from *Bacillus licheniformis* (CBL). Enzymatic hydrolysis by the microbial proteases increased the antioxidant properties of egg white protein. The egg white protein hydrolysates prepared with the commercial protease CAO presented higher antioxidant activity than the hydrolysates obtained with the other proteases. According to the central composite rotatable design (CCRD) analysis, the hydrolysates prepared with 30.0 $mg.mL^{-1}$ egg white protein and 20.0 U of protease per mL of reaction mixture presented a high radical scavenging effect, with an ORAC value of 1,193.12 ± 84.62 and a DPPH value of 19.05 ± 0.81 Trolox EQ $\mu mol.g^{-1}$. Using the ORAC assay, maximum antioxidant activity was observed in the hydrolysates presenting DH 50.0%, obtained after 120 min of hydrolysis. In contrast, using the DPPH assay, the highest values of antioxidant activity were detected in the hydrolysates obtained in the first 30 min, where the degree of hydrolysis was greater than 60.0%.

Keywords: enzymatic hydrolysis, egg white protein, proteases, antioxidant.

1. Introduction

Enzymatic hydrolysis under controlled conditions is an effective way to improve the functional properties of a protein without affecting its nutritive value (Mannheim & Cheryan, 1992; Li et al., 2012). Modification of protein structure, including the sequence and composition of amino acids, the molecular weight and the charge distribution on the molecule, can directly affect functional properties (Li et al., 2012). Processes involving protein hydrolysis have been studied for the production of bioactive peptides.

Bioactive peptides can be defined as specific protein fractions with amino acid sequences that promote a positive impact on several biological activities. They are recognized as functional food ingredients that prevent lifestyle-related diseases through their antihypertensive, antioxidant, antimicrobial and anti-adipogenic activities (Dávalos et al., 2004; Sakanaka et al., 2004; Expósito & Recio., 2006; Tsou et al., 2010). In intact proteins, these peptides are inactive but could be released by the enzymatic hydrolysis of food proteins (You & Wu, 2011). Among the various protein sources, egg white protein is widely used as functional and nutritional ingredients in food products. Egg white protein can form a significant component of the diet and are also an excellent source of biologically active substances (Mine, 2007; Huang, Majumder & Wu, 2010; Chen et al., 2011). The hydrolysis of egg white protein might result in the release or enhancement of their biological activity, such as the inhibitory and antioxidant activities of the angiotensin I-converting enzyme (ACE) (Chen et al., 2011; Lin et al., 2011; You & Wu, 2011).

Bioactive peptides from food proteins have been obtained by hydrolysis with trypsin, pepsin or chymotrypsin. However, the economical perspective of the process often limits the type and amount of enzymes that can be used in industrial protein hydrolysis (Li, Youravong & H-Kittikun, 2010). Microbial proteases have also been reported to be capable of hydrolyzing proteins. Proteases from *Aspergillus oryzae* and *Bacillus licheniformis*, which have considerably different substrate specialties and reaction characteristics, are commonly used for protein hydrolysis (Zhang et al., 2010).

To maximize the antioxidant activities of the protein hydrolysates, the determination of variables that exert a significant impact on the hydrolysis process is necessary. The application of statistical experimental design techniques has been extensively cited in the

scientific literature for the production of hydrolysates with antioxidant properties (Vastag et al., 2010; Zhou et al., 2011, Tavares et al., 2011; Teng et al., 2011; Sun et al., 2011; Contreras et al., 2011). The central composite rotatable design (CCRD) methodology has proven to be valuable tool for the investigation of individual and interactive effects of hydrolysis parameters on the progress of enzymatic hydrolysis by different proteins (Vastag et al., 2010; Contreras et al., 2011).

In this context, the main objective of this study was to evaluate the hydrolysis potential of egg white protein with different microbial proteases to study the antioxidant properties of the hydrolysates. For this purpose, we selected three microbial proteases: acid protease from *Aspergillus oryzae* LBA 01, produced under solid state fermentation; Flavourzyme® 500L from *Aspergillus oryzae*; and Alcalase® 2.4L from *Bacillus licheniformis*. The antioxidant activity was evaluated by two methodologies: the oxygen radical absorbance capacity (ORAC) method and DPPH radical scavenging. Optimization of egg white hydrolysates production involved selection of the most appropriate protease, determination of the optimal hydrolysis parameters (substrate and protease concentrations) using CCRD and determination of the hydrolysis time under the optimal conditions determined by CCRD.

2. Materials and Methods

2.1. Reagents

Azocasein, trichloroacetic acid (TCA), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (97.0%) (AAPH), fluorescein, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased where commercially available.

2.2. Enzymes

Proteases from *A. oryzae* LBA 01 (AO) (strain from the culture collection of the Laboratory of Food Biochemistry, Faculty of Food Engineering, State University of Campinas) were obtained by solid state fermentation using wheat bran as a substrate in 500 mL Erlenmeyer flasks containing 40 g of cultivation medium. The cultivation parameters were as previously determined: moisture (50.0%), temperature (23°C), inoculum (10^7 spores.g^{-1}), peptone (2.0% w/w), and yeast extract (2.0% w/w). The crude extract was obtained after incubation for 72 h by the addition of 150 mL of acetate buffer for 1 h and subsequent filtration. The crude extract was concentrated by precipitation with ammonium sulfate (80.0%), dialyzed and freeze-dried. The partially purified preparation was used for protein hydrolysis.

The commercial proteases Flavourzyme® 500L, from *A. oryzae* (CAO), and Alcalase® 2.4L, from *Bacillus licheniformis* (CBL), were purchased from Sigma Aldrich.

2.3. Determination of protease activity

The protease activity was measured using azocasein as a substrate according to Charney & Tomarelli (1948), with modifications. The reaction mixture contained 0.5 mL of azocasein (Sigma) 0.5% (w/v) in 0.05 M acetate buffer, pH 5.0, to which 0.5 mL of the enzyme solution was added. The reaction was carried out in optimum conditions of temperature and pH for each enzyme (AO: 55°C and pH 5.0; CAO: 50°C and pH 5.0 and CBL: 60°C and pH 7.0) and stopped after 40 min by the addition of 0.5 mL of 10.0% TCA. Test tubes were centrifuged at 17,000 x *g*/15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL of 5N KOH and, after agitation, the absorbance

was measured at 428 nm. The control was prepared by adding 0.5 mL TCA (10.0%) prior to the addition of the enzymatic solution. One unit of enzyme activity (U) was defined as the amount of enzyme required to cause an increase of 0.01 in absorbance at 428 nm under the assay conditions.

2.4. Preparation of protein hydrolysates: enzyme selection

Egg white protein was donated by Cooper Ovos and was used as a substrate for enzymatic hydrolysis. The proteins were suspended in buffer to a final concentration of 25.0 $mg.mL^{-1}$. The mixtures (50 mL) were distributed in 125 mL Erlenmeyer flasks and incubated. To select for the most appropriate enzyme for the production of hydrolysates, the enzyme concentrations were adjusted to 0 (control), 20.0 and 40.0 U per mL per reaction, according to the activity of each protease, as previously determined. The hydrolysis was carried out for 240 min in the optimum conditions of temperature and pH for each enzyme. After hydrolysis, the proteases were inactivated in a water bath at 100°C for 20 min. The peptide solution was separated from the residue by centrifugation at 17,000 x *g* at 5°C for 20 min. The supernatants were collected and freeze dried for determination of the antioxidant activity.

2.5. Optimization of hydrolysis by response surface methodology

The software Statistica® 8.0 from Statsoft Inc. (Tulsa, Oklahoma, USA) was employed for experimental design, data analysis, and model building. A central composite rotatable design (CCRD) with three replicates at the central point and four axial points (with 11 runs) was used to determine the response pattern and to subsequently establish a model. The 2 variables used in this study were substrate ($mg.mL^{-1}$) (x_1), and protease ($U.mL^{-1}$ of reaction) (x_2) concentrations, while the dependent variables were the DPPH radical scavenging activity and ORAC activity. The coded and real values of these variables are given in Table 1.

Table 1 – Independent variables and levels of egg white protein hydrolysis with proteases to increase antioxidant activity.

Independent variables	Levels				
	-α*	**-1**	**0**	**+1**	**+α**
[ ] Substrate (mg.mL^{-1})	10.0	13.0	20.0	27.0	30.0
[ ] Protease (U.mL^{-1})	9.8	20.0	45.0	70.0	80.2

*α =1.41

The experiments were randomized to maximize the effects of unexplained variability in the observed responses because of extraneous factors. A second-order model equation was used for this model, represented by Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (1)$$

where *Y* is the estimated response, *i* and *j* have values from 1 to the number of variables (*n*), β_0 is the intercept term, βi is the linear coefficient, βij is the quadratic coefficient, and *xi* and *xj* are the coded independent variables. The coefficient of determination, R^2, and *F* test (analysis of variance-ANOVA) were used to verify the quality of fit of the second-order model equation. This step was carried out with the enzyme that was most appropriate for egg white protein hydrolysis.

2.6. Determination of hydrolysis time

A kinetic study was used to determine the hydrolysis time when antioxidant activity was at a maximum. For this study, hydrolysis was carried out for 360 min and samples were collected at different times of incubation. The reactions were conducted similarly to that described above.

2.7. Determination of the degree of hydrolysis (DH)

The DH in the egg white protein hydrolysates was determined according to the method described by Pericˇin et al. (2009) with modifications. A mixture containing 1.0 mL of hydrolysates and 1.0 mL of 0.44 mol.L^{-1} trichloroacetic acid (TCA) was incubated for 30 min at room temperature. Then, the mixture was centrifuged at 17,000 x *g* for 15 min. The

obtained 0.22 mol.L^{-1} TCA-soluble protein fraction and the supernatant of the hydrolysate mixture (without addition of TCA) were each analyzed to determine the protein content by the method of Lowry et al. (1951), using bovine serum albumin as a standard protein. The DH value, expressed as a percentage, was calculated as the ratio of 0.22 mol.L^{-1} TCA-soluble protein to total protein in the supernatant of the hydrolysate mixture.

2.8. Determination of antioxidant activities

2.8.1. ORAC assay

The ORAC method was used to determine antioxidant activity, with fluorescein (FL) as the ‘‘fluorescent probe’’, according to Dávalos, Gómez-Cordovés & Bartolomé (2004) and described by Macedo et al., (2011). The automated ORAC assay was carried out on a Novo Star Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reactions were performed at 37°C, as the reactions were initiated by the thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. FL solution (0.4 µg.mL^{-1}) in phosphate buffer (PBS) (75 mM, pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 µM Trolox solution, prepared daily in distilled water and diluted to 1500–1.5 µmol.L^{-1} solutions for the preparation of the Trolox standard curve. In each well, 120 µL of FL solution was mixed with either 20 µL of sample, blank (distilled water), or standard (Trolox solutions), prior to the addition of 60 µL AAPH (108 mg.mL^{-1}). Fluorescence was measured immediately after the addition of AAPH, and measurements were taken every 1 min for 75 min. The measurements were taken in triplicate. ORAC values were calculated using the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. ORAC values were expressed as µmol of Trolox equivalent.g^{-1} for protein hydrolysates (Trolox EQ µmol.g^{-1}) (Cao, Sofic & Prior, 1996).

2.8.2. DPPH radical scavenging activity

The DPPH radical scavenging activity of the hydrolysates was determined as described by Bougatef et al. (2009). A 500 µL aliquot of each 5 $mg.mL^{-1}$ sample was mixed with 500 µL of 99.5% ethanol and 125 µL of 0.02% DPPH in 99.5% ethanol. The mixture was then kept at room temperature in the dark for 60 min, and the reduction of DPPH radicals was measured at 517 nm using a UV-visible spectrophotometer. The DPPH radical scavenging activity was calculated as follows (Equation 2):

$$Radical\ scavenging\ activity(\%) = \left[\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right] * 100 \quad (2)$$

The control was conducted in the same manner, except that distilled water was used instead of sample. Trolox was used as a standard. The test was carried out in triplicate. The results of the tests were expressed as µmol of Trolox equivalent$\cdot g^{-1}$ of protein hydrolysates (Trolox EQ $\mu mol.g^{-1}$).

2.9. Calculations and statistics

The statistical analyzes were performed using the Minitab® 16.1.1 software package from Minitab Inc. (USA). The values are expressed as the arithmetic mean. Tukey's test was used to check the significant differences between the analyzed groups. When $p < 0.05$, the differences were considered significant.

3. Results and Discussion

3.1. Selection of the protease to produce the egg white protein hydrolysates

The enzyme preparations of CAO, CBL and AO had 4,295.83 $U.mL^{-1}$, 4,293.58 $U.mL^{-1}$ and 4,640.23 $U.g^{-1}$ of protease activity, respectively.

Egg white protein was separately hydrolyzed with CAO, CBL and AO at the optimal conditions. The antioxidant activity of the hydrolysates was evaluated using ORAC and DPPH assays. The ORAC assay is a reliable test based upon the inhibition of peroxyl-radical-induced oxidation, initiated by the thermal decomposition of azocompounds such as AAPH (2,2'-azobis(2-methylpropionamide)-dihydrochloride). The ORAC assay is largely used to assess the total antioxidant capacity of proteins, plant or food extracts and pure antioxidant compounds (Prior & Cao, 1999; Huang, Majumder & Wu, 2010). As shown in Table 2, the antioxidant capacity (ORAC values) of egg white protein exhibited an increase between 6.7 and 12.3-fold after hydrolysis with the different microbial proteases and was influenced by the type of enzyme.

Table 2 – Trolox equivalents and linearity ranges for the ORAC assay (net AUC *vs*. concentration) performed on the control and egg white protein hydrolysate samples.

Enzyme	Protease ($U.mL^{-1}$)	ORAC (Trolox EQ $\mu mol.g^{-1}$)	Sample concentration range ($mg.mL^{-1}$)	Slope	Intercept	R^2
CAO	Control	47.18 ± 7.45[a]	1.05-4.20	0.34	-0.04	0.99
	20.0	559.99 ± 33.94[b]	0.25-1.00	0.04	-0.10	0.99
	40	581.63 ± 46.42[b]	0.30-1.20	0.04	-0.18	0.99
CBL	Control	75.94 ± 3.18[a]	1.10-4.40	0.28	-0.84	0.99
	20.0	509.47 ± 16.40[b]	0.30-1.20	0.04	-0.13	0.99
	40.0	457.76 ± 116.88[b]	0.40-1.60	0.08	-0.77	0.96
AO	Control	47.18 ± 7.45[a]	1.05-4.20	0.34	-0.04	0.99
	20.0	423.43 ± 56.80[b]	0.50-2.00	0.06	-0.32	0.99
	40.0	469.65 ± 47.80[b]	0.50-2.00	0.05	-0.24	0.99

[a, b, c] The results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the values of each enzyme treatment (not between different enzymes). CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

The protease concentration did not show significant differences ($p < 0.05$) in the levels evaluated. The highest increase was observed when the protease CAO was used, which presented 581.63 ± 46.42 Trolox EQ µmol.g^{-1}, a value that is 12.3-fold higher than the control.

The DPPH radical is a stable free radical that shows maximal absorbance at 517 nm in ethanol and has been widely used to test the ability of natural compounds to act as free radical scavengers or hydrogen donors as a means of evaluating their antioxidant potential. When the DPPH radical encounters a proton donating substance such as an antioxidant, the radicals are scavenged and the absorbance is reduced (Zhu, Zhou & Qian, 2006; Yang et al., 2011). Egg white protein hydrolysates prepared with the proteases showed higher DPPH radical scavenging activity when compared with the control samples, except for the hydrolysates prepared with AO, which showed a decrease in antioxidant capacity ($p < 0.05$, Table 3). The highest increase was observed when the protease CAO was used, which presented 1.62 ± 0.10 Trolox EQ µmol.g^{-1}, a value that is 16.2-fold higher than the control.

Table 3 – Hydrolysis of egg white protein with proteases, and antioxidant activities obtained by the DPPH assay.

Enzyme	Protease (U.mL^{-1})	DPPH (Trolox EQ µmol.g^{-1})
CAO	Control	0.10 ± 0.06^{a}
	20.0	1.25 ± 0.21^{b}
	40.0	1.62 ± 0.10^{c}
CBL	Control	0.20 ± 0.01^{a}
	20.0	0.64 ± 0.12^{b}
	40.0	0.87 ± 0.04^{c}
AO	Control	4.42 ± 0.11^{a}
	20.0	1.89 ± 0.30^{b}
	40.0	*Undetected*

$^{a, b, c}$ Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the values of each enzyme treatment (not between different enzymes). The DPPH assay was carried out at 5 mg·mL^{-1}. CAO: commercial protease Flavourzyme® 500L from *A. oryzae*; CBL: commercial protease Alcalase® 2.4L from *B. licheniformis*; AO: protease from *A. oryzae* LBA 01.

In both antioxidant assays, it became clear that the application of different microbial proteases produced hydrolysates with different antioxidant capacities. These differences could be attributed to a wide variety of smaller peptides and free amino acids that are generated, depending directly on the enzyme specificity and on the hydrolysis conditions (Tsou et al., 2010; Contreras et al., 2011).

The commercial protease CAO was the most appropriate enzyme for the production of hydrolysates. Therefore, CAO was selected to perform the optimization studies of egg white protein hydrolysis.

3.2. Optimization of egg white protein hydrolysis by response surface methodology

To optimize the process of hydrolysis, it is necessary to determine the conditions and variables that significantly affect the antioxidant activities of protein hydrolysates. The CCRD matrix with the independent variables (substrate, and protease concentrations) and the mean results for antioxidant activity are presented in Table 4. For the ORAC assay, the highest value obtained for antioxidant activity was observed in run 2 (1,160.42 Trolox EQ $\mu mol.g^{-1}$), and the lowest was 481.43 Trolox EQ $\mu mol.g^{-1}$, detected in run 1. Similar results were observed in DPPH assay, where the highest value was 17.45 Trolox EQ $\mu mol.g^{-1}$, detected in run 2, and the lowest antioxidant activity was observed in run 1 (13.14 Trolox EQ $\mu mol.g^{-1}$). Although for both assays, the maximum and minimum values for antioxidant activity have been identified in the same run, the ORAC values of egg white protein hydrolysates did not show a linear logarithmic relation with the DPPH values ($y = 0.005x + 11.81$; $R^2 = 0.59$) as ORAC and DPPH assays are involved in different reaction mechanisms, as previously described.

Table 4 - Central composite rotatable design (CCRD) matrix with coded and real values for the variables and responses for antioxidant activity of egg white protein hydrolysates obtained with the protease CAO.

Runs	x_1 Substrate ($mg.mL^{-1}$)	x_2 Protease ($U.mL^{-1}$)	Antioxidant activity (Trolox EQ $\mu mol.g^{-1}$)	
			ORAC	DPPH
1	-1 (13.0)	-1 (20.0)	481.43	13.14
2	+1 (27.0)	-1 (20.0)	1160.42	17.45
3	-1 (13.0)	+1 (70.0)	831.42	15.32
4	+1 (27.0)	+1 (70.0)	694.95	15.78
5	-1.41 (10.0)	0 (45.0)	576.47	14.43
6	+1.41 (30.0)	0 (45.0)	807.86	16.58
7	0 (20.0)	-1.41 (9.8)	715.42	16.84
8	0 (20.0)	+ 1.41 (80.2)	681.90	16.46
9	0 (20.0)	0 (45.0)	708.81	15.46
10	0 (20.0)	0 (45.0)	679.12	15.68
11	0 (20.0)	0 (45.0)	-	15.43

The results and significances of the linear, quadratic, and interactions for the model equations are shown in Table 3. As shown in Table 5, only the linear terms of the substrate concentration (x_1) and the interaction between the independent variables ($x_1 \times x_2$) had significant effects at $p < 0.05$.

Table 5 – Coefficient estimates for antioxidant activity of egg white protein hydrolysates by the regression model in CCRD.

Factors	Coefficients		Standard error		*t*-value (5)		*p*-value	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Intercept	693.97	15.96	56.45	0.40	12.29	40.09	<0.001	0.000
x_1 (L)	108.72	0.98	28.23	0.24	3.85	4.00	0.018	0.010
x_1 (Q)	23.26	-0.39	37.34	0.29	0.62	-1.35	0.567	0.236
x_2 (L)	-20.36	-0.01	28.23	0.24	-0.72	-0.01	0.511	0.994
x_2 (Q)	26.51	0.18	37.34	0.29	0.71	0.63	0.517	0.559
$x_1 \times x_2$	-203.87	-0.96	39.92	0.35	-5.11	-2.78	0.007	0.039

The analysis of variance (ANOVA) test indicated that the *p*-values for antioxidant activities in the ORAC and DPPH assays were less than 0.01, demonstrating that the proposed models showed high significance at a 99.99% confidence level. The coefficient of determination value (R^2) of the model could be used to check the variability of the experimental data. For the two tests, the R^2 values indicated that the models were be able to explain 89.0% (ORAC assay) and 75.0% (DPPH assay) of the experimental data variability. The computed *F*-values for regressions were greater than the tabulated *F*-values, reflecting the statistical significance of the models. The lack of fit of the models, for both assays, was not significant at a confidence level of 95.0% (Table 6).

Table 6 – ANOVA of the regression model for antioxidant activity (Trolox EQ μmol.g^{-1}) of egg white protein hydrolysates.

Source of variation	Sum of squares		Degrees of freedom		Mean of squares		*F* test	
	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH	ORAC	DPPH
Regression	260,804.9	11.30	2	2	130,402.45	5.65	27.82	12.02
Residual	32,813.2	3.78	7	8	4,687.60	0.47		
Lack of fit	32,372.5	2.76	6	6	5,395.42	0.46	12.24	0.90
Pure error	440.7	1.02	1	2	440.70	0.51		
Total	293,618.1	15.08	9	10				

ORAC - Regression: $F_{0.05;2,7}$ (*F* tabulated) = 4.74, R^2 = 0.89, *p*-value = 0.0005/ Residual: $F_{0.05;6,1}$ (*F* tabulated) = 234.0, *p*-value = 0.215.

DPPH - Regression: $F_{0,05;2,8}$ (*F* tabulated) = 4.46, R^2 = 0.75, *p*-value = 0.004 / Residual: $F_{0.05;6,2}$ (*F* tabulated) = 19.33, *p*-value = 0.611.

Equations 3 (ORAC) and 4 (DPPH) represent the models with significant factors for experimental data:

$$\textit{Antioxidant activity} = 733.78 + 108.72x_1 - 203.87x_1x_2 \quad (3)$$

$$\textit{Antioxidant activity} = 15.80 + 0.98x_1 - 0.96x_1x_2 \quad (4)$$

where x_1 is the coded value of the variable substrate concentration, and x_2 is the coded value of the variable protease concentration.

The effects of the independent variables (protease and substrate concentrations) are illustrated in surface response and contour plots (Fig. 1).

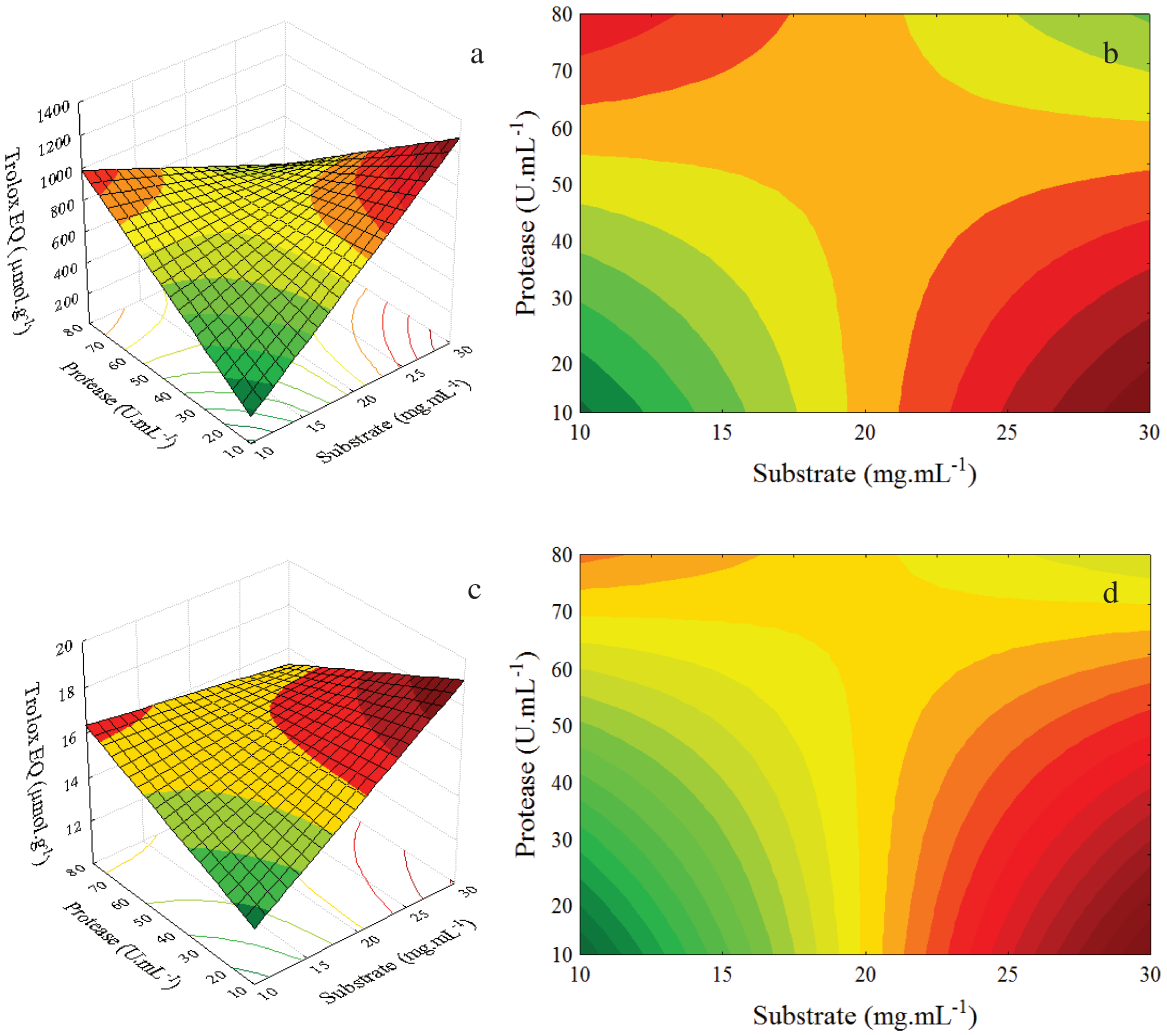

Fig. 1 - Response surfaces (a and c) and contour diagrams (b and d) for the antioxidant activity of egg white protein hydrolysates in ORAC and DPPH assays, respectively, as a function of substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$) concentrations.

The main goal of CCRD is to efficiently hunt for the optimum values of variables such that the response is maximized or minimized. In general, response surface plots and contour plots show the optimum conditions (Cao et al., 2012). As shown in Fig. 1, the antioxidant activity, as observed by ORAC and DPPH assays, increased with the increase of the substrate concentration and decreased at higher levels of protease. Under the proper condition, hydrolysis might release the antioxidant peptides, resulting in an increase in the antioxidant activity of the hydrolysates. Other treatments might produce without

antioxidant activity or hydrolyze the antioxidant peptides into amino acids and, thus, decrease the antioxidant activity of the hydrolysate (Zhuang & Sun, 2011).

The accuracy of the models was further tested by conducting hydrolysis experiments using intermediate values for increased antioxidant activity for the two responses, ORAC and DPPH, according to the superposition of the contour diagrams (Fig. 2).

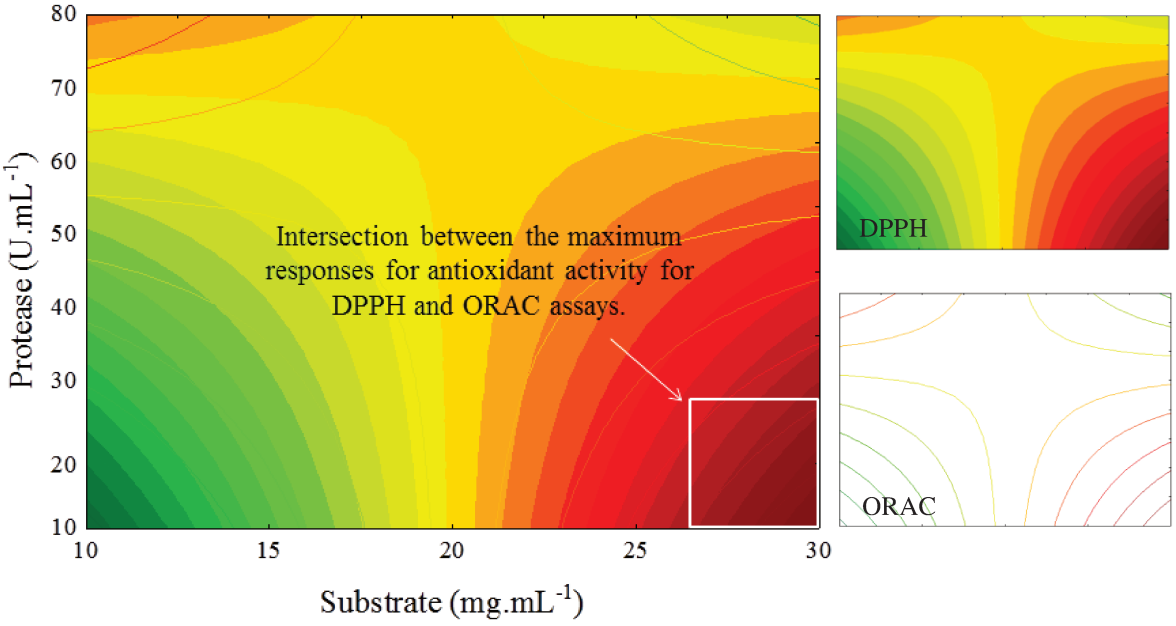

Fig. 2 – Superposition of contour diagrams for antioxidant activity (DPPH, and ORAC assays) for determination of the optimum conditions for egg white protein hydrolysis.

The selected parameters were 30.0 $mg.mL^{-1}$ substrate concentration and 20.0 U of protease per mL of reaction. According to the regression models (Equations 3 and 4), the predicted values for ORAC and DPPH in these conditions are 1,174.54 and 18.53 Trolox EQ $\mu mol.g^{-1}$, respectively. To confirm the validity of the model, three assays were performed under the optimal conditions given above. The experimental ORAC and DPPH values agreed with the values predicted by the model within a 95.0% confidence interval (Table 7).

Table 7 - Maximum antioxidant activity of egg white protein hydrolysates and corresponding values of two processing parameters, substrate ($mg.mL^{-1}$) and protease ($U.mL^{-1}$), obtained using CCRD.

Optimum processing conditions				
	ORAC		**DPPH**	
Independent variables	Substrate ($mg.mL^{-1}$)	Protease ($U \cdot mL^{-1}$)	Substrate ($mg.mL^{-1}$)	Protease ($U \cdot mL^{-1}$)
Experimental coded value	+1.41	-1	+1.41	-1
Experimental real value	30.0	20.0	30.0	20.0
Predicted response	1,174.54[a]		18.53[b]	
Experimental response[1]	1,193.12 ± 84.62[a]		19.05 ± 0.81[b]	

[1]Values are expressed as the mean ± standard deviation (n = 3) and those with different letters are significantly different ($p < 0.05$).

3.3. Effect of incubation time in antioxidant activity of the egg white protein hydrolysates

To study the effects of hydrolysis time on antioxidant properties, egg white protein were hydrolysed by CAO for 5–360 min at pH 5.0 and 50°C. In the ORAC assay, the antioxidant activity increased as the hydrolysis time increased (Fig. 3). All egg white protein hydrolysates showed significant ($p < 0.05$) increases in antioxidant activity compared to intact egg white protein (control). The hydrolysates obtained at 120 min of hydrolysis had the highest antioxidant activity, resulting in 653.42 Trolox EQ $\mu mol.g^{-1}$.

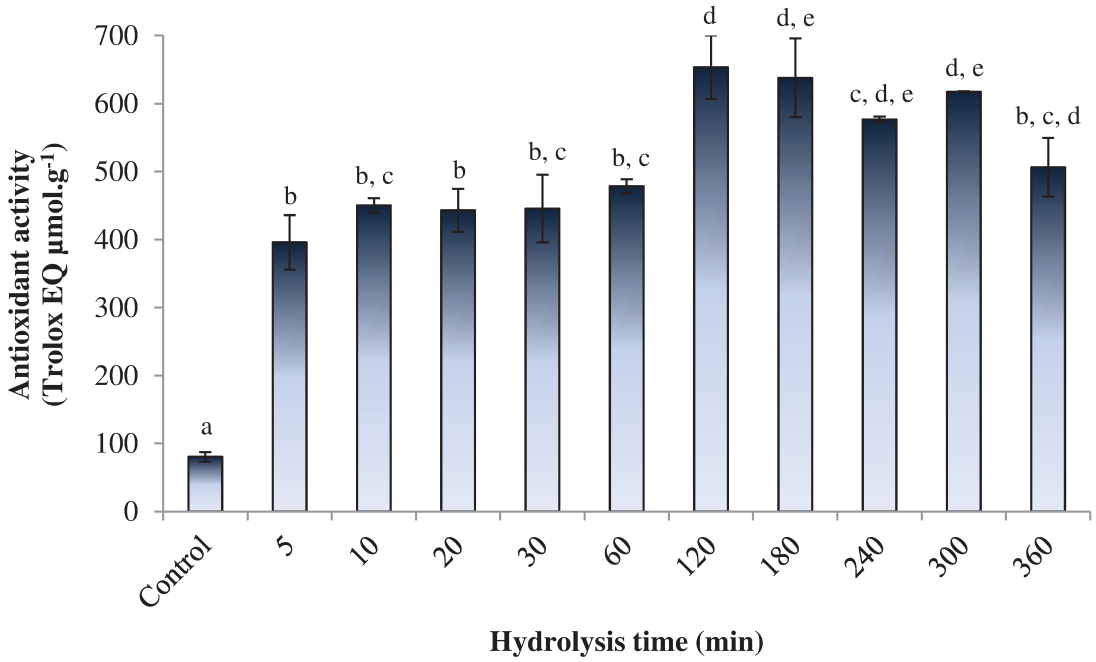

Fig. 3 – Antioxidant activity (ORAC values) of egg white protein hydrolysates after 360 min of hydrolysis. The results are presented as the means (n =3), and those with different letters are significantly different ($p < 0.05$).

In the DPPH assay, the antioxidant activity did not show a regular profile during hydrolysis, but rather, presented alternating increases and decreases over time (Fig. 4). During the hydrolysis, peptides with antioxidant properties were continuously formed and degraded, depending on their molecular structure, which is primarily affected by hydrolysis conditions (Vastag et al., 2010). The irregular profiles for egg white protein hydrolysates in the DPPH assay suggest a continuous formation and degradation of peptides with antioxidant properties. In contrast with the results obtained in the ORAC assay, the maximal antioxidant values in the DPPH assay were found at the initial hydrolysis times. After 5 min of hydrolysis, the antioxidant activity, expressed in Trolox EQ µmol·g^{-1}, increased by 51.04%, and the DPPH radical scavenging (%) increased by 47.2% compared to the control sample. The highest values for DPPH radical scavenging were detected at 5 and 20 min of hydrolysis, exhibiting an inhibition of about 55.0% at 5 mg.mL^{-1}. DPPH values expressed as Trolox EQ µmol·g^{-1} and DPPH radical scavenging of egg white protein hydrolysates showed a linear logarithmic relation (y = 4.24x + 4.64; R^2 = 0.97).

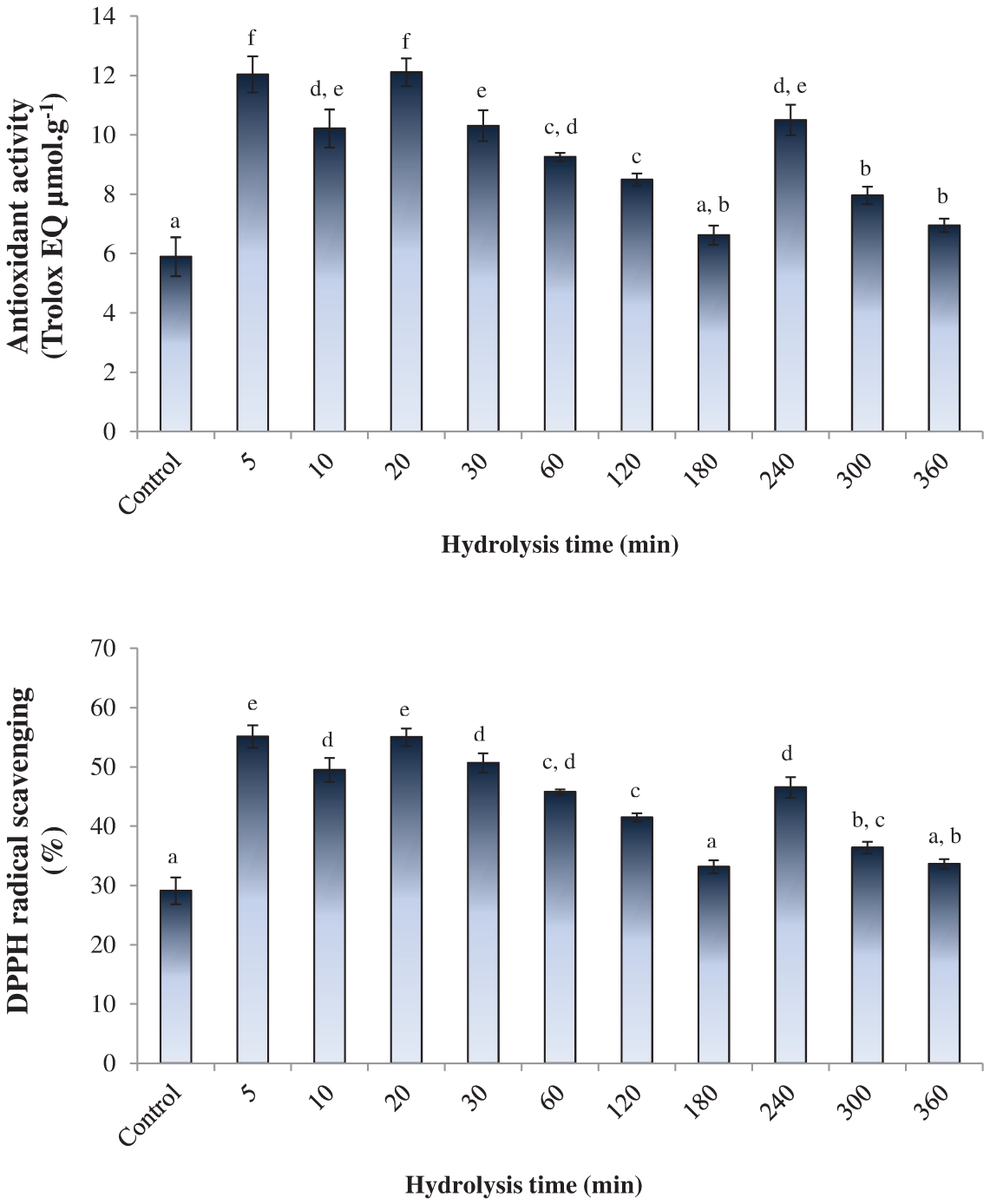

Fig. 4 – Antioxidant activity expressed in Trolox EQ µmol.g^{-1} and DPPH radical scavenging of egg white protein hydrolysates for 360 min of hydrolysis. The results are presented as the means (n = 3), and those with different letters are significantly different ($p < 0.05$).

The DH is generally used as a parameter for monitoring proteolysis and is the most widely used indicator for comparing different protein hydrolysates (Hsu et al., 2010). In several studies, the antioxidant activity showed a considerable correlation with the DH of the protein substrate. In some studies, an increase in the DH of the protein hydrolysates corresponded with an increase in antioxidant activity. However, some studies have reported a decrease in the antioxidant activity with increasing DH. The egg white protein hydrolysis was characterized by high values of the DH during the initial hydrolysis times (5 to 30 min), reaching a steady-state phase after 60 min of hydrolysis, where there was no significant difference ($p < 0.05$) between the DH values. The minimum and maximum DH values were observed at 30 and 180 min of hydrolysis, ranging from 48.62% to 66.04% (Fig. 5). In our study, the maximum antioxidant activity of egg white protein hydrolysates was observed when the DH was below 50.0%. According to the ORAC assay, the extensive hydrolysis (DH greater than 60.0%) of egg white protein did not guarantee high enhancement of antioxidant activity. According to Tsou et al. (2010), the limited hydrolysis was required to maintain the structure or sequence of active peptides and to ensure functionality. On the other hand, in the DPPH assay, the high values of DH reflected higher antioxidant activity. Li et al. (2012) studied the antioxidant activities of grass carp protein hydrolysates prepared with Alcalase® 2.4L and papain using four methodologies: ABTS radical scavenging, DPPH radical scavenging, power reduction, and Fe^{2+} chelation by the hydrolysates. The authors reported that when the DH increased, the metal chelating activity of the hydrolysates increased, while their reducing power and DPPH scavenging activity decreased. Theodore, Raghavan & Kristinsson (2008) prepared protein hydrolysates from catfish protein isolates with a commercial protease and had degrees of hydrolysis of 5.0, 15.0, and 30.0%. In your studies, the protein hydrolysates showed a decreased ability in DPPH radical scavenging and an increase in the degree of hydrolysis. The opposite results were observed by the ORAC assay; the antioxidant activity increased as the DH increased. Wiriyaphan, Chitsomboon & Yongsawadigul (2012) investigated the antioxidant properties of protein hydrolysates from surimi wastes obtained by different proteases using the 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS) radical, ferric reducing antioxidant power (FRAP) and inhibition of β-carotene bleaching assays. The results showed that the antioxidant activity may not be a direct function of the DH, but greatly

depends on the amino acid composition, specific amino acid sequence of the hydrolysates, type of protease and the antioxidant assay. For example, in ABTS radical scavenging, the authors reported high antioxidant activities in hydrolysates prepared by pepsin (5.0% DH) and *Virgibacillus* sp. SK33 proteinase (13.0% DH), while the ABTS radical scavenging activity of the hydrolysates prepared by Alcalase (27.0% DH) and trypsin (11.0% DH) were similar but lower than those prepared from pepsin. On the other hand, by FRAP assay, the hydrolysates prepared from pepsin at 5.0% DH showed the highest reducing power, followed by hydrolysates prepared from *Virgibacillus* sp. SK33 (13.0% DH), trypsin (11.0% DH) and Alcalase (27.0% DH).

The protease activity showed a decrease, retaining 63.2% of residual activity after incubation for 360 min.

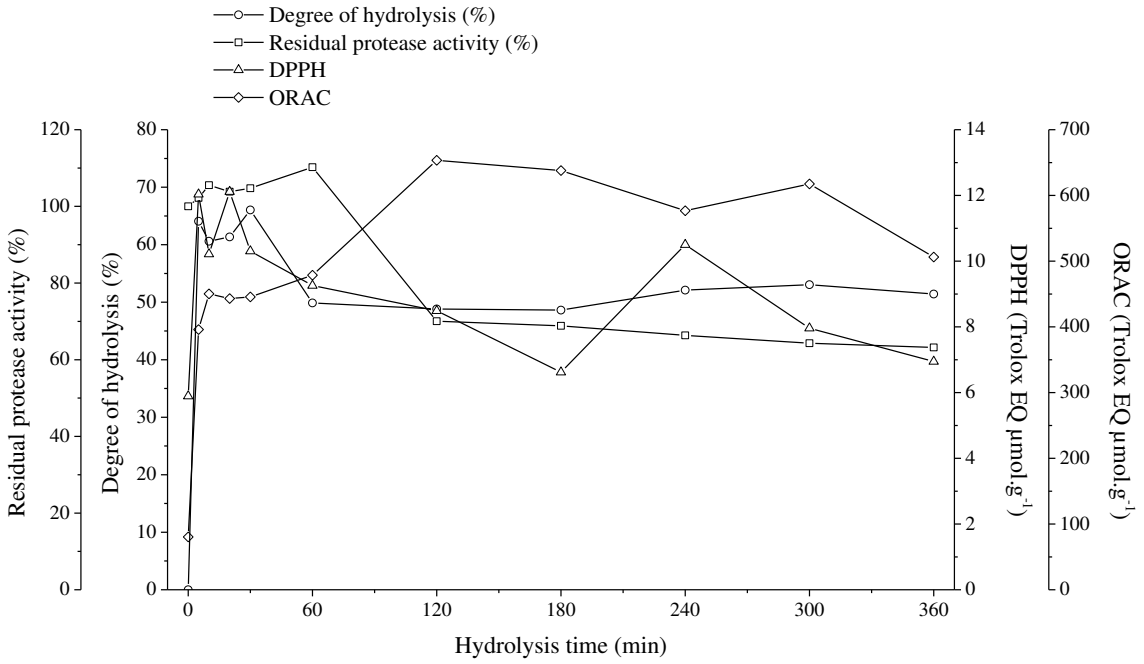

Fig. 5 – Degree of hydrolysis (%), ORAC and DPPH values of egg white protein hydrolysates and residual protease CAO activity for 360 min of hydrolysis.

4. Conclusion

The egg white protein hydrolysates prepared with the commercial protease Flavourzyme® 500L from *A. oryzae* (CAO) presented higher antioxidant activity compared with hydrolysates obtained with the protease from *A. oryzae* LBA 01 (AO) and the commercial protease Alcalase® 2.4L from *Bacillus licheniformis* (CBL). According to the CCRD, the hydrolysates obtained with 30.0 $mg.mL^{-1}$ egg white protein and 20.0 U of CAO protease per mL of reaction mixture presented strong radical scavenging effects, with an ORAC value of 1,193.12 ± 84.62 and DPPH value of 19.05 ± 0.81 Trolox EQ $\mu mol.g^{-1}$. Using the ORAC assay, maximum antioxidant activity was observed in the hydrolysates presenting 50.0% DH obtained after 120 min of incubation. In contrast, the DPPH assay indicated that the highest value of antioxidant activity was obtained in the first 30 min, where the degree of hydrolysis was greater than 60.0%. This study furthers existing knowledge of the use of microbial enzymes for producing bioactive peptides with antioxidant activities of egg white protein. Furthermore, this study suggests that the egg white protein hydrolysates can potentially be utilized as a natural source of antioxidants.

Acknowledgments

The work described in this paper was substantially supported by grants from São Paulo Research Foundation – FAPESP (Project No. 2011/10429-9), which is gratefully acknowledged. Acknowledgements to the National Counsel of Technological and Scientific Development – CNPq by the granting of scholarship.

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Conclusões gerais

A produção de proteases pela linhagem *A. oryzae* LBA 01 por fermentação em estado sólido, mostrou que a utilização de farelo de trigo como substrato permitiu a obtenção de extrato enzimático bruto de proteases com atividade enzimática superior aos obtidos com farelo de algodão e farelo de soja. Nos estudos de otimização da produção, as condições mais adequadas para fermentação, utilizando farelo de trigo como substrato, foram: umidade inicial do meio de cultivo de 50,0%, inóculo de 10^7 esporos.g^{-1}, adição de peptona 2,0% (p/p) e extrato de levedura 2,0% (p/p) e incubação a 23°C durante 72h;

A caracterização bioquímica da protease de *A. oryzae* LBA 01, utilizando delineamento composto central rotacional (DCCR), mostrou que a enzima foi mais ativa na faixa de pH 5,0 a 5,5 e temperatura de 55 a 60°C. A protease mostrou-se mais estável na faixa de pH de 4,5 a 6,0 após incubação na faixa de 35 a 45°C durante1h;

Os valores de *Km* e *V*max da protease de *A. oryzae* LBA 01 foram estimados em 2,5 mg.mL^{-1} e 5.139,3 U.g^{-1} para o substrato azocaseína; 4,9 mg.mL^{-1} e 5.446,3 U.g^{-1} para caseína e 0,7 mg.mL^{-1} e 2.764,5 U.g^{-1} para hemoglobina, respectivamente, indicando que a protease apresentou alta afinidade pelo substrato hemoglobina;

A protease de *A. oryzae* LBA 01 não foi inibida na presença de EDTA, indicando que íons metálicos não são necessários para atividade enzimática. A protease foi inibida na presença de $ZnSO_4$, $CuSO_4$, KCl, $CaCO_3$, cisteína, iodoacetamida e N-bromosuccinimida, na concentração de 5,0mM, apresentando atividade residual de 23,88%, 13,98%, 52,01%, 46,80%, 33,20% e 5,10%, respectivamente;

A fermentação da linhagem de *A. oryzae* LBA 01 em meio sólido de farelo de trigo nas condições otimizadas e posterior concentração do sobrenadante por precipitação com sulfato de amônio, diálise e liofilização, permitiu a obtenção de uma preparação concentrada de proteases com atividade enzimática (4.640,23 U.g^{-1}) comparável com as preparações comerciais de Flavourzyme® 500L de *A. oryzae* (4.295,83 U.mL^{-1}) e Alcalase® 2.4L de *Bacillus licheniformis* (4.293,58 U.mL^{-1});

Na obtenção de hidrolisados com atividade antioxidante, a protease de *A. oryzae* LBA 01 mostrou-se eficaz na hidrólise de proteínas de soja, soro de leite e clara de ovo, aumentando a capacidade antioxidante das mesmas em até 23 vezes quando comparadas às proteínas não hidrolisadas;

A protease comercial Flavourzyme® 500L de *A. oryzae* foi selecionada como mais adequada para hidrólise de proteína isolada de soja e proteínas da clara de ovo e obtenção de hidrolisados com maior propriedade antioxidante quando comparados com aqueles obtidos com as proteases de *A. oryzae* LBA 01 e protease comercial Alcalase® 2.4L de *Bacillus licheniformis*;

Para as proteínas de soro de leite bovino, a aplicação de protease de *A. oryzae* LBA 01 resultou em hidrolisados com maior atividade antioxidante;

As condições de hidrólise da proteína isolada de soja com a protease comercial Flavourzyme® 500L de *A. oryzae*, definidas por DCCR, foram: concentração de substrato de 90,0 $mg.mL^{-1}$ e adição de 70,0 U de protease por mL de reação ($U.mL^{-1}$), resultando em 775,17 ± 115,10 e 11,83 ± 0,31 Trolox EQ $\mu mol.g^{-1}$, para os ensaios de ORAC e DPPH, respectivamente. Os hidrolisados com maior atividade antioxidante foram obtidos entre 120 e 180 min de incubação, onde o grau de hidrólise atingiu em média 50,0%;

Os parâmetros de hidrólise das proteínas do soro de leite bovino utilizando a protease de *A. oryzae* LBA 01, definidos a partir do DCCR, foram concentração de substrato de 80,0 $mg.mL^{-1}$ e adição 70,0 $U.mL^{-1}$, resultando em 424,32 e 16,39 Trolox EQ $\mu mol.g^{-1}$, para os ensaios de ORAC e DPPH, respectivamente. Quando incubados no período de 60 a 240 min, o grau de hidrólise atingiu cerca de 44,0% e os hidrolisados apresentaram atividade antioxidante máxima nas condições de estudo avaliadas;

De acordo com a análise do DCCR, os hidrolisados obtidos com 30,0 $mg.mL^{-1}$ de proteínas de clara de ovo e 20,0 $U.mL^{-1}$ de protease comercial Flavourzyme® 500L de *A. oryzae* apresentaram maiores atividades antioxidantes, com valores de ORAC e DPPH de 1.193,12 ± 84,62 e 19,05 ± 0,81 Trolox EQ $\mu mol.g^{-1}$, respectivamente. No estudo da cinética de hidrólise, os resultados para os ensaios de ORAC e DPPH foram divergentes. Nos ensaios de ORAC, a máxima atividade antioxidante foi observada nos hidrolisados de proteínas de clara de ovo obtidos com 120 min de incubação, onde o grau de hidrólise foi

em média 50,0%. Para os ensaios de DPPH, os maiores valores de atividade antioxidante foram observados nos primeiros 30 min de incubação, onde o grau de hidrólise foi superior a 60,0%;

Os resultados obtidos neste trabalho indicam o potencial da aplicação da hidrólise enzimática utilizando proteases microbianas e diferentes fontes de proteínas, como processo viável para obtenção de hidrolisados proteicos com atividade antioxidante.

Sugestões para trabalhos futuros

A utilização de hidrolisados proteicos como fontes naturais de antioxidantes, e outras substâncias bioativas, mostra-se uma alternativa interessante para substituição de compostos sintéticos, visto que há uma tendência recorrente em substituí-los por componentes naturais. No entanto, mais estudos são necessários para determinação do real efeito destes hidrolisados em sistemas complexos. Nesse contexto, as sugestões para trabalhos futuros são:

- Avaliar o potencial de linhagens microbianas, incluindo fungos e bactérias, na produção de proteases com características diferenciadas de atuação e aplicá-las na hidrólise de proteínas para estudo da atividade biológica dos hidrolisados;

- Realizar estudos comparativos de atividade antioxidante dos hidrolisados de proteína isolada de soja, soro de leite bovino e clara de ovo, obtidos neste estudo, com antioxidantes sintéticos disponíveis no mercado;

- Incorporar os hidrolisados proteicos em uma matriz alimentar complexa sujeita à oxidação, como óleos insaturados, e avaliar o efeito antioxidante durante certo período de armazenamento;

- Estudar a utilização de outras fontes proteicas, com ênfase nas de baixo valor agregado, como resíduos da filetagem de peixes, farelos de soja, arroz, trigo, algodão e amendoim, cascas de ovo, soro de leite caprino, farinhas de carne e sangue, para hidrólise enzimática e estudo das bioatividades dos hidrolisados;

- Avaliar outras bioatividades, incluindo atividade antimicrobiana e antiadipogênica, dos hidrolisados proteicos;

- Utilizar o fracionamento por ultrafiltração para obtenção de peptídeos com atividade biológica de forma concentrada e em tamanhos selecionados;

- Purificar os peptídeos com atividade biológica por cromatografia de troca iônica e filtração em gel;

- Identificar as sequências peptídicas responsáveis pelas bioatividades utilizando cromatografia líquida de alta eficiência e espectrometria de massas.

Anexos

Anexos Capítulo II

1. Fermentação em estado sólido para produção de proteases por *A. oryzae* LBA 01.

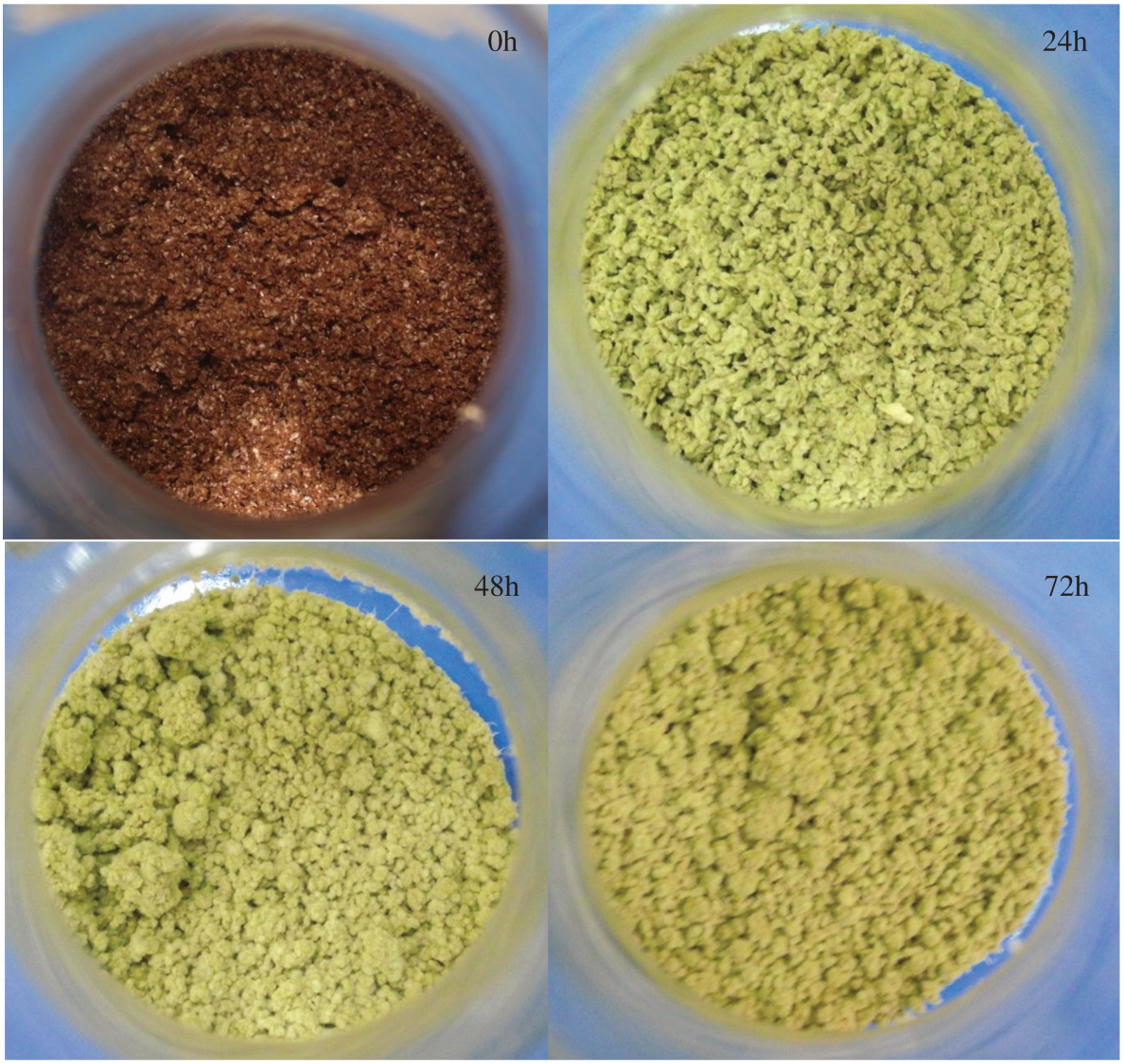

Figura 1 – Fermentação em estado sólido utilizando farelo de trigo como substrato para produção de proteases por *A. oryzae* LBA 01 durante 72h de incubação.

2. Caracterização bioquímica do extrato enzimático bruto de proteases[1] de *A. oryzae* LBA 01

2.1. Determinação do pH e temperatura ótima de atividade utilizando DCCR

Tabela 1 – Matriz do DCCR 2² com os valores codificados, reais e respostas para os ensaios de determinação do pH e temperatura ótima de atividade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01.

Ensaios	x_1	x_2	pH	Temperatura (°C)	Atividade proteolítica ($U.g^{-1}$)
1	-1	-1	5,6	34,4	32,31
2	+1	-1	8,4	34,4	3,86
3	-1	+1	5,6	55,6	48,56
4	+1	+1	8,4	55,6	1,26
5	-1,41	0	5,0	45,0	49,13
6	+1,41	0	9,0	45,0	2,44
7	0	-1,41	7,0	30,0	1,24
8	0	+1,41	7,0	60,0	5,06
9	0	0	7,0	45,0	3,66
10	0	0	7,0	45,0	4,95
11	0	0	7,0	45,0	5,51

Tabela 2 – Coeficientes de regressão do DCCR (modelo real) para determinação do pH e temperatura ótima de atividade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01.

Fatores	Coeficientes	Erro padrão	*t* (5)	*p* valor
Média	302,55	90,62	3,34	0,021
pH (L)	-84,42	17,00	-4,97	0,004
pH (Q)	6,15	1,08	5,71	0,002
Temperatura (L)	1,63	2,10	0,77	0,473
Temperatura (Q)	0,01	0,02	0,48	0,654
pH x Temperatura	-0,32	0,17	-1,85	0,123

[1] Corresponde ao sobrenadante obtido pela adição de tampão acetato pH 5,0 (200mM) e posterior filtração, após 72h de fermentação em estado sólido utilizando farelo de trigo como substrato nas condições de cultivo otimizadas. O extrato enzimático bruto ao qual se refere a caracterização bioquímica apresentada nos anexos, não foi concentrado, dialisado e liofilizado como o utilizado na caracterização bioquímica do Capítulo 2 e na hidrólise de proteínas dos Capítulos 3, 4 e 5.

Tabela 3 – ANOVA para atividade proteolítica na determinação do pH e temperatura ótima de atividade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01.

Fonte de variação	Soma dos quadrados	Graus de liberdade	Quadrado médio	*F* calculado
Regressão	3.534,66	5	706,93	27,21
Resíduos	129,91	5	25,98	
Total	3.664,57	10		

$F_{0,1;5,5}$ = 3,45, R^2 = 0,96, *p*-valor = 0,001

Modelo quadrático gerado a partir dos coeficientes de regressão e variáveis reais:

Atividade proteolítica (U.g^{-1}) = 302,55 – 84,42pH + 6,15pH² + 1,63T + 0,01T² - 0,32pH×T

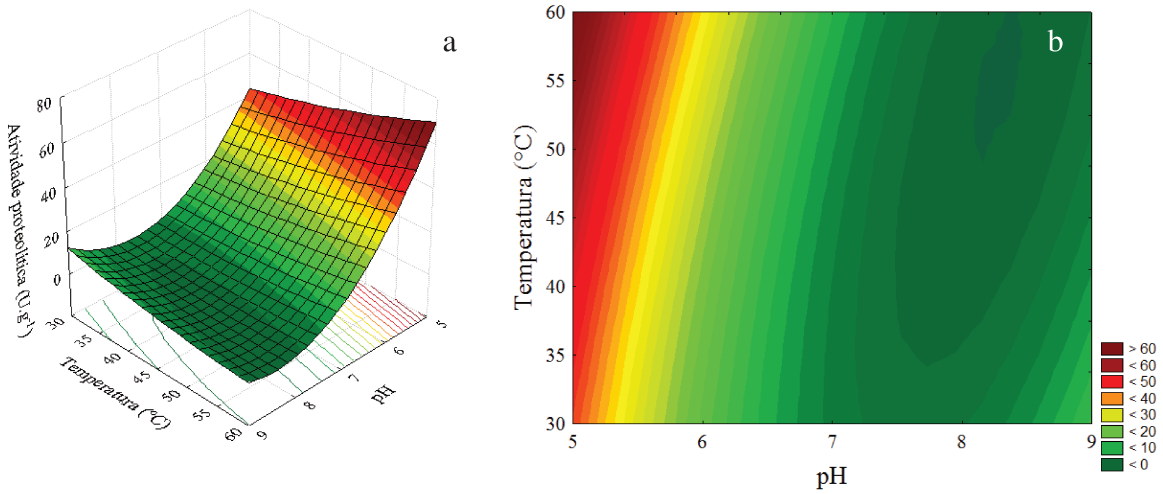

Figura 2 – Superfície de resposta (a) e curva de contorno (b) para atividade ótima do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 em função do pH e da temperatura.

2.2. Determinação do pH e temperatura de estabilidade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 utilizando delineamento experimental

Tabela 4 – Matriz do DCCR 2² com os valores codificados, reais e respostas para os ensaios de determinação do pH e temperatura de estabilidade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 após 1 e 2h de tratamento.

Ensaios	x_1	x_2	pH	Temperatura (°C)	Atividade proteolítica 1h ($U.g^{-1}$)	Atividade proteolítica 2h ($U.g^{-1}$)
1	-1	-1	5,0	35,0	77,66	83,85
2	+1	-1	9,0	35,0	26,01	16,03
3	-1	+1	5,0	60,0	11,12	7,67
4	+1	+1	9,0	60,0	2,45	2,87
5	-1,41	0	4,0	47,5	62,38	60,04
6	+1,41	0	10,0	47,5	2,47	1,16
7	0	-1,41	7,0	30,0	69,30	72,19
8	0	+1,41	7,0	65,0	2,10	4,05
9	0	0	7,0	47,5	20,62	10,87
10	0	0	7,0	47,5	19,35	11,57
11	0	0	7,0	47,5	17,64	11,23

Tabela 5 – Coeficientes de regressão do DCCR 2² para determinação do pH e temperatura de estabilidade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 após 1 e 2h de tratamento.

Fatores	Coeficientes		Erro padrão		*t* (5)		*p*-valor	
	1h	2h	1h	2h	1h	2h	1h	2h
Média	19,21	11,22	3,89	3,34	4,93	3,36	0,004	0,020
pH (L)	-20,55	-21,39	2,72	2,33	-7,56	-9,17	<0,001	<0,001
pH (Q)	5,40	8,01	2,89	2,48	1,87	3,23	0,120	0,023
Temperatura (L)	-20,72	-21,31	2,72	2,33	-7,62	-9,13	<0,001	<0,001
Temperatura (Q)	7,04	11,77	2,89	2,48	2,44	4,75	0,058	0,005
pH x Temperatura	10,83	15,71	3,99	3,42	2,71	4,59	0,042	0,005

Tabela 6 – ANOVA para atividade proteolítica na determinação do pH e temperatura de estabilidade do extrato enzimático bruto de proteases de *A. oryzae*.

Fonte de variação	Soma dos quadrados		Graus de liberdade		Quadrado médio		*F* calculado	
	1h	2h	1h	2h	1h	2h	1h	2h
Regressão	7757,51	9274,35	5	5	1551,50	1854,87	80,84	107,09
Resíduos	95,96	86,61	5	5	19,19	17,32		
Total	7853,47	9360,96	10	10				

1h de tratamento: $F_{0,1;5,5} = 3,45$, $R^2 = 0,97$, p-valor < 0,0001
2h de tratamento: $F_{0,1;5,5} = 3,45$, $R^2 = 0,99$, p-valor < 0,0001

Modelos quadráticos gerados a partir dos coeficientes de regressão e variáveis codificadas:

Atividade proteolítica (1h) = $19,21 - 20,55x_1 + 5,40x_1^2 - 20,72x_2 + 7,04x_2^2 + 10,83x_1.x_2$

Atividade proteolítica (2h) = $11,22 - 21,39x_1 + 8,01x_1^2 - 21,31x_2 + 11,77x_2^2 + 15,71x_1.x_2$

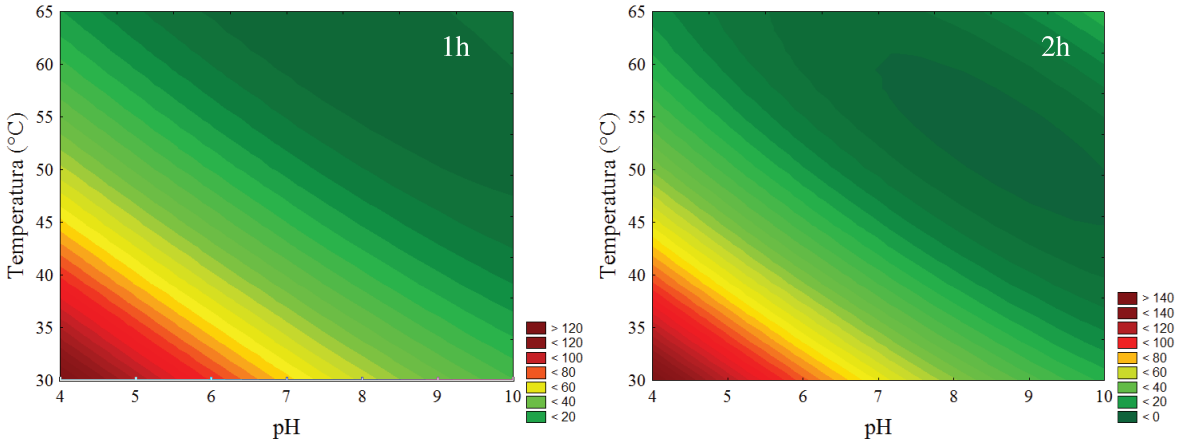

Figura 3 - Curvas de contorno para estabilidade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 após 1 e 2h de tratamento em função do pH e da temperatura.

2.3. Determinação da estabilidade do extrato enzimático bruto de proteases de *A. oryzae* LBA 01 nas condições ótimas de atividade (pH 5,0 a 55°C).

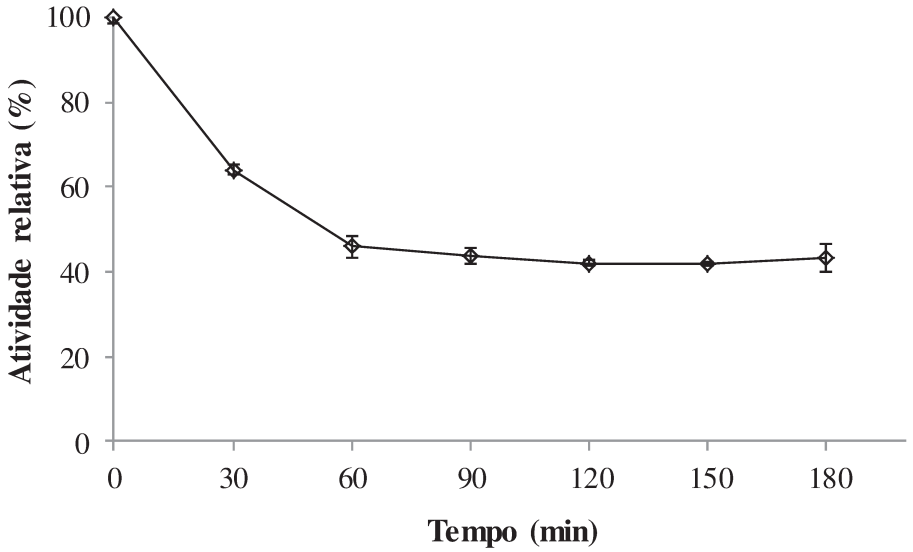

Figura 4 – Estabilidade do extrato enzimático bruto de protease de *A. oryzae* LBA 01 nas condições de pH e temperatura ótima de atividade durante 180 minutos de incubação.

2.4. Determinação dos parâmetros cinéticos *Km* e *V*máx do extrato enzimático bruto de proteases de *A. oryzae* LBA 01.

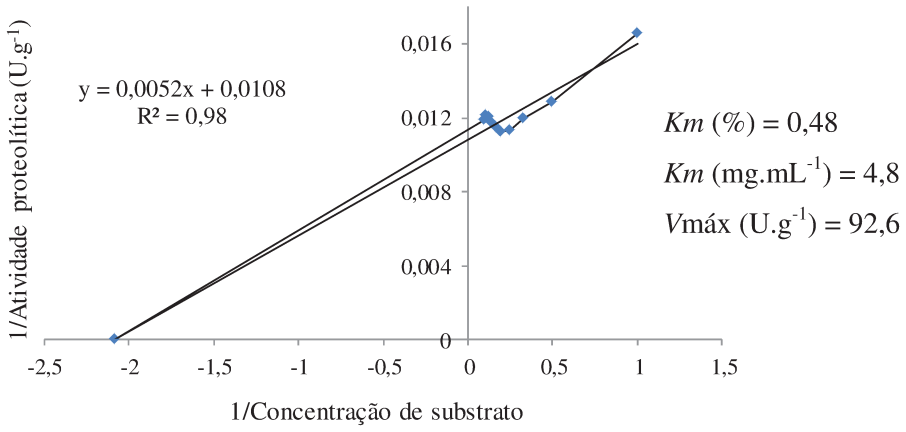

Figura 5 - Determinação das constantes *Km* e *V*máx da protease de *A. oryzae* de acordo com o gráfico de Lineweaver-Burk, utilizando-se o substrato azocaseína.

Anexos Capítulo III

1. Estudo das condições de hidrólise de proteína isolada de soja utilizando delineamento experimental

Tabela 1 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2² para o ensaio de ORAC de hidrolisados de proteína isolada de soja obtidos com protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	457,66	402,67	12,02
2	934,92	844,35	9,69
3	836,30	801,71	4,14
4	864,02	793,85	8,12
5	567,31	557,27	1,77
6	823,75	864,02	-4,89
7	536,55	587,41	-9,48
8	811,86	833,88	-2,71
9	627,35	710,64	-13,28
10	689,74	710,64	-3,03
11	667,63	710,64	-6,44

Tabela 2 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2² para o ensaio de DPPH de hidrolisados de proteína isolada de soja obtidos com protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	9,18	9,75	-6,16
2	9,87	9,75	1,20
3	11,29	11,47	-1,65
4	11,00	11,47	-4,32
5	11,08	10,70	3,47
6	10,86	10,70	1,56
7	9,51	9,31	2,12
8	12,10	11,75	2,92
9	11,79	11,45	2,92
10	11,38	11,45	-0,59
11	11,17	11,45	-2,49

Tabela 3 - Matriz do DCCR 2² com os valores codificados, reais e respostas (% de inibição de radicais DPPH) para os ensaios de determinação dos efeitos da concentração de substrato e da protease Flavourzyme® 500L de *A. oryzae* na hidrólise de proteína isolada de soja.

Ensaios	x_1	x_2	Substrato ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)	Inibição DPPH (%)
1	-1	-1	50,0	20,0	53,16
2	+1	-1	130,0	20,0	56,14
3	-1	+1	50,0	70,0	62,68
4	+1	+1	130,0	70,0	61,04
5	-1,41	0	33,6	45,0	63,88
6	+1,41	0	146,4	45,0	61,54
7	0	-1,41	90,0	9,8	52,87
8	0	+1,41	90,0	80,2	67,76
9	0	0	90,0	45,0	65,10
10	0	0	90,0	45,0	62,52
11	0	0	90,0	45,0	61,41

Tabela 4 – Coeficientes de regressão do DCCR 2² para determinação dos efeitos da concentração de substrato e da protease comercial Flavourzyme® 500L de *A. oryzae* na obtenção de hidrolisados de proteína isolada de soja com atividade antioxidante (% de inibição de radicais DPPH).

Fatores	Coeficientes	Erro padrão	*t* (5)	*p*-valor
Média	63,01	1,56	40,32	0,000
Substrato (x_1) (L)	-0,24	0,95	-0,26	0,805
Substrato (x_1) (Q)	-0,96	1,14	-0,85	0,436
Protease (x_2) (L)	4,43	0,96	4,64	0,006
Protease (x_2) (Q)	-2,16	1,14	-1,89	0,116
Substrato × Protease ($x_{1\times}x_2$)	-1,15	1,35	-0,85	0,431

Tabela 5 – ANOVA para atividade antioxidante (% de inibição de radicais DPPH) na determinação dos efeitos da concentração de substrato e protease comercial Flavourzyme® 500L de *A. oryzae* na hidrólise de proteína isolada de soja.

Fontes	Soma dos quadrados	Graus de liberdade	Quadrado médio	*F* calculado
Regressão	179,31	2	89,65	15,04
Resíduos	47,70	8	5,96	
Total	227,01	10		

$F_{0,1;2,8} = 3,11$, $R^2 = 0,79$, p-valor = 0,002

Modelo gerado a partir dos coeficientes de regressão e variáveis codificadas estatisticamente significativas:

$$Inibição\ DPPH\ (\%) = 63,01 + 4,43x_2 - 2,16x_2^2$$

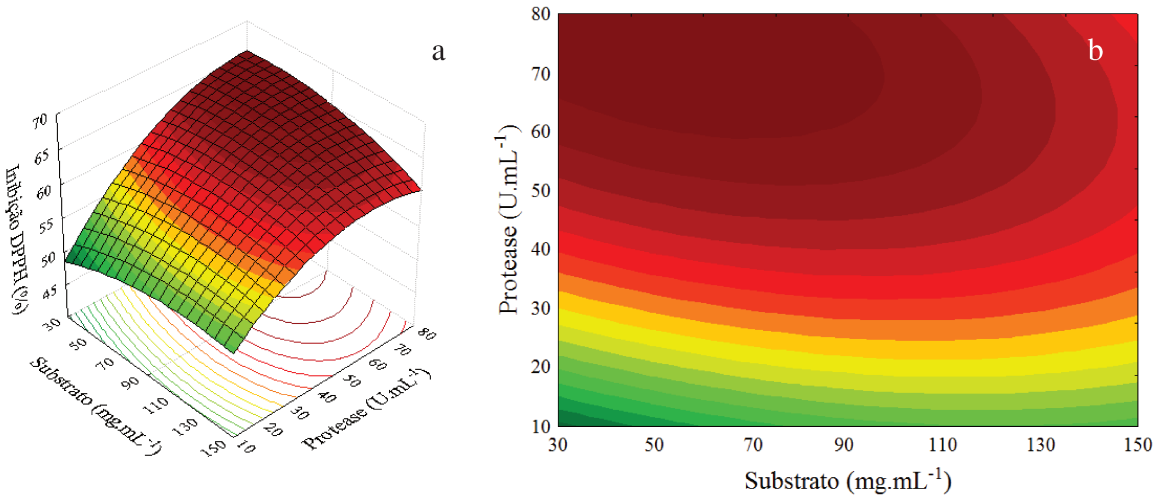

Figura 1 - Superfície de resposta (a) e curva de contorno (b) para determinação da atividade antioxidante (% de inibição de radicais DPPH) dos hidrolisados de proteína isolada de soja em função da concentração de substrato e da protease comercial Flavourzyme® 500L de *A. oryzae*.

Tabela 6 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (% de inibição de radicais DPPH) do DCCR 2^2 de hidrolisados de proteína isolada de soja obtidos com protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	53,16	55,79	-4,94
2	56,14	55,79	0,62
3	62,69	64,66	-3,15
4	61,04	64,66	-5,94
5	63,88	62,10	2,79
6	61,54	62,10	-0,92
7	52,87	52,07	1,50
8	67,76	64,62	4,64
9	65,10	62,10	4,61
10	62,52	62,10	0,67
11	61,41	62,10	-1,13

Anexos Capítulo IV

1. Estudo das condições de hidrólise de soro de leite bovino utilizando delineamento experimental

Tabela 1 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2² para o ensaio de ORAC de hidrolisados de soro de leite bovino obtidos com a protease *A. oryzae* LBA 01.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	197,27	186,36	5,53
2	152,62	111,37	27,03
3	394,45	412,64	-4,61
4	330,58	337,66	-2,14
5	341,25	314,87	7,73
6	206,13	209,14	-1,46
7	60,84	102,48	-68,43
8	434,82	421,54	3,05
9	259,28	262,01	-1,05
10	282,03	262,01	7,10
11	222,82	262,01	-17,59

Tabela 2 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2² para o ensaio de DPPH de hidrolisados de soro de leite bovino obtidos com a protease *A. oryzae* LBA 01.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	8,64	9,94	-14,95
2	6,81	5,98	12,15
3	16,52	17,06	-3,24
4	12,63	13,10	-3,74
5	17,07	15,92	6,70
6	9,95	10,35	-4,06
7	5,06	4,88	3,51
8	15,48	14,92	3,63
9	11,14	11,41	-2,43
10	11,85	11,41	3,74
11	11,23	11,41	-1,62

Tabela 3 - Matriz do DCCR 2² com os valores codificados, reais e respostas (% de inibição de radicais DPPH) para os ensaios de determinação dos efeitos da concentração de substrato e da protease de *A. oryzae* LBA 01 na hidrólise de soro de leite bovino.

Ensaios	x_1	x_2	Substrato ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)	Inibição DPPH (%)
1	-1	-1	80,0	20,0	39,25
2	+1	-1	220,0	20,0	30,37
3	-1	+1	80,0	70,0	74,11
4	+1	+1	220,0	70,0	57,04
5	-1,41	0	51,3	45,0	76,17
6	+1,41	0	248,7	45,0	45,57
7	0	-1,41	150,0	9,8	22,24
8	0	+1,41	150,0	80,2	69,44
9	0	0	150,0	45,0	52,62
10	0	0	150,0	45,0	54,57
11	0	0	150,0	45,0	51,42

Tabela 4 – Coeficientes de regressão do DCCR 2² para determinação dos efeitos da concentração de substrato e da protease de *A. oryzae* LBA 01 na obtenção de hidrolisados de soro de leite bovino com atividade antioxidante (% de inibição de radicais DPPH).

Fatores	Coeficientes	Erro padrão	*t* (5)	*p*-valor
Média	52,88	2,10	25,10	<0,0001
Substrato (x_1) (L)	-8,66	1,29	-6,70	0,001
Substrato (x_1) (Q)	3,22	1,54	2,09	0,091
Protease (x_2) (L)	16,06	1,29	12,42	<0,0001
Protease (x_2) (Q)	-4,34	1,54	-2,81	0,037
Substrato × Protease ($x_{1\times}x_2$)	-2,05	1,82	-1,12	0,313

Tabela 5 – ANOVA para atividade antioxidante (% de inibição de radicais DPPH) na determinação dos efeitos da concentração de substrato e da protease de *A. oryzae* LBA 01 na hidrólise de soro de leite bovino.

Fontes	Soma dos quadrados	Graus de liberdade	Quadrado médio	*F* calculado
Regressão	2.883,12	4	720,78	51,89
Resíduos	83,32	6	13,89	
Total	2.966,44	10		

$F_{0,1;4,6} = 3,18$, $R^2 = 0,97$, p-valor < 0,0001

Modelo gerado a partir dos coeficientes de regressão e variáveis codificadas estatisticamente significativas:

$$Inibição\ DPPH\ (\%) = 52,88 - 8,66\ x_1 + 3,22\ x_1^2 + 16,06\ x_2 - 4,34\ x_2^2$$

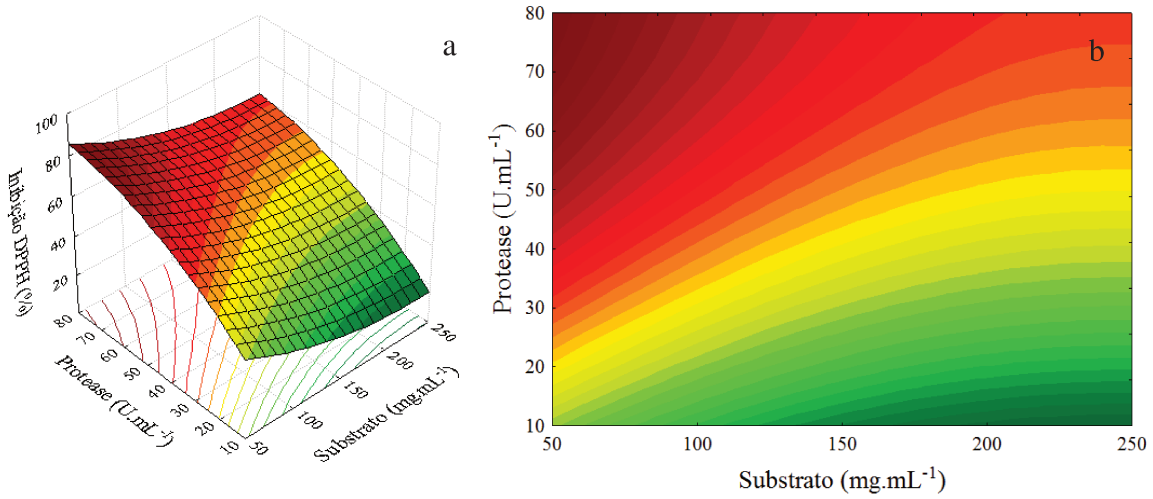

Figura 1 - Superfície de resposta (a) e curva de contorno (b) para determinação da atividade antioxidante (% de inibição de radicais DPPH) de hidrolisados de soro de leite bovino em função da concentração de substrato e da protease de *A. oryzae* LBA 01.

Tabela 6 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (% de inibição de radicais DPPH) do DCCR 2^2 de hidrolisados de soro de leite bovino obtidos com protease de *A. oryzae* LBA 01.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	39,25	44,37	-13,04
2	30,37	27,05	10,95
3	74,11	76,48	-3,20
4	57,04	59,16	-3,71
5	76,17	71,50	6,13
6	45,57	47,08	-3,30
7	22,24	21,62	2,81
8	69,44	66,90	3,66
9	52,62	52,88	-0,50
10	54,57	52,88	3,10
11	51,42	52,88	-2,85

Anexos Capítulo V

1. Estudo das condições de hidrólise de proteínas da clara de ovo utilizando delineamento experimental

Tabela 1 - Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2^2 para o ensaio de ORAC de hidrolisados de proteínas de clara de ovo obtidos com a protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	481,43	421,19	12,51
2	1160,42	1046,36	9,83
3	831,42	828,92	0,30
4	694,95	638,63	8,10
5	576,47	580,03	-0,62
6	807,86	887,53	-9,86
7	715,42	733,78	-2,57
8	681,90	733,78	-7,61
9	708,81	733,78	-3,52
10	679,12	733,78	-8,05

Tabela 2 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (Trolox EQ µmol.g^{-1}) do DCCR 2^2 para o ensaio de DPPH de proteínas de clara de ovo obtidos com a protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	13,14	13,87	-5,53
2	17,45	17,74	-1,70
3	15,32	15,79	-3,07
4	15,78	15,82	-0,25
5	14,43	14,43	0,01
6	16,58	17,19	-3,66
7	16,84	15,81	6,11
8	16,46	15,81	3,98
9	16,77	15,81	5,75
10	15,68	15,81	-0,83
11	15,43	15,81	-2,44

Tabela 3 - Matriz do DCCR 2² com os valores codificados, reais e respostas (% de inibição de radicais DPPH) para os ensaios de determinação dos efeitos da concentração de substrato e da protease comercial Flavourzyme® 500L de *A. oryzae* na hidrólise de proteínas da clara de ovo.

Ensaios	x_1	x_2	Substrato ($mg.mL^{-1}$)	Protease ($U.mL^{-1}$)	Inibição DPPH (%)
1	-1	-1	13,0	20,0	54,74
2	+1	-1	27,0	20,0	71,08
3	-1	+1	13,0	70,0	63,36
4	+1	+1	27,0	70,0	65,98
5	-1,41	0	10,0	45,0	59,34
6	+1,41	0	30,0	45,0	68,33
7	0	-1,41	20,0	9,8	68,89
8	0	+1,41	20,0	80,2	68,59
9	0	0	20,0	45,0	67,82
10	0	0	20,0	45,0	64,24
11	0	0	20,0	45,0	63,01

Tabela 4 – Coeficientes de regressão do DCCR 2² para determinação dos efeitos da concentração de substrato e protease comercial Flavourzyme® 500L de *A. oryzae* na obtenção de hidrolisados de proteínas da clara de ovo com atividade antioxidante (% de inibição de radicais DPPH).

Fatores	Coeficientes	Erro padrão	*t* (5)	*p*-valor
Média	65,03	1,45	44,61	0,000
Substrato (x_1) (L)	3,96	0,89	4,43	0,007
Substrato (x_1) (Q)	-1,22	1,06	-1,14	0,303
Protease (x_2) (L)	0,39	0,89	0,43	0,682
Protease (x_2) (Q)	1,23	1,06	1,16	0,298
Substrato × Protease ($x_{1\times}x_2$)	-3,43	1,26	-2,71	0,042

Tabela 5 – ANOVA para atividade antioxidante (% de inibição de radicais DPPH) na determinação dos efeitos da concentração de substrato e da protease comercial Flavourzyme® 500L de *A. oryzae* na hidrólise de proteínas da clara de ovo.

Fontes	Soma dos quadrados	Graus de liberdade	Quadrado médio	*F* calculado
Regressão	172,39	2	86,20	12,06
Resíduos	57,16	8	7,15	
Total	229,55	10		

$F_{0,05;2,8}$ = 4,46, R^2 = 0,75, *p*-valor = 0,004

Modelo gerado a partir dos coeficientes de regressão e variáveis codificadas estatisticamente significativas:

$$Inibição\ DPPH\ (\%) = 65{,}03 + 3{,}96x_1 - 3{,}43\ x_1x_2$$

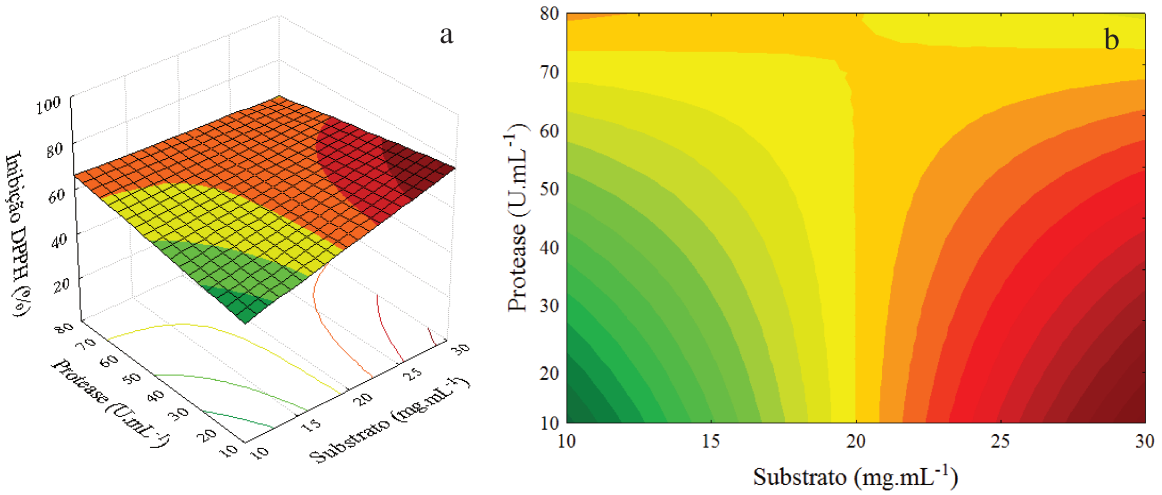

Figura 1 - Superfície de resposta (a) e curva de contorno (b) para determinação da atividade antioxidante (% de inibição de radicais DPPH) de hidrolisados de proteínas da clara de ovo em função da concentração de substrato e da protease comercial Flavourzyme® 500L de *A. oryzae*.

Tabela 6 – Valores experimentais, previstos pelo modelo e erros relativos de atividade antioxidante (% de inibição de radicais DPPH) do DCCR 2^2 de hidrolisados de proteínas da clara de ovo obtidos com protease comercial Flavourzyme® 500L de *A. oryzae*.

Ensaios	Valor experimental	Valor previsto	Erro relativo (%)
1	54,74	57,65	-5,32
2	71,08	72,42	-1,89
3	63,36	64,50	-1,81
4	65,98	65,57	0,63
5	59,34	59,44	-0,16
6	68,33	70,63	-3,38
7	68,89	65,03	5,60
8	68,59	65,03	5,19
9	67,82	65,03	4,11
10	64,24	65,03	-1,23
11	63,01	65,03	-3,21

Anexos Capítulos III, IV e V

1. Obtenção de hidrolisados proteicos com atividade antioxidante utilizando pepsina

Na determinação da combinação mais adequada de enzima:substrato, foram realizados testes com uma preparação comercial de pepsina (Sigma-P7125). Os dados não foram utilizados nos artigos, pois a padronização da concentração de enzima foi realizada em função da atividade proteolítica utilizando azocaseína como substrato nas condições ótimas de atividade de cada preparação enzimática. O substrato azocaseína não apresenta solubilidade em valores de pH abaixo de 5,0 e o pH ótimo de atividade da pepsina comercial é 2,4. Devido à limitação da técnica, a atividade proteolítica da pepsina foi realizada em pH 5,0, apresentando 1.022,0 ± 25,24 $U.g^{-1}$, um valor aproximadamente 4 vezes inferior ao das demais preparações avaliadas. Para padronização da concentração de enzima em função da atividade, grande quantidade de pepsina precisava ser adicionada às soluções de proteínas para hidrólise, tornando o processo inviável. Os resultados de atividade antioxidante dos hidrolisados obtidos com pepsina foram inferiores quando comparados aos obtidos com as outras preparações de proteases, pelo fato das hidrólises não terem sido conduzidas nas condições de pH ótimo da pepsina.

Tabela 1 – Efeito da concentração de preparação comercial de pepsina na hidrólise de proteína isolada de soja, soro de leite bovino e proteínas de clara de ovo e atividade antioxidante dos hidrolisados para os ensaios de ORAC e DPPH.

Substrato	Protease ($U.mL^{-1}$)	Atividade antioxidante (Trolox EQ $\mu mol.g^{-1}$)* ORAC	Atividade antioxidante (Trolox EQ $\mu mol.g^{-1}$)* DPPH
Proteína isolada de soja	Controle	393,78 ± 41,55	*Não detectado*
	10,0	403,23 ± 24,18	3,21 ± 0,36
	20,0	504,96 ± 28,67	3,81 ± 0,05
	40,0	387,42 ± 24,83	0,80 ± 0,31
Soro de leite bovino	Controle	28,01 ± 2,66	1,76 ± 0,06
	10,0	45,69 ± 2,88	1,45 ± 0,12
	20,0	49,33 ± 5,07	1,45 ± 0,11
	40,0	36,20 ± 4,91	1,74 ± 0,16
Proteínas da clara de ovo	Controle	131,24 ± 6,57	3,79 ± 0,49
	10,0	151,56 ± 10,73	4,58 ± 0,52
	20,0	161,28 ± 2,60	1,04 ± 0,22
	40,0	190,72 ± 6,37	3,29 ± 0,27

*Os ensaios foram realizados em triplicata e os resultados estão apresentados como média ± desvio padrão. As hidrólises foram conduzidas durante 4h em pH 5,0 e a temperatura de 37°C.