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JESSIKA GONÇALVES DOS SANTOS

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Orientadora: **HÉLIA HARUMI SATO**

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Resumo

Os efeitos da ampliação de escala e de diferentes variáveis de processo (temperatura e agitação) sobre a produção de proteases por *Bacillus licheniformis* LBA 46 foram avaliados utilizando meio de cultura composto por melaço de cana-de-açúcar (32 g/L), água de maceração de milho (6 g/L), extrato de levedura (2 g/L) e soro de leite (20 g/L), pH 7. Os resultados obtidos mostraram que a fermentação em reator de bancada contendo 3 L de meio resultou em 2.448 U/mL de protease após 48 h de fermentação e que as condições mais adequadas de temperatura e agitação foram 30 ºC e 300 rpm, respectivamente. O extrato bruto de proteases foi submetido à precipitação com sulfato de amônio (80% de saturação), diálise e liofilização para posterior caracterização bioquímica. A preparação enzimática semi-purificada apresentou atividade ótima na faixa de 60 e 65 °C em pH 7 e reteve 78%, 39% e 9% da atividade inicial após 120 min de tratamento a 50, 60 e 70 ºC, respectivamente. A protease foi purificada 3,33 vezes por precipitação com 80% de sulfato de amônio e cromatografia em coluna de DEAE-Sepharose, apresentando uma massa molecular de 40 kDa estimada por eletroforese por SDS-PAGE. A protease purificada apresentou atividade ótima na faixa de 50 e 60 °C. A enzima purificada apresentou alta atividade (> 80%) na faixa 6,5 a 9, tendo atividade ótima em pH 8,5; bem como mostrou-se estável na faixa de pH 5 a 10 após 24 h de incubação a 4 ºC, retendo mais de 86% da atividade inicial. A enzima purificada também se mostrou estável após 1 h de incubação a 40 ºC e reteve 85% da atividade inicial após 1 h a 50 ºC. O tratamento da protease semi-purificada (obtida por precipitação com 80% de sulfato de amônio) em pH 4, 7 e 9, com homogeneização a alta pressão (50-200 MPa) não aumentou sua atividade e estabilidade em 40, 60 e 90 °C. Os hidrolisados de proteína de arroz preparados com a protease de *B. licheniformis* LBA 46 e protease comercial Alcalase 2.4L apresentaram atividade antioxidante superior para as respostas avaliadas pelos métodos de ORAC e FRAP em comparação com proteína de arroz não hidrolisada. Os hidrolisados de proteína de ervilha preparados com a protease de *B. licheniformis* LBA 46 e protease comercial Alcalase 2.4L apresentaram atividade antioxidante para todas as respostas avaliadas pelos métodos DPPH, ORAC e FRAP. A atividade antioxidante dos hidrolisados de proteína de ervilha medida em termos de DPPH e FRAP foi menor em comparação com a atividade antioxidante da amostra de proteína não hidrolisada. No entanto, a atividade antioxidante medida em termos do ORAC teve um aumento significativo de 7,33 e 9,27 vezes para os hidrolisados da protease LBA e para os hidrolisados da Alcalase 2.4L, respectivamente, comparada com a proteína não hidrolisada. As condições de hidrólise foram validadas e, na

condição selecionada como mais adequada (pH 10 e 100 U/mL de protease), foi possível confirmar que os modelos definidos foram capazes de predizer os resultados alcançados.

Palavras-chave: *Bacillus licheniformis*; Protease; Fermentação; Homogeneização; Otimização; Antioxidante; Hidrolisados.

Abstract

The effects of scale amplification and different process variables (temperature and agitation) on the production of proteases by *Bacillus licheniformis* LBA 46 were evaluated using culture medium composed of sugar cane molasses (32 g/L), corn steep liquor (6 g/L), yeast extract (2 g/L) and dried whey (20 g/L), pH 7. The results showed that the fermentation in a bench reactor containing 3 L of medium resulted in 2,448 U/mL of protease after 48 h of fermentation and that the most suitable conditions of temperature and agitation were 30 ºC and 300 rpm, respectively. The crude protease extract was submitted to ammonium sulphate precipitation (80% of saturation), dialysis and lyophilization for subsequent biochemical characterization. The semi-purified enzymatic preparation presented optimum activity in the 60 and 65 ºC range at pH 7 and retained 78%, 39% and 9% of the initial activity after 120 min of treatment at 50, 60 and 70 ºC, respectively. The protease was purified 3.33 times by precipitation with 80% ammonium sulfate and DEAE-Sepharose column chromatography, presenting a molecular mass of 40 kDa estimated by SDS-PAGE electrophoresis. The purified protease presented optimum activity in the range of 50 and 60 ºC. The purified enzyme presented high activity (> 80%) in the range of 6.5 to 9, and had optimal activity at pH 8.5; as well as showed to be stable in the pH range of 5 to 10 after 24 h of incubation at 4 ºC, retaining more than 86% of the initial activity. The purified enzyme also showed to be stable after 1 h of incubation at 40 ºC and retained 85% of the initial activity after 1 h at 50 ºC. Treatment of semi-purified protease (obtained by precipitation with 80% ammonium sulphate) at pH 4, 7 and 9, with high pressure homogenization (50-200 MPa) did not increase its activity and stability at 40, 60 and 90 ºC. Rice protein hydrolysates prepared with the *B. licheniformis* protease LBA 46 and commercial protease Alcalase 2.4L showed superior antioxidant activity for the responses evaluated by the ORAC and FRAP methods compared to unhydrolyzed rice protein. Pea protein hydrolysates prepared with *B. licheniformis* protease LBA 46 and commercial protease Alcalase 2.4L showed antioxidant activity for all responses evaluated by the DPPH, ORAC and FRAP methods. The antioxidant activity of pea protein hydrolysates measured in terms of DPPH and FRAP was lower in comparison with the antioxidant activity of the non-hydrolyzed protein sample. However, the antioxidant activity measured in terms of the ORAC had a significant increase of 7.33 and 9.27 times for the LBA protease hydrolysates and for the Alcalase 2.4L hydrolysates, respectively, compared to the non-hydrolyzed protein. The hydrolysis conditions were validated and, in the condition

selected as the most appropriate (pH 10 and 100 U/mL protease), it was possible to confirm that the defined models were capable to predict the results achieved.

Keywords: *Bacillus licheniformis*; Protease; Fermentation; Homogenization; Optimization; Antioxidant; Hydrolysates.

Sumário

Introdução geral

As proteases catalisam a hidrólise de ligações peptídicas em proteínas e peptídeos e representam um dos grupos mais importantes de enzimas comerciais e industriais (Rao et al., 1998; Bach et al., 2012). Os micro-organismos, em geral, representam uma excelente fonte de proteases. Embora as enzimas proteolíticas possam ser obtidas de animais e plantas, os micro-organismos são os principais produtores devido à vantagem econômica e técnica ocasionada pela sua ampla gama de características fisiológicas, sua capacidade de produzir enzimas e outros metabólitos em altas concentrações, além de sua diversidade bioquímica e possibilidade de manipulação genética (Schallmey et al., 2004; Laxman et al., 2005; Sumantha et al., 2006).

Estas enzimas microbianas possuem uma variedade de funções bioquímicas, fisiológicas e reguladoras. Em comparação com as enzimas produzidas por animais ou extraídas de plantas, as enzimas microbianas têm a enorme vantagem de poderem ser produzidas em altas quantidades utilizando técnicas de fermentação já estabelecidas (Stanbury et al., 2003). As principais linhagens microbianas utilizadas na produção de enzimas ainda são espécies de *Bacillus* sp., utilizadas principalmente para produzir serina proteases alcalinas e proteases neutras (Schallmey et al., 2004; Ward, 2011; Pant et al., 2015).

As linhagens de micro-organismos selecionadas como produtoras de alta atividade da enzima, e promissoras em escala industrial, podem ser cultivadas por fermentação submersa ou sólida em diferentes tipos de reatores, a composição do meio de cultura e as condições podem ser otimizadas visando alta produtividade, facilidade de extração e purificação e menor custo (Stanbury et al., 2003). Fatores como aeração, agitação, temperatura, pH e tempo de incubação influenciam o metabolismo microbiano e consequentemente a produção de metabólitos como as enzimas (Potumarthi et al., 2007; Abidi et al., 2008; Chandrasekaran et al., 2015). Diversos estudos de otimização da composição do meio de cultura e das condições de fermentação de micro-organismos para a produção de enzimas têm sido descritos na literatura (Agrawal et al., 2005; Hmidet et al., 2009; Mahajan et al., 2012; Bagagli e Sato, 2013; Contesini, 2014; Pant et al., 2015), pois cada micro-organismo possui condições especiais para produção máxima de determinada enzima.

Potumarthi et al. (2007) estudaram o efeito da aeração e agitação na fermentação de *Bacillus licheniformis* NCIM-2041 em reatores agitados para produção de protease alcalina com meio de cultura composto por 5 g/L de caseina, 10 g/L de extrato de malte, 10 g/L de polipeptona, 10 g/L de Na_2CO_3, pH 9.5. Os autores obtiveram atividade máxima após 72 h de

fermentação sob as condições de 3 vvm de aeração e 200 rpm de agitação obtendo atividade de 102 U/mg. Chandrasekaran et al. (2015) estudaram a otimização da produção de proteases de fungos do gênero *Aspergillus* em meio de cultura com 1% de extrato de levedura, 0,02% de $MgSO_4$, 2% de glicose, 0,1% de K_2HPO_4, pH 7.0. Após incubação dos micro-organismos a 28 ºC por 5 dias em agitador incubador rotatório, a máxima produção de protease foi observada em pH 8 para *Aspergillus flavus* (30 U/mL) e pH 7 para *Aspergillus niger* (26 U/mL). As temperaturas ótimas para a fermentação de *A. flavus* e *A. niger* foram 30 ºC e 35 ºC sendo obtida atividade máxima de 26 U/mL e 32 U/mL, respectivamente.

Na literatura são encontrados diversos estudos sobre os efeitos do tratamento de enzimas sob alta pressão visando à inativação, ativação e aumento da estabilidade (Lacroix et al., 2005; Welti-Chanes et al., 2009; Carbonell et al., 2013; Tribst et al., 2013; Tribst et al., 2014; Pinho et al., 2014). Em geral, durante a homogeneização a alta pressão de (HAP), o fluido é forçado através de uma abertura estreita na válvula do homogeneizador. Alguns dos efeitos da HAP são atribuídos principalmente à cavitação, à pressão de cisalhamento e às forças de turbulência, mas a modificação das estruturas protéicas utilizando HAP também é causada pelo tempo de tratamento, impacto (mudança na direção do fluxo), fricção e calor, que ocorre simultaneamente durante a aplicação do tratamento. Essas forças têm a capacidade de alterar a configuração e a função das proteínas (Roach e Harte, 2008).

O tratamento da enzima por alta pressão pode resultar em inativação reversível ou irreversível, parcial ou completa, ativação ou aumento da estabilidade devido à alteração da conformação da estrutura proteica, mudanças na interação enzima-substrato e nos mecanismos de reação ou também devido ao efeito de um passo específico de limitação na taxa catalítica global (Cheftel, 1992; Masson et al., 2001; Eisenmenger e Reyes-de-Corcuera, 2009).

As proteases microbianas, especialmente as proteases alcalinas, são utilizadas na preparação de hidrolisados protéicos de alto valor. Muitas espécies de *Bacillus* sp. produzem grande quantidade de proteases que são capazes de hidrolisar proteínas liberando peptídeos com propriedades benéficas (Ward et al., 2009), tais como peptídeos com atividade antioxidante, resultantes da hidrólise de proteínas de alimentos (Korhonen e Pihlanto, 2006; Ricci et al., 2010). A bioatividade dos peptídeos formados depende principalmente da fonte de proteínas utilizadas como substrato, da especificidade da protease e do tipo de tratamento antes da hidrólise (Gauthier e Pouliot, 2003).

Os vegetais são uma excelente fonte de proteínas e, por isso, seus hidrolisados podem contribuir como fontes alternativas de peptídeos bioativos promotores de saúde (Thamnarathip et al., 2016). A proteólise limitada de algumas proteínas pode aumentar a sua utilização, por exemplo, produtos hidrolisados com diferentes propriedades funcionais podem ser produzidos através da aplicação de condições de hidrólise específicas utilizando determinada protease como catalisador. As propriedades bioativas de alguns hidrolisados de proteínas são dependentes do processo e da enzima empregada. A otimização das condições do processo é uma poderosa ferramenta capaz de reduzir o tempo para obter a resposta necessária.

O presente estudo visou estudar o efeito da temperatura e da agitação na fermentação submersa da linhagem de *B. licheniformis* LBA 46 e na produção da protease em reator de bancada; avaliar o efeito do tratamento de HAP na atividade e estabilidade da protease semi-purificada; purificar e determinar as características da protease, e aplicar a preparação semi-purificada da protease de *B. licheniformis* LBA 46 e da protease comercial na hidrólise das proteínas concentradas de arroz e ervilha visando a produção de peptídeos com atividade antioxidante. O trabalho encontra-se dividido em forma de capítulos como descrito a seguir.

O Capítulo I consiste em uma Revisão Bibliográfica que aborda os aspectos gerais e produção das proteases microbianas com enfoque na importância e uso dessas enzimas para produção de hidrolisados proteicos. Este capítulo foi publicado na revista Food Research International.

O Capítulo II descreve o estudo do efeito da temperatura e agitação na fermentação do micro-organismo *B. licheniformis* LBA 46 em reator de bancada utilizando o planejamento fatorial visando maior produção de proteases. Foram estudados os parâmetros cinéticos e termodinâmicos da protease semi-purificada e a purificação, bem como determinação de algumas características bioquímicas da protease purificada.

O Capítulo III apresenta a avaliação dos efeitos da aplicação da tecnologia de alta pressão de homogeneização, na atividade e estabilidade da preparação de protease semi-purificada de *B. licheniformis* LBA 46, em diferentes condições de tratamento (pH e pressão).

Os Capítulos IV e V têm como proposta estudar as melhores condições (pH e concentração das preparações de protease semi-purificada de *B. licheniformis* LBA 46 e Alcalase 2.4L comercial) para a hidrólise das proteínas de arroz e de ervilha, respectivamente, avaliando-se a produção de peptídeos com atividade antioxidante por meio dos ensaios DPPH, FRAP e ORAC (TE μmol/g).

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Capítulo I

Microbial proteases: Production and application in obtaining protein hydrolysates

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Abstract

The catalytic properties of the proteases have already allowed for their introduction into several industrial processes, such as food, chemical, pharmaceutical, etc. Recent advances in the biotechnology, particularly in the production of protein hydrolysates, have provided an important development of this area. The enzymatic hydrolysis allows for the use of different food protein sources that, after hydrolysis, can be used also as sources of bioactive peptides. Microbial proteases have interesting characteristics in the sense of low cost of production, good stability and specificity representing a powerful tool in the development and production of new protein hydrolysates with characteristics that can be explored industrially. This review aims to describe the production of proteases, the use of microbial proteases in enzymatic hydrolysis, in different industries and to explain the characteristics of such enzymes.

Keywords: Bacteria; Biotechnology; Protease; Peptide; Hydrolysis; Bioactivity.

1. Introduction

Proteases catalyze the hydrolysis of peptide bonds in proteins and peptides and represent one of the most important groups of commercial and industrial enzymes (Bach et al., 2012; Rao, Tanksale, Ghatge, & Deshpande, 1998; Sumantha, Larroche, & Pandey, 2006). According to the Enzyme Commission (EC), proteases belong to group 3 of the hydrolases and subgroup 4, hydrolysis of peptide bonds, but can still be classified according to the source of isolation (animal, vegetable or microbial), catalytic action (endo or exopeptidase), active site, charge, molecular size and substrate specificity (Sumantha et al., 2006).

Microorganisms represent an excellent source of proteases, being also the main source of production. Although the proteolytic enzymes can be obtained from animals and plants, microorganisms are the main producers due to their economic and technical advantages, besides their biochemical diversity and the possibility of genetic manipulation (Laxman et al., 2015; Sumantha et al., 2006).

These microbial enzymes possess a variety of biochemical, physiological and regulatory functions. Over the centuries, microbial proteases have played a key role in the production of traditional fermented foods, and now the industrial enzyme sector, dominated by microbial protease products, supplies the world with biocatalysts for use in many industries (Ward, Rao, & Kulkarni, 2009).

According to Pant et al. (2015) the main enzymes produced by microbial sources are proteases. Due to their versatility, microbial proteases could be used mainly in the cleaning, food and textile industries. Alkaline proteases, in particular, have been used in the cleaning (Gupta, Beg, & Lorenz, 2002; Jaouadi, Ellouz-Chaabouni, Rhimi, & Bejar, 2008; Maurer, 2004; Paul et al., 2014; Prakasham, Rao, Rao, & Sarma, 2005), textile (Maurer, 2004; Ward et al., 2009) and leather (Dayanandan, Kanagaraj, Sounderraj, Govindaraju, & Rajkumar, 2003; Gupta et al., 2002; Kumar & Takagi, 1999) industries and in food processing (Gupta et al., 2002; Kumar & Takagi, 1999; Valdez-Peña et al., 2010).

The cleavage of peptide bonds can be carried out via enzymatic or chemical. Hydrolysis using a chemical, alkaline or acidic process is more difficult to control and generates hydrolysates with modified amino acids (Tavano, 2013). Proteases are capable of promoting highly specific and selective proteins modifications (Sumantha et al., 2006). The limited proteolysis of proteins may increase its range of use, for example, hydrolyzed products with different functional properties can be produced through the application of specific hydrolytic conditions, and they could be used in the several kinds of industries. These

functional properties play an important role because they will determine the main characteristics of the final product, defining its use.

2. Microbial proteases

2.1 General aspects

The market for industrial enzymes expanded substantially during the 1960s, when alkaline proteases were initially marketed for use in detergents. The most important microbial strains used in the production of proteases are species of *Bacillus* sp. (Rao et al., 1998; Ward et al., 2009).

Many bacteria belonging to the *Bacillus* genus are important producers of enzymes for industry and research (Schallmey, Singh, & Ward, 2004; Widsten & Kandelbauer, 2008). Also, industrial microorganisms are attractive for a variety of reasons, including high growth rates leading to short fermentation times, their ability to secrete proteins in the extracellular environment, and are GRAS (generally recognized as safe), such the species of *Bacillus subtilis* and *Bacillus licheniformis* (Parrado, Rodriguez-Morgado, Tejada, Hernandez, & Garcia, 2014; Ward et al., 2009).

B. licheniformis have the ability to grow using various nutrient sources, as well as produce and excrete various hydrolytic enzymes and produce, mainly, alkaline and neutral proteases (Parrado et al., 2014; Ward et al., 2009).

Due to their enormous diversity, the classification of proteases is mainly based on the type of reaction catalyzed, their structure, and their active site. The protease groups can also be divided according to their site of action in: exopeptidases which cleave peptide bonds near the terminal groups and the endopeptidases which cleave peptide bonds distal to the terminal groups in the middle of the chain (Sumantha et al., 2006; Ward, 2011). Based on their active site, proteases are mainly referred to as aspartic, cysteine, serine and metallo proteases (Table 1).

Table 1. Main properties of proteases.

Classification	Molar mass (kDa)	Otimum range of pH	Optimum range of temperature (ºC)
Aspartic proteases	30-45	3-5	40-55
Cysteine proteases	34-35	2-3	40-55
Metallo proteases	19-37	5-7	65-85
Serine proteases	18-35	6-1	50-70

Adapted from Rao et al. (1998) and Sumantha et al. (2006).

The structure of the active site of the protease defines its binding to the substrate and determines how the substrate will fit to the enzyme and, consequently, is the responsible for defining the specificity of the substrate by the protease (Turk, 2006). This specificity is what determines the position where the enzyme will be able to hydrolyse and is important in the choice of protease according to the protein that will be hydrolyzed, modulating the degree of hydrolysis as well as the profile of released amino acids (Tavano, 2013).

Proteases can also be named according to the pH range in which they act: acidic, neutral and alkaline. Acid proteases are effective at low pH values and act on the breakdown of bonds involving aromatic amino acids bulky side chains at both sides of the cleaving bond (Rao et al., 1998; Sumantha et al., 2006).

Neutral proteases are active in the pH range of 5 - 8 and have the characteristic of producing protein hydrolysates with less bitterness than animal proteases, thus representing an important application for the food industry. These proteases have high affinity for hydrophobic amino acids and have low thermal tolerance, which is advantageous from the point of view of reaction control and specific conditions for the production of hydrolysates with low degree of hydrolysis (Rao et al., 1998).

Alkaline proteases, however, have activity in the alkaline pH ranging between 7 and 11 having their optimum at pH 10. These enzymes are able to hydrolyse bonds containing tyrosine, phenylalanine, leucine (close to the terminal carboxyl group), aspartate, histidine and serine (in their active site) (Gupta et al., 2002; Rao et al., 1998). Table 2 shows the properties of some proteases from different microbial sources.

Table 2. Protease producing microorganism and enzymes characteristics.

Microorganism	Molecular weight (kDa)	Optimum temperature (C°)	Optimum pH	Reference
Bacteria				
Bacillus subtilis PE 11	15	60	10	(Adinarayana, Ellaiah, & Prasad, 2003)
Streptomyces nogalator AC 80	66	28	7.5-8.5	(Mitra & Chakrabartty, 2005)
Xenorhabdus nematophila	39	30	8	(Mohamed, 2007)
Bacillus sp.	68	65	8	(Srinivasan, Das, Balakrishnan, Philip, & Kannan, 2009)
Bacillus circulans BM15	30	40	7	(Venugopal & Saramma, 2007)
Bacillus cereus VITSN04	32	30	8	(Sundararajan, Kannan, & Chittibabu, 2011)
Fungi				
Aspergillus fumigatus TKU003	124	40	8	(Wang, Chen, & Yen, 2005)
Aspergillus flavus AP2	46	45	8	(Hossain, Das, Marzan, Rahman, & Anwar, 2006)
Aspergillus niger	38	40	10	(Devi, Banu, Gnanaprabhal, Pradeep, & Palaniswamy, 2008)
Aspergillus oryzae	35	60	10	(Murthy & Naidu, 2010)

2.2 Production of proteases

2.2.1 Isolation of protease producing microorganisms

The first stage in the production of proteases is the stage of microbial isolation. The microorganisms screening is generally performed for selection of microorganisms. The fermentation products are quantified to determine the best producing microorganisms, which must be able to produce the enzyme in a desirable yield. The microorganisms used in the production of proteases are generally isolated at sites with specific and/or adverse conditions that reflect the type and characteristics of the enzyme that will be produced. Table 3 shows some examples of isolation sources of protease producer microorganisms.

Table 3. Different sources of isolation of protease producing microorganisms.

Identification of the selected microorganism	Source of isolation	Reference
Bacteria		
Aeromonas veronii	Milk processing unit	(Divakar, Priya, & Gautam, 2010)
Bacillus cereus	Gut of fish *Mugil cephalus*	(Esakkiraj, Immanuel, Sowmya, Iyapparaj, & Palavesam, 2009)
Bacillus cereus	Chemicals contaminated area	(Xu, Jiang, Sun, & He, 2010)
Bacillus cereus	Contaminated soil and sea sediment	(Shah, Mody, Keshri, & Jha, 2010)
Bacillus pumilus	Contaminated soils of a wood factory	(Rahman, Mahamad, Salleh, & Basri, 2007)
Bacillus sphaericus and *Bacillus subtilis*	Contaminated water	(Fang, Liu, Wang, & Lv, 2009)
Bacillus subtillis	Marine sedments	(Uttatree & Charoenpanich, 2016)
Brevibacillus	Soil from cattle farm	(Anbu, 2016)
Pseudomonas aeruginosa	Soil contaminated with crude oil	(Tang, Pan, Li, & He, 2008)
Pseudomonas aeruginosa	Contaminated soils of a wood factory	(Geok et al., 2003)
Pseudomonas aeruginosa	Soil near to a solvent extraction unit	(Gupta & Khare, 2006)
Streptomyces sp.	Soil from arid region	(Mehta, Thumar, & Singh, 2006)
Streptomyces sp.	Amazon lichens	(Silva et al., 2015)
Fungi		
Aspergillus clavatus	Wastewater	(Hajji, Kanoun, Nasri, & Gharsallah, 2007)
Aspergillus flavus and *Aspergillus terreus*	Soil around leather industry	(Chellapandi, 2010)
Aspergillus sp.	Soil	(Chandrasekaran, Kumaresan, & Manavalan, 2015)
Beauveria felina	Soil from soy meal manufacturing industry	(Agrawal, Patidar, Banerjee, & Patil, 2005)
Mucor sp.	Herbivorous dung	(Alves, Campos-Takaki, Okada, Ferreira-Pessoa, & Milanez, 2005)
Penicillium chrysogenum	Soil	(Ikram-Ul-Haq, Mukhtar, & Umber, 2006)
Penicillium sp.	Soil from soy meal manufacturing industry	(Agrawal, Patidar, Banerjee, & Patil, 2004)

According to Fang et al. (2009), for an enzyme to be used as a catalytic agent, it is important that it has good stability, especially in the presence of organic solvents. Several studies have aimed at the isolation of microorganisms producing proteases resistant to incubation in organic solvents (Fang et al., 2009; Geok et al., 2003; Gupta & Khare, 2006; Mahanta, Gupta, & Khare, 2008; Rahman et al., 2007).

2.2.2 Fermentation

The type of fermentation, submerged (SmF) or solid (SSF), has influence on microbial growth and also on the production of enzymes (Biesebeke et al., 2002). The main difference between them is the amount of water. In SmF the microorganisms grow in liquid media with high free water available (Soccol et al., 2017), and in SSF the microorganisms grow in natural or inert solid support materials in the absence of very low free water content (Pandey, 1992; Thomas, Larroche, & Pandey, 2013).

The fermentative processes of bacteria and fungi that result in the production of proteases have duration of 2 - 4 and 3 - 5 days, respectively. Concentrations of enzymes up to 30 g/L can be obtained which are separated from the fermented medium by centrifugation (bacteria) or filtration (fungi) (Ward et al., 2009). The excretion of these hydrolytic enzymes is not directly related to microbial growth, and may be inducible by environmental fermentation conditions. Low levels of nutrients and a higher ratio between carbon/nitrogen resulted in higher enzymatic production of *B. licheniformis* (Parrado et al., 2014).

2.2.3 Culture medium

According to Téllez-Luis, Ramírez and Vázquez (2004), the culture medium represents 30% of the cost of a fermentation process and its composition reflects on microbial growth and metabolite production, therefore, plays a significant role in the production of enzymes. Other factors such as temperature, pH and incubation time also influence microbial metabolism (Abidi, Limam, & Nejib, 2008). Each microorganism has its own conditions for maximum production of enzymes.

Much research must be done to establish the proper medium in a fermentation process; however, some points are common to any type of culture medium. All microorganisms need water, energy sources, carbon, nitrogen, minerals, vitamins and air, in the case of aerobic microorganisms (Stanbury, Whitaker, & Hall, 1995).

2.2.4 Temperature and pH

The temperature and pH are important control parameter within the fermentative process for maximum microbial growth and consequent enzymatic production (Ellaiah, Srinivasulu, & Adinarayana, 2002; Sharma, Kumar, Panwar, & Kumar, 2017; Thomas et al., 2013; Ward et al., 2009). According to Sharma et al. (2017), the pH of the culture affects the enzymatic processes and the transport of several components by the cell membrane. It is possible that, within the optimum pH, the relative metabolic efficiency is high, since proton motive force in chemiosmosis is affected by the medium pH value. Variations in pH values during fermentation are able to provide information on the start and end of the protease production period (Ellaiah et al., 2002; Kumar & Takagi, 1999).

The temperature of the process is a parameter that influences the cellular metabolism (Zheng, Du, Guo, & Chen, 2001) and the kinetics of molecules such as proteins. The reaction rates and collision, strength of molecular interactions and other physico-chemical characteristics of proteins are also affected (Elias, Wieczorek, Rosenne, & Tawfik, 2014).

2.2.5 Agitation and aeration

Aeration is used in the submerse fermentation to provide sufficient oxygen to meet the metabolic needs of the growing microorganism. Stirring is necessary to ensure the homogeneity of the cells in suspension and the nutrients of the medium. The variation in stirring speed influences the mixing and availability of nutrients. The amount of oxygen and carbon dioxide dissolved in the medium and foaming are also affected by rates of agitation and aeration (Ellaiah et al., 2002; Stanbury et al., 1995).

Hmidet et al. (2009) studied the co-production of proteases and α-amylases by *B. licheniformis* NH1 in the presence of chicken feathers as a source of nitrogen and carbon using culture medium containing 7.5 g/L chicken feathers; 1 g/L yeast extract; 1.4 g/L K_2HPO_4; 0.7 g/L KH_2PO_4; 0.1 g/L $MgSO_4$; 0.5 g/L NaCl, pH 7, after 48 h at 200 rpm and 37 ºC. The caseinolytic activity of protease reached 3,500 U/mL. Parrado et al. (2014) also studied the fermentation process of *B. licheniformis* ATCC 21415 using chicken feathers as substrate. The culture medium was composed of 10 g/L tryptone; 10 g/L NaCl and 5 g/L yeast extract and at low feather concentrations (0.2 to 1%, w/v). The protease activity ranged from 200 to 500 U/mL after 170 h of fermentation at 37 °C and 200 rpm.

Zanphorlin et al. (2011) studied the production of proteases from the thermophilic fungus *Myceliophthora* sp. The microorganism was cultivated in Erlenmeyer flasks

containing media composed of 4.75 g wheat bran; 0.25 g casein hydrated with 7 mL of distilled water and 3 mL of salt solution containing 0.1% $(NH_4)_2SO_4$; 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% NH_4NO_3. The protease activity reached 8 U/mg protein after 72 h of fermentation at 45 ºC.

Abraham, Gea and Sánchez (2014) used leather processing industrial material containing hair residues as a fermentation medium and as a source of microorganisms for production of proteases. Fermentation occurred in an air-tight reactor of 4.5 L and after 14 days the protease activity reached 56,270 U/g of dry sample and specific activity in the crude extract was 5,491 AU/cm² that was effective for the removal of cow hair.

Contesini (2014) obtained 3,000 U/mL protease activity after 96 h of fermentation with *B. licheniformis* LBA 46 in Erlenmeyer flasks containing a culture medium consisting of 40 g/L of cane molasses; 6 g/L corn steep liquor; 2 g/L yeast extract and 20 g/L dried whey, pH 7 at 30 ºC and 200 rpm, achieving a productivity of 31.5 U/mL.h.

Anbu (2016) studied the isolation and production of a protease resistant to organic solvents. The microorganism was identified as *Brevibacillus laterosporus*. The fermentation occurred in Erlenmeyer flasks containing 10 g/L peptone; 0.5 g/L $(NH_4)_2SO_4$; 0.3 g/L $MgSO_4.7H_2O$; 1 g/L $CaCl_2.2H_2O$; 1 g/L NaCl and 10 mL glycerol, pH 7. Incubation conditions were 37 ºC, 72 h and 180 rpm. After 48 h, an activity of approximately 1,600 U/mL at pH 7 and 37 °C was observed, which increased to approximately 2,000 U/mL when measured at 40 °C.

Singh, Vohra and Sahoo (2004) achieved a 44% increase in protease activity with the use of fed-batch cultures to maximize protease activity in a bioreactor, compared to batch process. The microorganism *B. sphaericus* was fermented (300 rpm at 30 ºC) to produce of alkaline proteases in culture medium composed of 10 g/L glucose; 5 g/L biopeptone; 5 g/L yeast extract; 1 g/L KH_2PO_4; 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L Na_2CO_3 in a 7 L fermenter, pH 10. The highest protease activity, 680,000 U/mL, was obtained after 30 h of fermentation.

3. Protein hydrolysis

Enzymatic hydrolysis is one of the most promising methods to produce bioactive peptides from proteins. In many cases, it has been used to alter the functional properties of intact proteins and can improve bioactivity, due to its specificity and ability to modify a wide variety of functional groups (Balakrishnan et al., 2011; Bhaskar, Sudeepa, Rashmi, & Selvi, 2007; Pihlanto, 2006; Sarmadi & Ismail, 2010). Depending on the sequence of amino acids,

these peptides can exhibit diverse activities. According to Agyei and Danquah (2011) many of the most common peptides have been produced using gastrointestinal enzymes, mainly pepsin or trypsin. The costs associated with such enzymatic reactions are high, so it is necessary to use cheaper sources of proteases, such as those derived from by-products and microorganisms. Some examples of proteases produced by microorganisms and utilized for protein hydrolysis are presented in Table 4.

Table 4. Microbial proteases commercially used and their respective producing microorganism.

Commercial protease name	Conditions of optimum activity		Microorganism producer
	pH	Temperature (ºC)	
Actinase E	7-8	37	*Streptomyces griséus*
Alcalase	6.5-8.5	60	*Bacillus licheniformis*
Corolase	7-8	55	*Bacillus subtilis*
Flavourzyme	5	50	*Aspergillus oryzae*
Neutrase	7	40-50	*Bacillus amyloliquefaciens*
Proleather FG-F	10	60	*Bacillus subtilis*
Protamex	8	60	*Bacillus* sp.
Proteinase A	6	25	*Saccharomyces cerevisiae*
Proteinase K	7-9	37	*Engyodontium album (*formerly *Tritirachium album)*
Thermolysin	5-8.5	65-85	*Bacillus thermoproteolyticus*

Traditionally, microbial proteases, especially alkaline proteases, have been used in the preparation of high-value protein hydrolysates. Hydrolysates have a variety of applications and can be used in infant food formulations, specific therapeutic food products, fortifying fruit juices and soft drinks, as functional food additives, animal feed, etc. (Chalamaiah, Kumar, Hemalatha, & Jyothirmayi, 2012; Ward et al., 2009).

The limited proteolysis of inexpensive materials, such as soy protein, may increase the range and value of their use, for example, soy hydrolysate products with different functional properties or flavor may be produced by selection of specific hydrolytic conditions, such as the use of alkaline proteases from *Bacillus* sp. as catalysts (Ward et al., 2009). Studies have shown that protein hydrolysates produced by microbial proteases may exhibit antioxidant (Beermann & Hartung, 2013; Bernardini et al., 2011; Kitts & Weiler, 2003), antithrombotic (Hartmann & Meisel, 2007), antihypertensive (Beermann & Hartung, 2013; Kitts & Weiler, 2003; Ricci, Artacho, & Olalla, 2010; Udenigwe & Aluko, 2012), anticarcinogenic (Jang, Jo, Kang, & Lee, 2008), satiety regulator or immunomodulatory characteristics (Agyei &

Danquah, 2012; Kitts & Weiler, 2003) and can affect the cardiovascular, immune, nervous and digestive systems.

4. Beneficial Properties

The most common way of producing bioactive peptides is through the enzymatic hydrolysis of protein. The current trend in peptide research has been to produce peptides that have bioactive and functional characteristics and that can be used industrially. The type of protein used in the production of protein hydrolysates, as well as the degree of hydrolysis, can be modulated according to the type of functional properties to be exploited (Centenaro, Prentice-Hernandez, Salas-Mellado, & Netto, 2009). Table 5 presents some examples of proteases used to hydrolyse different sources of proteins and bioactivity of the peptides produced.

Table 5. The properties of peptides produced after enzymatic hydrolysis of various proteins and the enzymes used.

Property of peptides	Protein source	Enzyme used	Reference
Antioxidant	Soy	Flavourzyme	(Moure, Domínguez, & Parajó, 2005)
	Peanut	Alcalase	(Chen, Zhao, Zhao, Cong, & Bao, 2007)
	Alfalfa leaf	Alcalase	(Xie, Huang, Xu, & Jin, 2008)
	Corn gluten meal	Alcalase	(Li, Han, & Chen, 2008)
	Whey	Corolase PP and Thermolysin	(Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011)
	Walnut	Alcalase and Neutrase	(Chen, Yang, Sun, Niu, & Liu, 2012)
	Rice	Validase FP, Alcalase and Neutrase	(Zhou, Canning, & Sun, 2013)
	Dairy whey	Protease from *Aspergillus oryzae* LBA 01 and	(Castro & Sato, 2014)
	Egg	Flavourzyme	(Castro & Sato, 2015a)
	Pea	Alcalase	(Girgih et al., 2015)

Table 5. (cont.)

Property of peptides	Protein source	Enzyme used	Reference
ACE inhibitory	Sardine	Alcalase	(Matsui et al., 1993)
	Salmon byproducts	Alcalase	(Ahn, Jeon, Kim, & Je, 2012)
	Date seed flour	Alcalase, Flavourzyme and Thermolysin	(Ambigaipalan, Al-Khalifa, & Shahidi, 2015)
	Sweet sorghum grain	Alcalase	(Wu, Du, Jia, & Kuang, 2016)
Antimicrobial	Casein	Thermolysin	(Guinane et al., 2015)
	Goat whey	Alcalase	(Osman, Goda, Abdel-Hamid, Badran, & Otte, 2016)
Anticancer	Beef	Alcalase, Thermolysin, Proteinase A and K	(Jang, Jo, Kang, & Lee, 2008)
	Rice bran	Alcalase	(Kannan, Hettiarachchy, Lay, & Liyanage, 2010)
Anti-inflammatory	Bovine whey	Alcalase	(Ma, Liu, Shi, & Yu, 2016)
	Egg ovomucin	Alcalase	(Sun, Chakrabarti, Fang, Yin, & Wu, 2016)
Antidiabetic	Hard-to-cook bean	Alcalase	(Oseguera-Toledo, Gonzalez de Mejia, & Amaya-Llano, 2015)
	Common bean	Alcalase	(Rocha et al., 2015)
Hypoglycemic	Asian melon type *(Momordica charantia)*	Alcalase	(Yuan, Gu, & Tang, 2008)
Hypocholesterolemic	Soy	Alcalase	(Zhong, Zhang, Ma, & Shoemaker, 2007)
Hypoallergenic	Soybean	Proleather FG-F	(Tsumura, Kugimiya, Bando, Hiemori, & Ogawa, 1999)

4.1 Antioxidant activity

Any excessive amount of reactive radicals can result in cellular damage which, in turn, initiates several diseases. Antioxidants are substances used to remove these reactive species, inhibiting and/or reducing damage caused by theirs deleterious action (Barbosa et al., 2010; Hancock & Neill, 2001) to biological macromolecules such as DNA, proteins and lipids (Ames, Shigenaga, & Hagen, 1993).

The mechanism of action of peptides with antioxidant action is not fully understood, but it is assumed that they may function as inhibitors of lipid peroxidation, scavengers of free radicals, transition metal chelators or as cell protective agents (Kitts & Weiler, 2003; Sarmadi & Ismail, 2010). Kitts and Weiler (2003) and Wang and Mejia (2005) verified that amino acids such as histidine, proline, tyrosine and tryptophan have antioxidant activity and are present in the peptide sequences, mainly at the amino terminal position. Histidine exhibits a strong radical scavenging capacity due to the decomposition of its imidazole ring (Bellia et al., 2008). In addition, the hydrophobic amino acids are related to the antioxidant activity of the peptides (Peña-Ramos, Xiong & Arteaga, 2004), mainly due to their excess of electrons that can be used to remove free radicals or to reduce metal cations (Bellia et al., 2008).

Castro and Sato (2015b) studied the hydrolysis of soy protein using combinations of Flavourzyme, Alcalase and Yeastmax A, at pH 7 and 50 ºC. The use of Flavourzyme combined with Alcalase showed a higher synergistic effect with increases of 10.9% and 13.2% in reduction of DPPH radical compared to the hydrolysates produced with individual enzymes. The peptides obtained with a mixture of the three enzymes studied showed the greatest inhibition of the autoxidation of linoleic acid.

Saiga, Tanabe and Nishimura (2003) tested the hydrolysis of proteins from pork muscle using Actinase E and papain at pH 7 and 37 ºC. The peptides exhibited high antioxidant activity, almost the same in terms of residual DPPH radical (%), which was approximately 30%. Peña-Ramos et al. (2004) studied the antioxidant activity of whey protein hydrolysates obtained using Flavourzyme, Protamex and Alcalase at pH 7 and 50 ºC. The fractions with small peptides showed the highest antioxidant activity measured as thiobarbituric acid reactive-substances inhibition effect (24 - 27%)

The bovine plasma protein hydrolysates were subject of study of Seo et al. (2015). By using of Alcalase in the hydrolysis, the authors optimized the conditions (values of pH ranging of 7.82 - 8.32, 54.1 ºC and 338.4 - 398.4 min) achieving more than 80% and 24% of DPPH radical scavenging activity and Fe^{2+} chelating activity, respectively.

Peptides hydrolysates were produced using poultry processing leftovers (broiler necks), using a mixture of enzymes: Alcalase, Neutrase, Flavourzyme and Protamex. The Box-Behnken statistical tool was used to optimize the protein recovery. Under the optimal conditions, hydrolysis time (3 h), hydromodule (2.25 L/kg) and multienzyme composition dosage (0.25%), the hydrolysates exhibited high antioxidant ORAC and TEAC responses, varying in the range of 297 - 325 and 662 - 683 μmol TE/g (Nikolaev et al., 2016).

4.2 Antihypertensive activity

The angiotensin I-converting enzyme (ACE, EC 3.4.15.1) has been associated with the renin-angiotensin system and is responsible to raise blood pressure by conversion of angiotensin I to the angiotensin II (strong vasoconstrictor) and inactivates the bradykinin (vasodilator) (Fig. 1). Any inhibition of this enzyme can exert an antihypertensive effect (Campbell, 2003; Korhonen & Pihlanto, 2006; Murray & FitzGerald, 2007).

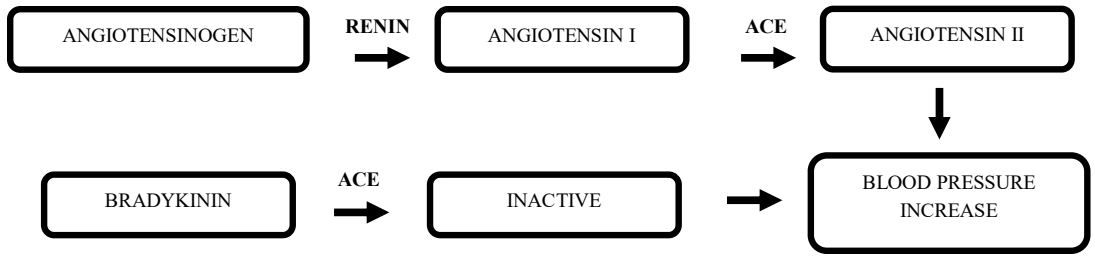

Fig. 1. The renin-angiotensin system.

ACE inhibitory peptides are commonly short peptides strongly influenced by the carboxyl terminal sequence of the peptides, generally with a proline, lysine or arginine residues in this position. Proline, which is a resistant amino acid, is not degraded by digestive enzymes, and can enter the bloodstream where it can act in a protective way (Pan, Luo, & Tanokura, 2005; Yamamoto, Ejiri, & Mizuno, 2003). Suetsuna (1998) observed that the peptides containing tyrosine and phenylalanine in the carboxyl terminal position assist in reducing blood pressure. The peptides containing tyrosine showed short and prolonged decrease effect and those containing phenylalanine presented rapid decrease, but short effect. ACE inhibitor potency is expressed as IC_{50}, which is the concentration of inhibitor capable of inhibiting 50% of ACE activity (Erdmann, Cheung, & Schröder, 2008).

Li, Shi, Liu and Le (2006) hydrolyzed mung bean proteins with Alcalase and Neutrase observing a larger ACE inhibitory activity for a hydrolysate generated by Alcalase (IC_{50}: 0.64 mg protein/mL) after 2 h of hydrolysis, pH 8 at 55 ºC. Yust et al. (2003) also evaluated the

use of Alcalase for hydrolysis. The authors produced a chickpea hydrolysate with ACE inhibitory activity (IC_{50}) of 0.18 mg/mL. Fujita, Yokoyama and Yoshikawa (2000) studied the ACE inhibitory activity from chicken muscle and ovalbumin digested by Thermolysin, observing IC_{50} values of 83 and 45 μg/mL, respectively.

Banerjee and Shanthi (2012) evaluated the ACE inhibitory capacity of peptides of bovine Achilles tendon collagen enzymatically hydrolyzed by bacterial collagenase (pH 7.5 at 37 ºC). Two biologically active peptides showing potent inhibition were obtained with IC_{50} values of 51.10 and 79.85 μM. The peptides retained 80% of activity, even after digestive enzyme treatment. Guo, Pan and Tanokura (2009) studied the hydrolysis optimization of whey protein by a mixture of proteases from *Lactobacillus helveticus* LB13. Whey protein hydrolysates showing 92.20% of ACE inhibition activity was obtained using optimized conditions of hydrolysis (pH 9.18, 38.9 ºC and 0.60 enzyme/substrate ratio).

Aluko et al. (2015) used Thermolysin to hydrolyse pea seed protein. After fractionation, some hydrolysates showed inhibition of in vitro renin activity that ranged from approximately 21 - 68% and inhibition of in vitro ACE activity in the range of 22 - 95%. The administration of 30 mg/kg dose in hypertensive rats resulted in a rapid drop in blood pressure after 2 h, reaching 37 mmHg. The best result was for reduction in pressure after 6 h, reaching 14 mmHg.

4.3 Antimicrobial activity

Antimicrobial peptides (AMPs) are small molecules that may present antibacterial, antifungal, antiparasitic, and antiviral activity and had been studied, isolated and characterized from milk proteins, a rich source of bioactive peptides (Hartmann & Meisel, 2007; Jenssen, Hamill, & Hancock, 2006). The main mechanism of action of AMPs involves their ability to cause damage to the cell membrane and may interact with microorganisms by electrostatic forces between their positive charges of amino acids and the negative charges exposed on the surface of the cell (Guilhelmelli et al., 2013).

Tan, Ayob, Osman and Matthews (2011) defined the pH 8.5 and 50 ºC as the best conditions to obtain high degree of hydrolysis (DH) peptides from palm keller expeller using Alcalase as catalyst. The authors concluded that the peptide with 70% DH showed better inhibitory effect compared to the 100% DH peptide against *Clostridium perfringens*, *Lisinibacillus sphaericus*, *Listeria monocytogenes* and *Bacillus* sp.: *B. cereus*, *B. coagulans*,

B. megaterium, *B. pumilus*, *B. stearothermophillus*, *B. subtilis* and *B. thuringiensis*. The minimum inhibitory concentration (MIC) was ranging of 100 - 800 µg/mL.

Tan, Ayob and Yaacob (2013) compared the action mode of palm kernel cake peptides formed by Alcalase (pH 9.59 and 50 ºC) and trypsin (pH 10 and 40 ºC). The antimicrobial activity was measured against *B. cereus*. Both peptides inhibited the bacterial growth by altering the membrane permeability of the bacterial cells. The MIC was 250 and 350 µg/mL for peptides hydrolyzed with Alcalase and trypsin, respectively. The peptides obtained with Alcalase exhibited a better action against the microorganism tested. Tan, Matthews, Di, and Ayob (2013) purified and characterized an antimicrobial peptide from palm kernel cake using Alcalase as catalyst. The hydrolysis was performed according to Tan et al., (2011). The authors found one peptide active fraction which could inhibit the growth of *B. thuringiensis*, *B. cereus*, *B. subtilis*, *L. sphaericus* and *C. perfringens*. The MIC varied from 250 to 500 µg/mL.

The production of antibacterial peptides from anchovy cooking wastewater using Protamex (pH 6.5 and 55 ºC) was studied by Tang, Zhang, Wang, Qian and Qi (2015). The peptide presented antibacterial activity against all bacteria tested: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *B. subtilis*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli* in bacterial growth medium and the MIC values were 16, 64, 16, 256, 32, 64, and 32 µg/mL, respectively. It also exhibited a dose-dependent bactericidal effect against *S. aureus* in reconstituted milk used as a representative food matrix.

Beef sarcoplasmic protein hydrolysates were produced using commercial proteases. The peptides with high ACE inhibitory activity produced by Alcalase, Proteinase K and a mixture of Proteinase A and Thermolysin (pH 7.5 and 37 ºC) were synthesized and its antimicrobial capacity using the paper disc diffusion method was measured. The peptides showing antimicrobial capacity are GLSDGEWQ against *S. typhimurium*, *B. cereus* and *E. coli*; GFHI against *E. coli* and *P. aeruginosa*; FHG against *P. aeruginosa* and DFHINQ against *B. cereus*, *P. aeruginosa* and *E. coli*, at concentrations of 100 - 400 µg/mL (Jang et al., 2008).

4.4 Functional properties of protein hydrolysates for food industry

According to Kinsella and Whitehead (1989) the functional properties of proteins are physicochemical properties that govern their behavior within food systems during preparation,

processing, storage and consumption. The various functional characteristics of the proteins can be potentiated and modulated after hydrolysis, such as viscosity, agitation and high dispersibility, solubility, foaming, emulsifying, which gives them advantages for use in various products in the food industry (Benítez, Ibarz, & Pagan, 2008).

Wheat gluten was hydrolyzed by Alcalase (pH 8.5 and 60 ºC) and some other digestive proteases (Kong, Zhou, & Qian, 2007). The Alcalase showed high hydrolytic efficiency, with a protein recovery of 81.3%, so the functional characterization was realized only for this protease. The DH had relation with most of the properties measured. The solubility increased after hydrolysis. The emulsifying capacity increased with the decrease of DH. There was an increase in the foaming capacity for the 5% DH sample, which was the most stable foaming, sustaining 40% of the initial foam during 1 h, compared to the 9.09% of the control sample. Similar results were found by Tamm, Herbst, Brodkorb and Drusch (2016) for pea protein hydrolysates in spray-dried emulsions. The authors concluded that using Alcalase (pH 8 and 75 °C) in the hydrolysis, only at very low DH (1%), the emulsions were stable.

The enzymatic treatment (pH 7 and 40 ºC) of defatted soy flour with three different microbial proteases (Flavourzyme, Novozym and Alcalase) improved the foaming and gelling properties, mainly protein hydrolysates obtained with Flavourzyme protease (Hrčková, Rusňáková, & Zemanovič, 2006).

Centenaro et al., (2009) studied the hydrolysis of corvina (*Micropogonias furnieri*) by Alcalase (pH 8 and 50 ºC) and evaluated some functional characteristics of the hydrolysates. Protein hydrolysates showed increased solubility and good foam stability.

Chickpea protein isolate was hydrolyzed using immobilized Flavourzyme (pH 7 and 50 ºC). All hydrolysates presented better functional properties (except emulsifying activity): higher solubility, oil absorption, emulsifying activity and stability and foaming capacity and stability, than the intact protein (Yust, Millan-Linares, Alcaide-Hidalgo, Millan, & Pedroche, 2013).

Ribotta, Colombo and Rosell (2012) observed an increase of low molecular weight peptides after treatment of pea protein with Alcalase (pH 6.5 and 35 ºC). The authors evaluated the application of the peptides in protein-cassava and corn starch gels. Regarding the viscosity profile, the hydrolyzed proteins induced a drop in peak viscosity, final viscosity and setback of the gels. The hydrolyzed pea proteins also affected the amylose retrogradation

due to interactions between the low molecular weight polypeptides and the amylose chains. The gels' water retention capacity was decreased after the incorporation of peptides.

Zhao et al. (2012) investigated the effect of various proteases on the formation and characteristics of rice dreg protein hydrolysate. The authors observed that the microbial proteases, Neutrase (pH 7 and 45 ºC) released more hydrophobic groups, in decreasing order of Neutrase > Alcalase > Flavourzyme > Protamex > trypsin. In the range of pH 3 - 11, the solubility of all hydrolysates was higher than 60% and more than 80% was found for peptides obtained using Protamex (pH 7 and 50 ºC) in pH value different of 5, while the native protein had only 20% of solubility.

5. Others important applications of proteases

5.1 Cleaning industry

In the last years, proteases have ceased to be additives to become one of the standard components of all kind of detergents. In 1956, BIO 40, the first detergent containing bacterial proteases, was produced by Gebrüder Schnyder (Rao et al., 1998; Vojcic et al., 2015). In 1960, the alcalase from *B. licheniformis*, produced by Novo Industria A/S entered the market under the name BIOTEX (Jisha et al., 2013; Rao et al., 1998).

After the fermentation, the biomass produced by the microorganism used is separated and the liquid supernatant becomes the source of proteases. This liquid is concentrated and dried. The proteases are used in powder form in the detergent composition, being prepared as the characteristic wax-containing granules which are present in the enzymatic detergents and protect the user from undesirable inhalation of protease dusts (Ward, 2011). One of the requirements for using proteases in formulating detergents is their high activity and stability at high pH and temperature values, as well as being resistant and compatible with chelating and oxidizing agents present in the formulation. The most suitable protease should have its pI coincident with the pH of the detergent (Rao et al., 1998). Alkaline proteases (e.g. subtilisin) are suitable for use in detergents, since they act at highly alkaline pH (Anwar & Saleemuddin, 1998).

Contesini (2014) evaluated the addition of the crude protease from *B. licheniformis* LBA 46 on washing performance of cotton fabrics. The detergents were heated for 30 min at 95 ºC to remove any enzymatic activity presented. The incubation conditions were 2h at 40 ºC. The author achieved a complete blood stain removal after use of 1000 U of the protease without detergent. In combination with the solid detergent, 100 U or

1000 U of enzyme were also effective. For tomato sauce stains, the use of the protease improved the detergent action.

Benmrad et al. (2016) isolated a purified serine protease from *Trametes cingulata* strain CTM10101 and studied the stability and compatibility of the enzyme with laundry detergents and for removal of blood stains from cotton fabrics. The laundry detergents used were heated for 1h at 65 ºC for inactivation of the endogenous proteases present. The protease was extremely stable and compatible with the liquid detergents studied, retaining above 99% initial activity after incubation at 40 °C for 120 h. The use of the enzyme improved the cleaning process and quickly removed the blood stains compared to using the detergent alone. The enzyme also showed better hydrolysis ability, substrate specificity, catalytic efficiency and tolerance to high organic solvent concentration when compared to commercial enzymes, Flavourzyme 500L (from *A. oryzae*) and Thermolysin type X (from *Geobacillus stearothermophilus*).

5.2 Food industry

The use of proteases in the food industry is already well known. These enzymes have been used for various purposes such as production of dairy products, bakery and clarification of xanthan gum, among others.

5.2.1 Cheesemaking

Proteolysis is one of the events responsible for the main biochemical modifications during the cheesemaking (Tavano, 2013). The main application of proteases in this type of industry is in the manufacture of cheeses. Milk coagulant enzymes can be divided into animal rennets, microbial milk coagulants, and genetically modified chymosin. The chymosin has high specificity for casein, so it is the most used in cheesemaking. Chymosin has been gradually replaced by some microbial proteases produced by GRAS microorganisms such as *Mucor michei*, *B. subtilis* and *Endothia parasitica* (Neelakantan, Mohanty, & Kaushik, 1999; Rao et al., 1998).

A purified protease from *P. fluorescens* RO98 was able to hydrolyse two hydrophobics bitter peptides in Cheddar and Gouda cheese in a reaction of 90 min at 30 ºC and pH 6.8 (Koka & Weimer, 2000).

5.2.2 Baking

The proteases are used in baking to modify gluten, which is an insoluble protein that determines the viscoelastic properties and the mass expansion capacity during the baking process (Rao et al., 1998; Ward, 2011). The manipulation of strength gluten flour can be achieved by using proteases and this increases the amount of products with distinct characteristics that can be obtained after the treatment of flours with these enzymes.

The development of flavor and taste are also achieved by use of proteases. Neutral protease are used for this purpose, for example, Neutrase, which is used in baking to degrade proteins in flour for biscuits, crackers and cookies (Sumantha et al., 2006; Ward, 2011). Proteases from *A. oryzae* proteases are employed to facilitate handling and machining during the production of various bakery products, reducing mixing time and improving loaf volume (Rao et al., 1998).

5.2.3 Clarification of xanthan gum

Xanthan gum is produced by *Xanthomonas campestris* and is used as a viscosity control agent in various industries such as food, pharmaceutical, agricultural and others (Contesini, 2014). The fermentation of the microorganism to produce xanthan gum generates a highly viscous broth, which makes it difficult to separate the biomass from the product. Centrifugation separation is not sufficient due to the high viscosity of the broth and the heat treatment also used for this purpose may cause thermal degradation of the gum. Removal of the cell mass is generally done by the enzymatic way, using proteases (Shastry & Prasad, 2005; Contesini, 2014).

The protease produced by *B. licheniformis* LBA 46 was used for *X. campestris* cells lysis. When the crude protease extract (42 U/mL of cell suspension) was employed, a 40% increase in transmittance of the medium was observed after 2 h of reaction at 65 °C and pH 7.0 (Contesini, 2014).

5.3 Leather industry

Proteases are used in animal leather processing as a substitute to traditional chemical processes involving toxic and dangerous chemical products (Singh, Mittal, Kumar, & Mehta, 2016). The mainly leather protein is the collagen. Some processes involving leather require removal of non-collagenous constituents, partially or completely. The degree of these modifications controls the physical characteristics of the leather (Khan, 2013). Proteases are

used to promote dehairing, degrade non-collagenous constituents of the skin and eliminate non-fibrillar proteins (Jisha et al., 2013). The use of these enzymes represents a good alternative in improving the quality of leather and reducing environmental pollution. Alkaline proteases are the most used in this process because they are more stable and active under conditions necessary for the hair removal. In addition, the enzymes reduce the immersion time by ensuring a faster absorption of water (Ellaiah et al., 2002; Jisha et al., 2013).

Dettmer, Cavalli, Ayub and Gutterres (2012) studied the optimization of the unhairing leather processing with protease from *B. subtilis*. The optimum activity conditions for the protease were in the ranging of pH 9 - 10 and temperature between 37 - 55 ºC. Some hair remained in the treated skins, but when compared with the conventional unhairing process, the protease use in the inter-fibrillary proteins removal was approximately 4-fold for glycosaminoglycans and 6-fold for proteoglycans.

6. Conclusion

Proteases are extremely versatile enzymes. There is a wide variety of microbial proteases that have diverse specificities and applications. Knowing the characteristics of these proteases is a very important factor for its use in the enzymatic hydrolysis of proteins and for general industrial applications. Biotechnological processes that require specific peptides are highly dependent on the use of microbial proteases that represent an important tool in the modification of protein structures and the obtaining of specific peptides. A greater use of these proteases and knowledge of their characteristics depend on the studies carried out in this sense, in search of innovations, discovery of new enzymes or improvement of the function of existing enzymes.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Capítulo II

Production, characterization and purification of alkaline protease from *Bacillus licheniformis* LBA 46

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Abstract

The production of protease from *Bacillus licheniformis* LBA 46 was studied in a 6 L bench reactor. In the effect of temperature and agitation study on the fermentation of the strain by experimental design, a higher protease production was obtained at 30 °C and 300 rpm using the culture medium composed of 32 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract and 20 g/L dried whey, pH 7. The increase of fermentation scale from Erlenmeyer flasks containing 100 mL of culture medium to the bench reactor containing 3 L of culture medium resulted in a 2.3-fold increase in enzyme activity, with 2,448 U/mL and yield of 51 U/mL.h after 48 h of fermentation at 30 ºC and 300 rpm. The semi-purified protease preparation, obtained by 80% ammonium sulfate precipitation, showed optimum activity at 60 and 65 ºC, pH 7. In the thermostability study, the semi-purified enzyme retained about 78%, 39% and 9% of the initial activity after 120 min of treatment at 50, 60 and 70 °C, respectively. The protease was purified 3.33 times by ammonium sulfate precipitation and DEAE-Sepharose column chromatography and had a molecular mass estimated at 40 kDa by SDS-PAGE. The purified protease showed optimum activity at 50 and 60 °C. The purified enzyme presented high activity (> 80%) in the pH range 6.5-9 and optimal activity in pH 8.5. The purified protease showed to be stable in the range between pH 5-10 after 24 h of incubation at 4 °C, presenting more than 86% of the initial activity. The purified enzyme was stable after 1 h incubation at 40 °C and retained 85% of the initial activity after 1 h at 50 °C.

Keywords: *Bacillus licheniformis*; Reactor; Protease; Fermentation; Purification.

1. Introduction

Industries of food, pharmaceutical, agricultural and medical have been taking advantage of using *Bacillus* sp. because of their wide range of physiological characteristics and ability to produce enzymes and other metabolites (Gupta et al. 2002; Schallmey et al. 2004; Voigt et al., 2004). *Bacillus subtilis* and *Bacillus licheniformis* species are attractive industrial microorganisms recognized as GRAS (generally recognized as safe), which have high growth rates leading to shorter fermentation times and posses the ability to secrete extracellular proteins (Ward et al. 2009; Ward, 2011; Parrado et al. 2014).

Since the advent of enzymology, one of the most important classes of hydrolytic enzymes, which have been extensively studied, is the microbial proteases (Furhan and Sharma 2014). Each enzyme has a peculiar characteristic of performance, which makes it different from the others. The alkaline proteases were initially marketed for use in detergents and the market for these industrial enzymes expanded substantially during the 1960s. Until nowadays they are one of the most widely studied groups of enzyme because of their extensive types of application in several sectors such as detergent, textile, leather and food industries (Kumar and Takagi 1999; Ward et al., 2009). The main microbial strains used in enzyme production are still *Bacillus* species, used principally to produce alkaline serine proteases and neutral proteases (Schallmey et al. 2004; Ward 2011; Pant et al. 2015). Due to these differences, another protease has been studied.

Microorganisms are widely known for their function of producing intracellular and extracellular enzymes on an industrial scale and for maximum efficiency they are cultivated in reactors under the best conditions (Moo-Young and Chisti 1994; Gupta et al. 2002). According to Potumarthi et al. (2007), mixing in the reactor is important during the production of proteases, which is transmitted by aeration and agitation. The temperature is another important function to control in a fermentative process, so there is a necessity of defining a better combination of these factors within the fermentation process for maximum efficiency and productivity. Based on this, the aim of this study was to verify the effects of modifying temperature and agitation conditions during the submerged fermentation of *B. licheniformis* LBA 46 in a bench reactor on protease production, using the experimental design process. The protease was also purified and characterized.

2. Material and Methods

2.1 Fermentation

2.1.1 Microorganism and culture medium

The microorganism used in this study was a strain of *B. licheniformis* LBA 46 from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, UNICAMP, Brazil. The culture medium used was proposed by Contesini (2014), with slight modifications, containing agroindustrial by-products as the carbon and nitrogen sources (32 g/L of sugar cane molasses (Fio de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®) and 20 of g/L dried whey (Alibra®), adjusted to pH 7).

2.1.2 Inoculum preparation

The microorganism was grown in nutrient agar slants and incubated at 30 °C for 18-24 h. After growth, a bacterial cell suspension (~10^8) was prepared adjusting the absorbance at 620 nm to 0.49-0.51. Accordingly, 2 mL aliquot of cell suspension was added aseptically to 3 Erlenmeyer flasks each containing 100 mL of culture medium, and incubated at 30 °C and 200 rpm for 36-40 h.

2.1.3 Submerged fermentation in a bench reactor

The fermentation of *B. licheniformis* LBA 46 was performed in a New Brunswich Bioflo II bench reactor with a capacity for 6 L and a working volume of 3 L. The inoculum represented 10% of the culture medium and was prepared according to the item 2.1.2. Foaming was controlled during fermentation using the anti-foam DC*FG-10 (Dow Corning®), which dripped automatically when the foam level reached the sensor. The air flow rate was maintained at 0.8 vvm. The pH value was monitored using a calibrated potentiometer. The total fermentation time was 72 h, and samples (15 mL) were collected every 12 h and centrifuged at 11,000 x *g* for 15 min at 5 °C. The cell-free supernatant was used as the enzyme extract for the determination of protease activity.

2.1.4 Optimization of temperature and agitation

A factorial design with 4 possible combinations and 3 central points was used to optimize and evaluate the effects of temperature and agitation for bench reactor fermentation,

resulting in a total of 7 tests, which were carried out at random order. Table 1 shows the coded and real values of the variables studied.

Table 1. Factorial design, coded and real values of the variables studied (temperature and agitation).

Assay	Temperature (ºC)	Agitation (rpm)
1	-1 (30.0)	-1 (200)
2	1 (37.0)	-1 (200)
3	-1 (30.0)	1 (300)
4	1 (37.0)	1 (300)
5	0 (33.5)	0 (250)
6	0 (33.5)	0 (250)
7	0 (33.5)	0 (250)

2.1.5 Kinetics of microbial growth and protease production

The microbial growth kinetics of *B. licheniformis* LBA 46 and the protease production were carried out in a 6 L bench reactor containing 3 L composed of 32 g/L of sugar cane molasses (Fios de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®) and 20 g/L dried whey (Alibra®), adjusted to pH 7, at 300 rpm, 30 °C and 0.8 vvm. Samples of the culture media were collected at different times and inoculated into petri dishes containing nutrient agar using the pour plate technique. Petri dishes were incubated at 30 °C for 24 h. Microbial growth was expressed as colony forming units (CFU)/mL. Protease activity, protein and reducing sugar were determined as described in 2.1.6, 2.1.7 and 2.1.8, respectively.

2.1.6 Protease activity determination

Protease activity was determined according to the method described by Charney and Tomarelli (1947) and modified by Castro and Sato (2014), using azocasein as the substrate. The reaction mixture contained 0.5 mL of 0.5% azocasein in 0.05 M sodium phosphate buffer, pH 7, and 0.5 mL of the enzymatic extract which were incubated for 40 min at 60 °C. The reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 17,000 x *g* for 15 min at 15 °C. An aliquot of 1 mL of the supernatant obtained was neutralized with 1 mL of 5 M KOH. One protease activity unit was defined as the amount of enzyme which caused an increase of 0.01 in absorbance at 428 nm.

2.1.7 Protein determination

Protein quantification was carried out by Lowry's method with some modifications (Hartree, 1972). The calculations were based on a standard curve of bovine serum albumin (BSA) and were expressed in mg/mL.

2.1.8 Reducing sugar determination

Reducing sugars were quantified by dinitrosalicylic acid, DNS (Miller, 1959). The calculations were based on a standard glucose curve and were expressed in mg/mL.

2.2 Determination of kinetic and thermodynamic parameters of semi-purified protease

2.2.1 Activation energy and temperature coeficient (Q_{10})

To determine the activation energy (E_a), measurements of protease activity were performed according to item 2.1.6 with incubation at different temperatures, 30 - 80 °C. E_a was calculated from the slope of the plot of 1000/T vs. ln (protease activity), E_a = - slope x R.

The value of the temperature coefficient, Q_{10}, was determined according to Eq. 1 (Dixon and Webb, 1979). This measure is used to relate the reaction rate with a 10 °C increase in the reaction temperature.

(Eq. 1)

$$Q_{10} = antilog\ \varepsilon\ (E_a\ x\ 10/RT^2)$$

where R is the gas constant (8.314 J/Kmol) and T is the absolute temperature (K).

2.2.2 Determination of K_m and V_{max}

Kinetic parameters (Michaelis Mentem constants, K_m and maximum velocity, V_{max}) were determined at the optimum temperature and pH of protease activity using different concentrations of azocasein as substrate (1-10 mg/mL).

2.2.3 Determination of kinetic and thermodynamic parameters for thermal inactivation

Kinetic parameters for thermal inactivation

To determine the thermal inactivation of the protease, the enzyme was incubated at temperatures of 50-70 °C for 120 min in the absence of substrate. Samples were collected

periodically throughout the incubation period and residual activity was determined at the temperature and optimal pH of protease activity.

The value of the deactivation constant (K_d) expressed as an exponential decay and found by plotting ln (A/A_0) vs. time was measured according to the Eq. 2.

(Eq. 2)

$$A = A_0 \, x \, e^{-k_d^t}$$

where A and A_0 is the protease activity at a determined time t and at an initial time, respectively.

The activation energies for denaturation (E_{ad}) were calculated by plotting ln (K_d) vs. 1/RT as described in Eq. 3. The time when the residual activity reaches 50% (apparent half-life) was estimated by Eq. 4. The *D*-value, which is defined as the time required for a 90% reduction in the initial enzyme activity at a specific temperature, was calculated as shown in Eq. 5.

(Eq. 3)

$$k_d = A_e^{(-E_{ad}/RT)}$$

(Eq. 4)

$$t_{1/2} = \ln(0.5)/k_d$$

(Eq. 5)

$$D = 2.303/k_d$$

Thermodynamic parameters for thermal inactivation

Thermodynamic parameters of protease were projected using the Eyring absolute rate (Eq. 6).

(Eq. 6)

$$k_d = (k_b \, x \, T/h) x \, e^{(-\Delta H/RT)} \, x \, e^{(\Delta S/R)}$$

where k_b is the Boltzmann constant (1.38 x 10^{-23} J/K); T is the absolute temperature (K); h is the Planck constant (6.63 x 10^{-34} J.s); ΔH is the enthalpy of activation (kJ/mol) and ΔS is the entropy of activation (J/mol K).

The enthalpy of activation, ΔH, was calculated using Eq. 7. The free activation energy, ΔG was calculated using Eq. 8 and the activation entropy, ΔS was determined according to Eq. 9. All terms were previously described in the equations above.

(Eq. 7)

$$\Delta H = E_{ad} - RT$$

(Eq. 8)

$$\Delta G = -RTln(k_d x\ h/k_b\ x\ T)$$

(Eq. 9)

$$\Delta S = (\Delta H - \Delta G)/T$$

2.3 Purification and characterization of purified protease

The protease of *B. licheniformis* LBA 46 strain was produced as described in item 2.1.3, using the otimized conditions of temperature and agitation. The supernatant was separated by centrifugation and fractionated with 80% ammonium sulfate. The precipitate was dissolved in 0.05 M phosphate buffer pH 7 and dialyzed against distilled water at 5 °C and freeze-dryed. The freeze-dryed protease (0.1 g/10 mL) was applied to an ion exchange 20 mL DEAE Sepharose column (HiPrep™ DEAE FF 16/10, GE, Little Chalfont, UK) equilibrated with 0.05 M sodium phosphate buffer, pH 7, and the proteins were eluted (5 mL/min) with a linear 0 to 1 M sodium chloride gradient (Äkta Purifier, GE, Little Chalfont, UK). Fractions containing protease activity were pooled and analyzed by SDS-PAGE (Vertical Slab Mini-Protean Electrophoresis System Bio-Rad Laboratories, Hercules, CA, USA) as described by Laemmli (1970). The run was performed at 110 V for 30 min. The molecular weight of the enzyme was estimated using molecular mass markers (Thermo Fisher Scientific Ruler™ Unstained Protein Ladder) ranging from 10 to 200 kDa. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

2.3.1 Determination of optimum pH of activity and stability of purified protease

The effect of pH on protease activity was determined by univariate assay under the conditions described in item 2.1.6 using 0.1 M acetate buffer (pH 4-5), 0.1 M sodium phosphate buffer (pH 6-8), 0.1 M Tris-HCl buffer (pH 9), 0.1 M carbonate-bicarbonate buffer (pH 10) and 0.1 M NaOH-bicarbonate buffer (pH 11).

The effect of pH on protease stability was determined using the same buffers and pH values already mentioned. The enzyme solutions were incubated at different pH values for 24 h at 4 °C in the absence of substrate. The residual enzyme activity was then determined according to item 2.1.6. The results were expressed as a percentage and relative activity.

2.3.2 Determination of optimum temperature of activity and stability of purified protease

The protease activity was tested, as described in item 2.1.6, at different temperatures (between 30 ºC and 80 ºC, pH 7). Relative activities were determined by defining the maximum enzyme activity, at a specific temperature, such as 100%.

The thermal stability of the enzyme was evaluated by pre incubation at various temperatures (between 30 ºC and 80 ºC, pH 7) for 1 h with subsequent cooling, and the residual enzymatic activity was determined as previously described in item 2.1.6.

2.4 Statistical analysis

The experimental design, matrix and statistical analysis were developed using the Statistica 7.0 program (Statsoft®/Dell, USA), Tukey's test and Pearson correlation were carried out in Minitab 16.1.1 (Minitab Inc., USA). The analyses were carried out in triplicates and analyzed considering *p*-value lower than 10% ($p \leq 0.10$).

3. Results and Discussion

3.1 Experimental design for the kinetics of protease production in a bench reactor

Preliminary tests were performed to verify the effects of temperature (in the range of 30-37 ºC) on extracellular protease production by *B. licheniformis* LBA 46. Using 100 mL of culture medium in Erlenmeyer flasks, it was found that temperature variations caused variations on the protease production. Therefore, the reactor studies were carried out using bench reactor in this temperature range. Fig. 1 presents the responses obtained in the factorial design for the effect of temperature and agitation study during the kinetics of protease production by *B. licheniformis* LBA 46 in a bench reactor for 72 h of fermentation.

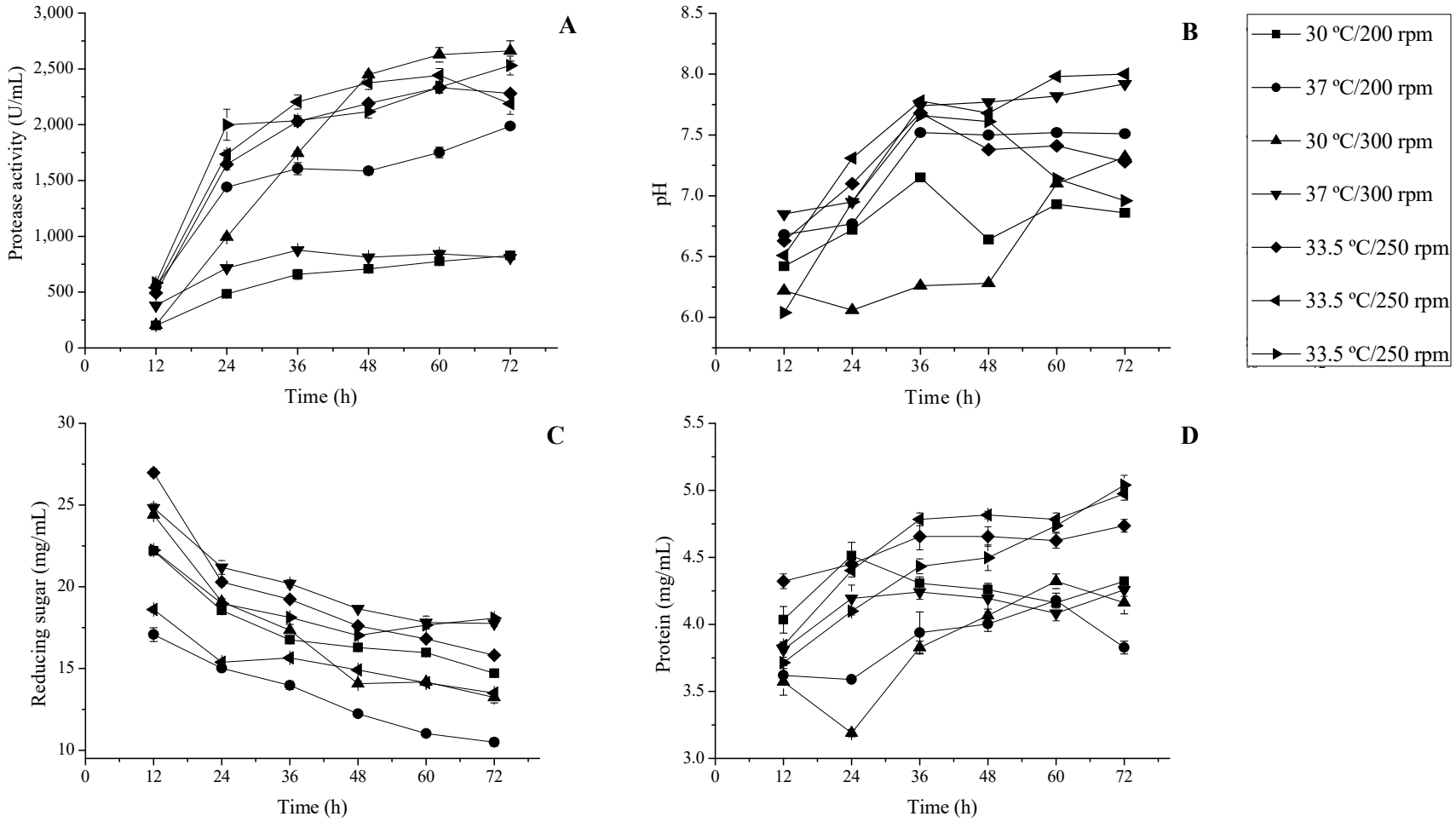

Fig. 1. Kinetics of the protease production (A), pH values (B), reducing sugars (C) and protein (D) measured during fermentation of *B. licheniformis* LBA 46 in a bench reactor during 72 h of fermentation.

Due to the great variability that involves bioprocesses, with enzymes and microorganisms, the *p*-values ≤ 0.10 were considered significant. The estimated enzymatic activity for the kinetics at 72 h (Fig. 1A) showed that, the values remained high (> 1000 U/mL) in 71.43% of the assays analyzed. Under the conditions evaluated, the microorganism produced protease in the range from 30-37 ºC and 200-300 rpm.

It can be observed that, in the majority of the analyzed times, the central points (assays 5-7) and test 3 presented values of activity greater than the other tests. This information indicates that there is no adequate fit for a 1st order model, so there is a necessity of evaluating the curvature. According to the *p*-value obtained ($p \leq 0.10$) the analysis of curvature was significant. Table 3 presents the estimated regression coefficients for each variable, their interaction and statistical analysis of each effect for significance assessment.

For protease activity, the temperature, the agitation and the interaction between them showed effect on the factorial design, and the calculated *p*-value confirmed the presence of all significant effects after 48 h of fermentation with 90% of confidence (Table 3). The maximum protease activity was reached under the conditions of assay 3 (30 ºC and 300 rpm), which presented activities of 2,448.83, 2,627.33 and 2,661.17 U/mL after 48, 60 and 72 h of fermentation, corresponding to protease productivity values equals to 51, 46.8 and 36.7 U/mL.h, respectively. The values for the coefficients of temperature and agitation from 48 h of fermentation were high, negative for temperature and positive for agitation, which means that when the temperature decreased and the agitation increased the protease activity was at its highest. The assay 2 fits perfectly with these conditions, and it was chosen for the protease production.

Table 2. Regression coefficients, standard error, t_{calc} and *p*-value during the protease production by *B. licheniformis* LBA 46 in a bench reactor during 72 h of fermentation.

Variables	Coefficient	Standard error	t_{calc}*	*p*-value
12 h of fermentation				
Mean	333.96	22.97	14.54	0.005
Curvature	204.17	35.09	5.82	0.028
Temperature	130.96	22.97	5.70	0.029
Agitation	-40.54	22.97	-1.76	0.219
Interaction	-42.87	22.97	-1.87	0.203
24 h of fermentation				
Mean	909.13	92.10	9.87	0.0101
Curvature	884.43	140.69	6.29	0.0244
Temperature	169.46	92.10	1.84	0.2072
Agitation	-53.38	92.10	-0.58	0.6208
Interaction	-308.88	92.10	-3.35	0.0786
36 h of fermentation				
Mean	1,221.50	49.44	24.71	0.0016
Curvature	868.19	75.52	11.50	0.0075
Temperature	19.83	49.44	0.40	0.7271
Agitation	88.67	49.44	1.79	0.2148
Interaction	-453.83	49.44	-9.18	0.0117
48 h of fermentation				
Mean	1,301.13	66.60	19.54	0.0026
Curvature	926.53	101.73	9.11	0.0118
Temperature	-277.38	66.60	-4.17	0.0531
Agitation	329.29	66.60	4.94	0.0386
Interaction	-541.04	66.60	-8.12	0.0148
60 h of fermentation				
Mean	1,499.17	30.91	48.50	0.0004
Curvature	871.31	47.21	18.45	0.0029
Temperature	-202.42	30.91	-6.55	0.0225
Agitation	235.67	30.91	7.62	0.0168
Interaction	-690.08	30.91	-22.33	0.0020
72 h of fermentation				
Mean	1,571.21	88.34	17.79	0.0031
Curvature	761.35	134.95	5.64	0.0300
Temperature	-173.54	88.34	-1.96	0.1884
Agitation	163.63	88.34	1.85	0.2052
Interaction	-752.79	88.34	-8.52	0.0135

*t_{calc} calculated with 3 degrees of freedom
$R^2 > 0.96$ for all responses

Fig. 1 (B, C, D) shows the pH values, reducing sugars and protein measured during fermentation of by *B. licheniformis* LBA 46 in a bench reactor. The pH of the culture medium provides some important information. The initial pH (7) of the culture medium decreased to 6-6.8 after 12 h and then increased to reach 6.8 - 8.0 after 72 h in the most assays. The pH

initially dropped, probably due to glucose utilization during growth phase with the increase in the number of microbial cells, but when the enzymatic production was initiated, the pH started to increase. The culture medium used is a complex medium, which presents a variety of proteins and peptides from the yeast extract, dried whey protein and corn steep liquor. According to Chu et al. (1992), the acidification or alkalinization of the medium during the microbial growth reflects the substrate consumption. When microbial cells are using organic nitrogen (amino acids and proteins), the medium becomes more alkaline resulting in rising pH. Another explanation for the increase in pH is the production of free amino acids during the fermentative process.

Consumption of sugars and protein synthesis were consistent with cell growth. The sugars were consumed and decreased with the advance of the fermentation, expected behavior, because the sugars are fermented by the microorganisms during their growth to supply their metabolic needs. According to Fig. 1C, the consumption of sugars had a similar profile for the 7 assays. Protein content of the culture medium increased in all assays reaching about 3.5-5 mg/mL after 72 h of fermentation (Fig. 1D), representing the increase in the protease production.

The Pearson's coefficient (Table 3) was used to verify the correlation between values of protease activity, sugars and protein. Regarding the sugar content, all correlations were significant ($p \leq 0.10$), that is, the activity increases with the consumption of sugars by the microorganism in all the assays studied. As for protein content, from assay 3 to 7, significant correlations were observed, where the higher the protease activity the higher the protein content.

Table 3. Correlation analysis between protease activity (U/mL), reducing sugars (mg/mL) and protein content (mg/mL) during protease production by *B. licheniformis* LBA 46 in a bench reactor during 72 h of fermentation.

	Assays						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Correlation between protease activity (U/mL) and reducing sugars (mg/mL)							
Pearson coefficient	-0.995	-0.833	-0.985	-0.894	-0.987	-0.901	-0.923
***p*-value**	0.000*	0.039*	0.000*	0.016*	0.000*	0.014*	0.009*
Correlation between protease activity (U/mL) and protein content (mg/mL)							
Pearson coefficient	0.318	0.487	0.843	0.887	0.926	0.956	0.894
***p*-value**	0.539	0.328	0.035*	0.018*	0.008*	0.003*	0.016*

*The correlations between the analyzed parameters were considered significant when the *p*-value ≤ 0.10.

Potumarthi et al. (2007) evaluated the alkaline protease production by *B. licheniformis* NCIM-2042 in an 11 L reactor containing 5 g/L casein, 10 g/L malt extract, 10 g/L polypeptone, 10 g/L Na_2CO_3, pH 9.5. The effect of aeration (1, 2, 3 vvm) and agitation (200, 300 and 400 rpm) were tested and the maximum protease production, 340 U/mL, was achieved using 300 rpm and 2 vvm, after 120 h of incubation at 35 °C.

Chuprom et al. (2016) studied the enhancement of halophilic protease production by *Halobacterium* sp. strain LBU50301 using statistical design response to optimize the medium composition. Using 18.62 g/L gelatin, 9.13 g/L MgSO4.7H_2O, 27.95% (w/v) NaCl, pH 7.88. The protease production was 231.33 U/mL in a laboratory fermenter. According to the authors, the higher production of proteases was obtained in the reactor since the reactor systems provide more precise control of parameters such as pH, aeration and stirring speed.

On the other hand, the values of enzyme activity found in this study were higher. The protease of *B. licheniformis* LBA 46 was produced in greater quantity when produced in bench reactor.

3.2 Kinetics of microbial growth and protease production

In the fermentation of *B. licheniformis* LBA 46 in a 6 L bench reactor in the best conditions of temperature (30 °C) and agitation (300 rpm), according to assay 3, the protease was produced in the exponential phase of growth reaching maximum activity (~3,000 U/mL) after 48 h of fermentation. The reducing sugar content in the culture medium decreased to 17.9 mg/mL and 16.2 mg/mL after 36 and 48 h of fermentation, respectively. Cell growth decreased after 48 h of fermentation.

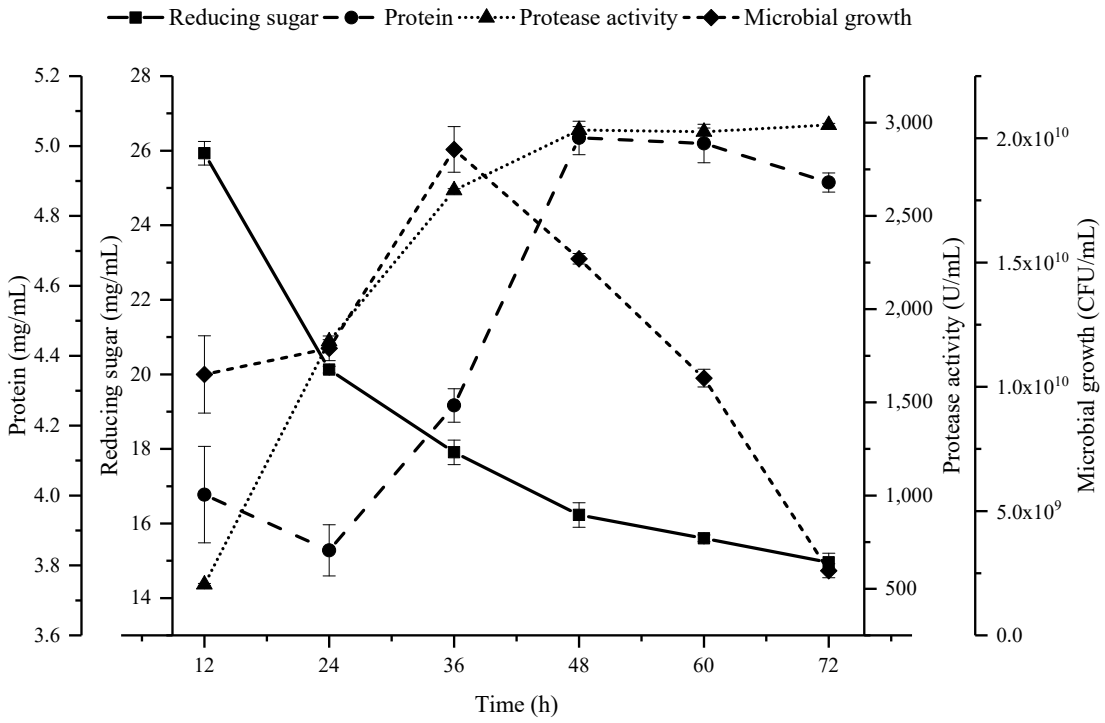

Fig. 2. Fermentation of *B. licheniformis* LBA 46 in a bench reactor at temperature of 30 °C and agitation of 300 rpm: microbial growth, protease production, reducing sugar and protein content.

The production of proteases by *Bacillus* species is controlled by a series of events during the phase transition between exponential and stationary phases. The production of enzymes is related to the growth phase of the microorganism. According to Strauch and Hock (1993); Jisha et al. (2013) and Contesini (2014), proteases from *Bacillus* sp. are mainly produced during the stationary phase of microbial growth. The extracellular enzyme production pattern depends on the *Bacillus* strains (Jisha et al., 2013).

Dias et al. (2008) observed maximum proteolytic activity of the enzyme produced by *B. subtilis* ATCC 6633 (839.8 U/mg) and *Bacillus* sp. UFLA 817 (975.9 U/mg) after 24 h of fermentation coinciding with the end of the exponential phase of microbial growth. The protease production by *Bacillus cereus* VITSN04 also had its maximum activity (200 U/mL) associated with exponential growth phase (Sundararajan et al., 2011). Rao e Narasu (2007) observed that maximum activity (215 U/mL) of the protease produced by *Bacillus firmus* 7728 was reached in the stationary phase after 48 h of growth.

3.3 Determination of kinetic and thermodynamic parameters of semi-purified protease

3.3.1 Activation energy and Q_{10} value

Fig. 3 presents the protease activity in different temperatures (30-80 ºC). It can be observed that the semi-purified protease from *B. licheniformis* LBA 46 showed high activity between 55-65 ºC, with the optimum value at 60-65 ºC.

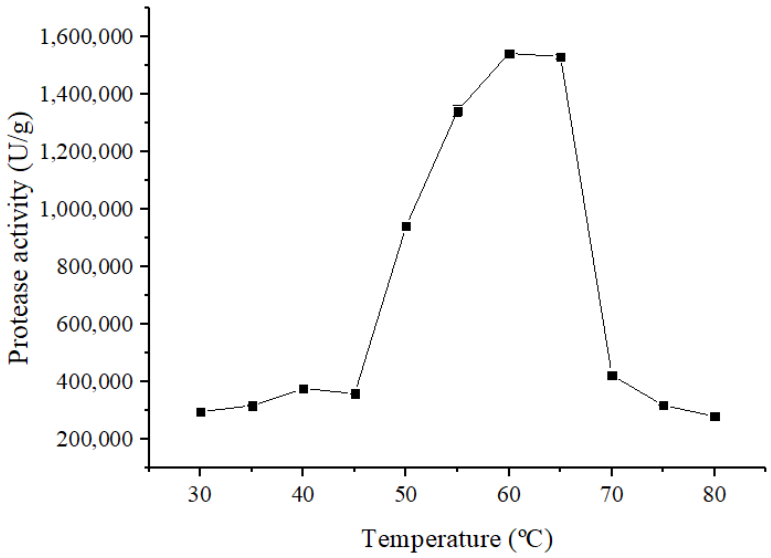

Fig. 3. Effect of temperature in the activity of semi-purified protease from *B. licheniformis* LBA 46.

The Arrenius plot is presented in Fig. 4. For the range of temperature analyzed (30-80 ºC) a linear variation could be observed with the increase in temperature, which suggests that the protease have a single conformation in the temperature of transition (Castro et al. 2014).

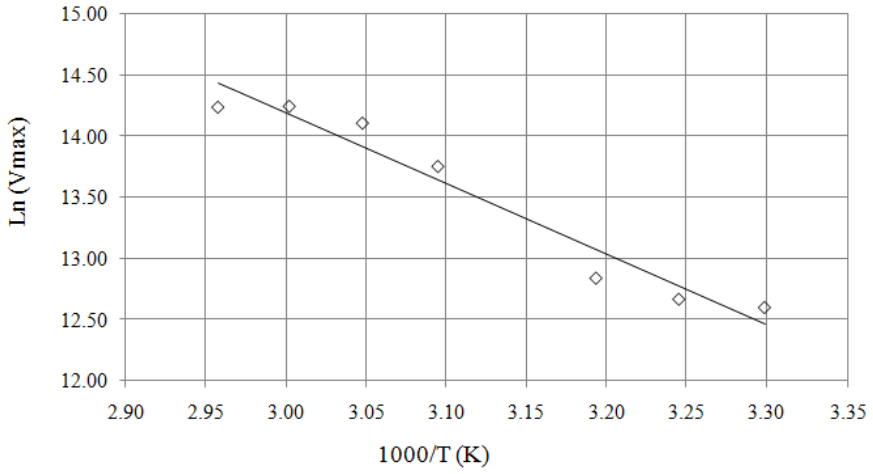

Fig. 4. Arrenius plot for the determination of E_a of semi-purified protease from *B. licheniformis* LBA 46.

The E_a value (47.96 kJ/mol) was high, positive and showed good correlation ($R^2 = 0.95$), within the temperature range studied. Abdel-Naby (2017) related E_a of 17.31 kJ/mol for alkaline protease from *Bacillus stearothermophilus*. Souza et al. (2015) also found E_a (19.03 kJ/mol) positive for acid protease from *Aspergillus foetidus*. For an alkaline protease from *Nacordiopsis alba*, Gohel and Singh (2012), determined a E_a value of 36.80 kJ/mol.

The Q_{10} value is a kinetic parameter used to determine whether the catalytic reactions are controlled by temperature or other factors. According to Elias et al. (2014), the enzymatic reactions have values of Q_{10} ranging from 1 to 2. Values outside this range can be interpreted as indicative of the involvement of factors other than temperature in the control of the reaction rate. The Q_{10} value, evaluated between 30-80 ºC, was determined to be in the range of 1.59-1.87, representing the rate of reaction which was affected only by temperature increase.

3.3.2 Kinetic parameters, K_m and V_{max}

The kinetic parameters were calculated according to double reciprocal Lineweaver-Burk plot. The K_m value indicates the protease-substrate affinity and a low K_m value indicates higher affinity of the enzyme for the substrate. The V_{max} value could be defined as the maximum value of initial velocity when all actives sites are occupied by the substrate.

The enzyme showed, with good correlation ($R^2 = 0.91$), Michaelis-Menten-type kinetics with K_m = 1.60 mg/mL and a high V_{max} = 2 x 10^6 U/g. A similar K_m value (1.92

mg/mL) was reported by Souza et al. (2015) for acid protease from *A. foetidus* utilizing azocasein as substrate. A lower K_m value (0.44 mg/mL) was related for serine protease from *Aspergillus niger* (Castro et al., 2014) also using azocasein as substrate. Using casein as substrate, Abdel-Naby (2017) determined a higher K_m value (3.7 mg/mL) than the one founded here for alkaline protease from *B. stearothermophilus*.

3.3.3 Thermal inactivation of semi-purified protease

The thermostability of semi-purified protease from *B. licheniformis* LBA 46 was studied in the range of 50-70 ºC (Fig. 5 and 6). The enzyme showed higher stability in temperature of 50 ºC, retaining above 80% of the initial activity after 120 min. The protease was rapidly inactivated at 70 °C in the absence of substrate losing 84% of the initial activity after 30 min of incubation. After 120 min of treatment at 60 and 70 °C, the enzyme retained about 39% and 9% of the initial activity, respectively.

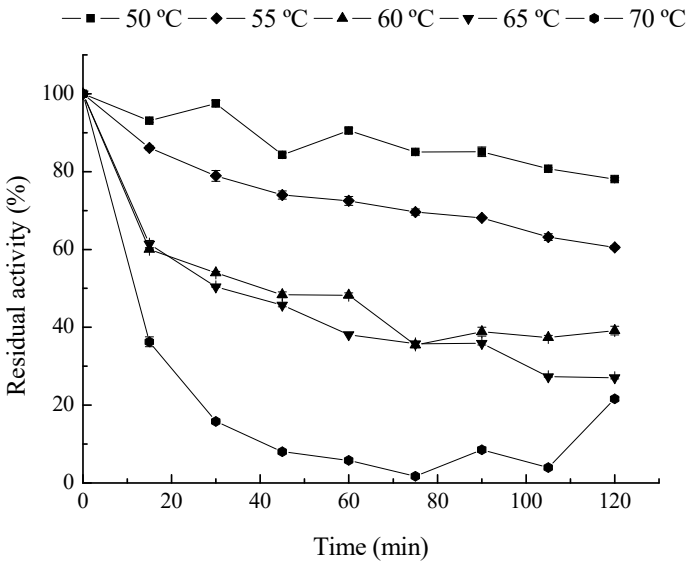

Fig. 5. Thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

The half-life of an enzyme is defined as the amount of time required at a given temperature, capable of reducing its initial activity by half. According to Table 5, the semi-purified protease of *B. licheniformis* LBA 46 has high thermal resistance, requiring 693.15 min to reduce half of its activity at 50 ºC and that value fell as the temperature increased, reaching 23.90 min to 70 °C. The *D*-value, which is the time required for a 90% reduction in the initial enzyme activity was also reduced with increasing temperature, ranging from

2,302.60 to 79.40 min between 50 and 70 °C. In relation to the inactivation rate constants (K_d), the values increased with an increase in temperature, ranging from 1.0 x 10^{-3} - 29 x 10^{-3} min^{-1}. The energy required for thermal inactivation (144.50 kJ/mol) was calculated using Arrenius plot (Fig. 7). Abdel-Naby (2017) determined a similar value for E_a (105.5 kJ/mol) by studying an alkaline protease from *B. stearothermophilus*.

Table 4. Thermodynamic and kinetic parameters for thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

Temperature (ºC)	K_d (min^{-1})	$t_{1/2}$ (min)	D (min)	R^2	E_{ad} (kJ/mol)
50	0.0010	693.15	2,302.60	0.87	
55	0.0030	231.05	767.53	0.93	
60	0.0070	99.02	328.94	0.82	144.50
65	0.0090	77.02	255.84	0.90	
70	0.0290	23.90	79.40	0.96	

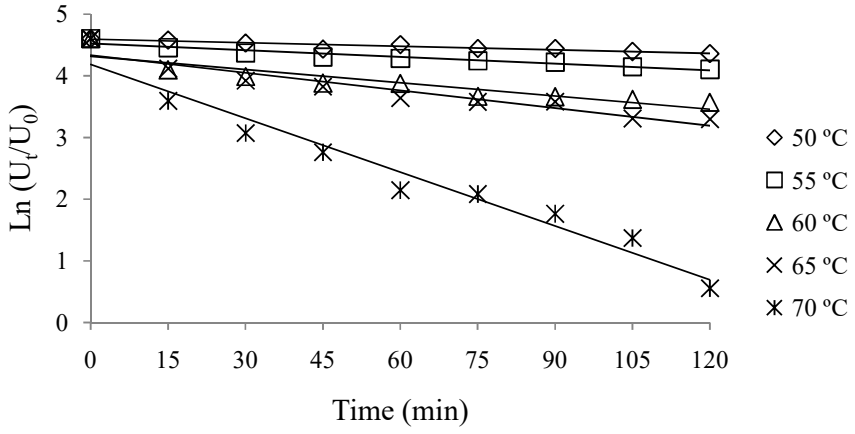

Fig. 6. Pseudo-first-order plots for irreversible thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

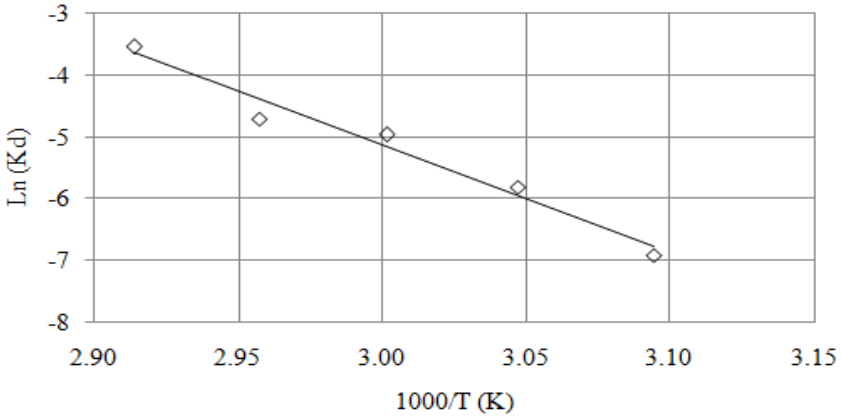

Fig. 7. Arrhenius plot to calculate E_{ad} for irreversible thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

Thermal inactivation of enzymes is accompanied by the breakdown of many non-covalent bonds, which represents an increase in the value of ΔH. According to Batista et al. (2014) high ΔH values are linked to high thermal stability of the enzyme. The opening or unfolding of the enzyme caused by heating increases its disordered state which can be measured by the value of ΔS. An enzymatic reaction can also be evaluated by measuring the change in ΔG value during the conversion of a complex enzyme-substrate into a product (Riaz et al., 2007). A low ΔG value suggests that this conversion was more spontaneous; however, high ΔG values indicate high enzyme stability (Batista et al., 2014).

Table 5. Thermodynamic parameters for thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

Temperature (ºC)	ΔH (kJ/mol)	ΔS (J/mol.K)	ΔG (kJ/mol)
50	141.81	101.79	108.92
55	141.77	103.98	107.65
60	141.73	104.28	106.99
65	141.69	99.83	107.93
70	141.65	103.21	106.23

The values of ΔH, ΔS and ΔG practically did not vary within the temperatures analyzed. The parameters of kinetic inactivation are important since they serve to define and model the use of enzymes in certain industrial applications.

3.4 Purification of the protease extract

The protease extract from *B. licheniformis* LBA 46 was purified 3.33 fold using 80% ammonium sulfate precipitation and using DEAE Sepharose column chromatography. The purified protease showed a specific activity of 628.96 U/mg (Table 6).

Table 6. Summary purification of protease from *B. licheniformis* LBA 46.

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery* (%)	Purification fold**
Crude extract	5,000	6,355,416.67	33,620.33	189.03	100	1
Ammonium sulfate precipitation	400	539,933.33	4,300.32	125.56	8.50	0.66
DEAE-Sepharose	10	327.90	0.52	628.96	0.005	3.33

Calculations were based on the provided volumes
*Recovery = total activity*100/crude extract total activity
**Purification fold = specific activity/crude extract specific activity

3.4.1 Biochemical characterization of purified protease extract

The molecular weight of purified protease extract from *B. licheniformis* LBA 46 was estimated as 40 kDa by SDS-PAGE (Fig. 8).

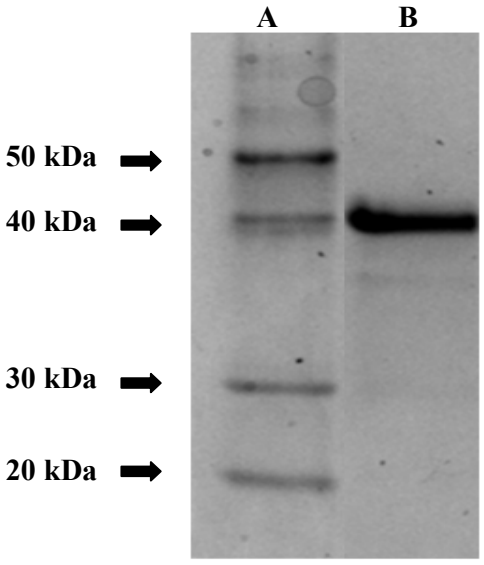

Fig. 8. SDS-PAGE of purified protease extract from *B. licheniformis* LBA 46. (A) molecular mass markers and (B) purified protease.

Jalkute et al. (2017) purified the protease from *Bacillus safensis* CK about 7-fold by DEAE cellulose column chromatography and estimated the molecular weight of the protease at 40 kDa by SDS-PAGE. Annamalai et al. (2013) purified a 33 kDa protease from *Bacillus alveayuensis* CAS 5 using a DEAE cellulose and Sephadex G-50 columns. Jellouli et al. (2011) purified a 30 kDa protease from *B. licheniformis* MP1 using Sephadex G-100 and Mono Q-Sepharose columns. Another protease from *B. licheniformis* purified by Lin et al. (1992) showed molecular weight equal to 33 kDa. Other studies also described proteases with low molecular weight from *Bacillus* sp: 17.10 kDa (Kim and Kim 2005), 20.10 kDa (Rai et al. 2009) and 15 kDa (Adinarayana et al. 2003).

The purified protease extract of *B. licheniformis* LBA 46 presented high activity (> 80%) in the range of pH 6.5-9, optimal activity at pH 8.5 and low activity at pH 4.0 (15%). The purified protease was stable in the range of pH 5-10 after 24 h at 4 °C, retaining more than 86% of the initial activity (Fig. 9A). The purified protease extract presented optimum activity at 50 and 60 ºC at pH 7.0. The enzyme was stable at 40 °C for 1 h in pH 7 and retained 85% and 30% of the initial activity after 1 h of treatment at 50 °C and 60 °C, pH 7, respectively (Fig. 9B).

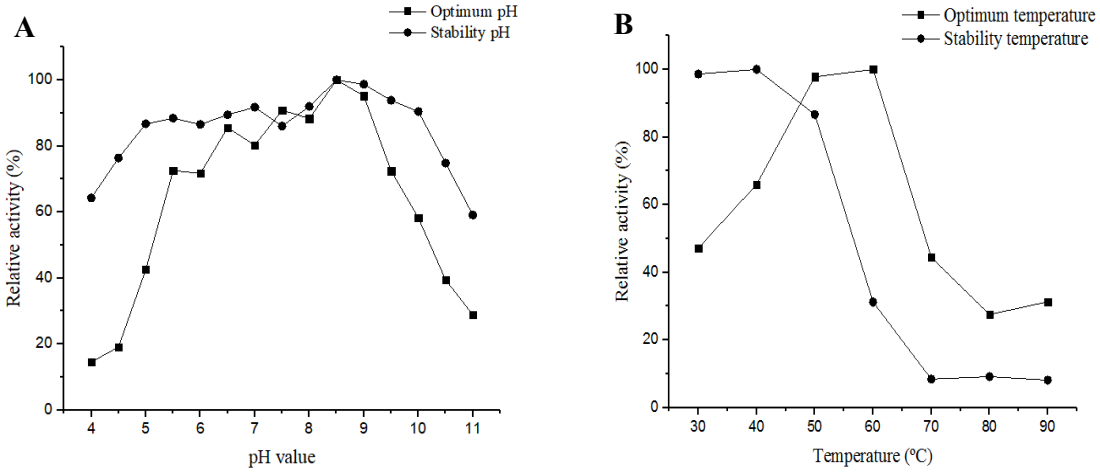

Fig. 9. Optimum and stability pH (A) and temperature (B) of purified protease extract from *B. licheniformis* LBA 46.

4. Conclusion

The highest protease activity was obtained after fermentation of *B. licheniformis* LBA 46 in a culture medium composed of 32 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract and 20 g/L dried whey, at 30 ºC for 48 h (2,448 U/mL) and 72 h (2,661 U/mL) at 300 rpm, obtaining productivities of 51 and 36.7 U/mL.h, respectively. The

scale up of Erlenmeyer flasks containing 100 mL of culture medium to the 6 L bench reactor containing 3 L of culture medium provided a 2.3-fold increase in protease activity. The semi-purified protease showed optimal activity at 60-65 °C with high catalytic activity (~1,500,000 U/g) and was stable at 50 °C retaining more than 80% of the initial activity after 120 min. The purified protease presented optimum activity at 50-60 °C and maximum activity in pH 8.5 at 60 ºC. The purified protease was stable in the range of pH 5-10 after 24 h at 4 °C. This protease has interesting characteristics with potential industrial application.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Capítulo III

Effect of high pressure homogenization process on the activity and stability of protease from *Bacillus licheniformis* LBA 46 in different pH values

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Abstract

Alkaline proteases have great importance in the pharmaceutical, textile, food industries and research studies. High pressure is an emerging and relatively new technology that is capable of modifying various molecules. The use of high pressure homogenization (HPH) has been widely evaluated in the modulation of enzyme activity, either to inactivate, improve or stabilize its activity. The effect of HPH treatment on *Bacillus licheniformis* LBA 46 semi-purified protease activity was investigated at different pH values and temperatures of activity. The semi-purified protease was inactivated at pH 4 (40, 60 and 90 °C) and showed low activity at pH 7 (40 and 90 °C) and pH 9 (40 and 90 °C). The treatment of the semi-purified protease at pH 4, 7 and 9 with HPH (50-200 MPa) did not increase protease activity and stability at 40, 60 and 90 °C.

Keywords: Pressure; Homogenization; Protease; Denaturation; Inactivation.

1. Introduction

Pressure is a thermodynamical parameter that has gained importance in a range of fields related to biochemistry and biology (Kunugi, 1992). Various studies have been performed to discover or change macromolecules structures, such as proteins, and have explored the use of high pressure, in different aspects, in the field of enzymatic technology.

High pressure homogenization (HPH) is a promising emergent technology, non-thermal, convenient, versatile, and environmentally friendly, that could guarantee food safety and stability with reduced sensory and nutritional changes (McKay, Linton, Stirling, Mackle, & Patterson, 2011; Salaberria, Fernandes, Diaz, & Labidi, 2015; Tribst, Franchi, Cristianini, & Massaguer, 2009). HPH can lead to alterations in the protein structure, stability and/or enzymatic activity (Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012a).

In general, during HPH, the fluid is forced through a narrow opening in the valve homogenizer. The pressure gradient formed between the inlet and outlet of the liquid in the valve generates, as a result, cavitation, turbulence and shear pressure, which are simultaneously induced, leading to an instantaneous temperature increase whose magnitude depends on the intensity of the applied pressure (Dumay, et al., 2013; Paquin, 1999; Panozzo et al., 2014; Porto, Augusto, Terekhov, Hamaker, & Cristianini 2015). The fluid disruption rate is proportional to approximately the third power of the turbulent velocity of the fluid flowing through the homogenizer orifice, which is directly proportional to the applied pressure. The operating parameters, pressure, temperature, number of cycles or passes, valve design and flow rate are responsible for affecting the homogenized components (Dhankhar, 2014). The temperature increases during the process, approximately 1.1 °C per 10 MPa (Tribst & Cristianini, 2012b).

Some of the effects of HPH are mainly attributed to cavitation; shear pressure and turbulence forces, but, the modification of protein structures using HPH is also caused by treatment time, impact (change in flow direction), friction and heat, which occur simultaneously during the application of the treatment. These forces have the ability to alter configuration and function of proteins (Roach & Harte, 2008).

Proteases are enzymes that catalyze the hydrolysis of peptide bonds resulting in proteins and smaller peptides. This group of enzymes plays a vital role in various industrial applications and represents one of the most important groups of commercial enzymes (Li, Yi, Marek, & Iverson 2013; Nijland & Kuipers, 2008; Rao, Tanksale, Ghatge, & Deshpande, 1998; Schallmey, Singh, & Ward, 2004). They are classified as acid, neutral and alkaline,

depending on the pH in which they have maximum activity. Proteases are also the main enzymes produced by microbial sources and occupy 60-65% of the global industrial market (Esakkiraj, Immanuel, Sowmya, Iyapparaj, & Palavesam, 2009; Pant et al., 2015; Zanphorlin et al., 2011).

Enzyme activity is determined by its appropriate conformations that are defined by conditions such as pH and temperature. Pressure can cause changes in secondary, tertiary and quaternary structures of enzymes causing an increase in hydrophobic sites, exhibiting amino acid and sulphydryl groups. Pressure can also induce changes in the protein functionality and dissociate protein aggregates. The degree of denaturation and/or aggregation of proteins depend on the intensity of mechanical forces (Dumay et al., 2013; Floury, Legrand, & Desrumaux, 2004; Freudig, Tesch, & Schubert, 2003; Liu et al., 2009; Paquin, 1999).

Concerning stability, the flexibility of the molecule side chains can also play an important role on the pressure stability of proteins, depending basically on intermolecular interactions (covalent, hydrophobic, electrostatic and van der Waals); hydration of charged groups; bonded water release; stabilization of hydrogen bonds and on the proteins conformational capacity to balance the loss of weak interactions (Boonyaratanakornkit, Park, & Clark, 2002).

There is already a large array of commercially used proteases and there is an incredible landscape of potential uses for proteases and interest for the enhancement of their production, activity and specificity. Based on the importance of such enzymes, the aim of this study was evaluate the behavior of a novel alkaline semi-purified protease from *B. licheniformis* against the treatment of HPH at different pH values.

2. Material and Methods

2.1 Protease

The protease utilized in this study was produced by *B. licheniformis* LBA 46, from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas. The microorganism was fermented in 6 L bench reactor containing culture medium as proposed by Contesini (2014) with slight modifications composed of 32 g/L of sugar cane molasses (Fio de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®) and 20 g/L dried whey (Alibra®) adjusted to pH 7 at 30 ºC and 300 rpm during 48 h. The crude extract was semi-purified using 80% ammonium sulfate precipitation, dialysed, freeze-drying and used for these experiments.

2.2 Protease activity and stability on different values of pH and temperature

The protease activity was measured (according to item 2.3) in different values of pH: 4 (value below the protease stability), 7 (higher value for the protease activity) and 9 (optimum value for the protease stability). The buffers used were 0.05 M acetate (pH 4), 0.05 M sodium phosphate (pH 7) and 0.05 M boric acid-borax (pH 9). Three temperatures were used, 40 ºC (value below the optimum for the protease activity), 60 ºC (optimum value for the protease activity) and 90 ºC (temperature of inactivation). For the evaluation of the protease stability, the controls and the processed samples, in each different pH value, were stored under refrigerated conditions (5 ºC) for 24 h and the residual activity was evaluated in each pH value used at 40, 60 and 90 ºC. Protease activity at pH 7 and 60 °C was considered 100%.

2.3 Protease activity

The protease activity was determined according to Charney and Tomarelli's method (1947), modified by Castro and Sato (2014) using azocasein as the substrate. The protease was dissolved at the appropriate pH and its analysis also occurred with the substrate at the same pH. The buffers used are described in item 2.2. The reaction mixture contained 0.5 mL of 0.5% azocasein (in the adequate pH) and 0.5 mL of the enzyme solution (in the adequate pH), which were incubated for 40 min at the temperature chosen for measuring the enzymatic activity. The reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 17,000 x *g* for 15 min at 15 °C. An aliquot of 1 mL of the supernatant obtained was neutralized with 1 mL of 5 M KOH. One unit of protease activity (U) was defined as the amount of enzyme which causes an increase of 0.01 in absorbance at 428 nm.

2.4 High pressure homogenization process

The equipment utilized was a Panda Plus High Pressure Homogenizator (GEA-Niro-Soavi, Parma, Italy) with a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa at a 9 L/h flow rate.

The protease solution (0.1% w/v) was prepared in 0.05 M acetate buffer (pH 4), in 0.05 M sodium phosphate buffer (pH 7) and in 0.05 M boric acid-borax buffer (pH 9) and each single mixture was processed under the pressures of 0, 50, 100, 150 and 200 MPa. The enzymatic solutions were collected and immediately cooled in an ice bath. The enzymatic solution without treatment was used as the control. The activity of protease samples treated at

pH 4, 7 and 9 was measured respectively at pH 4, 7 and 9 at different temperatures (40, 60 and 90 ºC). The inlet and outlet temperature of each mixture were also measured using a digital thermometer.

2.5 UV- absorption spectra analysis of native and HPH processed protease

The UV-absorption spectra analysis was measured for the unprocessed and processed enzymatic solutions in different pH values just after the HPH treatment. The spectra was scanned from 200 to 400 nm (Liu et al., 2010a) using a UV-VIS spectrophotometer DU-800 (Beckman Coulter®, Brea, CA) to determine the absorption peak value and its wavelength.

2.6 Statistical analysis

The analysis, ANOVA and Tukey's test, were carried out in Minitab 16.1.1 (Minitab Inc., USA) at 95% significance level. The process and the measurements of the activity were all performed in triplicates.

3. Results and Discussion

3.1 Protease characterization

The enzyme characterization was performed evaluating the protease activity at different pH value and temperatures (Fig. 1). The semi-purified protease from *B. licheniformis* LBA 46 presented 100 and 87% activity at 60 ºC at pH 7 and 9, respectively. After incubation at refrigeration temperature (5 ºC) for 24 h, the activity was decreased to 89 and 86.8%, respectively. The protease presented very low activity in pH 4 at 40, 60 and 90 ºC (~1.5% of residual activity) and showed low activity at 40 ºC (15-17%) and at 90 ºC (6-12%) in pH 7 and 9 just after preparation of the enzyme solution and also after 24 h of refrigeration at 5 ºC.

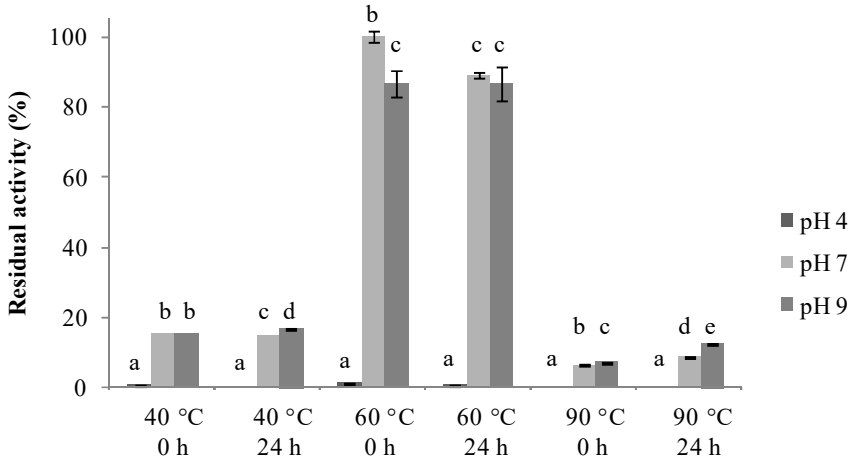

Fig. 1. Residual activity of semi-purified protease in different conditions (pH and temperature) just after enzyme solution preparation and after 24 h of refrigerated storage (5 °C). Different letters = difference ($p \leq 0.05$) between the activity at the same temperature at different time and pH.

3.2 Effect of HPH process on semi-purified protease from *Bacillus licheniformis* LBA 46 activity and stability

Fig. 2 (A, B, C) illustrates, respectively, the protease residual activity determined at 40, 60 and 90 °C, after HPH treatment (50-200 MPa) in different pH values (4, 7 and 9). The enzyme residual activity was determined at the same pH used in the high pressure treatment, as described in item 2.4. At pH 4, the residual activity was approximately 1%, determined at 40, 60 and 90 °C. Fig. 2A shows that protease treated at pH 7 and 9 showed no increase in activity at 40 °C at these pH values, obtaining residual activity of approximately 15-17%.

Fig. 2B shows that protease at pH 7 and 9, just after HPH treatment, retained 70 - 80% and 80 - 88% of residual activity at pH 7 and 9, respectively at 60 ºC. After HPH treatment, the protease showed low residual activity of approximately 5-7% in pH 7 and 9 at 90 ºC (Fig. 2C).

The treatment at 0 MPa, when the solution passes through the valve into the equipment without pressure application, can promote small changes in enzyme configuration due to shear, friction and others stress caused by the process. According to Benjakul and Morrissey (1997), the conformation and charge distributions are altered under acidic conditions making the enzyme unable to bind to the substrate properly. Klomklao, Kishimura, Nonami and Benjakul (2009) related that in a very acidic or alkaline solution, most enzymes

experience an irreversible denaturation which causes loss of stability. This indicated that the pH changed the enzyme native molecule configuration and, consequently, the enzyme susceptibility to HPH treatment is also altered.

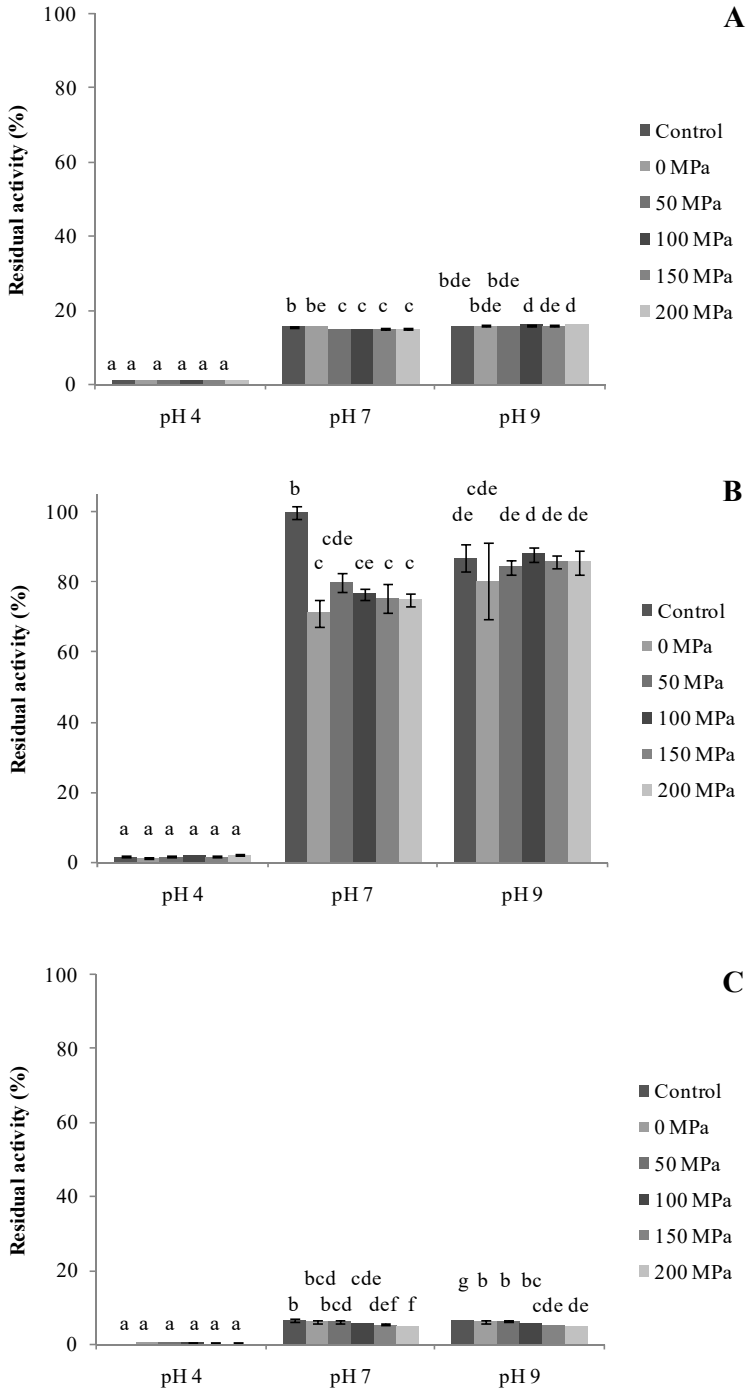

Fig. 2. Effects of HPH treatment (50-200 MPa) on semi-purified protease from *B. licheniformis* LBA 46 in different pH values (4, 7 and 9). Percentage of residual activity determined at 40 (A), 60 (B) and 90 °C (C), just after the processing. Different letters = difference ($p \leq 0.05$) between the samples at the same temperature and different pH.

Treatment of samples using HPH results in heating because the rapid decompression promotes intense shear and friction (Tribst, Augusto, & Cristianini, 2012b). The samples were cooled before being placed in the homogenizer device to minimize any damage caused by heating during the process. Treatment of the protease solution at pH 9 at 18 °C using HPH at 200 MPa increased the temperature to 36.7 °C. This protease is stable at 40 °C for 1 h at pH 7 indicating that the modifications observed in its activity were caused only by HPH. Table 1 presents the inlet and outlet temperatures of homogenized samples after the homogenization process for each pH value studied.

Table 1. Temperature increase during HPH at pH 4, 7 and 9 (inlet temperature ~ 18 °C).

Pressure (MPa)	pH value					
	4	7	9	4	7	9
	Outlet temperature (°C)			Temperature increment (°C)		
0	23.0	24.3	26.7	5.0	6.3	8.7
50	23.3	27.3	28.7	5.3	9.3	10.7
100	24.6	32.0	32.7	6.6	6.0	14.7
150	29.0	33.7	35.7	11.0	15.7	17.7
200	29.0	36.0	36.7	11.0	18.0	18.7

No statistical differences ($p \leq 0.05$) were observed between the triplicates of processed samples, indicating good repeatability of the process. The temperature increase could be related to differences in the residence time of the different protease solutions within the homogenization valve during the process.

Fig. 3 (A, B, C) shows that after 24 h of incubation at 5 °C, the protease samples adjusted at pH 4 and HPH treated did not result in increased activity compared to the control. The protease was unstable at pH 4 obtaining approximately 1% of the initial activity under the tested conditions.

HPH treatment of the protease at pH 7 and 9 with subsequent refrigeration for 24 h did not result in increased activity determined at any of the temperatures analyzed. The protease retained approximately 15-17% of the initial activity at 40 ºC (Fig. 3A), 80-90% at 60 °C (Fig. 3B), less than 12% at 90 °C (Fig. 3C).

Similar protease behavior was observed at 40 and 90 °C (Fig. 2 and 3) and could be explained because; in these temperatures, the enzyme is outside its zone of activity, having low activity at 40 and being inactivated at 90 °C. Then, the treatment was not able to modify the enzyme temperature of activity; which remained 60 ºC.

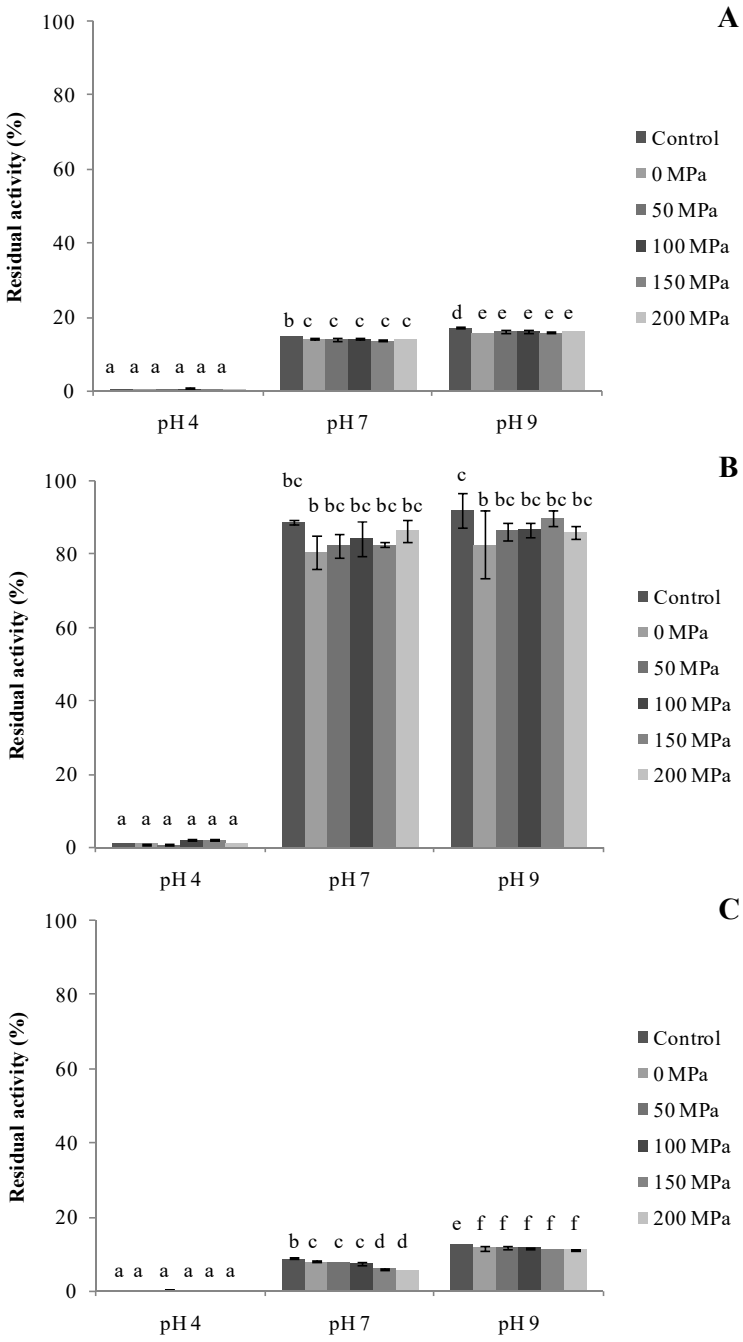

Fig. 3. Effects of HPH treatment (50-200 MPa) on semi-purified protease from *B. licheniformis* LBA 46 in different pH values (4, 7 and 9). Percentage of residual activity determined at 40 (A), 60 (B) and 90 °C (C), after 24 h of refrigerated storage at 5 ºC. Different letters = difference ($p \leq 0.05$) between the samples at the same temperature and different pH.

According to Cheftel (1992), the pressurization can generate reversible or irreversible, partial or complete enzyme inactivation because of the changes generated in the protein structure. High pressures could lead to the inactivation or reduction of enzymatic activity (Yaldagard et al., 2008). HPH treatment is a process able to improve (Tribst & Cristianini, 2012a; Tribst & Cristianini, 2012b), decreased (Tribst, Cota, Murakami, & Cristianini, 2014, Carbonell, Navarro, Izquierdo, & Sentandreu, 2013) or promote no changes (Liu et al., 2010a) in enzymatic activity. These results could be generated due to the resistance to modification of enzymatic structure, since it can be observed that after the homogenization, the enzymatic activity of the treated samples remained very close to the enzymatic activity of the native enzyme. Probably the treatment, even at high pressures (up to 200 MPa), was not sufficient to cause the unfolding of the enzyme, alterations in molecular conformation or reduction of the exposed active sites, resulting in no activity alterations just after the application of treatment and after the refrigerated storage. The protease from *B. licheniformis* LBA 46 requires more energy input to change its performance, which might be related to the enzyme conformation or to the capability of HPH to cause reversible modification. According to Tribst et al. (2012a) enzymes with no quaternary structure and with good thermal resistence are resistant to HPH processing.

Tribst et al. (2012a) evaluated the HPH effect on activity and stability of one protease commercial from *Bacillus subtillis* in the range of 0 - 2,000 bar at pH 7.5. The authors observed no increased on the activity measured at 55 and 70 °C, but, at 20 °C, the activity was improved around 30% after 2,000 bar treatment. Tribst, Augusto, & Cristianini (2013) studied the effect of multiples cycles of pressure on the activity of commercial enzymes. The authors verified an increase of 20% in the activity at 20 °C and pH 7.5, after homogenization of a neutral commercial protease from *B. subtilis* with multiple steps using 200 MPa. On the other hand, the processes at 150 and 200 MPa with up to three passes caused 60% reduction in the enzymatic activity at 55 °C.

Liu et al. (2010a) studied the effect of HPH microfluidization on the activity, stability and conformation of trypsin from porcine pancreas and observed that the enzyme activity was not affected by three passes treatment at in the range of 80-160 MPa, however, the pH and thermal stability was improved. The higher thermal stability measured was after 80 MPa and 100 min at 45 °C. The optimal temperature of activity of trypsin increased from 35 to 45 °C and pH optimum of activity was not altered by the treatment. Liu et al. (2010b) described a 90% of reduction on papain activity treated with HPH microfluidization at 180 MPa, pH 6.

The protease presented different degrees of reduction on the enzymatic activity after treatment in the range of 120-180 MPa.

Pinho et al. (2014) evaluated the activity of the protease produced by *Pseudomonas fluorescens* in skimmed milk after the HPH treatment at 200, 250 and 300 MPa. At a pressure of 200 MPa, the enzymatic activity was equal to that of the control sample, and at 250 and 300 MPa, a partial inactivation of the enzyme occurred, with a maximum reduction of 72.50%.

Observing the influence of HPH treatment in a commercial calf rennet (composed of 94% chymosin and 6% pepsin) for the milk coagulation process, using 50, 100, 150 and 190 MPa at pH 5.1, Leite Júnior et al. (2014) concluded that the process decreased the calf rennet proteolytic activity on the day of treatment (reduction of 52% at 190 MPa) and during the storage. The authors observed that the non treated sample showed a continuous reduction activity profile while the homogenized showed no significant activity reduction. The HPH did not affect the milk-clotting activity of calf rennet using 50-190 MPa.

Leite Júnior et al. (2015a) studied the effect of HPH under range of pressures between 0 and 190 MPa on commercial porcine pepsin and observed that milk-clotting and proteolytic activities were not altered, just after pressure treatment. The milk-clotting activity was improved after 150 MPa treatment and 60 days of storage, 15% higher than the control samples. For the proteolytic activity, a continuous decline was observed for all samples, reaching approximately 85% after 14 days. After 30 and 60 days of storage, the proteolytic activity for all samples showed a similar diminution behavior. On the contrary, studying the protease of *Rhizomucor miehei*, Leite Júnior et al. (2015b) observed that this enzyme was resistant to homogenization, obtaining a slight increase of 6% in its proteolytic activity only after 3 cycles at 190 MPa.

According to the literature and studies presented for HPH treatment on the behaviour of proteases, could be observed that the results are relative to source of protease, temperature, pH, time and pressure applied, among others. The application of homogenization in different types of enzymes have been studied demonstranting that the effect of homogenization varies for each kind of enzyme (bacterial, fungal, plant, animal) and can also be affected by the treatment conditions (Carbonell et al., 2013; Tribst et al., 2014; Tribst & Cristianini, 2012a; Tribst & Cristianini, 2012b).

3.3 UV- absorption spectra analysis of untreated and HPH processed protease

According to Liu et al. (2010a) alterations on the UV-absorption spectra after HPH treatment can indicate that the process was able to promote changes on the protein molecule structure. Fig. 4 shows the results for UV-absorption spectra analysis at different pH values. Absorption was noted in the interval of 230-240 nm and a slight absorption peak at the interval of 270-280 nm for the distinct pH values. In the spectrum of proteins and peptides, absorption between 220 and 240 nm is caused because of the peptide bonds and carboxyl groups. Other materials, as impurities in solvents, can also exhibit absorption at this range of wavelength. The peak at 280 nm is caused for the absorbance of aromatic amino acids, as tryptophan, tyrosine and, to a small extent, cystine (Schmid, 2001; Málnási-Csizmadia, 2013).

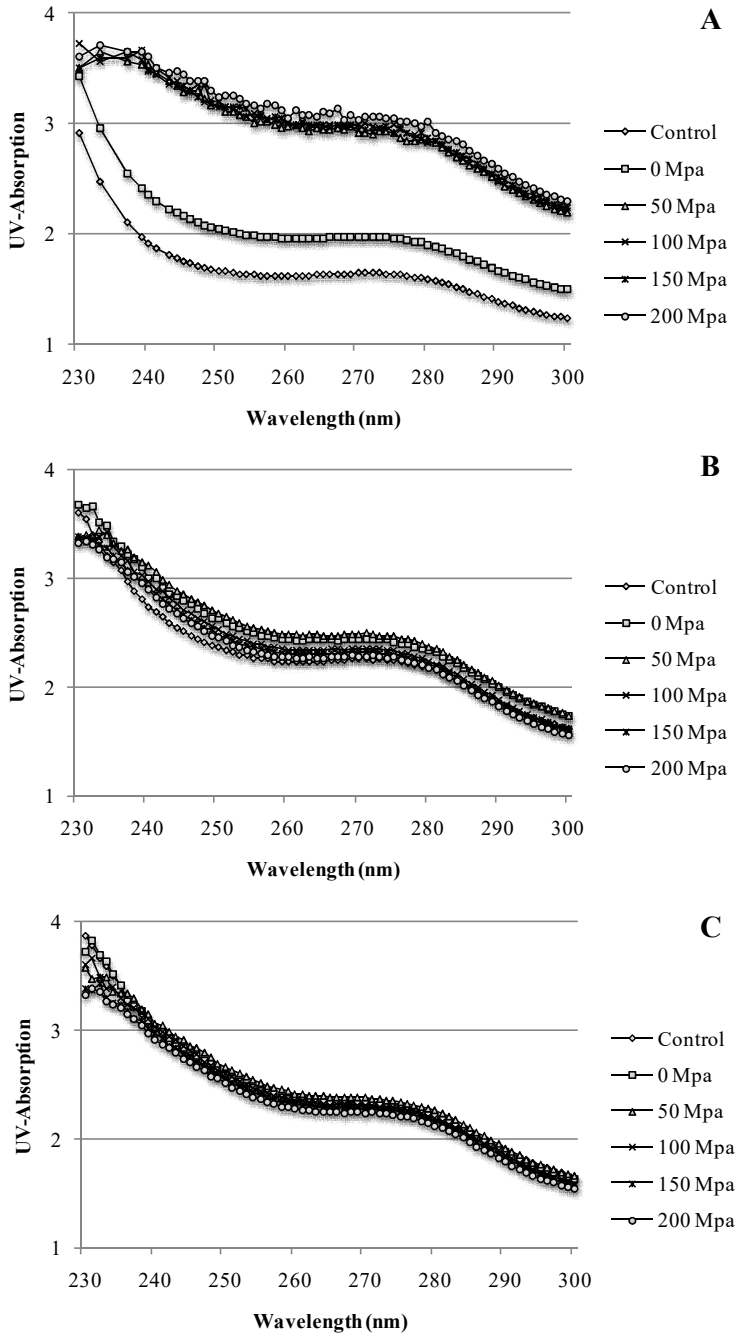

Fig. 4. UV-absorption spectra between 230 and 300 nm of treated and untreated semi-purified protease at pH 4 (A), 7 (B) and 9 (C).

There was a normalization of the curves behavior. The protease treated at pH 7 and 9 showed similar UV-absorption curves indicating that the treatment was not able to change the enzyme structure in a detectable way using this measurement. HPH-treated protease (50-200 MPa) at pH 4 presented different UV-absorption curves profile when compared to the control

sample (untreated) and the treated sample without application of pressure (0 MPa) (Fig. 3A). The semi-purified protease from *B. licheniformis* LBA 46 is unstable at pH 4 and the HPH treatment was not able to increase the activity or stability of the enzyme.

In general, no relation was observed between UV-absorption spectra and protease activity, for the control and the homogenized samples at different pH values.

4. Conclusion

Treatment under high pressure is classified within emerging technologies and has been used in the treatment of different foods. Due to the low energy employed in the process, the pressure can modify different balances involved in the reaction mechanisms, thus explaining its ability to modify various types of chemical bonds in biological molecules such as proteins. The untreated semi-purified protease from *B. licheniformis* LBA 46 showed low activity and stability at pH 4 at 40, 60 and 90 ºC and also presented low activity at pH 9 at 40 and 90 ºC. The HPH treatment (50-200 MPa) of this protease at pH 7 and 9 decreased the enzyme activity at 60 ºC and the treatment at pH 4 and 9 did not increased the activity and stability of the enzyme at 40 ºC and 90 ºC. It means that homogenization is not adequate to improve the activity of this enzyme; since the protease was resistant to treatment the HPH cannot change its performance.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Capítulo IV

Optimization of enzymatic hydrolysis of rice protein by different enzymes using response surface methodology

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Abstract

The optimization of the enzymatic hydrolysis of rice protein was determined. The semi-purified protease of *Bacillus licheniformis* LBA 46 and the commercial protease Alcalase 2.4L were used to produce rice protein hydrolysates using pH (6-10) and protease concentration (50-150 U/mL) as independent variables. Both enzymes demonstrated the ability to hydrolyse rice protein producing peptides with antioxidant activity and degree of hydrolysis up to 50% for almost all of the hydrolysates. For the analysis of ORAC and FRAP, the behavior of the enzymes in relation to the independent variables was similar, having the same optimization range of pH (9-10) and protease concentration (50-150 U/mL). The optimization conditions were validated and, in the chosen condition (pH 10 and 100 U/mL of protease), it was possible to confirm that the defined model was predictive for ORAC and FRAP responses. The experimental values for ORAC and FRAP under these conditions were 940 and 18.78 TE μmol/g for the rice protein hydrolysates prepared with LBA protease and 1,001.94 and 19.31 TE μmol/g for the rice protein hydrolysates prepared with Alcalase 2.4L. After optimization of the enzymatic hydrolysis conditions, the antioxidant activity values increased when compared to non-hydrolyzed rice protein: 324.97 TE μmol/g (ORAC) and 6.14 TE μmol/g (FRAP). For the hydrolysis performed with the LBA enzyme, there was an increase of 2.9-fold for ORAC and 3.05-fold for FRAP assay. For the hydrolysis performed with Alcalase 2.4L, there was an increase of 3.08-fold for ORAC and 3.14-fold for FRAP assay.

Keywords: Optimization; Contour Curve; Rice; Antioxidant; Protease.

1. Introduction

Plant has an excellent source of proteins and bioactive materials, so its hydrolysates can contribute as alternative sources of protein and health-promoting ingredients (Thamnarathip, Jangchud, Nitisinprasert, & Vardhanabhuti, 2016). According to USDA (2017), the global annual production of rice (*Oryza sativa* L.) is estimated at about 480 million metric tons between the years 2016 and 2017. This grain is also the considered the staple food for 3.5 billion people around the world, mainly in Asia, where rice provides 50% of the dietary caloric supply and a relevant amount of the protein intake for millions of people (Muthayya, Sugimoto, Montgomery, & Maberly, 2014).

The microbial proteases, specially the alkaline proteases, are used in the preparation of high value protein hydrolysates. Many species of *Bacillus* sp. produce high quantity of proteases, which are capable of releasing peptides with beneficial properties (Ward, Rao, & Kulkarni, 2009), such as peptides with antioxidant functions resulted by enzymatic hydrolysis of food protein (Korhonen & Pihlanto, 2006; Ricci, Artacho, & Olalla, 2010).

The traditional univariate method of testing is not efficient and not cost effective to study the effects of the studied variables. The response surface methodology (RSM) appears as a better alternative method used to optimize process (Phongthai, Lim, & Rawdkuen, 2016). Multivariate equations can describe the effects of the independent variables (test factors) on the dependent variable (response) studied and also to determine relationships between them (Madamba, 2002).

The bioactive properties of some protein hydrolysates, such as the rice protein, are dependent of the process and the enzyme employed. Process optimization is a powerful tool capable of reducing the time required to obtain the required response. The objective of this study was to establish the most adequate conditions for the production of rice protein hydrolysates with antioxidant activity measured in terms of DPPH, FRAP and ORAC assays and to make a comparative study between proteases from *Bacillus licheniformis* (LBA 46 and Alcalase 2.4L) which were used as reaction catalysts.

2. Material and Methods

2.1 Materials

Rice protein concentrate (RPC), 83.20% protein, dry weight basis, was obtained from Growth Supplements (Brazil). The chemicals, azocasein, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron chloride ($FeCl_3.6H_2O$), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2,20-azobis(2-methylpropionamidine) dihydrochloride (97%) (AAPH), fluorescein and (7)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and chemicals used were of analytical grade.

2.2 Enzymes

The alkaline protease was produced by *B. licheniformis* LBA 46 and belongs to the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas. The culture medium used in the fermentation process was composed by 32 g/L of sugar cane molasses (Fio de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®) and 20 g/L of dried whey (Alibra®), adjusted to pH 7 as proposed by Contesini (2014) with slight modifications. The cultivation conditions were 30 ºC, 300 rpm and 48 h of fermentation. The crude extract was partially purified using 80% of ammonium sulfate precipitation, dialysed, freeze-drying and storage for enzymatic hydrolysis.

The commercial protease Alcalase 2.4L obtained from *B. licheniformis* was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3 Experimental design

The parameters of study, pH (X_1) and protease concentration (U/mL) (X_2), were evaluated using a Central Composite Rotatable Design (CCRD) with 4 factorial points, 3 replicates at the central point and 4 axial points (11 assays). The responses were based on the antioxidant activity analysis using the following methods: DPPH radical scavenging, ORAC and FRAP. A second order model equation was used to explain the performance of the system as follows:

(Eq. 1)

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

where Y is the dependent variable or response; i and j have values varying from 1 to the number of variables (n), β_0 is the intercept, βi and β_{ij} are the linear and quadratic coefficients, respectively; Xi and Xj are the coded values of independent variables. ANOVA, coefficient of determination R^2 and F-test at $p \leq 0.10$ were used to measure the quality of the model's fit. Table 1 provides the coded and uncoded levels for CCRD.

Table 1. Coded and uncoded levels for CCRD.

Factors	Levels				
	-1.41 (-α)	**-1**	**0**	**+1**	**+1.41 (+α)**
X_1: pH	6	6.58	8	9.42	10
X_2: Protease (U/mL)	50	64.54	100	135.46	150

2.4 Preparation of protein hydrolysates

The rice protein hydrolysates were prepared according to the method described by Castro and Sato (2014). The substrate utilized for enzymatic hydrolysis was RPC. A concentration of 10% RPC was suspended in 50 mL of the appropriate buffer according to the values described in the CCRD in 125 mL Erlenmeyer flasks. The concentration of both enzymes was adjusted according to their activities in each pH using 0.1 M sodium phosphate buffer (pH 6, 6.58 and 8) and 0.1 M sodium bicarbonate-NaOH buffer (pH 9.42 and 10). The enzymatic hydrolysis was performed at 60 °C for 120 min. After hydrolysis, the proteases were inactivated at 100 °C for 20 min. The mixtures were centrifuged at 17,000 x *g* and 5 °C for 20 min, and the supernatants containing the peptides, were collected, freeze-dried and used for the posterior analysis.

2.5 Determination of protease activity

The protease activity was performed according to the method described by Charney and Tomarelli (1947) and modified by Castro and Sato (2014) using azocasein as substrate. Azocasein and enzymatic solutions were prepared in different buffers, 0.1 M sodium phosphate (pH 6, 6.58 and 8) and 0.1 M sodium bicarbonate-NaOH (pH 9.42 and 10). An aliquot of 0.5 mL of 0.5% azocasein and 0.5 mL of the enzymatic solution was incubated for 40 min at 60 °C. Then, the reaction was paralyzed by adding 0.5 mL 10% TCA. The mixture was centrifuged at 17,000 x *g* for 15 min at 15 °C. An aliquot of 1 mL of the supernatant was added to 1 mL of 5 M KOH and the absorbance was measured at 428 nm. One unit of protease activity (U) was defined as the amount of enzyme which causes an increase of 0.01 in absorbance under the assay conditions.

2.6 Determination of TCA soluble protein content

The TCA soluble protein content was applied as the indirect way to measure the degree of hydrolysis (DH) using the method proposed by Peričin, Radulović-Popović, Vaštag, Madarev-Popović and Trivić (2009). An aliquot of 0.5 mL of the hydrolysate (0.8 mg/mL) was added to 0.5 mL of 0.44 mol/L TCA. The mixture was incubated for 30 min at room temperature, centrifuged at 17,000 x *g* and 15 ºC for 15 min and the supernatant was utilized to measure the protein content. The Lowry's method (with modifications) was utilized to measure protein and the bovine serum albumin was the protein standard (Hartree, 1972). The results were calculated as the ratio of the TCA soluble protein content and the total protein content in the supernatant of the hydrolysate mixture and expressed as a percentage.

2.7 Determination of the antioxidant activities

2.7.1 DPPH radical-scavenging

The DPPH radical-scavenging was determined using the method described by Bougatef et al. (2009). An aliquot of 0.5 mL of each sample (5 mg/mL) was mixed with 0.5 mL of 99.5% ethanol and 125 µL of 0.02% DPPH solution in 99.5% ethanol. The mixture was incubated for 60 min at room temperature in the dark. The measurements were performed at 517 nm using a UV-visible spectrophotometer. A calibration curve of standard Trolox (1.5-37.5 µM) was used to calculate the DPPH radical-scavenging (Trolox equivalent/g of protein hydrolysates - TE µmol/ g). The results were also represented as a DPPH inhibition (%) as in Eq. 2.

(Eq. 2)

$$\frac{\text{Absorbance of control} - \text{Absorbance (sample} - \text{control sample)}}{\text{Absorbance of control}} \text{ x } 100$$

Distilled water was used instead of sample in the control. The control sample was performed with no DPPH solution.

2.7.2 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to the method of Dávalos, Gómez-Cordovés and Bartolomé (2004) and described by Castro and Sato (2014). The automated ORAC assay was carried out on a Novo Star Microplate reader (BMG LABTECH, Germany)

with fluorescence filters (excitation wavelength of 485 nm and an emission wavelength of 520 nm) over 120 min at 37 ºC. The reaction mixture contained 20 µL of sample (1 mg/mL), blank (distilled water) or standard (Trolox solutions); 120 µL of 0.4 mg/mL fluorescein and 60 µL of 108 mg/mL AAPH in 75 mM sodium phosphate buffer (pH 7.4). The area under the fluorescence decay curve (AUC) was calculated according to Eq. 3:

(Eq. 3)

$$\text{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$$

where f_i is the fluorescence reading at time i and f_0 is the fluorescence obtained after the addition of AAPH.

The net AUC was calculated according to:

(Eq. 4)

$$\text{Net AUC} = \text{AUC}_{(\text{sample})} - \text{AUC}_{(\text{blank})}$$

The ORAC values were calculated using the calibration curve which was constructed with standard Trolox (1.5-1,500 µM) and a blank without antioxidants. The results were expressed as TE µmol/g of protein hydrolysates.

2.7.3 Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed according to the method described by Benzie and Strain (1996) and modified by Wiriyaphan, Chitsomboon and Yongsawadigul (2012). An aliquot of 100 µL of each sample (5 mg/mL) was mixed with 1 mL of fresh FRAP reagent (25 mL of 300 mM acetate buffer, pH 3.6; 2.5 mL of 10 mM TPTZ in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$). The mixture was incubated for 15 min at 37 ^oC. The absorbance was measured at 593 nm. The results were calculated using a curve of Trolox (1.5-300 µM) and expressed in TE µmol/g of protein hydrolysates.

2.8 Statistical analysis

The Statistica 7.0 program (Statsoft®/Dell, USA) was used for experimental design, matrix and statistical analysis. The differences were considered significant at $p \leq 0.10$. All assays were performed in three independent replicates.

3. Results and Discussion

To optimize the hydrolysis process, it is necessary to determine the conditions, and variables that significantly affect the responses evaluated (Castro & Sato, 2015). The combined effect of hydrolysis pH (X_1) and protease (X_2) on DPPH, ORAC and FRAP responses to the rice protein hydrolysis by the protease produced by *B. licheniformis* LBA 46 (LBA protease) and Alcalase 2.4L, are presented in Tables 2 and 3, respectively.

Table 2. Experimental values of responses for CCRD for the LBA protease hydrolysates.

Assay	Independent variables		Dependent variables			
	X_1 (pH)	X_2 (Protease - U/mL)	DPPH		ORAC	FRAP
			TE µmol/g	%	TE µmol/g	
1	-1 (6.58)	-1 (64.54)	6.22	49.47	754.87	9.13
2	1 (9.42)	-1 (64.54)	4.75	37.95	795.55	18.72
3	-1 (6.58)	1 (135.46)	6.19	49.50	861.58	10.69
4	1 (9.42)	1 (135.46)	5.88	47.04	1,019.94	17.56
5	-1.41 (6.0)	0 (100)	5.62	44.93	661.50	8.08
6	1.41 (10)	0 (100)	4.70	37.60	903.21	16.07
7	0 (8.0)	-1.41 (50)	4.98	39.83	658.10	9.83
8	0 (8.0)	1.41 (150)	5.33	42.60	814.31	10.67
9	0 (8.0)	0 (100)	5.24	41.89	704.73	10.50
10	0 (8.0)	0 (100)	5.09	40.68	700.99	9.88
11	0 (8.0)	0 (100)	5.32	42.55	695.08	9.73

Table 3. Experimental values of responses for CCRD for the Alcalase 2.4L hydrolysates.

Assay	Independent variables		Dependent variables (TE µmol/g)			
	X_1 (pH)	X_2 (Protease - U/mL)	DPPH		ORAC	FRAP
			TE µmol/g	%	TE µmol/g	
1	-1 (6.58)	-1 (64.54)	6.74	53.91	784.76	9.02
2	1 (9.42)	-1 (64.54)	5.55	44.41	933.34	17.23
3	-1 (6.58)	1 (135.46)	6.66	53.23	873.85	9.68
4	1 (9.42)	1 (135.46)	6.42	51.34	921.81	16.67
5	-1.41 (6.0)	0 (100)	6.07	48.50	791.22	8.54
6	1.41 (10)	0 (100)	5.63	44.99	1,012.57	18.45
7	0 (8.0)	-1.41 (50)	5.40	43.21	762.79	10.54
8	0 (8.0)	1.41 (150)	5.87	46.97	827.08	11.69
9	0 (8.0)	0 (100)	5.68	45.40	730.19	11.64
10	0 (8.0)	0 (100)	5.90	47.18	735.72	11.30
11	0 (8.0)	0 (100)	5.65	45.19	722.14	11.30

The highest values for DPPH, ORAC and FRAP of rice protein hydrolysates using the LBA protease were found in assays 1 (6.22 TE μmol/g), 4 (1,019.94 TE μmol/g) and 2 (18.72 TE μmol/g), respectively. Unlike results for Alcalase 2.4L which showed better results for DPPH in assays 1 (6.74 TE μmol/g) and for ORAC and FRAP in assay 6 (1,012.57 and 18.45 TE μmol/g). Slightly higher values can be observed for the hydrolysis responses made with Alcalase 2.4L. The lowest values obtained using the LBA protease were presented in the assays, 6 (37.60 TE μmol/g), 5 (661.50 TE μmol/g) and 5 (8.08 TE μmol/g), for DPPH, ORAC and FRAP, respectively. For the hydrolysis with Alcalase 2.4L, the lowest values were founded in assays 7 for DPPH (43.21 TE μmol/g), 11 for ORAC (722.14 TE μmol/g) and 5 for FRAP (8.54 TE μmol/g). The limited variability of the central points (runs 9-11) indicated good reproducibility of the experimental data.

The evaluation of the DH is used to verify how much the protein was broken by the enzyme used during the hydrolysis, which is related to the catalytic action of the enzyme, being used to monitoring the proteolysis and to compare the different protein hydrolysates produced (Hsu, 2010). The hydrolysates presented similar DH values independent of the enzyme employed in the process. For the LBA protease hydrolysates, the lowest value was found for the hydrolysates obtained in assay 2 (49%). The other tests presented medium values between 52.45-55.83%. In relation to Alcalase 2.4L hydrolysates, the degree of hydrolysis ranged from 50.83-54.69%.

Tables 4 and 5 show the regression coefficients of the variables calculated for the responses studied. According to Solouk, Solati-Hashjin, Najarian, Mirzadeh and Seifalian (2011), the importance of variables and their effects within experimental planning can be explained by the magnitude and sign of the coefficients. The significance of the regression coefficients was determined based on their *p*-value. Statistical analysis showed that the variation in pH (6-10) and protease concentration (50-150 U/mL) studied had no effect on the antioxidant activity of the hydrolysates measured by the DPPH assay. So it was not possible to predict a mathematical model for this response.

The regression coefficients for LBA protease hydrolysates indicated that pH of hydrolysis had linear and quadratic effects ($p < 0.10$) on ORAC and FRAP responses. The linear term of protease had significant effect ($p < 0.05$) only on ORAC response. About Alcalase 2.4L hydrolysates, the regression coefficients indicated that pH of hydrolysis had linear and quadratic strong effects ($p < 0.01$) on ORAC and FRAP response. The linear and quadratic term of protease affected only ORAC response ($p < 0.10$).

Table 4. Regression coefficients, standard error, t_{calc} and *p*-value of rice protein hydrolysis using the LBA protease.

Factors	Coefficients			Standard error			t_{calc}*			*p*-value		
	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP
Intercept	5.21	699.87	10.03	0.23	39.45	1.15	22.22	17.74	8.71	0.000	0.000	0.000
pH	-0.39	67.68	3.48	0.14	24.20	0.71	-2.68	2.80	4.92	0.044	0.038	0.004
pH^2	0.12	66.22	1.75	0.17	28.87	0.84	0.73	2.29	2.08	0.500	0.070	0.093
Protease	0.20	69.12	0.20	0.14	24.20	0.71	1.39	2.86	0.28	0.224	0.036	0.790
Protease^2	0.12	43.01	0.83	0.17	28.87	0.84	0.71	1.49	0.99	0.510	0.197	0.370
pH * Protease	0.29	29.42	-0.68	0.20	34.17	1.00	1.44	0.86	-0.68	0.211	0.429	0.527

*t_{calc} calculated with 5 degrees of freedom

Table 5. Regression coefficients, standard error, t_{calc} and *p*-value of rice protein hydrolysis using Alcalase 2.4L.

Factors	Coefficients			Standard error			t_{calc}*			*p*-value		
	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP
Intercept	5.74	729.24	11.41	0.24	15.37	0.36	24.32	47.45	31.80	0.000	0.000	0.000
pH	-0.26	63.77	3.66	0.14	9.43	0.22	-1.77	6.77	16.62	0.137	0.001	0.000
pH^2	0.20	94.22	1.26	0.17	11.25	0.26	1.18	8.38	4.80	0.291	0.000	0.005
Protease	0.18	21.09	0.22	0.14	9.43	0.22	1.25	2.24	0.99	0.266	0.075	0.370
Protease^2	0.10	40.42	0.06	0.17	11.25	0.26	0.58	3.59	0.24	0.589	0.016	0.821
pH * Protease	0.24	-25.15	-0.31	0.20	13.31	0.31	1.16	-1.89	-0.98	0.297	0.117	0.371

*t_{calc} calculated with 5 degrees of freedom

The results of the analysis of variance (ANOVA) for the regression model are shown in Tables 6 and 7. The significance of each coefficient was verified using the test *F*, the *p*-value (associated probability) and the coefficient of determination, R^2. The *F*-values calculated for the regressions were greater than the tabulated *F*-values and are presented as *F*-ratio, demonstrating the statistical significance of the models. The R^2 value of the regression model can be used to determine the variability in the experimental data. The statistical parameter R^2 ranging from 0.71 to 0.98, meaning that the models are able to explain from 71 to 98% the variability of the experimental data. The *p*-values for ORAC and FRAP responses were less than 0.05, showing that the proposed models presented high significance at a 95% confidence level. The models with significant factors for the experimental data representing each of the responses are also presented in Tables 6 and 7.

Table 6. ANOVA including models, R^2 and probability values for the final reduced models for rice protein hydrolysis using the LBA protease.

Response: ORAC (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	R^2	***p*-value**
Regression	92,297.51	3	30,765.84	1.89	0.71	0.026
Residual	37,174.16	7	5,310.94			
Total	129,471.67					
Model: $Y = 740.15 + 67.68X_1 + 53.68X_1^2 + 69.12X_2$						
Response: FRAP (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	R^2	***p*-value**
Regression	110.29	2	55.14	5.48	0.81	0.001
Residual	25.90	8	3.24			
Total	136.19					
Model: $Y = 10.80 + 3.48X_1 + 1.5X_1^2$						

*F-ratio = $F_{calculated}/F_{tabulated}$

Table 7. ANOVA including models, R^2 and probability values for the final reduced models for rice protein hydrolysis using the Alcalase 2.4L.

Response: ORAC (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	**R^2**	***p*-value**
Regression	97,391.93	5	19,478.39	7.97	0.96	0.001
Residual	3,543.28	5	708.66			
Total	100,935.21					
Model: $Y = 729.23 + 63.77X_1 + 94.22X_1^2 + 21.09X_2 + 40.42X_2^2 - 25.15X_1X_2$						
Response: FRAP (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	**R^2**	***p*-value**
Regression	116.14	2	58.07	55.28	0.98	0.000
Residual	2.70	8	0.34			
Total	118.84					
Model: $Y = 11.47 + 3.66X_1 + 1.24X_1^2$						

*F-ratio = $F_{calculated}/F_{tabulated}$

The effects of the independent variables (pH and protease) are presented as response surface and contour curves, as shown in Fig. 1 and 2. In general, these graphs show the optimal conditions (Cao, Zhang, Ji, & Hao, 2012), which expose the values for the variables to find the maximum or minimum response, according to the needs of the study.

For rice protein hydrolysates prepared with the two enzymes evaluated, a similar behavior can be observed, where the ORAC and FRAP responses are maximal (red region) and minimal (green region) responses at similar intervals. The pH values that give maximum responses for both antioxidant activities are in the range of 9 to 10 and the protease is in the whole studied range, from 50 to 150 U/mL, representing that both enzymes evaluated have the catalytic power to produce rice protein hydrolysates with antioxidant activity even at the lowest evaluated concentration (50 U/mL). The enzyme produced in the laboratory by the microorganism *B. licheniformis* LBA 46, although producing peptides with values of antioxidant activity lower than the values obtained when the hydrolysis was performed with Alcalase 2.4L, acts in a similar way to this enzyme, since both are enzymes with pH range in the alkaline region.

The differences in the antioxidant activities observed for hydrolysates depend on the variety of peptides formed during the hydrolysis. Minor peptides and free amino acids generated depend directly on the enzyme specificity, mechanisms of action, hydrolysis conditions and may affect the magnitude of antioxidant activity (Contreras, Hernández-

Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Tsou, Kao, Tseng, & Chiang, 2010). According to Ozgen, Reese, Tulio, Scheerens and Miller (2006), the antioxidant activity is also dependent on the reaction time, pH and the complexity of the reaction.

Asp and Glu are amino acids that exhibit strong antioxidant activity and are important in the composition of protein hydrolysates (Saiga, Tanabe, & Nishimura, 2003). A study developed by Zhao et al. (2012) using Alcalase 2.4L on the hydrolysis of rice dreg protein (pH 8.5 and temperature of 55 °C) showed that Asp and Glu were the amino acids found in higher concentration on the hydrolysates produced. The authors also observed a high amount of low molecular weight peptides, 41.75% (1-3 kDa) and 26.75% (< 1 kDa), which are related to higher antioxidant activities.

Zhou, Canning and Sun (2013) described rice protein hydrolysates prepared by different microbial proteases (Validase protease from *Aspergillus oryzae*, pH 7, alkaline protease from *B. licheniformis*, pH 10 and neutral protease from *Bacillus subtillis*, pH 7) incubated at 55 ºC and ultrafiltrated. The authors reported ORAC values lower than that found in this study, ranging between 34.20-87.30 TE µmol/g. The hydrolysate fractions at 100 mg/mL registered 31.2-49.7% of DPPH reduction. In this study, the hydrolysates at 5 mg/mL showed DPPH reduction of 37.95-49.50% and 43.20-53.91% for hydrolysis performed by the LBA protease and Alcalase 2.4L, respectively.

Castro and Sato (2014) studied the optimization of whey protein hydrolysis by a protease from *A. oryzae* LBA 01 using pH 5 and temperature of 55 ºC. The independent variables were substrate (%) and protease concentration (U/mL). It was found a value range of 60.84-434.82 TE µmol/g for ORAC assay and 5.06-17.07 TE µmol/g for DPPH assay. These values are also lower than those presented in this study.

On the other hand, Castro and Sato (2015) studied egg white protein hydrolysates, achieving values higher of antioxidant activity than those presented in this study, but using a commercial protease Flavourzyme 500 L from *A. oryzae*. The factors studied were substrate (%) and protease concentration (U/mL) at pH 5 and 50 ºC. According to the results, the authors achieved a higher antioxidant activity, measured in terms of the ORAC and DPPH assays, 1,193.12 and 19.05 TE µmol/g, respectively.

Wattanasiritham, Theerakulkait, Wickramasekara, Maier and Stevens (2016) studied the native and denatured fractions of rice bran after hydrolysis with trypsin (pH 7.8) and papain (pH 7) at 37 ºC. The denatured fraction (albumin) hydrolyzed by trypsin, exhibited the highest antioxidant activity with an ORAC value of 4,067 TE µmol/mg protein when

compared to the native protein. In this case, after the denaturation, the enzyme could be penetrating cleaving a greater number of peptide bonds producing low molecular weight peptides which has high antioxidant activity.

Fig. 1. Response surfaces and contours diagrams for ORAC (A) and FRAP (B) assays as a function of the pH value and protease concentration (U/mL) for rice protein hydrolysates prepared with LBA protease.

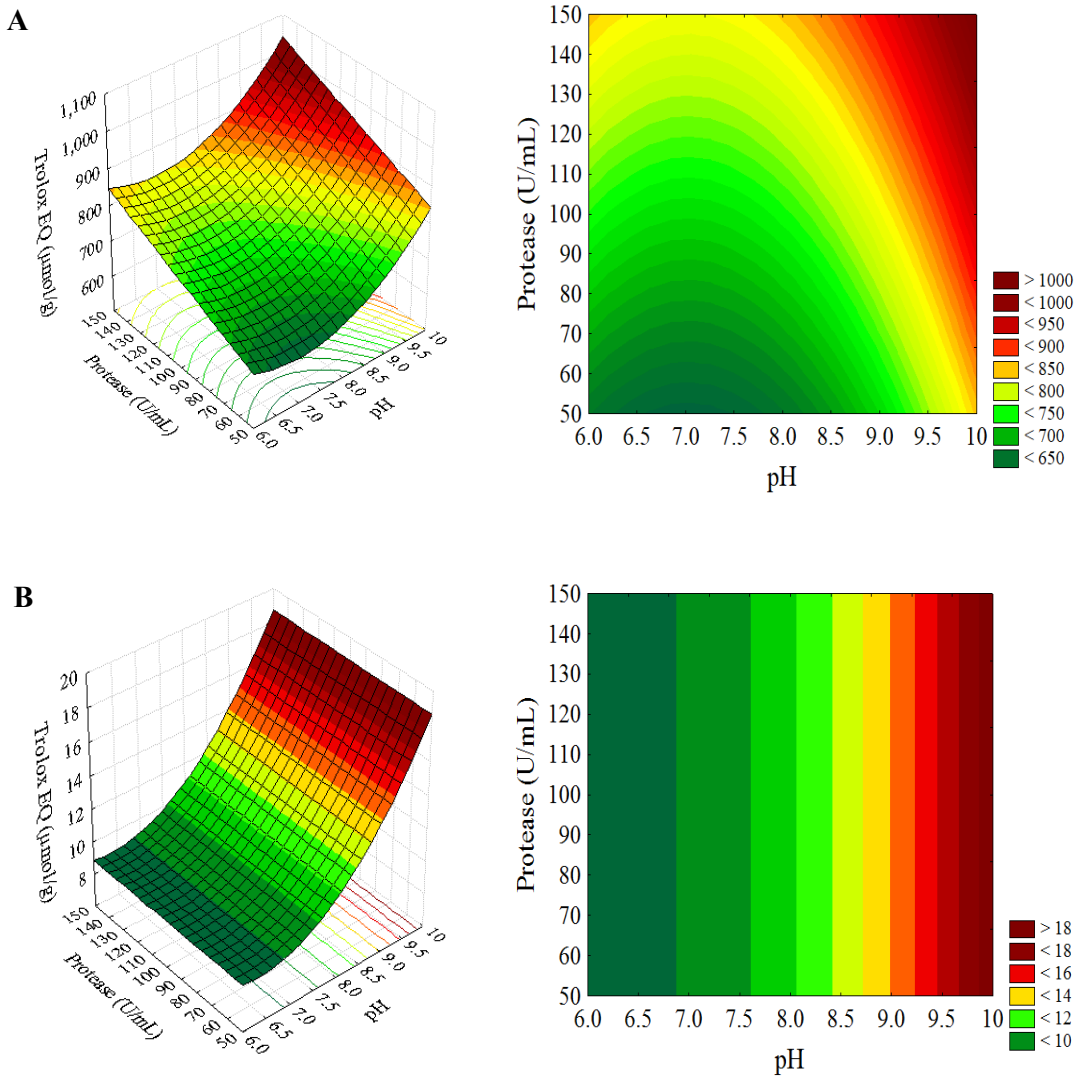
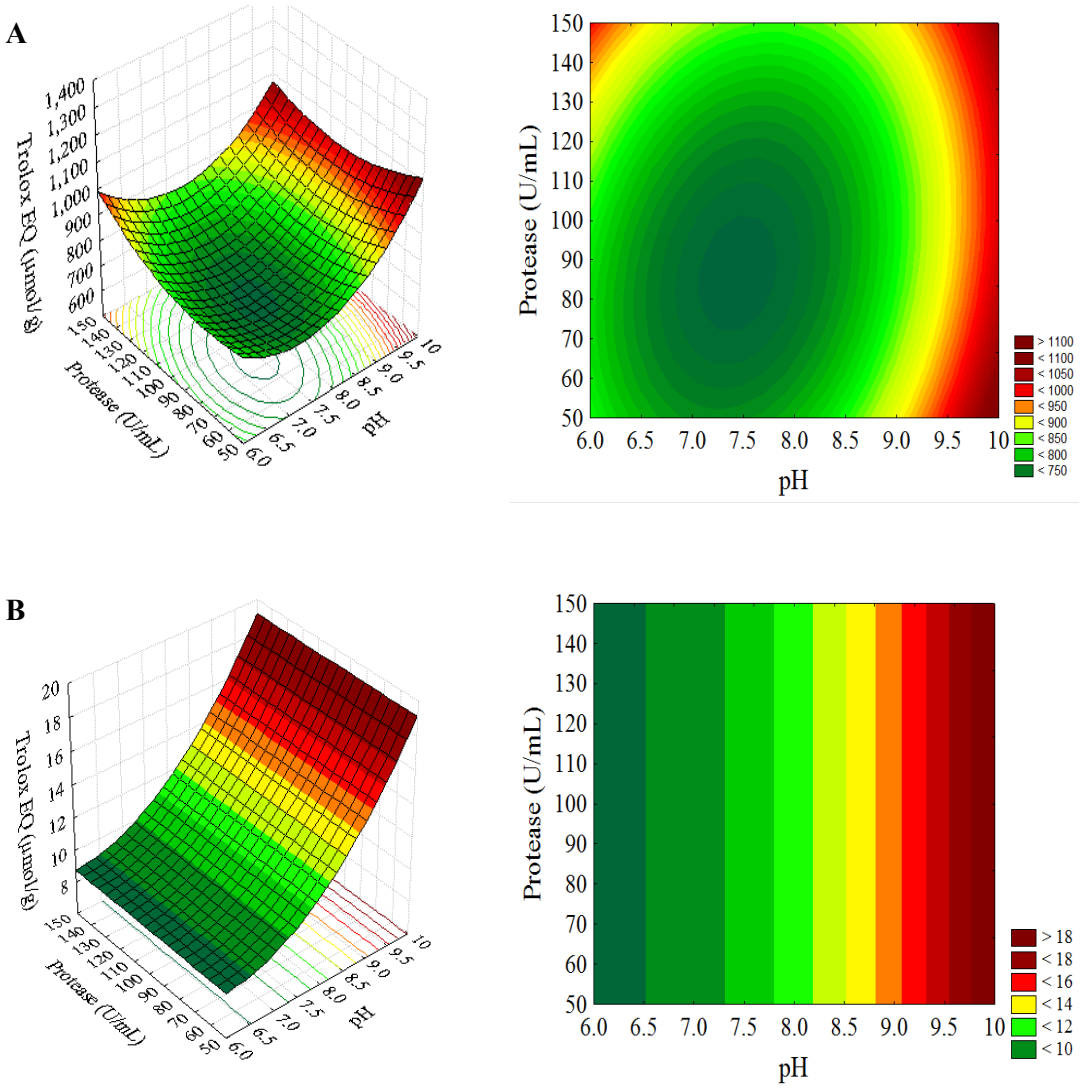


Fig. 2. Response surfaces and contours diagrams for ORAC (A) and FRAP (B) assays as a function of the pH value and protease concentration (U/mL) for rice protein hydrolysates prepared with Alcalase 2.4L.



Based on the range of values already studied and taking into account that the antioxidant activity analysis are susceptible to variation, to optimize the responses obtained for the antioxidant activity, the chosen pH was the highest (10) and the protease concentration was the intermediate (100 U/mL), since this variable had practically the same effect throughout the CCRD. Due to the fact that the responses to the hydrolysis of the rice protein were similar, the optimization parameters were the same for the two enzymes used. According to the regression models presented in Tables 6 and 7, the predicted values for ORAC and FRAP under these conditions were 942.30 and 18.70 TE µmol/g for the rice protein

hydrolysates prepared with LBA protease and 1,006.46 and 19.10 TE μmol/g for the rice protein hydrolysates prepared with Alcalase 2.4L. The hydrolysis assays, in conditions defined as optimal according to the interval presented in the contour curves, were performed in triplicate to confirm the validity of the model. The experimental values obtained are in agreement with the expected values ($p \leq 0.10$) confirming once again the validity of the model (Tables 8 and 9).

Table 8. Validation tests to determine the adequacy of the models obtained for maximum antioxidant activities of rice protein hydrolysates prepared with LBA protease.

	Optimum conditions			
	ORAC (TE μmol/g)		**FRAP** (TE μmol/g)	
Independent variables	pH	Protease (U/mL)	pH	Protease (U/mL)
Experimental coded value	1.41	0	1.41	0
Experimental real value	10	100	10	100
Predicted response	942.30a		18.70b	
Experimental response	940.05 ± 20.18a		18.78 ± 0.15b	

*Results presented as mean (n = 3) ± standard deviation. Values with different letters are significantly different. ($p \leq 0.05$).

Table 9. Validation tests to determine the adequacy of the models obtained for maximum antioxidant activities of rice protein hydrolysates prepared with Alcalase 2.4L.

	Optimum conditions			
	ORAC (TE μmol/g)		**FRAP** (TE μmol/g)	
Independent variables	pH	Protease (U/mL)	pH	Protease (U/mL)
Experimental coded value	1.41	0	1.41	0
Experimental real value	10	100	10	100
Predicted response	1,006,46a		19.10b	
Experimental response	1,001.94 ± 24.58a		19.31 ± 0.19b	

*Results presented as mean (n = 3) ± standard deviation. Values with different letters are significantly different. ($p \leq 0.05$).

Each method of determining antioxidant capacity is based on different mechanism. The FRAP assay is a method that relies on oxide-reduction reactions. At low pH, the complex Fe^3-TPTZ is reduced to FE^2 producing an intense blue color showing the maximum absorbance at 593 nm. The presence of a reducing agent, antioxidant, favors a reduction of the complex and the blue color becomes stronger (Benzie & Strain, 1996). The ORAC method is based on the peroxil radical capture. The radical reacts with a fluorescent probe to produce a non-fluorescent product. The antioxidant activity is measured by maintenance of the fluorescence (Dávalos et al., 2004).

The unhydrolyzed rice protein sample (control) presented values of 324.97 ± 13.25 (ORAC) and 6.14 ± 0.11 (FRAP) all in TE μmol/g. As can be seen in Tables 8 and 9, after optimization of the enzymatic hydrolysis conditions, the antioxidant activity values increased regardless of the enzyme used in the hydrolysis. For the hydrolysis performed with the LBA protease, there was an increase of 2.9-fold for ORAC and 3.05-fold for FRAP assay. For the hydrolysis performed with Alcalase 2.4L, the increase was slightly higher, from 3.08-fold for ORAC and 3.14-fold for FRAP.

4. Conclusion

The hydrolysis of rice protein for obtaining peptides with antioxidant activity was optimized with pH 10 and 100 U/mL protease. The protease from *B. licheniformis* LBA 46 was shown to be as efficient in the production of bioactive peptides with antioxidant activity as the commercial protease Alcalase 2.4L, presenting similar responses after the validation: 942.28 TE μmol/g (ORAC) and 18.70 TE μmol/g (FRAP), using the LBA protease; 1,006.46 TE μmol/g for (ORAC) and 19.10 TE μmol/g for (FRAP) using Alcalase 2.4L. For both protease preparations used in this study, there was an increase in the antioxidant activity of rice protein hydrolysates when compared to the control sample (non-hydrolyzed sample). This work amplifies the existing knowledge about the use of microbial proteases in the production of rice protein peptides with antioxidant activity and suggests, in view of the results found, that these hydrolysates have the potential to be used as a natural source of antioxidants.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Capítulo V

Antioxidant properties of pea protein hydrolysates obtained by response surface methodology: A comparative evaluation of alkaline proteases

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Abstract

The objective of this study was to establish the most adequate hydrolysis conditions of pea protein based on the hydrolysates antioxidant properties. Response surface methodology (RSM) was used to model and optimize responses: DPPH, ORAC and FRAP assays. The semi-purified protease from *Bacillus licheniformis* LBA 46 and commercial protease Alcalase 2.4L were compared using the same pH range (6-10) and protease concentration (50-150 U/mL). It was possible to define a mathematical model to predict the results for all responses. The behavior of the enzymes in relation to the independent variables was similar, having the same optimization range of pH (9-10) and protease concentration (50-150 U/mL). The hydrolysis conditions, pH value (10) and enzyme concentration (100 U/mL) were selected as the main processing conditions for the hydrolysis of the pea protein. After validation, it was possible to confirm that the defined models were predictive. The experimental values for the DPPH, ORAC and FRAP under the chosen conditions were 3.64, 568.7 and 9.18 TE μmol/g for LBA protease hydrolysates and 3.77, 719.67 and 9.17 TE μmol/g for Alcalase 2.4L hydrolysates, respectively. Compared to the non-hydrolyzed pea protein, the antioxidant activity measured in terms of the DPPH (8.04 TE μmol/g) and FRAP (25.07 TE μmol/g) was reduced. But, the antioxidant activity measured by ORAC assay, after optimization, was higher than the activity for the intact protein (77.61 TE μmol/g), increasing 7.33 and 9.27-fold for LBA protease and commercial protease Alcalase 2.4L, respectively.

Keywords: Pea; Antioxidant; Optimization; RSM; Hydrolysates.

1. Introduction

Peas belong to the re-emerging legumes group which is rich in starch, fiber, vitamins, minerals and also an excellent source of valuable proteins (Costa, Queiroz-Monici, Reis, & Oliveira, 2006). Pea has been highlighted as an alternative source for production of protein-rich intermediates (Reinkensmeier, Bußler, Schlüter, Rohn, & Rawel, 2015). The protein content of peas ranges from 20% to 30% among different pea varieties and different environmental conditions (Barac et al., 2010; Dahl, Foster, & Tyler, 2012; Hood-Niefer, Warkentin, Chibbar, Vandenberg, & Tyler, 2012; Nesterenko, Alric, Silvestre, & Durrieu, 2013). The protein hydrolysis could promote interesting changes in foods improving their sensory attributes and physical-chemical properties (Aaslyng, Larsen, & Nielsen 1999; Lamsal, Reitmeier, Murphy, & Johnson, 2006), besides this, the enzymatic hydrolysis enhances the protein digestibility in various food products and particularly in legumes, which are a great source of proteins (Messina, 1999).

Enzymes are usually highly specific in their mode of action and different enzymes could be used to produce hydrolysates with different characteristics. The biological properties of peptides are dependent of several parameters, such as: the proteins source used as substrate, the protease specificity and preliminary treatment, which could modify the structure of native protein (Gauthier & Pouliot, 2003).

The limited proteolysis of food proteins, such as pea protein, may increase its range of use, for example, hydrolyzed products with different functional properties can be produced through the application of specific hydrolytic conditions, using proteases as catalysts of the reaction. The response surface methodology (RSM) is a statistical technique used to obtain important information in less time and with less costs, leading to the development of more efficient processes. This technique relates to independent variables (input parameter) and dependents/responses (output parameter) (Montgomery, 1984).

The purpose of this study was to establish the most adequates conditions for the enzymatic hydrolysis of pea protein concentrate (pH and protease concentration) based on antioxidant properties, performing a comparative analysis between the protein hydrolysates obtained using the semi-purified protease from *Bacillus licheniformis* LBA 46 and the commercial protease Alcalase 2.4L.

2. Material and Methods

2.1 Materials

Pea protein concentrate (PPC), 78.70% protein dry weight basis, was obtained from Growth Supplements (Brazil). The chemicals, azocasein, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron chloride ($FeCl_3.6H_2O$), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), 2,20-azobis (2-methylpropionamidine) dihydrochloride (97%) (AAPH), fluorescein and (7)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals and reagents used were of analytical grade.

2.2 Enzymes

The protease used in this study was produced by *B. licheniformis* LBA 46, from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas. The conditions of production were 30 ºC, 300 rpm and 48 h of fermentation utilizing 32 g/L of sugar cane molasses (Fio de Ouro®); 6 g/L of corn steep liquor (Corn Products®); 2 g/L of yeast extract (Prodex-Lac SD®) and 20 g/L of dried whey (Alibra®), adjusted to pH 7 as culture medium as proposed by Contesini (2014) with slight modifications. The crude extract was partially purified using 80% of ammonium sulfate precipitation, dialyzed, freeze-drying and used for protein hydrolysis experiments.

The commercial protease Alcalase 2.4L obtained from *B. licheniformis* was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3 Experimental design

The optimization of enzymatic hydrolysis was based on 2 variables of study: pH (X_1) and protease concentration (U/mL) (X_2). The parameters were optimized using a Central Composite Rotatable Design (CCRD) with 4 factorial points, 3 replicates at the central point and 4 axial points (11 assays). The dependent variables (responses) were the antioxidant properties of the proteins hydrolysates evaluated by the methods: DPPH radical scavenging, ORAC and FRAP. The behavior of the system was explained by the following second order model equation:

(Eq. 1)

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

where Y is the dependent variable; i and j have values from 1 to the number of variables (n), β_0, βi, β_{ij} are the intercept, linear and quadratic coefficient, respectively; and Xi and Xj are the coded values of independent variables. The accuracy and the quality of model's fit were checked by the coefficient of determination R^2 and analysis of variance (ANOVA) combined with the application of F-test at $p \leq 0.10$. Table 1 provides the coded and uncoded levels for CCRD.

Table 1. Coded and uncoded levels for CCRD.

Factors	Levels				
	-1.41 (-α)	**-1**	**0**	**+1**	**+1.41 (+α)**
X_1: pH	6	6.58	8	9.42	10
X_2: Protease (U/mL)	50	64.54	100	135.46	150

2.4 Preparation of protein hydrolysates

The pea protein hydrolysates were prepared according to the method described by Castro and Sato (2014). The PPC was used as the substrate for enzymatic hydrolysis. The enzyme concentrations were adjusted according to the previously determined protease activity in each pH value (0.1 M of sodium phosphate buffer for pH 6, 6.58 and 8; 0.1 M of sodium bicarbonate-NaOH buffer for pH 9.42 and 10). The 10% PPC was suspended in appropriate buffer and 50 mL aliquots of the mixtures were distributed in 125 mL Erlenmeyer flasks. The enzymatic hydrolysis was performed at 60 °C for 120 min. After hydrolysis, the proteases were inactivated in a water bath at 100 °C for 20 min. The mixtures were centrifuged at 17,000 x *g* at 5 °C for 20 min, and the supernatants containing the peptides were collected and freeze-dried for the following analysis.

2.5 Determination of protease activity

The protease activity was determined for each pH value used in this study according to the method described by Charney and Tomarelli (1947) modified by Castro and Sato (2014) using azocasein as substrate. The reaction mixture containing 0.5 mL of 0.5% azocasein and 0.5 mL of the enzymatic solution was incubated for 40 min at the optimum temperature for enzyme activity (60 °C). Azocasein and enzymatic solutions were prepared in different

buffers: 0.1 M of sodium phosphate (pH 6, 6.58 and 8) and 0.1 M of sodium bicarbonate-NaOH (pH 9.42 and 10). The reaction was stopped by adding 0.5 mL of 10% TCA. The reaction mixture was centrifuged at 17,000 x *g* for 15 min at 15 °C. An aliquot of 1 mL of the supernatant obtained was neutralized with 1 mL of 5 M KOH and absorbance was measured at 428 nm. One unit of protease activity (U) was defined as the amount of enzyme which causes an increase of 0.01 in absorbance at 428 nm under the assay conditions.

2.6 Determination of TCA soluble protein content

The TCA soluble protein content of the hydrolysates, an indirect way to measure the degree of hydrolysis (DH), was determined using a method of Peričin, Radulovic-Popovic, Vaštag, Madarev-Popovic and Trivic (2009). An aliquot of 0.5 mL of hydrolysate (0.8 mg/mL) was added to 0.5 mL of 0.44 mol/L TCA. The mixture was incubated for 30 min at room temperature and then centrifuged at 17,000 x *g* for 15 min. The supernatant was used to measure protein content by the Lowry's method (Hartree, 1972) using bovine serum albumin as the standard. The results were expressed as a percentage of the ratio between TCA soluble protein content and the total protein content in the supernatant of the hydrolysate.

2.7 Determination of antioxidant activities

2.7.1 DPPH radical-scavenging

The DPPH radical-scavenging activity of the protein hydrolysates was determined as described by Bougatef et al. (2009). An aliquot of 0.5 mL of each sample (5 mg/mL) was mixed with 0.5 mL of 99.5% ethanol and 125 µL of 0.02% DPPH in 99.5% ethanol. The mixture was incubated for 60 min at room temperature in the dark, 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 the equation bellow:

(Eq. 2)

$$\frac{\text{Absorbance of control} - \text{Absorbance (sample} - \text{control sample)}}{\text{Absorbance of control}} \text{ x } 100$$

For the control, distilled water was used instead of sample. The DPPH radical-scavenging activity was also calculated using a calibration curve of standard Trolox (1.5-37.5 µM) and the results were expressed as µmol of Trolox equivalent/g of protein hydrolysates (TE µmol/g).

2.7.2 Oxygen radical absorbance capacity (ORAC)

The ORAC assay, based on the method determined by Dávalos, Gómez-Cordovés and Bartolomé (2004) and described by Castro and Sato (2014), was carried out in a microplate reader (BMG LABTECH, Germany). The decay of fluorescence was measured with excitation and emission wavelengths of 485 and 528 nm, respectively, for 80 min at 37 ºC. The final assay mixture was contained by 20 µL of sample (1 mg/mL), blank (distilled water) or standard (Trolox solutions), 120 µL of 0.4 mg/mL fluorescein and 60 µL of 108 mg/mL AAPH in 75 mM sodium phosphate buffer (pH 7.4). The area under the fluorescence decay curve (AUC) was calculated according to equation 3:

(Eq. 3)

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$$

where f_i is the fluorescence reading at time i and f_0 is the fluorescence obtained after the addition of AAPH.

The net AUC was calculated according to:

(Eq. 4)

$$\mathrm{Net\ AUC} = \mathrm{AUC}_{(\mathrm{sample})} - \mathrm{AUC}_{(\mathrm{blank})}$$

The ORAC values were calculated using the difference between the area under the fluorescein decay curve and the Net AUC. The equation obtained using the standard Trolox (1.5-1,500 µM) was used for calculating the samples concentrations and the ORAC values were expressed as TE µmol/g of protein hydrolysates.

2.7.3 Ferric reducing antioxidant power (FRAP)

The FRAP assay was determined according to method Benzie and Strain (1996), modified by Wiriyaphan, Chitsomboon and Yongsawadigul (2012). FRAP reagent was prepared by the mixture of 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution (prepared in 40 mM HCl), and 2.5 mL of 20 mM $FeCl_3.6H_2O$. An aliquot of 100 µL of each sample (5 mg/mL) was mixed with 1 mL of fresh FRAP reagent. The solution was incubated for 15 min at 37 ^oC and the absorbance was read at 593 nm. A curve of calibration using Trolox (1.5-300 µM) was performed and the results were expressed as TE µmol/g of protein hydrolysates.

2.8 Statistical analysis

The experimental design, matrix and statistical analysis were developed using the Statistica 7.0 program (Statsoft®/Dell, USA). The differences were considered significant at $p \leq 0.10$. The experiments were randomized and the analyses were carried out in triplicate.

3. Results and Discussion

To optimize the hydrolysis process, it is necessary to determine the conditions and variables that interfere with the response antioxidant activity of the hydrolysates. In each of the 11 points studied, mean values were determined for the analysis of DPPH, ORAC and FRAP (Tables 2 and 3). In the hydrolysis, use the protease produced by *B. licheniformis* LBA 46 (LBA protease) in the DPPH, ORAC and FRAP analysis presented their highest values in the assays, 5 (4.54 TE µmol/g), 6 (537.84 TE µmol/g) and 6 (9.65 TE µmol/g), respectively. For the hydrolysis with Alcalase 2.4L, these assays were 3 for DPPH (4.33 TE µmol/g), 4 for ORAC (704.15 TE µmol/g) and 6 for FRAP (9.07 TE µmol/g).

Table 2. Experimental values of responses for CCRD for the LBA protease hydrolysates.

Assay	Independent variables		Dependent variables (TE µmol/g)			
			DPPH		ORAC	FRAP
	X_1 (pH)	X_2 (Protease - U/mL)	TE µmol/g	%	TE µmol/g	
1	-1 (6.58)	-1 (64.54)	3.84	30.71	353.27	5.76
2	1 (9.42)	-1 (64.54)	2.93	23.41	449.03	7.56
3	-1 (6.58)	1 (135.46)	4.47	35.70	381.74	7.15
4	1 (9.42)	1 (135.46)	3.57	28.56	515.07	9.23
5	-1.41 (6.0)	0 (100)	4.54	36.27	438.04	7.33
6	1.41 (10)	0 (100)	3.43	27.43	537.84	9.65
7	0 (8.0)	-1.41 (50)	2.43	19.44	321.56	7.15
8	0 (8.0)	1.41 (150)	3.04	24.29	351.69	8.56
9	0 (8.0)	0 (100)	2.98	23.82	349.62	7.71
10	0 (8.0)	0 (100)	2.74	21.89	341.19	8.03
11	0 (8.0)	0 (100)	2.67	21.37	394.42	8.39

Table 3. Experimental values of responses for CCRD for the Alcalase 2.4L hydrolysates.

Assay	Independent variables		Dependent variables (TE µmol/g)			
			DPPH		ORAC	FRAP
	X_1 (pH)	X_2 (Protease - U/mL)	TE µmol/g	%	TE µmol/g	
1	-1 (6.58)	-1 (64.54)	4.23	33.84	547.61	6.42
2	1 (9.42)	-1 (64.54)	3.41	27.24	721.95	7.78
3	-1 (6.58)	1 (135.46)	4.33	34.64	561.58	6.14
4	1 (9.42)	1 (135.46)	4.01	32.02	704.15	8.67
5	-1.41 (6.0)	0 (100)	4.33	34.59	573.50	6.42
6	1.41 (10)	0 (100)	3.71	29.62	674.13	9.07
7	0 (8.0)	-1.41 (50)	3.24	25.87	555.91	6.08
8	0 (8.0)	1.41 (150)	4.11	32.89	583.94	6.92
9	0 (8.0)	0 (100)	3.96	31.62	529.89	7.02
10	0 (8.0)	0 (100)	3.52	28.15	496.87	6.37
11	0 (8.0)	0 (100)	3.72	29.72	496.64	6.67

The lowest values obtained using the LBA protease were presented in the assays 7 (19.44 TE µmol/g), 7 (321.56 TE µmol/g) and 1 (5.76 TE µmol/g) for DPPH, ORAC and FRAP, respectively. For the hydrolysis with Alcalase 2.4L, the lowest assays were 7 for DPPH (25.87 TE µmol/g), 11 for ORAC (496.64 TE µmol/g) and 7 for FRAP (6.08 TE µmol/g).

The limited variability of the central points (assays 9-11) indicated good reproducibility of the experimental data.

A key parameter for monitoring the protein hydrolysis reaction is the DH. According to Peričin et al. (2009) the specific properties of hydrolysates are dependent on DH, which is influenced by enzyme specific activity, substrate characteristics and reaction conditions. In this work, the hydrolysates presented similar DH values that were all between 52.60-56.40%, regardless of the enzyme used.

Tables 4 and 5 show the regression coefficients of the variables calculated for the responses studied. The regression coefficients for antioxidant activity of the protein hydrolysates obtained using LBA protease and evaluated by ORAC method indicated that pH had strong and significant linear and quadratic effects ($p < 0.01$). For the FRAP response, the linear coefficient of pH effect was also significant ($p < 0.01$). For the DPPH response, the linear term of pH had a negative and significant effect ($p < 0.01$). The linear term of protease concentration had significant effect ($p < 0.10$) for all responses.

Table 4. Regression coefficients, standard error, t_{calc} and *p*-value of pea protein hydrolysis using the LBA protease.

Factors	Coefficients			Standard error			t_{calc}*			*p*-value		
	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP
Intercept	2.80	361.70	8.05	0.14	14.66	0.32	19.54	24.68	25.30	0.000	0.000	0.000
pH	-0.42	46.36	0.90	0.09	8.99	0.20	-4.80	5.16	4.59	0.005	0.004	0.006
pH^2	0.68	66.57	0.04	0.10	10.73	0.23	6.51	6.21	0.15	0.001	0.002	0.884
Protease	0.27	17.17	0.63	0.09	8.99	0.20	3.03	1.91	3.24	0.029	0.114	0.023
Protease^2	0.05	-9.54	-0.28	0.10	10.73	0.23	0.52	-0.89	-1.22	0.625	0.414	0.276
pH * Protease	0.00	9.39	0.07	0.12	12.69	0.28	0.04	0.74	0.25	0.970	0.493	0.809

*t_{calc} calculated with 5 degrees of freedom

Table 5. Regression coefficients, standard error, t_{calc} and *p*-value of pea protein hydrolysis using Alcalase 2.4L.

Factors	Coefficients			Standard error			t_{calc}*			*p*-value		
	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP	DPPH	ORAC	FRAP
Intercept	3.73	507.65	6.69	0.11	22.19	0.14	33.63	22.88	48.44	0.000	0.000	0.000
pH	-0.25	57.52	0.95	0.07	13.61	0.08	-3.74	4.23	11.26	0.013	0.008	0.000
pH^2	0.18	67.59	0.56	0.08	16.24	0.10	2.23	4.16	5.59	0.077	0.009	0.003
Protease	0.24	4.48	0.23	0.07	13.61	0.08	3.57	0.33	2.66	0.016	0.756	0.045
Protease^2	0.01	40.48	-0.06	0.08	16.24	0.10	0.11	2.49	-0.62	0.913	0.055	0.562
pH * Protease	0.12	-7.94	0.29	0.10	19.22	0.12	1.29	-0.41	2.45	0.252	0.696	0.058

*t_{calc} calculated with 5 degrees of freedom

For the protein hydrolysates obtained using Alcalase 2.4L, the regression coefficients indicated that pH had strong linear and quadratic significant effects ($p < 0.01$) on the antioxidant activities evaluated by ORAC and FRAP assays. The linear term of protease concentration affected only the FRAP response and the quadratic term affected only the ORAC response ($p < 0.10$). Just like LAB protease hydrolysates, for the DPPH response, the linear term of pH had a negative and significant effect ($p < 0.05$), the quadratic term had a positive and significant effect ($p < 0.10$) and the linear term of protease had a positive and significant effect ($p < 0.05$).

Statistical analysis showed that the variation in the independent variables, pH (6-10) and protease concentration (50-150 U/mL) studied had effect on the antioxidant activity measured in all of the assays, which demonstrates an empirical relationship between a response and the studied variables. Thus, it was possible to predict a mathematical regression model for all responses.

Tables 6 and 7 present the ANOVA details for the regression models and were developed when the experimental data adjusted to the response surface, showing if the analysis of the response data was significant for all regression terms (linear, quadratic and interaction) and residual variances for all responses. Significant models were created for all responses. The significance of each coefficient was checked using the coefficient of determination, R^2, the associated probability, p-value and F-value. The higher the F-value and the lower the p-value, more significant the variables are. The value of R^2 is defined as the ratio between the explained variance and the total variance and is a degree of adjustment. It is also the proportion of variability in the response variable, which is explained by regression analysis (Madamba, 2002).

The analysis of variance (ANOVA) showed that 82-97% of the total variation was explained by the models. All F-values calculated for the regressions were greater than the tabulated F-values (p-values < 0.005), reflecting the statistical significance of the model equations (Table 6 and 7).

Table 6. Analysis of variance (ANOVA), models, R^2 and probability values for the final reduced models for pea protein hydrolysis using the LBA protease.

Response: DPPH (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R*²**	***p*-value**
Regression	4.70	3	1.57	11.02	0.93	0.000
Residual	0.32	7	0.04			
Total	5.02					
Model: $Y = 2.85 - 0.42X_1 + 0.67X_1^2 + 0.27X_2$						
Response: ORAC (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R*²**	***p*-value**
Regression	48,934.72	3	16,311.57	9.10	0.92	0.000
Residual	4,085.04	7	583.58			
Total	53,019.76					
Model: $Y = 352.76 + 46.36X_1 + 69.35X_1^2 + 17.17X_2$						
Response: FRAP (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R*²**	***p*-value**
Regression	9.58	2	4.79	5.93	0.82	0.001
Residual	2.07	8	0.26			
Total	11.65					
Model: $Y = 7.87 + 0.90X_1 + 0.63X_2$						

*F-ratio = $F_{calculated}/F_{tabulated}$

Table 7. Analysis of variance (ANOVA), models, R^2 and probability values for the final reduced models for pea protein hydrolysis using the Alcalase 2.4L.

Response: DPPH (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R²***	***p*-value**
Regression	1.18	3	0.39	3.63	0.83	0.005
Residual	0.25	7	0.03			
Total	1.43					
Model: $Y = 3.74 - 0.25X_1 + 0.18X_1^2 + 0.24X_2$						
Response: ORAC (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R²***	***p*-value**
Regression	61,160.31	3	20,386.77	5.96	0.87	0.001
Residual	7,799.51	7	1,114.22			
Total	68,959.82					
Model: $Y = 507.65 + 57.52X_1 + 67.59X_1^2 + 40.48X_2$						
Response: FRAP (TE µmol/g)						
Factors	**Sum of squares**	**Degrees of freedom**	**Mean of squares**	***F*-ratio**	***R²***	***p*-value**
Regression	10.09	4	2.52	15.45	0.97	0.000
Residual	0.31	6	0.05			
Total	10.40					
Model: $Y = 6.63 + 0.95X_1 + 0.58X_1^2 + 0.23X_2 + 0.29X_1X_2$						

*F-ratio = $F_{calculated}/F_{tabulated}$

The antioxidant activity for ORAC and FRAP assays increased with increasing substrate concentration and decreased at higher levels of protease.

Contour and surface plots were generated using significant parameters for each response (Fig. 1 and 2). In general, these graphs show the optimum conditions (Cao, Zhang, Ji, & Hao, 2012), which expose the values for the variables allowing to find the maximal (red region) or minimal (green region) responses, according to the need of the study. The results show a similar behavior of the enzymes. For the DPPH response, the optimal region was in the range of lower pH values, between 6-6.5 for LBA protease and in the range of 6-7.5 for Alcalase 2.4L. The variable protease concentration showed a positive effect across the range of values studied. Regarding the ORAC and FRAP responses, the best values of antioxidant activity were found within the highest pH values, mainly in the 9.5-10 range. The concentration of enzyme used in the hydrolysis had a positive effect throughout the range of values studied. These responses signify the behavior of both enzymes evaluated in this study.

The protease preparation obtained from *B. licheniformis* LBA 46, produced peptides with antioxidant activity value slightly lower than the value obtained in comparison with Alcalase 2.4L, when the test used was ORAC. For the DPPH and FRAP assays the pea protein hydrolysates showed practically the same values, indicating that the hydrolysis with LBA protease can be carried out obtaining similar results to the Alcalase 2.4L.

According to Tsou, Kao, Tseng and Chiang (2010) smaller peptides and free amino acids generated by hydrolysis depend on the enzyme specificity, mechanisms of action and hydrolysis conditions and may affect the magnitude of the antioxidant activity. In this case it can be said that the specificity of the enzymes studied is very similar, firstly because they are alkaline proteases, their behavior within the CCDR and the responses obtained in the antioxidant activities.

Girgih et al. (2015) studied the enzymatic Alcalase hydrolysis (pH 9 at 50 ºC) of pea protein pretreated with high pressure (HP) and heat treatment. The untreated pea protein hydrolysate presented 500 μM TE/g for ORAC and 10% of reduction of DPPH, respectively. After HP, the ORAC and DPPH activities of hidrolysates were improved. The thermal treatment improved the antioxidant activity of the hydrolysates according to the FRAP assay.

Pownall, Udenigwe and Aluko (2010) evaluated the Thermolysin hydrolysis (pH 8 at 55 ºC) of pea protein isolate. The pea protein hydrolysates (1mg/mL) had about 21% of DPPH scavenging activity. On the other hand, Humiski and Aluko (2007) obtained pea protein hydrolysates (1 mg/mL) with lower values (7-11%) of DPPH scavenging activity using Flavourzyme (pH 7 at 50 ºC) and Alcalase (pH 8.5 at 50 ºC) in the pea protein hydrolysis.

Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez and Recio (2011) studied the production optimization of whey protein hydrolysates using Corolase PP (pH 7.5) and Thermolysin (pH 8) at 37 ºC. The authors evaluated the independent variables enzyme/substrate ratio and time of hydrolysis. Both enzymes were capable to increase the antioxidant activity measured by ORAC assay that ranged from 0.704 to 1.122 μmol Trolox/mg using Corolase PP and from 0.832 to 2.321 μmol Trolox/mg protein, using Thermolysin.

Fig. 1. Response surfaces and contours diagrams for the DPPH (A), ORAC (B) and FRAP (C) assay as a function of the pH value and protease (U/mL) concentration for pea protein hydrolysates prepared with LBA protease.

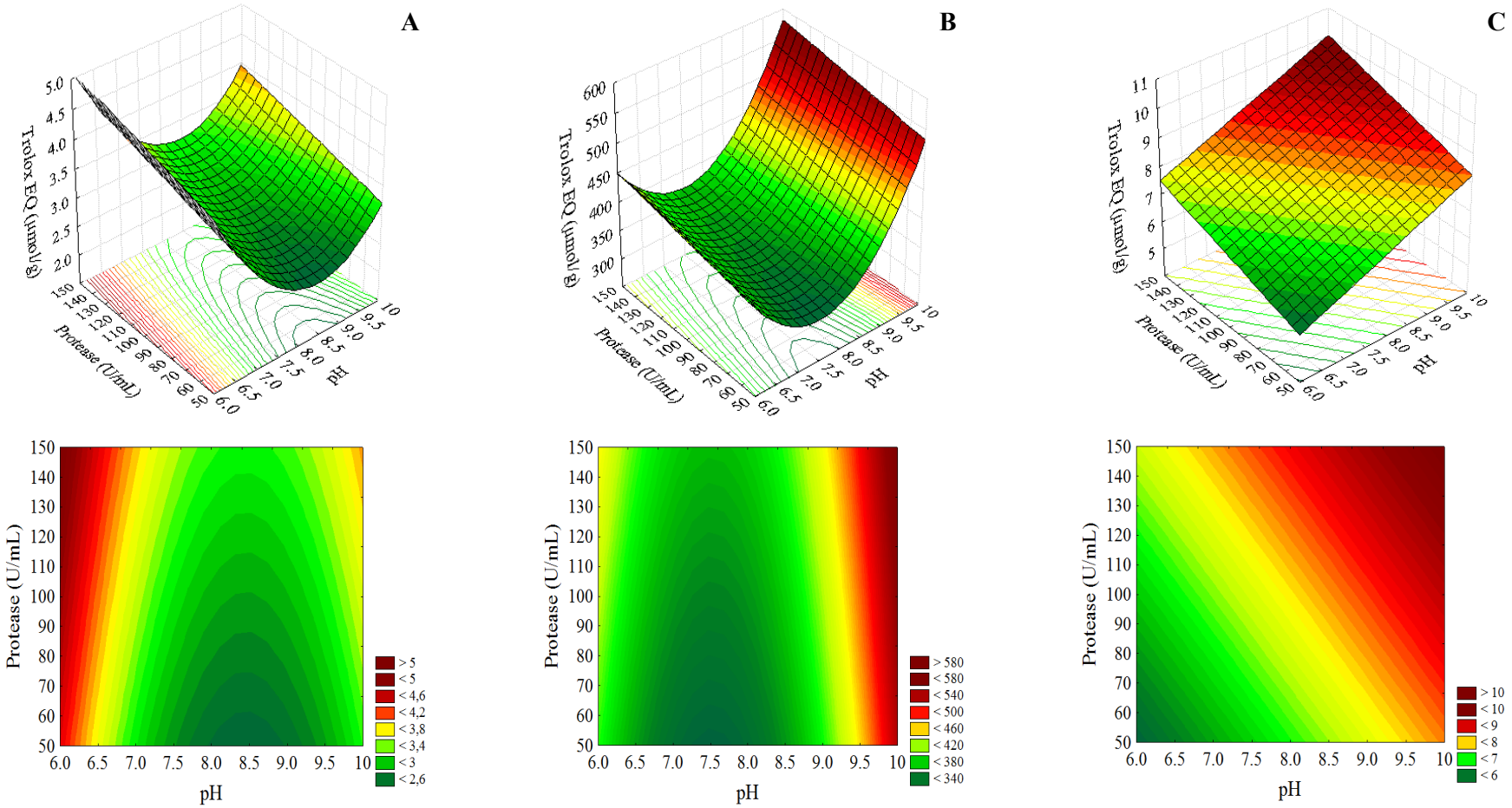
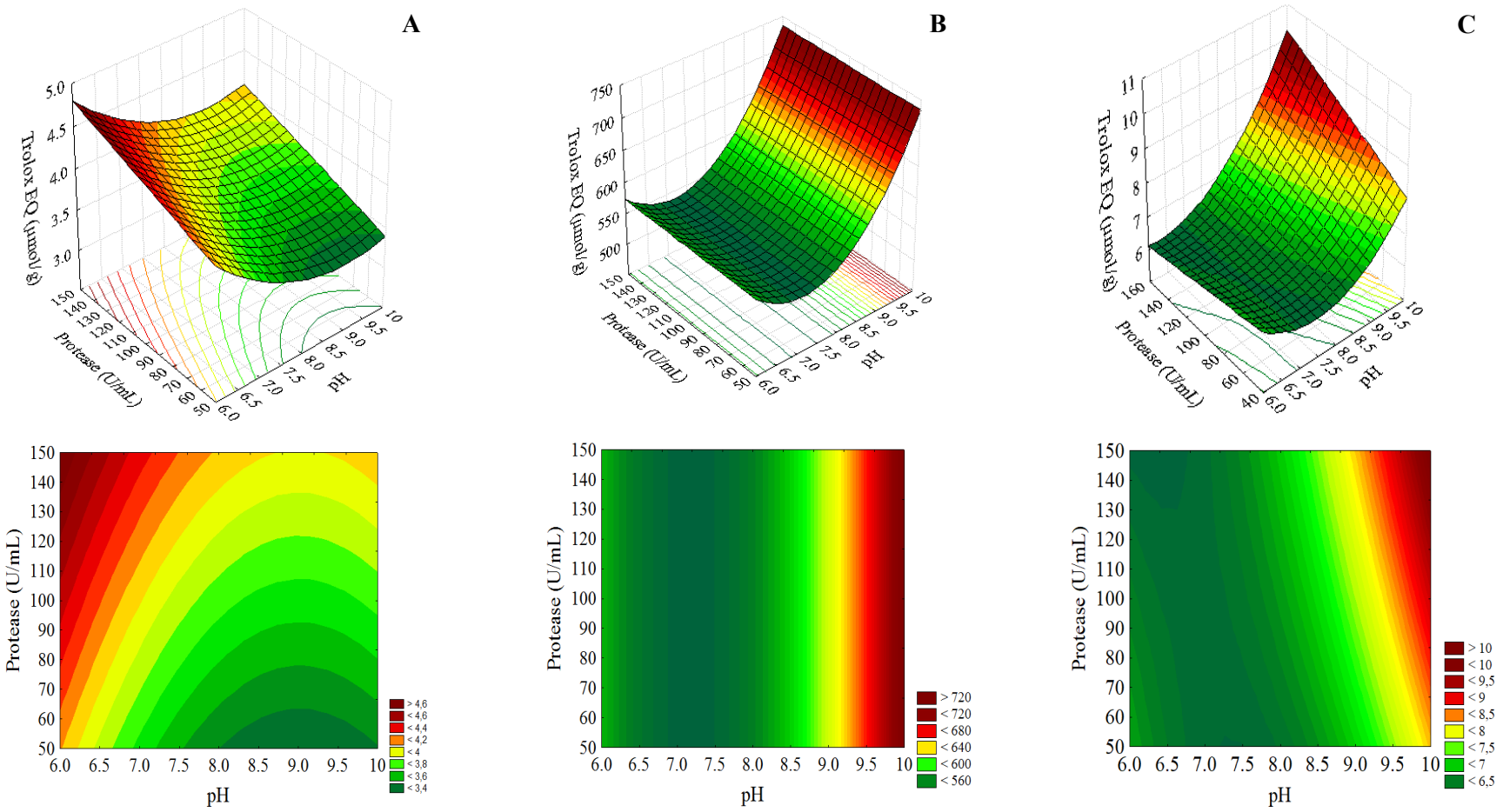


Fig. 2. Response surfaces and contours diagrams for the DPPH (A), ORAC (B) and FRAP (C) assay as a function of the pH value and protease (U/mL) concentration for pea protein hydrolysates prepared with Alcalase 2.4L.



In this study, the optimal response range for ORAC and FRAP was similar for both enzymes, with the maximum responses for the assays performed in the axial points of the planning. For the antioxidant activity evaluated by DPPH method, the best response was obtained when the pH value was lower within the entire range of enzyme concentration evaluated. The decision to choose the optimization points was then attempted to respond to most of the dependent variables studied, choosing the highest pH value (10) and the intermediate enzyme concentration (100 U/mL), which according to Fig. 1 and 2 are within the optimal range for ORAC and FRAP responses and the intermediate value for DPPH.

According to the regression models presented in Tables 6 and 7, predicted values for DPPH, ORAC and FRAP under these conditions were 3.60, 556 and 9.14 TE μmol/g for LBA protease hydrolysates and 3.74, 723.13 and 9.12 TE μmol/g for Alcalase 2.4L hydrolysates, respectively. To confirm the validity of the models, each hydrolysis was performed 3 times and the assays were done in triplicate. The experimental values obtained are in agreement with the predicted values (in a 95% confidence interval) confirming that the model is predictive (Tables 8 and 9).

Table 8. Validation tests to determine the adequacy of the models obtained for maximum antioxidant activities of pea protein hydrolysates by the LBA protease.

	Optimum conditions					
	DPPH		FRAP		ORAC	
Independent variables	pH	Protease (U/mL)	pH	Protease (U/mL)	pH	Protease (U/mL)
Experimental coded value	1.41	0	1.41	0	1.41	0
Experimental real value	10	100	10	100	10	100
Predicted response	3.60a		9.14c		556b	
Experimental response	3.64 ± 0.14a		9.18 ± 0.12c		568.70 ± 15.76b	

*Results presented as mean (n = 3) ± standard deviation. Values with different letters are significantly different. ($p \leq 0.05$).

Table 9. Validation tests to determine the adequacy of the models obtained for maximum antioxidant activities of pea protein hydrolysates prepared with Alcalase 2.4L.

	Optimum conditions					
	DPPH		FRAP		ORAC	
Independent variables	pH	Protease (U/mL)	pH	Protease (U/mL)	pH	Protease (U/mL)
Experimental coded value	1.41	0	1.41	0	1.41	0
Experimental real value	10	100	10	100	10	100
Predicted response	3.74a		9.12c		723.13b	
Experimental response	3.77 ± 0.06a		9.17 ± 0.10c		719.67 ± 20.27b	

*Results presented as mean (n = 3) ± standard deviation. Values with different letters are significantly different. ($p \leq 0.05$).

The non-hydrolyzed pea protein (control) presented values of 8.04 ± 0.19 (DPPH), 25.07 ± 0.46 (FRAP) and 77.61 ± 10.20 (ORAC) all in TE μmol/g. As can be seen in Tables 8 and 9, after optimization of the enzymatic hydrolysis conditions, the antioxidant activity measured in terms of the DPPH and FRAP was reduced in comparison to the antioxidant activity measured in the intact protein intact sample. However, the activity measured in terms of the ORAC had a significant increase of 7.33 and 9.27-fold for the LBA protease and Alcalase 2.4L, respectively. These differences can be explained based on the mechanisms of action of each antioxidant assay.

The DPPH, FRAP and ORAC assays have different reaction mechanisms. The antioxidant analysis based on the DPPH radical has been widely used to test the ability of natural compounds to act as free radical scavengers or hydrogen donors by means of a redox reaction (Castro & Sato, 2015). After elimination of the radicals by the test substance, the absorbance is reduced changing the color from purple to violet at 517 nm. The FRAP assay is another method based on oxidation-reduction reactions. At low pH, the complex Fe^3-TPTZ is reduced to FE^2 producing an intense blue color showing the maximum absorbance at 593 nm. The antioxidant agent in study is the responsible to reduce the blue complex, increasing the antioxidant activity (Benzie & Strain, 1996). The mechanism of action of the ORAC assay is based on the peroxyl radical capture, usually used to test the hydrogen atom donating activity potential of antioxidant specie (Zheng et al., 2012). The radical reacts with a fluorescent indicator producing a non-fluorescent product. In the presence of any antioxidant, the fluorescence is preserved (Dávalos et al., 2004). Therefore, a higher value of antioxidant activity measured in ORAC assay is not necessarily related to the hydrolysates antioxidant ability measured in DPPH and FRAP assays.

4. Conclusion

The hydrolysis of pea protein and production of antioxidant peptides was studied using experimental design and the most adequate conditions were pH 10 and 100 U/mL protease. The protease from *B. licheniformis* LBA 46 was shown to be as efficient in the production of bioactive peptides with antioxidant activity as the commercial protease Alcalase 2.4L presenting relatively similar values for the responses after the validation: 3.64 TE μmol/g for the (DPPH) and 9.18 TE μmol/g (FRAP) using the LBA protease; 3.77 TE μmol/g (DPPH) and 9.17 TE μmol/g (FRAP) using Alcalase 2.4L. In comparation with the non-hydrolyzed pea protein, the antioxidant activity measured in terms of the DPPH and FRAP was reduced. However, the antioxidant activity measured by ORAC assay had a significant increase of 7.33 and 9.27 fold for the LBA and Alcalase 2.4L pea protein hydrolysates, respectively, demonstrating that antioxidant activity assays do not always have their correlated responses, since they have different mechanisms of action. In this study, the catalytic action of microbial proteases on the pea protein hydrolysis was demonstrated, providing further knowledge about the subject.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Discussão geral

No estudo do efeito da temperatura (30-37 ºC) e agitação (200-300 rpm) na produção de protease por fermentação submersa pela linhagem de *B. licheniformis* LBA 46 em reator de bancada, por planejamento experimental, foram obtidos altos valores de atividade de protease (maiores que 1.000 U/mL) para 71,43% dos ensaios analisados. Devido aos altos valores de atividade observados na maioria dos ensaios do planejamento, a avaliação dos resultados foi realizada baseada na curvatura, que foi significativa ($p \leq 0.10$). As condições de 30 ºC e 300 rpm foram escolhidas para a produção da protease em reator de bancada com valores de atividade de 2.448,83; 2.627,33 e 2.661,17 U/mL após 48, 60 e 72h de fermentação.

Potumarthi et al. (2007) avaliaram a produção de protease alcalina por *B. licheniformis* NCIM-2042 em um reator de 11 L contendo 5 g/L de caseina, 10 g/L de extrato de malte, 10 g/L de polipeptona, 10 g/L de Na_2CO_3, pH 9.5. Os efeitos da aeração (1, 2, 3 vvm) e agitação (200, 300 and 400 rpm) foram testados e a maxima produção de protease, 340 U/mL, foi alcançada usando 300 rpm e 2 vvm, após 120 h de incubação à 35 °C.

Após o estudo da inativação térmica da protease semi-purificada na faixa de 50-70 ºC, pode-se observar que a enzima reteve cerca de 78%, 39% e 9% da atividade inicial após 120 min de tratamento a 50 ºC, 60 ºC e 70 ºC, respectivamente.

O extrato da protease de *B. licheniformis* LBA 46 foi purificado 3,33 vezes por precipitação com 80% de sulfato de amônio e cromatografia em coluna de DEAE-Sepharose. A extrato purificado da protease mostrou atividade específica de 655,80 U/mg de proteína e a massa molecular de 40 kDa estimada por SDS-PAGE.

A literatura presenta valores variados de peso molecular para proteases purificadas de *Bacillus* sp.: 17,10 kDa (Kim e Kim 2005), 20,10 kDa (Rai et al. 2009), 15 kDa (Adinarayana et al. 2003), 30 kDa (Jellouli et al. (2011), 33 kDa (Annamalai et al., 2013), etc.

O extrato purificado da protease de *B. licheniformis* LBA 46 apresentou atividade ótima em pH 8,5, alta atividade (> 80%) na faixa de pH 6,5 a 9 e baixa atividade a pH 4,0 (15%); foi estável na faixa de pH 5 a 10 após 24h a 4 °C, mantendo mais de 86% da atividade inicial; e apresentou atividade ótima na faixa de temperatura de 50 e 60 ºC, mostrando-se estável a 40 °C durante 1 h em pH 7 e mantendo 85% da atividade inicial após 1 h de tratamento a 50 °C, pH 7.

Para a análise do processamento da protease semi-purificada sob homogeneização a alta pressão (HAP), a enzima foi caracterizada antes do tratamento. A protease nativa, sem tratamento, apresentou baixa atividade em pH 4 a 40, 60 e 90 ºC (~ 1,5%). Em pH 7 e 9, sem

tratamento, a enzima apresentou baixa atividade a 40 °C (15-17%) e a 90 ° C (6-12%) imediatamente após a preparação da solução enzimática e após 24 h de refrigeração a 5 °C.

O tratamento de HAP não aumentou a atividade e a estabilidade da enzima a pH 4. Após o tratamento com HAP, a atividade residual da protease medida a 60 ºC, manteve-se entre 70-80% e 80-88% em pH 7 e 9, respectivamente. Observou-se que o tratamento com HAP não aumentou a atividade e estabilidade da enzima em pH 7 e 9 a alta temperatura de 90 °C obtendo-se atividade residual de aproximadamente 5-7%. O tratamento HAP da protease a pH 7 e 9 com refrigeração subseqüente durante 24h não resultou em aumento da atividade da protease determinada em nenhuma das temperaturas analisadas. Portanto, o tratamento de pressão não aumentou a atividade ou estabilidade da protease nas condições testadas.

De acordo com Cheftel (1992), a pressurização pode gerar inativação enzimática reversível ou irreversível, parcial ou completa por causa das mudanças geradas na estrutura protéica. Pressões elevadas podem levar à inativação ou redução da atividade enzimática (Yaldagard et al., 2008). O tratamento HPH é um processo capaz de melhorar (Tribst e Cristianini, 2012a; Tribst e Cristianini, 2012b), diminuir (Tribst et al., 2014, Carbonell et al., 2013) ou promover nenhuma alteração (Liu et al., 2010) na atividade enzimática.

A utilização da protease semi-purificada de *B. licheniformis* LBA 46 na hidrólise das proteínas de arroz e ervilha, foi comparada com a aplicação da enzima comercial Alcalase 2.4L. O grau de hidrólise foi superior a 50% em todas as amostras independentes da enzima e da proteína utilizada. Para os hidrolisados de proteína de arroz, a análise estatística mostrou que a variação no pH (6-10) e na concentração da enzima (50-150 U/mL) não teve efeito sobre a atividade antioxidante dos hidrolisados medida pelo ensaio DPPH. Portanto, não foi possível prever um modelo matemático para essa resposta. Entretanto, foram obtidos modelos matemáticos significativos capazes de explicar as variações nas respostas obtidas pelas análises de ORAC e FRAP.

Para os hidrolisados de proteína de ervilha, a análise estatística definiu que a variação no pH (6-10) e na concentração da enzima (50-150 U/mL) teve efeito sobre a atividade antioxidante dos hidrolisados medidos pelos ensaios de DPPH, ORAC e FRAP, obtendo-se assim um modelo matemático para cada uma das respostas.

Os valores de pH na faixa de 9 a 10 e concentração da enzima na faixa 50 a 150 U/mL resultaram em máxima atividade antioxidante, indicando que as duas enzimas avaliadas poderiam ser usadas na concentração de 50 U/mL para a hidrólise das proteínas de arroz e ervilha.

De acordo com os modelos de regressão obtidos, os valores previstos para ORAC e FRAP nas condições escolhidas (pH 10 e concentração de protease 100 U/mL) foram 942,30 e 18,70 TE μmol/g para os hidrolisados de proteína de arroz preparados com protease LBA e 1.006,46 e 19,10 TE μmol/g para os hidrolisados de proteína de arroz preparados com Alcalase 2.4L. Para os hidrolisados de proteína de ervilha, os valores preditos para DPPH, ORAC e FRAP foram 3,60; 556 e 9,14 TE μmol/g para protease LBA e 3,74; 723,13 e 9,12 TE μmol/g para Alcalase 2.4L. Os ensaios de hidrólise, nas condições definidas como ótimas, de acordo com o intervalo apresentado nas curvas de contorno, foram realizados em triplicata para confirmar a validade do modelo. Todos os valores experimentais obtidos estão de acordo com os valores esperados ($p \leq 0,10$), confirmando novamente a validade dos modelos.

Após a otimização das condições da hidrólise enzimática da proteína de arroz, os valores de atividade antioxidante aumentaram independentemente da enzima utilizada na hidrólise. Para a hidrólise realizada com a enzima LBA, houve um aumento de 2,9 vezes para o ORAC e 3,05 vezes para o FRAP. Para a hidrólise realizada com a Alcalase 2.4L, o aumento foi ligeiramente superior, de 3,08 vezes para o ORAC e 3,14 para o FRAP.

Para a proteína de ervilha, após a otimização das condições de hidrólise enzimática, a atividade antioxidante medida em termos de DPPH e FRAP foi reduzida em comparação com a atividade antioxidante medida na amostra intacta de proteína. No entanto, a atividade medida em termos do ORAC teve um aumento significativo de 7,33 e 9,27 vezes para os hidrolisados da protease LBA e para os hidrolisados da Alcalase 2.4L, respectivamente. Essas diferenças podem ser explicadas com base nos mecanismos de ação de cada ensaio antioxidante.

Zhou et al. (2013) avaliaram hidrolisados de proteína de arroz preparados por diferentes proteases microbianas (Validase de *Aspergillus oryzae*, pH = 7, protease alcalina de *B. licheniformis*, pH = 10 e protease neutra de *Bacillus subtillis*, pH = 7). Os autores relataram valores ORAC inferiores aos encontrados neste estudo, variando entre 34,20-87,30 TE μmol/g. As frações de hidrolisado com concentração de 100 mg/mL registraram 31,2-49,7% na redução do radical DPPH. Neste estudo, os hidrolisados com concentração de 5 mg/mL obtidos pela enzima LBA e Alcalase 2,4L mostraram 37,95-49,50% e 43,20-53,91% de redução no DPPH, respectivamente.

Pownall et al. (2010) avaliaram a hidrólise de proteína de ervilha isolada pela enzima Thermolisina (pH 8 a 55 ºC). Os hidrolisados de proteína de ervilha (1 mg/mL) apresentaram cerca de 21% na atividade de eliminação de DPPH. Por outro lado, Humiski e Aluko (2007)

obtiveram hidrolisados de proteína de ervilha (1mg/mL) com valores mais baixos (7-11%) para a eliminação de DPPH usando Flavourzyme (pH 7 a 50 ºC) e Alcalase (pH 8,5 a 50 ºC) na hidrólise.

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Conclusão geral

A linhagem de *B. licheniformis* LBA 46 foi cultivada em reator de bancada de 6 L. Os maiores valores de atividade de protease foram obtidos nas condições de temperatura e agitação de 30 ºC e 300 rpm após 48h (2.448,83 U/mL) e 72h (2.661,17 U/mL). O extrato purificado da protease apresentou atividade ótima a 50-60 ºC e pH 8,5; mostrou-se estável na faixa de pH de 5-10, e também após 1 h de incubação a 40 ºC.

O tratamento da protease semi-purificada de *B. licheniformis* LBA 46 com homogeneização a alta pressão de (HAP) na faixa de 50 a 200 MPa não resultou em aumento de atividade e estabilidade nas condições avaliadas.

Os resultados obtidos mostraram que a aplicação da técnica de planejamento experimental é um método interessante na produção de hidrolisados proteicos com propriedades antioxidantes. A melhor condição de hidrólise das proteínas de arroz e de ervilha para obtenção de peptídeos com atividade antioxidante foi alcançada em pH 10 e concentração de protease de 100 U/mL. A protease de *B. licheniformis* LBA 46 mostrou ser tão eficiente na produção de peptídeos bioativos com atividade antioxidante quanto a Alcalase 2.4L. Para ambas as preparações de protease utilizadas neste estudo, houve um aumento na atividade antioxidante dos hidrolisados de proteína de arroz quando comparados à amostra controle (não hidrolisada). A atividade antioxidante dos hidrolisados de ervilha medida pelos métodos DPPH e FRAP foi reduzida comparada com a proteína de ervilha não hidrolisada. No entanto, a atividade antioxidante dos hidrolisados medida pelo método ORAC apresentou um aumento em relação à proteína intacta.

Este trabalho proporcionou maior conhecimento sobre a produção, características e aplicações da protease de *B. licheniformis* LBA 46. Esta enzima possui potencial para maior exploração científica e possivelmente uso industrial.

Sugestões para trabalhos futuros

- ✓ Estudar outros parâmetros que afetam as condições de crescimento do *B. licheniformis* LBA 46;
- ✓ Purificar a enzima em maior quantidade e avaliar características bioquímicas como o efeito da presença de sais e outros compostos na atividade de protease;
- ✓ Testar a aplicação da HPH nas proteínas de arroz e ervilha;
- ✓ Realizar a cinética de hidrólise e verificar qual o melhor tempo para produção de hidrolisados protéicos de arroz e ervilha com atividade antioxidante;
- ✓ Avaliar características funcionais (emulsificante, estabilizante, resistência térmica, etc.) dos hidrolisados protéicos de arroz e ervilha;
- ✓ Avaliar outras características bioativas (antimicrobiano, antihipertensivo, etc.) dos hidrolisados protéicos de arroz e ervilha;
- ✓ Identificar e isolar as sequências dos peptídeos que apresentaram características interessantes.

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Anexo