UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

Estudo da hidrólise da proteína de soja utilizando proteases de *Chryseobacterium* sp. para o uso como antioxidante em alimentos

Cibele Freitas de Oliveira

Porto Alegre

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Estudo da hidrólise da proteína de soja utilizando uma protease produzida por *Chryseobacterium* sp.

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Orientador: Adriano Brandelli

RESUMO

A demanda por antioxidantes naturais vem aumentando devido à toxicidade de alguns antioxidantes sintéticos. Estudos vêm identificando antioxidantes de origem natural, como a proteína da soja, que é capaz de contribuir na melhoria de propriedades funcionais e biológicas de alimentos. A hidrólise enzimática da proteína de soja aumenta sua atividade antioxidante, assim como a capacidade emulsificante e a capacidade de formação de espuma. O objetivo deste trabalho foi o estudo da hidrólise da proteína de soja através de uma protease produzida por *Chryseobacterium* sp., a verificação da capacidade antioxidante e aplicação do hidrolisado em diferentes tipos de carnes para evitar a oxidação lipídica. A eficácia da hidrólise foi determinada através da proteína solúvel utilizando o método de Folin enquanto que a atividade antioxidante foi avaliada pelos métodos referentes à captura do radical DPPH e ABTS. Os hidrolisados foram adicionados em carne de porco e peixe e foi verificada a inibição da oxidação lipídica. A influência de três parâmetros (temperatura, pH, relação enzima/substrato) na hidrólise foi estudada através um experimento fatorial 2^3 . Como respostas foram avaliadas a atividade antioxidante (DPPH e ABTS), atividade quelante de ferro, proteína solúvel, capacidade de formação de espuma e capacidade emulsificante. Observou-se um aumento na concentração de proteína solúvel em função do tempo, sendo que os hidrolisados foram capazes de inibir tanto o radical DPPH quanto o ABTS. Os hidrolisados inibiram em parte a oxidação lipídica em carne suína e peixe. Ainda foi possível concluir que dependendo da finalidade para que se deseja o hidrolisado, diferentes condições devem ser utilizadas. Os resultados demonstram uma potencial aplicação da protease microbiana para gerar hidrolisados antioxidantes da proteína de soja.

Palavras-chave: Proteína, soja, atividade antioxidante, hidrólise, otimização

Dissertação de Mestrado em Ciência e Tecnologia de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, setembro, 2011

Study of hydrolysis of soy protein using a protease produced by *Chryseobacterium* sp.

Author: Cibele Freitas de Oliveira

Adviser: Adriano Brandelli

ABSTRACT

The demand for natural antioxidants has been increasing due to the toxicity of some synthetic antioxidants. Studies have identified naturally occurring antioxidants, such as soy protein, which can contribute to improve functional and biological properties of food. Enzymatic hydrolysis of soy protein increases its antioxidant activity, as well as emulsifying capacity and foaming capacity. The purpose of this work was to study the hydrolysis of soy protein, verifying the antioxidant capacity, application of the hydrolysate in different types of meat and optimization of hydrolysis. The efficiency of hydrolysis was determined by the soluble protein by the method of Folin while the antioxidant activity was evaluated by the methods related to the capture of the radical DPPH and ABTS. The hydrolysates were added to pork and fish and the extent of lipid oxidation was determined by TBARS. In optimizing of the hydrolysis three parameters were varied (T, pH, enzyme substrate), it was applied to a surface response methodology for conducting trials using a 2^3 factorial experiment. As answers were evaluated antioxidant activity (DPPH and ABTS), iron chelating activity, Lowry, foaming capacity and emulsifying capacity. There was an increase in soluble protein concentration versus time, and the hydrolysates were able to inhibit both the ABTS and the DPPH radical. The hydrolysates were able of inhibit lipid oxidation in pork and fish. Was still possible to conclude that depending on the finality that will be given to hydrolysates different treatment conditions should be used. The results demonstrate a significant potential for application of microbial protease to generate antioxidants of hydrolyzed soy protein.

Keywords: protein, soy, antioxidant activity, hydrolysis, optimization

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1. Introdução

As proteínas dos alimentos possuem propriedades físico-químicas que regem o seu desempenho e comportamento em sistemas de alimentação durante o processamento, armazenamento e consumo que são coletivamente denominadas propriedades funcionais (Achouri *et al*.,1998).

Vários estudos têm demonstrado a contribuição da soja na melhoria de certas propriedades funcionais e tecnológicas em sistemas alimentares (Lui, 2000). Hidrolisados da proteína de soja, utilizados na formulação de produtos, normalmente apresentam um incremento em algumas propriedades tais como: solubilidade, capacidade de formação de espuma e propriedades emulsificantes (Achouri *et al*.,1998). Ainda vale destacar que a hidrólise enzimática pode ser responsável pela diminuição da massa molecular do hidrolisado, aumento no número de grupos ionizáveis e exposição dos grupos hidrofóbicos (Jung *et al*., 2005).

Um fator importante relacionado com a hidrólise da proteína de soja é a geração de peptídeos bioativos, capazes de atuar como antioxidantes. Os processos oxidativos degradam lipídeos e proteínas e são os mecanismos principais de perda de qualidade dos produtos alimentícios (Haak *et al*., 2006). Óleos e gorduras são usados em alimentos por inúmeras razões, são nutrientes e também conferem textura e sabor aos alimentos, porém são facilmente oxidados. Por isso, antioxidantes sintéticos são largamente utilizados em produtos alimentícios para retardar a oxidação de lipídeos, porém, alguns antioxidantes possuem elevado grau de toxicidade (Sakanaka *et al*., 2005). Devido a isso, pesquisadores vêm identificando e produzindo antioxidantes naturais e de baixo custo. Estudos mostram que as proteínas presentes em alguns vegetais representam uma potencial fonte dietética de antioxidantes naturais. Dentre os vegetais pode-se destacar a

soja, sendo que a proteína presente na soja é capaz de eliminar radicais livres (Chen *et al.*, 1995).

As proteases microbianas têm sido alvo de muitos estudos, devido às possibilidades de aplicações industriais, em especial, nas indústrias de fertilizantes, farmacêuticas, de detergentes, ração animal e alimentos. Dessa forma, o presente trabalho visa o estudo da hidrólise da proteína de soja utilizando uma protease produzida por *Chryseobacterium* sp. kr6, a verificação da melhoria das propriedades funcionais, capacidade antioxidante desse hidrolisado e a verificação da influência de três parâmetros (temperatura, pH, relação enzima/substrato) na hidrólise deste substrato.

2. Objetivos

2.1 Objetivo geral:

- Estudo da hidrólise da proteína de soja utilizando uma protease produzida por *Chryseobacterium* sp. e potencial aplicação do hidrolisado.

2.2 Objetivos específicos:

- Obtenção e purificação parcial da protease;
- Maximização da hidrólise da proteína de soja;
- Verificação das propriedades funcionais e biológicas dos hidrolisados;
- Aplicação dos hidrolisados para evitar a oxidação em carnes.

3. Revisão Bibliográfica

3.1 Proteases

As proteases são uma classe única de enzimas com capacidade de hidrolisar ligações peptídicas em proteínas e fragmentos de proteínas, possuem grande importância fisiológica e comercial (Rao *et al*., 1998). Essas enzimas causam reações irreversíveis, que do ponto de vista biológico podem ser importantes em processos fisiológicos, patológicos e tecnológicos (Barret *et al*., 2001).

Por serem fisiologicamente necessárias estão distribuídas entre os animais, plantas e microrganismos. Devido ao rápido crescimento, reduzido espaço para cultivos e facilidade com que podem ser manipulados geneticamente, os microrganismos são a fonte preferida para a produção de proteases (Barret *et al.*,2001).

As proteases constituem aproximadamente 10% de todas as enzimas incluídas na lista do Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (Barret *et al*., 2001).

3.1.1 Classificação

Devido à grande diversidade de atividade e estrutura as proteases não são inseridas nos sistemas gerais de nomenclatura de enzimas. Essa classe de enzimas é classificada segundo três critérios principais: quanto ao tipo de reação catalisada, exopeptidases (clivam ligações nas extremidades das cadeias) e endopeptidases (clivam ligações no interior da cadeia); quanto à natureza química do sítio ativo, dividem-se em serina, aspartil, cisteína e metalopeptidases e quanto a relação evolutiva, onde são classificadas em famílias e subdivididas em clãs, de acordo com a convergência ou divergência de um ancestral comum (Barret *et al*., 2001).

As serina-proteases são caracterizadas pela presença de um resíduo de serina em seu sítio ativo, são amplamente distribuídas entre os vírus, procariotos e eucariotos, apresentando funções diversas. São divididas em 20 famílias e 6 clans, dos quais os dois maiores são os clãs tipo-(quimo) tripsina e tipo-subtilisina (Beynon & Bond, 1996). As serina-proteases são reconhecidas por sua inibição irreversível pelos compostos 3,4 – dicloroisocumarina – 3,4 – DSI, diisopropilfluorfosfato – DFP, 3 – carboxitrans 2,3 – epoxipropil – leucilamido e Fluoreto de fenilmetilsulfonil – PMSF (Barret *et al*., 1994)

As proteases aspárticas, também conhecidas como proteases ácidas, são endopeptidades que dependem de resíduos de ácido aspártico para sua atividade catalítica. A maiorias dessas proteases apresenta máxima atividade em baixos valores de pH. A pepstatina, um hexapeptídeo, é capaz de inibir aspartil proteases (Beynon *et al*., 1996).

A atividade das cisteína proteases depende da presença de uma cisteína (Cys) e histidina (His) no sítio ativo, sendo que a ordem desses resíduos difere dentro das famílias. As cisteína proteases são ativas na presença de agentes redutores, tais como HCN (Rao *et al*., 1998). São inibidas por agentes sulfidrilas como p-cloromercurilbenzoato – PCMB e Iodocetamida (Barret., 1994)

As metaloproteases encontram-se na maioria dos organismos vivos e são as enzimas que apresentam maior diversidade de sítios ativos. Cerca de 30 famílias de metaloproteases são conhecidas, sendo 17 com atividade endopeptidase, 12 com atividade exopeptidase e uma contendo ambas (Barret *et al*., 1994). São inibidas por agentes quelantes, tais como EDTA, mas não por agentes sulfidrícos ou DPF (Rao *et al*., 1998).

3.1.2 Aplicações

As proteases têm despertado atenção de inúmeros pesquisadores devido a suas múltiplas aplicações, dentre elas destacam-se as indústrias farmacêuticas, de detergentes, alimentos, processos biotecnológicos não poluentes (Brandelli, 2008). Na indústria de alimentos são amplamente utilizadas em laticínios, massas e na produção de hidrolisados protéicos. Na indústria farmacêutica proteases são utilizadas em formulações como auxiliares digestivos, combinações com antibióticos e para tratamento de lesões. Outra aplicação interessante é a substituição de compostos químicos na indústria e a utilização em processos de tratamentos de resíduos industriais, além da utilização na pesquisa científica (Rao *et al*.,1998).

3.2 *Chryseobacterium* sp. kr6

Tem-se encontrado microrganismos capazes de produzir proteases (Onifade *et al*., 1998). Estes são chamados de microrganismos proteolíticos e podem desenvolver-se em diferentes condições ambientais. *Bacillus* e *Aspergillus* são os principais microrganismos produtores de proteases comerciais (Zhang *et al*., 2010).

Estudos vêm identificando novos microrganismos proteolíticos, como a *Chryseobacterium* sp. kr6 (Sangali & Brandelli, 2000*;* Riffel *et al.*, 2003). Esta é uma bactéria gram-negativa, que foi isolada do efluente de uma indústria de processamento de aves e mantida em ágar farinha de penas (Riffel *et al*., 2003). Esse microrganismo é capaz de utilizar queratina como substrato, para seu crescimento produzindo proteases. Além da hidrólise de queratinas, as proteases produzidas por esta linhagem são capazes de hidrolisar outras proteínas como caseína, albumina e hemoglobina.

As proteases desempenham um papel importante no metabolismo da bactéria *Chryseobacterium* sp. kr6, visto que esta pode crescer em meio contendo farinha de penas como única fonte tanto de carbono quanto de nitrogênio (Silveira *et al*.,2008).

Segundo Silveira *et al.* (2008), as condições ótimas para a atividade da protease produzida pela *Chryseobacterium* sp. kr6 são, temperatura de 30°C, pH 8, sob agitação (100 rpm) por 48 horas, utilizando um meio composto por 0,5 $g.L^{-1}$ de NaCl, 0,4 $g.L^{-1}$ de KH_2PO_4, 0,015 $g.L^{-1}$ de $CaCl_2$ e 10 $g.L^{-1}$ de farinha de penas.

A Chryseobacterium sp. kr6 produz metaloproteases pertencentes à família M14 das peptidases, também conhecidas como carboxipeptidases. Sendo este microrganismo pertencente à família M14 associada à atividade proteolítica e ao gênero *Chryseobacterium* (Riffel *et al*., 2007).

3.3 Proteína de soja

Proteínas de soja são amplamente utilizadas como ingredientes nutricionais e funcionais em muitos alimentos (Hettiarachchy *et al*., 1997; Lui, 2000). É sabido que a proteína da soja possui várias funções fisiológicas, tais como reduzir o colesterol e a gordura corporal (Kito *et al*., 1993; Johnstone *et al*., 1995). Além disso, a *Food and Drug Administration* (FDA) indica que a proteína da soja auxilia na redução do risco de doença cardíaca coronária (FDA, 1999).

Algumas indústrias de alimentos têm utilizado como ingrediente a proteína de soja, com o objetivo de melhorar algumas características durante o processamento. O isolado protéico de soja possui várias propriedades funcionais (capacidade emulsificante, capacidade de formação de espuma), devido a isso é utilizado em uma ampla gama de produtos alimentares, incluindo carnes processadas, bebidas nutricionais, fórmulas infantis (Genovese & Lajolo, 1992).

De acordo com Genovese & Lajolo (1992) as propriedades das proteínas de soja dependem das condições de cultivo. O efeito do grau de maturação, cultivar, condições de estocagem, processamento e percentual de grãos danificados alteram as propriedades físico-químicas, principalmente a viscosidade, capacidade de formação de gel e emulsificação das proteínas da soja. No Brasil a qualidade dos grãos de soja é muito heterogênea devido a diferentes condições de cultivo, colheita e estocagem.

Muitas proteínas nativas apresentam limitada funcionalidade, devido a isso modificações na estrutura protéica são realizadas para ampliar as características das propriedades funcionais disponíveis. A hidrólise enzimática da proteína da soja pode modificar as propriedades funcionais da proteína, incluindo solubilidade, emulsificação e formação de espuma (Panyam & Kilara, 1996; Surowka *et al*., 2004; Jung *et al*., 2005). Trabalhos relatam que hidrolisados de proteína de soja possuem atividade antioxidante (Beermann *et al*., 2009; Chen *et al*., 1998; Pena-Ramos & Xiong., 2002).

A hidrólise enzimática pode ser responsável por uma diminuição na massa molecular do hidrolisado, um aumento no número de grupos ionizáveis, e a exposição de grupos hidrofóbicos (Jung *et al*., 2005). Mudanças nas propriedades funcionais, incluindo características reológicas são um resultado direto das modificações estruturais dos componentes principais da proteína de soja, glicinina e β-conglicinina, por proteases (endo ou exo-peptidases) (Jung *et al*., 2005).

Estudos mostram que a proteína de soja hidrolisada é muito mais solúvel no ponto isoelétrico do que a proteína nativa, pois ocorre a exposição dos grupos hidrofóbicos escondidos (Jung *et al*., 2005). Ainda a hidrólise enzimática aumenta a capacidade de absorção de gordura, isso pode ser atribuído ao desdobramento da estrutura da proteína

3.4 Compostos bioativos

Compostos bioativos são constituintes extra-nutricionais e ocorrem tipicamente em pequenas quantidades nos alimentos. Para ser considerado bioativo, o componente da dieta deve exercer um efeito biológico fisiologicamente significativo e a bioatividade deverá afetar a saúde de maneira benéfica (Moller *et al*., 2008).

Nos últimos anos tem sido dada uma maior importância para as proteínas na dieta. Já é reconhecido o valor das proteínas como fonte de aminoácidos essenciais, mas recentemente tem sido reconhecido que as proteínas da dieta exercem muitas outras funcionalidades *in vivo* por meio de peptídeos biologicamente ativos. Esses peptídeos se mostram inativos dentro de uma sequência de proteína, mas podem ser liberados pelas enzimas digestivas durante o trânsito gastrointestinal, fermentação ou amadurecimento, durante o processamento de alimentos e ainda por enzimas derivadas de microrganismos ou plantas (Moller *et al*., 2008; Kittis & Weiler, 2003). A hidrólise enzimática é a maneira mais comum para a produção de peptídeos bioativos (Moller *et al*., 2008).

Peptídeos bioativos têm sido definidos como fragmentos de proteínas específicos que exercem um impacto positivo sobre algumas funções do corpo e dessa forma acabem influenciando na saúde (Kittis & Weiler, 2003).

3.4.1 Peptídeos antioxidantes

Antioxidantes são definidos como substâncias que, quando presentes em baixas concentrações em relação ao substrato oxidável, são capazes de inibir ou retardar substancialmente a oxidação daquele substrato. Os antioxidantes não se tornam radicais livres pela doação de elétrons, pois eles são estáveis em ambas formas. Existem duas categorias básicas de antioxidantes denominadas sintético e natural (Wood *et al*., 2008).

Peptídeos ou fragmentos de proteínas são curtas cadeias de aminoácidos produzidos pela hidrólise enzimática ou pela digestão gastrointestinal dos alimentos. Alguns peptídeos possuem funções bioativas, entre elas destaca-se a capacidade antioxidante (Dziuba *et al*., 2003).

A oxidação de gorduras nos alimentos produz odor e gosto a ranço capaz de ser detectado pelo consumidor (Wood *et al*., 2008). Assim, a oxidação lipídica é a maior causa de perda de aroma, valor nutritivo e diminuição da vida útil de produtos contendo gordura. Com o intuito de diminuir este problema, antioxidantes sintéticos como butil-hidroxil tolueno (BHT) e butil-hidroxil anisolo (BHA) têm sido amplamente utilizados na indústria alimentícia. Porém os efeitos adversos causados por estes compostos estimularam o uso de antioxidantes naturais tais como catequinas, tocoferóis, ascorbato, ácido rosmarínico e vários extratos fenólicos de plantas. A procura por antioxidantes naturais se estendeu para proteínas e peptídeos de origem animal e vegetal como proteínas de soja, zeína, gérmen de trigo, albumina do ovo, proteínas lácteas (Xue *et al*., 2009; Zhang *et al*., 2010).

Segundo Beermann *et al.* (2009) hidrolisados protéicos de soja possuem atividade antioxidante e são capazes de reduzir a oxidação lipídica em produtos cárneos (Zhang *et al*., 2010). Estudos mostram que a proteína hidrolisada de soja possui

habilidade em inibir o radical livre 2,2-difenil-1-picrilhidrazila (DPPH) e o etilbenztisolina-6-ácido sulfônico (ABTS) (Zhang *et al*., 2010).

O maior efeito antioxidante da proteína de soja está atribuído a seis peptídeos compostos por 5-16 resíduos de aminoácidos (Chen *et al*., 1995). Peptídeos com massa molecular inferior a 1 kDa e prevalecente de resíduos de aminoácidos aromáticos possuem maior capacidade antioxidante (Beermann *et al*., 2009).

Embora vários estudos tenham mostrado o sequestro de radicais livres, a inibição da peroxidação lipídica e quelação de íons metálicos de transição, o mecanismo responsável pela atividade antioxidante de peptídeos não está completamente esclarecido. As propriedades antioxidantes estão mais relacionadas à composição, estrutura e hidrofobicidade dos aminoácidos (Sarmadi & Ismail, 2010).

3.6 Propriedades funcionais das proteínas

As propriedades funcionais das proteínas são definidas propriedades físico-químicas que afetam o seu comportamento no alimento durante o preparo, processamento e armazenamento, e contribuem para a qualidade e atributos sensoriais dos alimentos (Antunes *et al*., 2003). Suas propriedades funcionais podem ser classificadas em hidrofílicas, afinidade com a água; interfásicas, capacidade das moléculas de proteína se unirem formando uma película entre duas fases imiscíveis; intermoleculares, capacidade de formarem ligações entre si ou com outros componentes dos alimentos; reológicas, dependem de características físicas e químicas específicas das proteínas; e organolépticas, manifestam-se através dos órgãos dos sentidos, referindo-se a textura, cor, sabor e aroma (Araújo, 2006).

Fatores como temperatura, pH, processos de obtenção e isolamento de proteínas, entre outros, podem afetar essas propriedades funcionais. Muitos tratamentos físicos, químicos e enzimáticos têm sido amplamente aplicados para modificar as propriedades funcionais de proteínas vegetais, através da mudança da estrutura da proteína. Geralmente, a modificação enzimática é mais preferível devido a condições mais brandas de processo, mais fácil o controle da reação e formação mínima de subproduto (Mannheim & Cheryan, 1992).

A aplicação de hidrólise enzimática às proteínas podem tanto incrementar quanto diminuir sua funcionalidade, dependendo do grau de hidrólise aplicado, sendo geralmente aceito que uma hidrólise branda é benéfica, ao passo que uma hidrólise extensiva é considerada prejudicial (Haque, 1993). Sendo assim, o grau de hidrólise deve ser controlado para a obtenção de peptídeos de tamanho ideal com suas funcionalidades modificadas. A proteólise tem sido usada para modificar as propriedades funcionais e físico-químicas da proteína de soja (Jung, Murphy & Johnson, 2004), proteína de girassol (Martineza *et al*., 2005), proteína de canola (Vioque *et al*., 2002).

Certas propriedades funcionais de hidrolisados protéicos exercem um papel predominante no sentido em que irão determinar as características principais do produto final definindo seu uso. Logo, o tipo e a forma da proteína a ser utilizada como ponto de partida do hidrolisado protéico, assim como o grau de hidrólise aplicado, devem ser controlados em função das principais propriedades funcionais que se deseja explorar.

Proteínas de soja são amplamente utilizadas em muitos alimentos como ingredientes funcionais e nutricionais (Hettiarachchy & Kalapathy, 1997; Liu, 2000). Proteína isolada de soja é o mais refinado produto de proteína de soja, possuindo muitas propriedades funcionais. Por isso é usada em uma ampla gama de aplicações de

alimentos incluindo carnes processadas, bebidas nutricionais, formulações infantis e produtos lácteos.

3.7 Oxidação lipídica

Os lipídeos desempenham um importante papel quando se refere à qualidade de certos produtos alimentares, particularmente em relação às propriedades organolépticas que os tornam desejáveis (flavor, cor, textura). Por outro lado, conferem valor nutritivo aos alimentos constituindo uma fonte de nutrientes, energia, ácidos graxos essenciais e vitaminas lipossolúveis (Borges *et al*., 1999).

A oxidação lipídica é um fenômeno espontâneo e naturalmente inevitável, com uma implicação direta no valor comercial dos alimentos. A deterioração dos lipídeos está relacionada à oxidação, os processos oxidativos degradam lipídeos e proteínas e são mecanismos de perda de qualidade dos produtos alimentícios (Haak *et al*.,2006).

A oxidação lipídica é induzida pelo oxigênio na presença de iniciadores como temperatura, radicais livres, pigmentos fotossintéticos e íons metálicos (Laguerre *et al*., 2007). Este processo ocorre durante o armazenamento e processamento de alimentos, acarretando na rancidez e sabor desagradável dos mesmos.

Nos últimos anos, a preocupação constante de proporcionar aos consumidores produtos de alta qualidade levou à procura de medidas que permitem limitar o fenômeno de oxidação durante as fases de processamento e armazenagem dos produtos. Deste conjunto de ações, a adição de compostos antioxidantes é, sem dúvida, uma prática corrente, razão que justifica o atual interesse pela pesquisa de novos compostos com capacidade antioxidante. O baixo custo de obtenção, facilidade de emprego, eficácia, termo-resistência, "neutralidade" organoléptica e ausência reconhecida de

toxicidade, são fatores para a sua seleção e utilização a nível industrial (Borges *et al*., 1999).

Antioxidantes sintéticos são amplamente utilizados em produtos alimentícios para retardar a oxidação de lipídeos, porém, a procura por antioxidantes naturais tem aumentado por causa da toxicidade de alguns antioxidantes sintéticos (Sakanaka *et al*., 2005).

Fontes naturais tem sido uma alternativa para a obtenção de antioxidantes (Haak *et al*., 2006). Dentre essas substâncias, destacam-se aminoácidos e proteínas por serem solúveis em água e capazes de complexar íons metálicos. É importante destacar que algumas proteínas hidrolisadas de aminas e plantas possuem atividade antioxidante (Sakanaka *et al*., 2005). Estudos mostram que a caseína quando hidrolisada possui propriedades antioxidantes (Kitts, 2005).

4. Resultados

Os resultados deste trabalho estão apresentados na forma de artigos, sendo cada subtítulo deste capítulo correspondente a um artigo.

4.1 Antioxidant activity and inhibition of meat lipid oxidation by soy protein hydrolysates obtained with microbial protease. Submetido para publicação no periódico European Food Research and Technology.

4.2 Production of soy protein hydrolysates with improved functional properties by a microbial protease

Antioxidant activity and inhibition of meat lipid oxidation by soy protein hydrolysates obtained with microbial protease

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Abstract

Antioxidant peptides derived from edible proteins are interesting natural antioxidants that may be useful to improve functional properties of food. In this work, soy protein was hydrolyzed using a protease from *Chryseobacterium* sp. kr6. The antioxidant capacity of the resulting hydrolysates was evaluated by the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. An increase in soluble protein concentration was observed as the hydrolysis time increased. The hydrolysates showed antioxidant activity by both DPPH and ABTS assays. Soybean hydrolysates were applied in pork loin and fish meat systems and their efficiency in reducing thiobarbituric acid reactive substances (TBARS) was observed, indicating that the soy hydrolysates caused a significant inhibition of lipid peroxidation in both systems. These results demonstrated the potential of microbial protease to produce effective antioxidant peptides from soy protein.

Keywords: bioactive peptides; soy; antioxidant activity; hydrolysis; protease

Introduction

Lipid oxidation is a serious problem in foods because it produces off-flavors and also decreases the nutritional quality, safety and shelf-life [1]. The control of lipid oxidation in food products is desirable, and the beneficial effect of antioxidants during food storage has been described [2]. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) have been commercially used in various food products with the aim of increasing shelf-life and quality. However, there is a great concern about the use of such synthetic products, due to their toxicity and carcinogenicity [2,3] .

In this regard, there is an increasing interest in the identification and development of low cost natural antioxidants. Some plant proteins are potential dietary source of natural antioxidants. Among them, it is possible to emphasize the free radical scavenging ability of soybean protein [4]. The antioxidant properties of soy protein have been attributed to certain specific sequences of peptides encrypted in the protein. Furthermore, an increase in antioxidant capacity can be achieved with the hydrolysis of soy protein [5]. The hydrolysis with proteolytic enzymes often results in the formation of peptides with low molecular mass, increased number of ionizable groups, and exposure of hidden hydrophobic groups [6]. Despite soy protein-derived peptides may present interesting biological activities [7], little information is available on the use of microbial proteases to produce such bioactive peptides.

Proteases have been the target of many studies due to the possibilities of industrial applications in foods and animal feed [8]. Some microbial proteases have been successfully used to modify food proteins resulting in hydrolysates with improved nutritional and/or functional properties [9,10]. Thus, the aim of this work was to investigate the antioxidant activity of soybean protein hydrolysates using a novel

protease produced by *Chryseobacterium* sp. kr6, and their application to reduce lipid oxidation in meat systems.

Materials and methods

Microorganism and cultivation conditions

The keratinolytic strain *Chryseobacterium* sp. kr6, isolated from the effluent of a poultry processing industry, was kept in feather meal agar [9]. The microbial culture was performed in feather meal broth, containing the following components (g L^{-1}): NaCl (0.5), KH_2PO_4 (0.4), $CaCl_2$ (0.015) and feather meal (10.0). The pH was adjusted to 8.0. The cultures were performed in 250 mL erlenmeyer flasks (working volume of 50 mL) at 30°C and 150 rpm for 48 h. After the growth period, the culture was centrifuged for 20 min at 10,000 x *g* and 4°C and the supernatant was collected [21].

Enzyme isolation

Solid ammonium sulfate was added to the supernatant (obtained in the previous step) under stirring to reach 50% saturation. The mixture was centrifuged at 10,000 x *g* for 20 min at 4°C, the precipitate was dissolved in 50 mmol L^{-1} Tris-HCl pH 8; and centrifuged again to remove any insoluble material. The concentrated sample was applied to a gel permeation column of Sephadex G-100 (0.8 x 30 cm) equilibrated and eluted with 50 mmol L^{-1} Tris-HCl pH 8.0. Fractions showing proteolytic activity were pooled and used in the experiments [21].

Determination of enzyme activity

The enzyme activity was determined using azocasein as substrate as described elsewhere [11]. One unit of enzyme activity was defined as the amount of enzyme

required to produce a change in absorbance of 0.01 (420 nm) under the assay conditions (40 min at 45^{o}C, pH 8.0).

Enzymatic hydrolysis of soy protein

The peptides were prepared by hydrolysis of soy protein using the protease isolated from *Chryseobacterium* sp. kr6. The protein was dissolved in Tris-HCl (pH 8.0), the hydrolysis was performed using a ratio of 0.2 mL of enzyme (816.67 U/mL) to 0.1 g substrate in a water bath at 45°C under shaking at 150 rpm. During the reaction, aliquots (1 mL) were withdrawn at different time intervals (0, 30, 60, 120, 180, 240, 360 minutes). The hydrolysis was stopped by boiling in a water bath. The hydrolysates were centrifuged (10,000 x *g* for 20 min) to remove insoluble materials. The hydrolysates were frozen and stored at -18°C until further analysis [8]. Controls were prepared similarly, but without enzyme

Determination of soluble protein concentration

The concentration of soluble protein was determinate by the Folin phenol reagent method [12]. Bovine serum albumin (BSA) was used as standard. The measurements were performed using a spectrophotometer UV mini-1240 Shimadzu (Shimadzu do Brasil, Agua Branca, Brazil).

Determination of antioxidant activity by DPPH

The antioxidant activity was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) method as described by Brand-Williams et al. [13], which is based on the capture of the DPPH radical by antioxidants, producing a decrease in absorbance at 515 nm.

Determination of antioxidant activity by ABTS

The ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) cation radical was used for evaluation of antioxidant capacity, using the method described elsewhere [8].

Application in pork and fish meat

Antioxidant activity in pork and fish was determined by measuring thiobarbituric acid reactive substances (TBARS) [14]. Salmon fish and pork meats were used in this experiment. Salmon and pork samples (20 g) were homogenized in 100 mL of 0.1 mol L^{-1} Tris HCl pH 7.4. The test medium contained 100 μL of meat homogenate, 30 μL of Tris-HCl buffer pH 7.4, 30 μL ferrous sulfate (10 μmol L^{-1}) and 100 μL soy protein hydrolysates (obtained from 6 hours of hydrolysis) was incubated at 100°C for 120 min. The mixture was then tested for the formation of TBARS, by adding 200 μL of 8.1% sodium lauryl sulfate (SDS), 500 μL of acetic acid buffer pH 3.44 and 500 μL of 0.6% thiobarbituric acid (TBA). After additional incubation at 100°C for 60 min the reaction products were determined by measuring absorbance at 532 nm in a spectrophotometer. The TBARS concentration was calculated using a standard curve and results were expressed in nM of malondialdehyde (MDM). The fat and moisture contents in meats were determined according AOAC [15]. The experiments were performed in duplicate. The results were examined by Tukey test.

Results and Discussion

Antioxidant activity of soybean protein hydrolysates

Enzymatic hydrolysis of soy protein using a protease of *Chryseobacterium* sp. kr6, with an activity of 816.67 U/mL, was monitored for up to 6 h through the determination of soluble protein. Under the experimental conditions tested in this work (45°C, pH 8 and 150 rpm), an increased concentration of soluble proteins was observed as a function of reaction time (Fig. 1). Maximum values were reached from 180 min. This indicates that the protease is efficient to form peptides by hydrolysis of soy protein. This pattern is in agreement to that observed for casein hydrolysis by Alcalase, a commercial protease from *Bacillus licheniformis* [8] and soybean protein hydrolysis by neutral protease from *Bacillus subtilis* and validase from *Aspergillus oryze* [5].

The antioxidant activity of the hydrolysates was assessed by two methods, based on the scavenging of the ABTS radical and the DPPH radical. The results showed that the hydrolysates of soybean protein are able to scavenge both ABTS and DPPH radicals (Fig. 2). An increase in the ability to capture the ABTS radical was observed as the hydrolysis time increases (Fig. 2a). Maximum values were observed from 180 min of hydrolysis time. Yang et al. [16] observed that the antioxidant capacity of hydrolysates of soy sauce lees increased with the hydrolysis time. Zhang et al. [5] recently reported that soy hydrolysates showed the ability to inhibit about 29% the ABTS oxidation.

For DPPH assay, higher values of inhibition were in the range of 77-79%, with no major fluctuation among the hydrolysates obtained from 30 min (Fig. 2b), while for ABTS higher values of inhibition reached 88%. There was a limited ability to capture the DPPH radical in comparison with the ability to capture the ABTS radical. Previous study have showed that a series of food-derived peptides or protein hydrolysates were

able to scavenge the DPPH radical [5]. However it is difficult to make a direct comparison with other studies due to different specificity of enzyme catalytic sites, which could generate different peptides.

Some studies show that hydrolyzed soybean protein has antioxidant activity [17,18]. According to Chen et al. [4], the highest antioxidant effect of soybean protein is assigned to six peptides consisting of 5-16 amino acid residues. Peptides with molecular mass below 1 kDa and aromatic amino acid residues have been described to possess a higher antioxidant capacity [18].

Antioxidant capacity in meat systems

Some protein hydrolysates and specific peptides have been shown to effectively inhibit lipid peroxidation in different food products [19], suggesting that specific protein-derived peptides can be utilized as natural antioxidants to improve food quality and stability. The antioxidant activity of the soybean hydrolysates was tested in meat systems. Two different concentrations of the hydrolysates (10 and 2 mg mL^{-1}) obtained from 360 minutes of reaction were added to homogenates of pork (1.62% w.b. or 2.27% d.b. fat) and salmon (2.88% w.b., or 4.27% d.b. fat) and lipid oxidation was evaluated by measuring TBARS. When the hydrolysate was incorporated to samples of homogenized pork it was possible to observe a 63% reduction in lipid oxidation as compared with controls (Table 1). When the hydrolysate was diluted, a 53% reduction in oxidation was observed (No significant difference between treatments). Different results were obtained for salmon: a 65% reduction in lipid oxidation was observed in comparison with the controls, while the diluted hydrolysate caused only 12% decrease in lipid oxidation (Table 2).

Soy protein hydrolyzed with commercial enzymes was able to reduce lipid peroxidation of meat around 26% when 800 μg g^{-1} of hydrolyzate was used [5]. Casein peptides were also capable to act as antioxidants when incorporated in beef homogenates, reducing lipid oxidation by 100 % [8]. Sakanaka et al. [2], obtained an inhibition of 69.7 % on lipid oxidation of ground beef when applied casein peptides obtained by microbial hydrolysis at a concentration of 20 mg mL^{-1}. Lee and Hendricks [20] obtained 76.2 % inhibition of oxidation using carnosine (an endogenous dipeptide found in skeletal muscle of most vertebrates). The results from this research demonstrate the potential application of specific microbial proteases for generating antioxidant hydrolysates from soy proteins that may be used as effective natural antioxidants to improve quality and shelf-life of food products.

Conclusion

The protease produced by *Chryseobacterium* sp. kr6 shows a marked ability to hydrolyze soy protein. The hydrolysates obtained were able to inhibit the DPPH and ABTS radicals in values ranging 77-79% and 81-88%, respectively. The results suggest that bioactive peptides derived from soybean hydrolysis can be used as natural antioxidants in meats, inhibiting about 50 and 60% lipid peroxidation in pork and fish homogenates, respectively. These results demonstrate a potential application of microbial protease produced by *Chryseobacterium* sp. kr6 to generate antioxidant peptides from soy protein.

Acknowledgments

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Figure Legends

Figure 1. Soluble protein concentration (mg mL^{-1}) as a function of hydrolysis reaction time. Samples were incubated for up to 360 min in the presence (●) or absence (○, control) of protease from *Chryseobacterium* sp. kr6. Values are the means of three independent experiments (SD ≤ 2.60).

Figure 2. Antioxidant activity of soy protein hydrolysates measured by (A) % inhibition of ABTS radical and (B) % inhibition of DPPH radical. Samples were incubated for up to 360 min in the presence (●) or absence (○, control) of protease from *Chryseobacterium* sp. kr6. Values are the means of three independent experiments (SD ≤ 0.12).

Table 1. Application of soybean hydrolysate in pork to inhibit lipid oxidation (the efficiency was verified by TBARS).

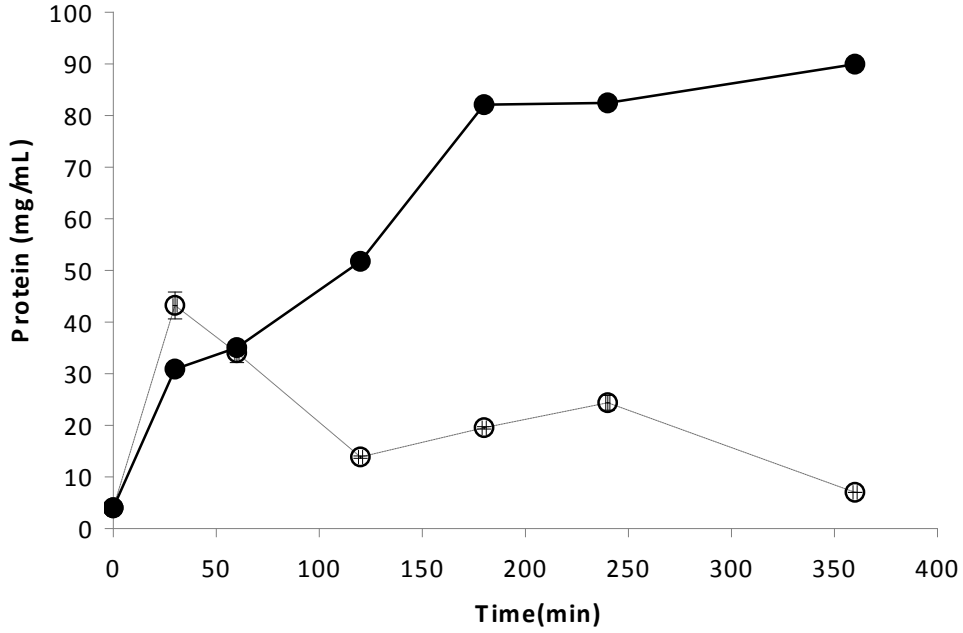
Treatment	MDA concentration (nmol L^{-1})
Control	4.39 ± 0.31^{a}
Hydrolysate (undiluted)	1.64 ± 0.11^{b}
Hydrolysate (1:5)	2.34 ± 0.82^{b}

The results represent the means ± standard deviations of three independent assays. Values followed by different small letters indicate significant difference ($P<0.05$) by the Tukey test.

Table 2. Application of soybean hydrolysate in salmon to inhibit lipid oxidation (the efficiency was verified by TBARS).

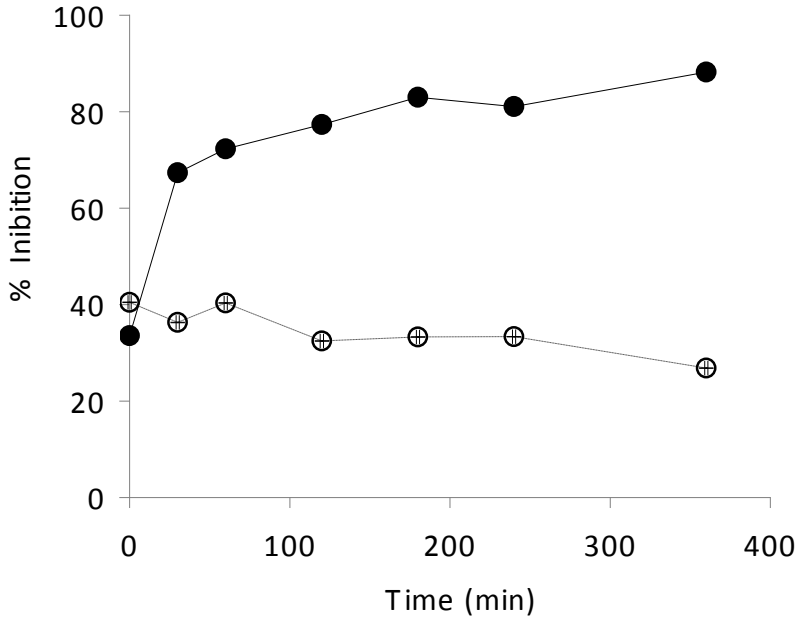
Treatment	MDA concentration (nmol L^{-1})
Control	12.52 ± 0.65^{a}
Hydrolysate (undiluted)	4.37 ± 1.03^{b}
Hydrolysate (1:5)	11.05 ± 0.84^{a}

The results represent the means ± standard deviations of three independent assays. Values followed by different small letters indicate significant difference ($P<0.05$) by the Tukey test.

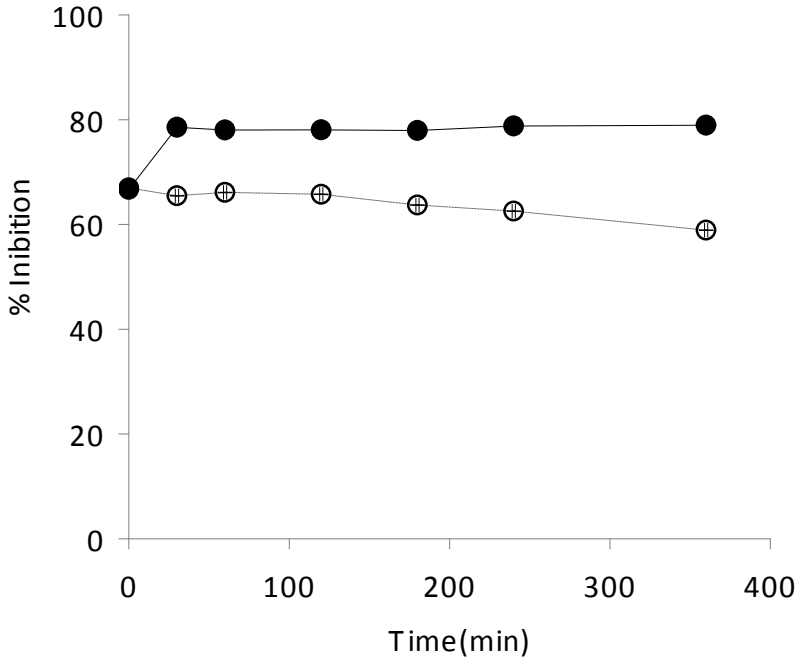


Oliveira et al., Fig. 1

(A)



(B)



Oliveira et al., Fig. 2

Production of soy protein hydrolysates with improved functional properties by a microbial protease

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Abstract

Soybean proteins are widely used in many foods as functional and nutritional ingredients. The hydrolysates of soy protein have been shown to possess antioxidant activities and increased functional properties. The purpose of this study was to evaluate the effect of three variables (pH, temperature and enzyme/substrate ratio) in the production of soy protein hydrolysates using a protease produced by *Chryseobacterium* sp. kr6. For this, a 2^3 factorial experiment was employed. Antioxidant activity of the hydrolysates was determined by ABTS, DPPH, reducing power and Fe-chelating activity methods. Foaming and emulsifying activities of the hydrolysates were also analyzed as responses. In the range studied all the variables (pH, temperature and E/S), as well as the interaction of these variables had significant effect on the enzymatic hydrolysis, considering the amount of soluble protein. The hydrolysate produced at 45 °C and using 8000 U/g (E/S) showed a capacity to inhibit the radical ABTS around 43 %. Models for soluble protein and ABTS were obtained. However, in the range studied, variables tested had no significant influence on the ability to scavenge the DPPH radical. For the foaming capacity the temperature and E/S ratio were significant, showing higher values at 45 °C and 8000 U/g. As for the emulsifying capacity only the E/S ratio had a significant effect. Different variables have different effect on the response examined, but the temperature and the E/S ratio had more significant effect in obtaining hydrolysates with specific characteristics.

Keywords: Protein; soy; protease; functional properties; hydrolysis; *Chryseobacterium*

1. Introduction

Proteins of plant and animal origin are important ingredients in diverse food formulations. Some functional properties of proteins can be improved by enzymatic hydrolysis under controlled conditions (Quaglia & Orban, 1990). Hydrolysis potentially influences the molecular size, hydrophobicity and exposition of polar groups of the protein (Adler-Nissen, 1986; Kristinsson & Rasco, 2000). The characteristics of the hydrolysates directly affect the functional properties and their use as food ingredients (Kristinsson & Rasco, 2000). Hydrolysates of soy protein have been shown to possess antioxidant activities (Beermann *et al*., 2009, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Pena-Ramos & Xiong, 2002). Furthermore, enzymatic hydrolysis also influences the emulsifying and foaming properties of food proteins (Gbogouri *et al*., 2004; Kristinsson & Rasco, 2000). These functional characteristics are important in the formulation of foods for consumer acceptance (Lamsal *et al*., 2006).

Microbial proteases have many applications in foods, they can be used to produce functional peptides from soy protein (Wang & Meija, 2005). Temperature, pH, enzyme/substrate (E/S) ratio are very important parameters in the production of protein hydrolysates by microbial proteases. Commercial proteases as neutral protease and validase are produced by *Bacillus subtilis* and *Aspergillus oryze*, respectively. These enzymes have been used to produce hydrolysates with improved properties (Zhang *et al*., 2010). However, novel enzymes may be useful since their specificities may generate peptides with unique characteristics. The keratinolytic strain *Chryseobacterium* sp. kr6 produces a novel metalloprotease that belongs to the family M14 (Riffel *et al*. 2006). This protease hydrolyses keratins, casein, albumin, showing potential for production of protein hydrolysates.

The purpose of this study was to evaluate the effect of three variables (pH, temperature and E/S ratio) in the production of soy protein hydrolysates using a protease produced by *Chryseobacterium* sp. kr6. The antioxidant, emulsifying and foaming capacity of the resulting hydrolysates were evaluated.

2. Materials and methods

2.1 Microorganism and cultivation conditions

The proteolytic strain *Chryseobacterium* sp. kr6 was kept in feather meal agar (Riffel *et al*., 2003). The microbial culture was performed in feather meal broth, containing the following components (g L^{-1}): NaCl (0.5), KH_2PO_4 (0.4), $CaCl_2$ (0.015) and feather meal (10.0). The pH was adjusted to 8.0. The cultures were performed in 250 mL erlenmeyer flasks (working volume of 50 mL) at 30°C for 48 h in a rotary shaker at 150 rpm. After the growth period, the culture was centrifuged for 20 min at 10,000 x *g* and 4°C and the supernatant was collected (Silveira *et al*., 2008)

2.2 Partial purification of the enzyme

Solid ammonium sulfate was added under stirring to culture supernatant to reach 50% (w/v) saturation. The mixture was centrifuged at 10,000 x *g* for 20 min at 4°C, the resulting pellet was dissolved in 50 mmol L^{-1} Tris-HCl pH 8.0 and centrifuged again to remove any insoluble material. The concentrated sample was applied to a Sephadex G-100 gel permeation column (0.8 x 30 cm) equilibrated and eluted with 50 mmol L^{-1} Tris-HCl pH 8.0. Fractions with proteolytic activity were pooled and used in the experiments (Riffel *et al*., 2006).

2.3 Determination of enzyme activity

The enzyme activity was determined using azocasein as substrate essentially as described elsewhere (Thys *et al*., 2004). One unit of enzyme activity was defined as the amount of enzyme required to produce a change in absorbance of 0.01 (420 nm) under the assay conditions (40 min at 45^{o}C, pH 8.0). Controls were prepared by the same procedure, except that the stop was added before the enzyme.

2.4 Enzymatic hydrolysis of soy protein

The peptides were prepared by hydrolysis of soy protein using the partially purified protease. The protein was dissolved in Tris-HCl the hydrolysis was performed using different concentration of enzyme in a water bath at different temperatures and rotation of 150 rpm. After 6h incubation, the hydrolysis was stopped by boiling in a water bath (5 min). The hydrolysates were centrifuged (10,000 x *g* for 20 min) to remove the insoluble materials and then stored at -18°C until further analyses (Rossini *et al*., 2008).

2.5 Experimental design

At this stage the purpose was to determine the influence of three variables, namely temperature, initial pH and E/S ratio. For this purpose, the response surface approach by using a central composite design with five coded levels was performed. For the three factors, this design was made up of a full 2^3 factorial design with its eight points augmented with three replications of the center points (all factors at level 0) and the six star points, that is, points having for one factor an axial distance to the center of ±α, whereas the other two factors are at level 0. The axial distance α was chosen to be 1.68 to make this design orthogonal. Real and codified values are shown in Table 1. A set of

17 experiments was carried out (Table 2). The central values (0 level) chosen for the experimental design were: temperature at 45°C; initial pH at 8.0 and E/S ratio at 4000 U/g. In developing the regression equation, the test factors were coded according to the following equation:

$$xi = (X_i - X_0)/\Delta X_i \quad (1)$$

where x_i is the coded value of the independent variable, X_i the natural value of the independent variable, X_0 the natural value of the independent variable at the center point and ΔX_i the step change value (ΔX_i is 1.5 for initial pH, 10 for temperature and 3571 U/g for concentration of enzyme/substrate). For a three factors system, the model equation is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (2)$$

where Y, predicted response, b_0, intercept; b_1, b_2, b_3, linear coefficients; b_{11}, b_{22}, b_{33}, squared coefficients and b_{12}, b_{13}, b_{23}, interaction coefficients. Results were analyzed by the Experimental Design Module of the Statistica 7.0 software (Statsoft, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables. The response surface plots were drawn to illustrate the main and interactive effects of the independent variables on hydrolysates production (Myers & Montgomery, 2002).

Hydrolysis occurred in 250 mL Erlenmeyer flask with a volume of 10 mL as described above for 6 hours. The hydrolysates were frozen and stored at -18°C until further analysis: soluble protein concentration, antioxidant activity, emulsifying activity, foaming capacity, metal chelating activity and reducing power, as described below. Each experiment was a control (without enzyme).

2.5 Determination of soluble protein concentration

The concentration of soluble protein was determinate by the Folin phenol reagent method (Lowry *et al*., 1951). Bovine serum albumin (BSA) was used as standard. The measurements were performed using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu do Brasil, Agua Branca, SP, Brazil).

2.6 Determination of antioxidant activity by ABTS (3-etilbenztiasolina-6-sulfonic acid)

The antioxidant activity was determined using the ABTS cation radical (3-etilbenztiasolina-6-sulfonic acid) essentially as described by Re *et al*., (1998).

2.7 Determination of antioxidant activity by DPPH (1,1-Diphenyl-2-picrylhydrazyl)

The DPPH method used was described by Brand-Williams *et al*.(1995), based on the capture of the radical DPPH (2,2-diphenyl-1-picryl-hydrazyl) by antioxidants, producing a decrease in absorbance at 515 nm.

2.10 Metal chelating activity

The chelating activity of Fe^{2+} was measured using the method described by Chang *et al*. (2007) with slight modifications. One milliliter of sample was mixed with 3.7 mL distilled water and then the mixture was reacted with 0.1 mL of 2 mmol L^{-1} FeSO4 (Fe^{2+}) and 0.2 mL of 5 mmol L^{-1} ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine). After 10 min the absorbance was read at 562 nm. One milliliter of distilled water, instead of sample, was used as a control. The results were expressed as:

$$\text{Chelating Activity (\%)} = [1 - (A/A_0)] \times 100 \quad (3)$$

where A is the absorbance of the test and A_0 is the absorbance of the control.

2.11 Determination of reducing power

Reducing power was measured by mixing the hydrolysates with 2.5 mL phosphate buffer (0.2 mol L^{-1}, pH 6.6) and 2.5 mL of potassium ferricyanide (10 mg mL^{-1}), and then the mixture was incubated at 50 °C for 20 min. Then, 2.5 mL trichloroacetic acid (10% v/w) was added and the mixture was centrifuged (3000 × *g* for 10 min). Supernatant (1 mL) was mixed with 2.5 mL of distilled water and 0.2 mL of ferric chloride (1 mg mL^{-1}), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated greater reducing power (Corrêa *et al*., 2011)

2.8 Emulsifying activity

The capacity of the hydrolysates to emulsify hydrophobic substances was tested. Each hydrolysate was mixed with 2 mL of soybean oil and allowed to stand for 24 h at room temperature (Willumsen & Karlson, 1997). After this time the emulsifying index (E24) was determined by the following equation:

$$E24 = (height.of.the.layer / height.of.the.total) * 100 \quad (4)$$

2.9 Determination of the foaming capacity

The foaming capacity was determined by the method of Fernandez and Macarulla (1997) and Coffman and Garcia (1997) with minor modifications. Aliquots (500 µL) of each hydrolysate were mixed with 10 mL of distilled water and homogenized for 2 minutes in a mixer at maximum speed. Foaming was calculated according to the percentage increase in volume by the equation:

$$\%\, foa\min g = \left(\frac{V2 - V1}{V1}\right) * 100 \qquad (5)$$

Where V_1 is the volume after homogenized and V_2 is the volume before homogenized.

3. Results and discussion

Enzymatic hydrolysis was performed in a time of 6 h, 17 experiments were performed at varying temperatures, pH and E/S ratio (Table 2).

In the range studied the variables pH, temperature and E/S, as well as the interaction of pH and E/S, T and E/S had significant effect on the enzymatic hydrolysis, considering the amount of soluble protein (Figure 1a). The results of the second-order response surface models were examined by analysis of variance (ANOVA) and Fischer's F-test (Table 3). The model adequacy was checked by F-test and by R^2 value. The first one showed the value $F_{(9,7)}$ = 5.75 greater than F tabulated ($Ft_{(9.7)}$ = 3.68), which demonstrate significance for the regression model (Myers & Montgomery, 2002). Regressions obtained indicated the R^2 value of 0.761 for soluble protein. A value of R^2 > 0.75 indicates the aptness of the model (Myers and Montgomery, 2002). The model could explain 76.1 % of the variability for response (soluble protein). The following regression equation was obtained:

$$Y = 21.560 - 13.697x_1 + 4.341x_2 + 8.023x_3 + 11.417x_1^2 + 2.896x_2^2 + 8.388x_3^2 - 1.681x_1x_2 + 11.469x_1x_3 + 3.281x_2x_3$$

The response surface curve was then plotted (Figure 1). While the temperature decreases and pH increase the higher the amount of soluble protein obtained (Figure 1b). The experiment 7, which used a temperature of 35 °C and a pH 9.5 buffer solution, resulted the highest concentration (73 mg/mL). These results are in agreement with the optimal conditions for protease activity produced by *Chryseobacterium* sp. kr6 (Silveira

et al., 2008). Note that when the temperature remains constant (at level 0), and pH and E/S ratio increase, higher concentration of soluble protein is obtained (figure 1c). When the pH remains constant (at level 0), with lower values of temperature and E/S ratio higher concentrations of soluble protein are obtained (Figure 1d). The same effect is observed at higher values of temperature and E/S ratio (Figure 1d), however these conditions may not be interesting (due to higher cost).

The antioxidant activity of hydrolysates was evaluated by the ABTS and DPPH methods, and also by the reducing power and iron chelating activity (Table 2). The statistical analysis showed that temperature, E/S and interaction between them, in the range studied, have a significant effect on the production of hydrolysates with the ability to kidnap the ABTS radical (Fig. 2a). In temperature of 45°C and using 8000 U/g (E/S), hydrolysates were obtained with a capacity of inhibition of ABTS radical around 43%, while the control (45°C without addition of enzyme) resulted in 10 % capacity of inhibition of the radical. According to Zhang *et al* (2010), hydrolyzed soy protein using a commercial enzyme has the ability to sequester the radical ABTS around 29%. The results of the second-order response surface models were examined by analysis of variance (ANOVA) and Fischer's F-test (Table 4). Fisher's *F*-test showed the value $F_{(9,7)}$ = 18.75 which is greater than *F* tabulated ($Ft_{(9.7)}$ = 3.68), which demonstrate significance for the regression model (Myers & Montgomery, 2002). Regressions obtained indicated the R^2 value of 0.922 for ability to kidnap the ABTS radical showing that the model could explain 92.2 % of the variability for response. The following regression equation was obtained:

$$Y= 21.313 - 4.908x_1 + 2.536x_2 + 5.578x_3 + 2.849x_1^{\ 2} - 1.032x_2^{\ 2} + 3.592x_3^{\ 2} - 1.193x_1x_2 + 4.530x_1x_3 - 1.025x_2x_3$$

The response surface curve was then plotted (Figure 2). Note that maximum activity was achieved at lower temperatures and at higher pH values (Figure 2b). The interaction between temperature and E/S ratio was significant (Figure 2a), at high temperatures and high E/S ratio, greater values of antioxidant activity were obtained and the same was observed at low values of temperature and E/S ratio.

The radical scavenging capacity is related to the amount of soluble protein, in other words, the higher hydrolysis greater the antioxidant capacity of the hydrolysate (Figures 1 and 2).

We also analyzed the ability to capture the DPPH radical, but in range studied the temperature, pH and E/S had no significant effect for this response. However, the hydrolysates showed a high sequestration capacity of the radical DPPH, around 77% regardless of the conditions tested. Nevertheless, the controls (protein treated in same conditions without enzyme) also showed high values (67 ± 2%). Previous studies have shown that a series of food-derived peptides or protein hydrolysates were able to interact and capture the DPPH radical (Zhang *et al*., 2009).

For the iron chelating activity, as only the temperature had a significant effect (Figure 3), R^2 value was small (<0.75) and *F* value was smaller than *F* tabulated, the model was not considered adequately . Iron acts as a catalyst for the generation of hydroxyl radicals through the Fenton reaction (Pownall et al., 2006), which may contribute to diseases related to oxidative stress. In addition, transition metals can stimulate lipid peroxidation in foods, resulting in rancidity. Consequently, the chelating metal ions can contribute to the antioxidant activity of hydrolysates (Corrêa *et al*., 2011). The iron chelating activity of soy protein hydrolysates were maximum when the temperature was 61.8 °C and 28.2 °C (86% and 84%, respectively), the highest and lowest temperatures tested (Table 2). The controls had an ability to chelate iron ions around 45.4 ± 1.8%. Sheep caseinate

subjected to hydrolysis with protease from *Bacillus* sp. strain P7 at 45 °C, pH 8 and 30 min of hydrolysis, presented 83% iron chelating activity (Corrêa *et al*., 2011). Pownall *et al.* (2010) studied the pea protein isolate and found that it has a high ability to chelate iron ions around of 95%.

In the range studied, variables tested were not significant for the reducing power, however, some of the hydrolysates showed reducing power. This assay is based on the ability of a compound to reduce Fe^{+3} to Fe^{+2}. Consequently, the reduction capacity of protein hydrolysates indicates that they could act as electron donors, reducing the oxidized intermediates of lipid peroxidation processes, and suggesting that the reducing power contributes to the antioxidant activity (Zhu *et al*., 2006). Germ wheat proteins treated with alcalase were capable of reducing Fe^{+3} (Zhu *et al*., 2006), as well as sheep caseinates that had an absorbance at 700 nm between 0.607-1.094 in this trial (Corrêa *et al*., 2011). In this work values obtained were between 0.088 and 0.465.

The use of proteolytic enzymes is often an attractive means to improve the functional properties of food proteins, without losing its nutritional value (Clemente, 2000). The functional properties of a protein determine their behavior during storage, processing and food preparation (Moure *et al*., 2006). In this work, the hydrolysates were evaluated for their ability to foaming and emulsifying capacity. The statistical analysis showed that only the temperature (linear effect) in the range tested, had a significant effect in obtaining hydrolysates with a capacity of foaming (Figure 4). This effect has a value of -21.6 (results not shown), showing that at lower temperatures there are increased capacity of foaming. Values for capacity of foaming varied between 14.9 and 65.5% (Table 2). For the control it was not observed ability to foaming. It is known that the foam is related to the amount of protein, but according to Lin *et al.* (1974) other

components also participate in the formation of foam. Note that the larger the amount of soluble protein, higher the foaming capacity of the hydrolysates (Table 2).

For the emulsifying capacity, the statistical analysis of results showed that over the range studied only the E/S ratio had a significant effect (Figure 5). As $R^2 = 0.274$ it was not possible to construct a model for predicting the emulsifying capacity. Surowka *et al.* (2004) also found significant effect of the E/S ratio to obtain hydrolyzed soy protein with emulsifying capacity, and noted that the higher levels of the enzyme Neutrase, the higher the emulsifying capacity.

4. Conclusions

In this work we studied the effect of three variables, temperature, pH, and enzyme/ substrate ratio. The results showed that enzymatic hydrolysis of soy proteins by protease kr6 may be a promising alternative in obtaining natural antioxidants for use in foods, in addition to providing interesting functional properties. Models for soluble protein and ABTS were obtained. Depending on the desired features of hydrolysates is necessary to use a specific temperature, pH and E/S ratio.

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Figure captions

Figure 1: (a) Bar graph of standardized estimated effects of the different variables tested in soluble protein concentration of hydrolysates. The variables tested were temperature (T), pH and the enzyme/ substrate relation. The point at which the effect estimates were statistically significant (at P = 0.05) is indicated by the vertical line

(b) Response surface of soluble protein concentration, depending on the temperature and pH (enzyme/substrate ratio at level 0)

(c) Response surface of soluble protein concentration, depending on the pH and the enzyme / substrate relation (temperature at level 0).

(d) Response surface of soluble protein concentration, depending on the temperature and the enzyme/ substrate relation (pH at level 0)

Figure 2: (a) Bar graph of standardized estimated effects of the different variables tested in the the ability to capture the radical ABTS. The variables tested were temperature (T), pH and the enzyme/ substrate relation. The point at which the effect estimates were statistically significant (at P = 0.05) is indicated by the vertical line.

(b) Response surface of the capacity to remove the ABTS radical of the hydrolysates, depending on the temperature and the enzyme / substrate relation (pH at level 0).

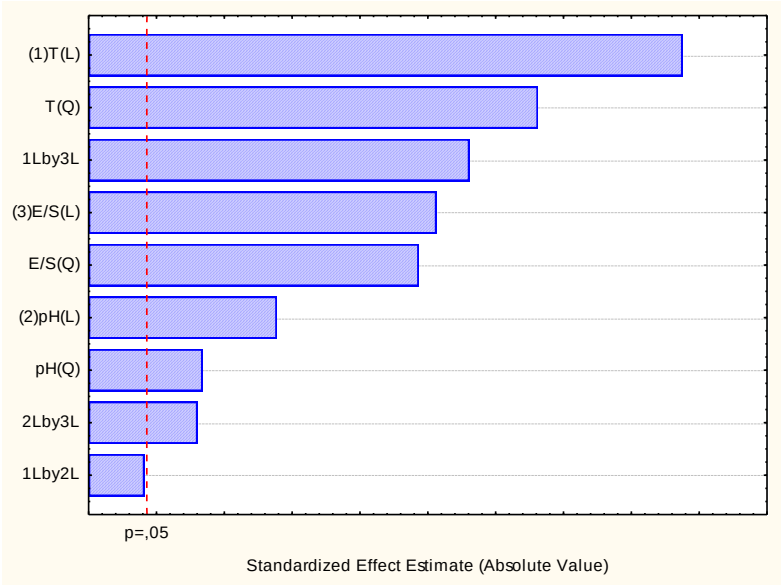
Figure 3: Bar graph of standardized estimated effects of the different variables tested in iron chelating activity of the hydrolysates. The variables tested were temperature (T),

pH and the enzyme/ substrate relation. The point at which the effect estimates were statistically significant (at P = 0.05) is indicated by the vertical line.

Figure 4: Bar graph of standardized estimated effects of the different variables tested in the capacity of foaming. The variables tested were temperature (T), pH and the enzyme/ substrate relation. The point at which the effect estimates were statistically significant (at P = 0.05) is indicated by the vertical line.

Figure 5: Bar graph of standardized estimated effects of the different variables tested in emulsifying capacity of the hydrolysates. The variables tested were temperature (T), pH and the enzyme/ substrate relation. The point at which the effect estimates were statistically significant (at P = 0.05) is indicated by the vertical line.

Figure 1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T(Q)
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(3)E/S(L)
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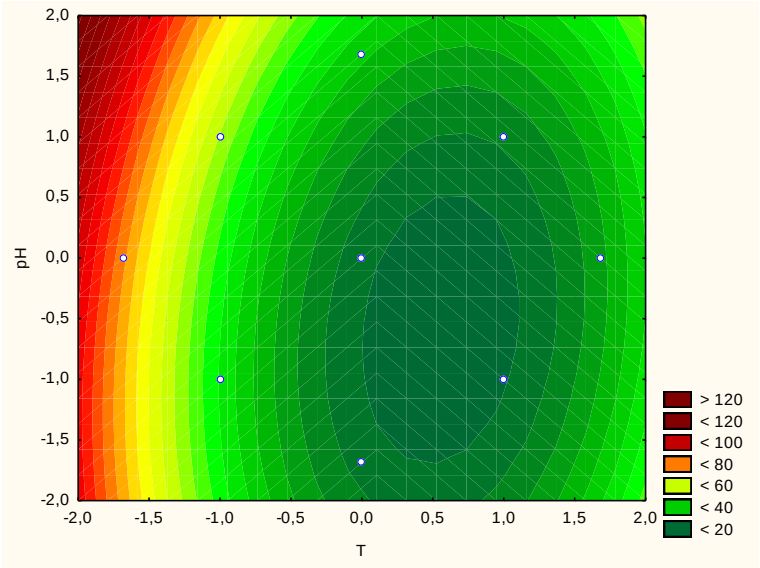
p=,05
Standardized Effect Estimate (Absolute Value)

(a)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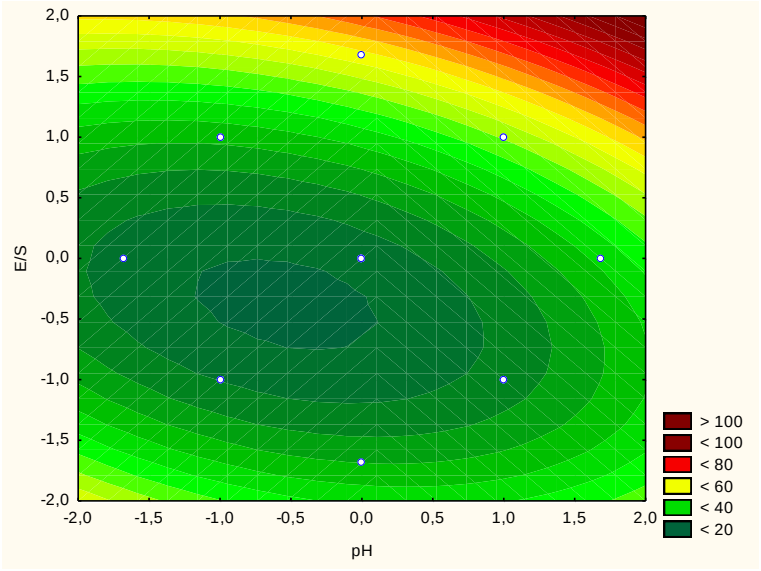
< 20

(b)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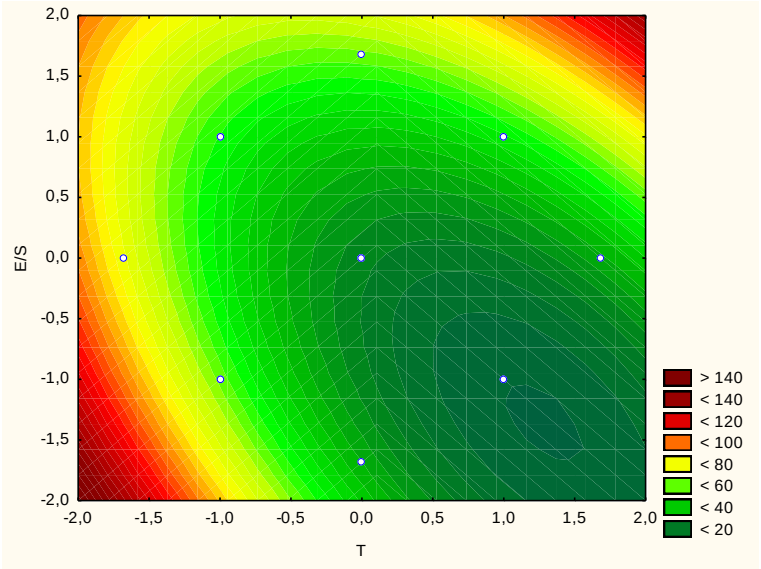
(c)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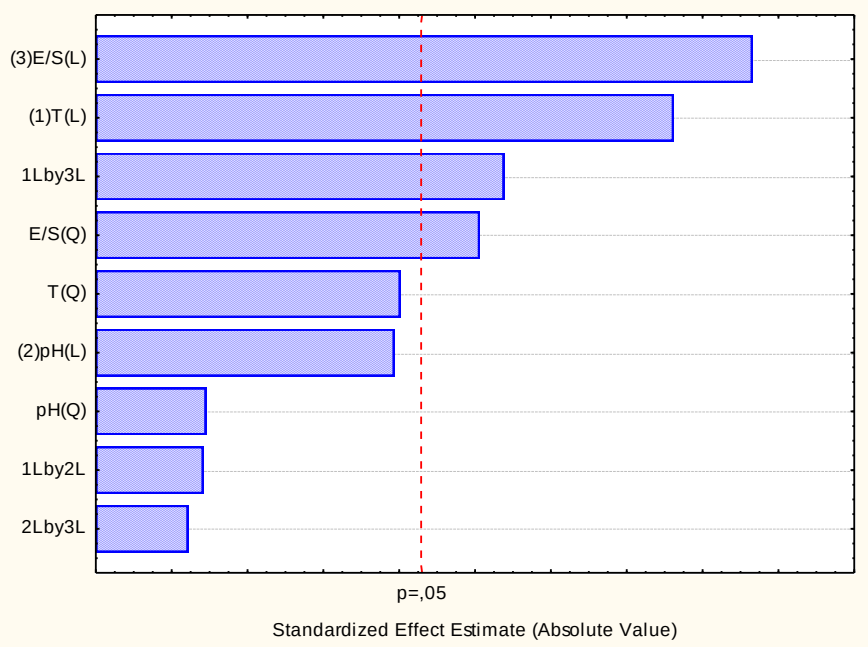
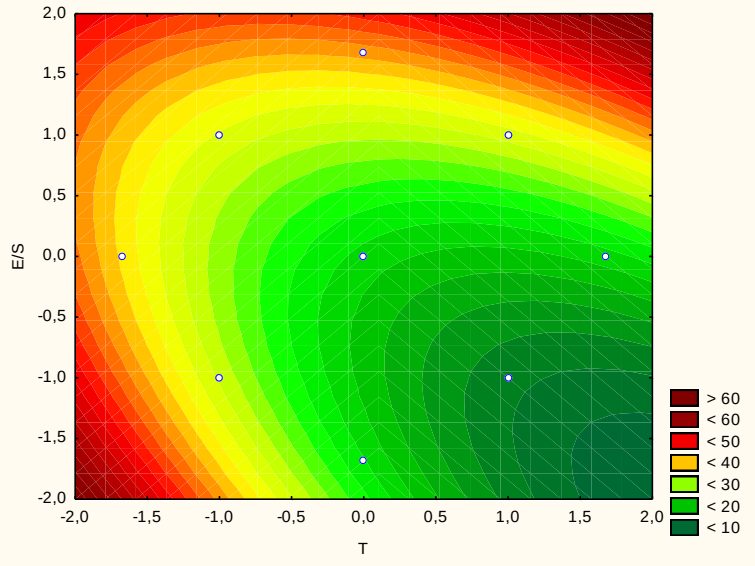
(d)

Figure 2


(3)E/S(L)
(1)T(L)
1Lby3L
E/S(Q)
T(Q)
(2)pH(L)
pH(Q)
1Lby2L
2Lby3L
p=,05
Standardized Effect Estimate (Absolute Value)

(a)


2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
E/S
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
2,0
T
> 60
< 60
< 50
< 40
< 30
< 20
< 10

(b)

Figure 3

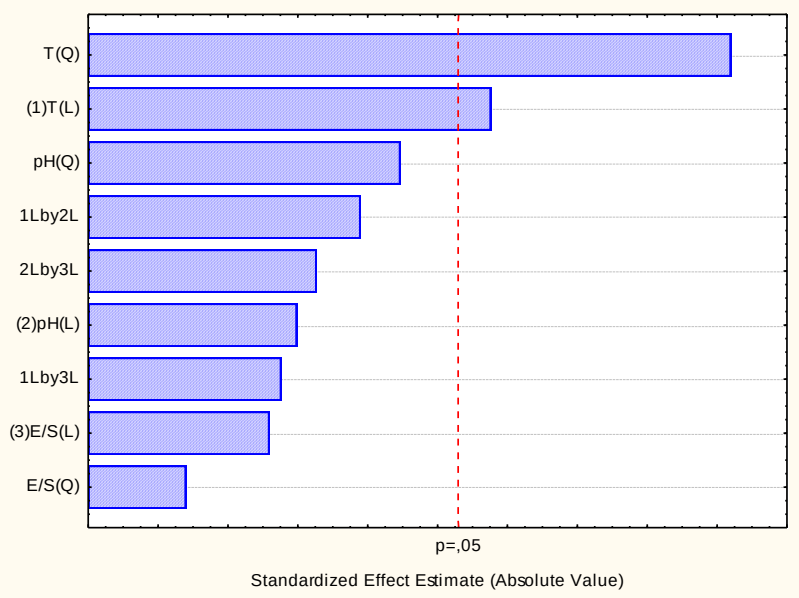

T(Q)
(1)T(L)
pH(Q)
1Lby2L
2Lby3L
(2)pH(L)
1Lby3L
(3)E/S(L)
E/S(Q)
p=,05
Standardized Effect Estimate (Absolute Value)

Figure 4

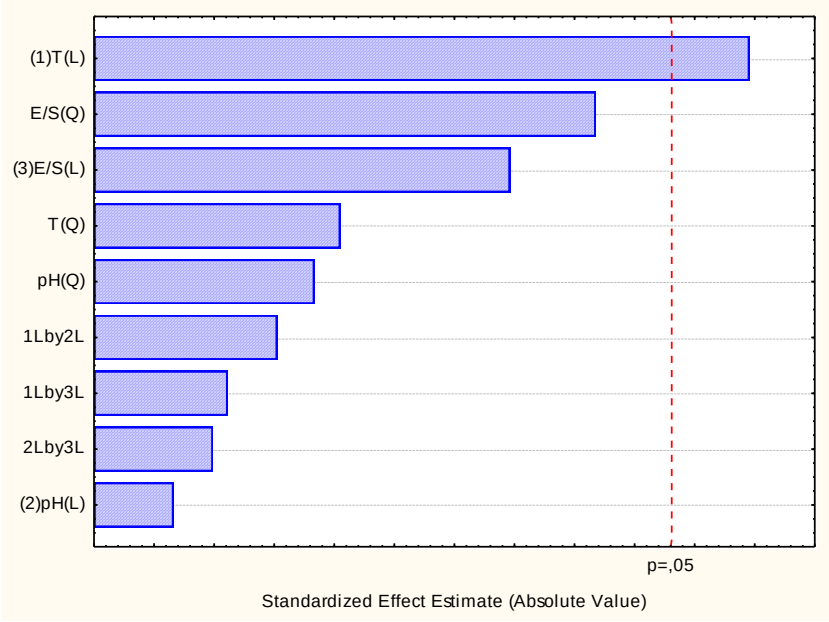

(1)T(L)
E/S(Q)
(3)E/S(L)
T(Q)
pH(Q)
1Lby2L
1Lby3L
2Lby3L
(2)pH(L)
p=,05
Standardized Effect Estimate (Absolute Value)

Figure 5

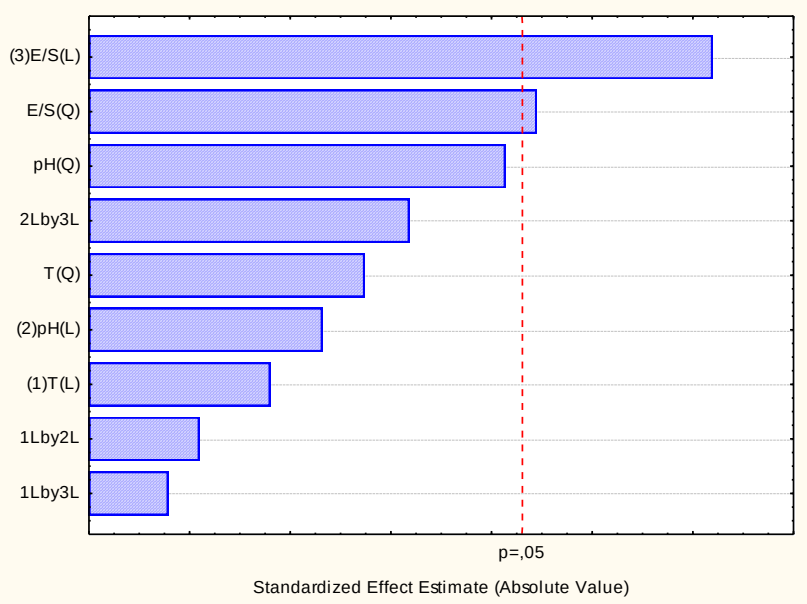

(3)E/S(L)
E/S(Q)
pH(Q)
2Lby3L
T(Q)
(2)pH(L)
(1)T(L)
1Lby2L
1Lby3L
p=,05
Standardized Effect Estimate (Absolute Value)

Table 1 Values of independent variables at different levels of the 2^3 factorial design

Independent variable	**Symbol**	**-1.68**	**-1**	**0**	**+1**	**+1.68**
Temperature (°C)	X_1	28.2	35	45	55	61.8
pH	X_2	5.48	6.5	8	9.5	10.52
Enzyme/substrate (U/g)	X_3	100	1649	4000	6321	8000

Table 2 Experimental design and results of the 2^3 factorial design

Test	T(°C) (X_1)	pH (X_2)	E/S (U/g) (X_3)	soluble protein (mg.mL^{-1})	ABTS (%)	DPPH (%)	Reducing power (Absorbance-700 nm)	Fe^{2+}chelating ability (%)	Determination of the foaming (%)	Emulsifying activity (%)
1	35	6.5	1649	67.931	24.682	72.431	0.140	39.752	51.092	4.672
2	55	6.5	1649	14.733	12.156	75.782	0.094	10.836	18.416	5.726
3	35	9.5	1649	65.636	35.623	74.261	0.309	79.221	41.945	8.382
4	55	9.5	1649	16.683	14.915	73.287	0.093	18.023	21.436	5.287
5	35	6.5	6321	51.938	28.948	74.445	0.191	46.105	54.172	7.396
6	55	6.5	6321	55.582	31.125	74.986	0.248	36.772	28.963	4.552
7	35	9.5	6321	73.739	32.429	74.931	0.464	60.298	51.097	5.167
8	55	9.5	6321	59.685	33.192	73.284	0.294	18.745	37.092	4.581
9	28.2	8.0	4000	66.736	40.402	72.028	0.265	84.582	52.663	4.867
10	61.8	8.0	4000	22.487	18.659	70.649	0.271	86.123	19.794	10.533
11	45	5.5	4000	10.531	13.990	72.527	0.088	3.691	14.882	2.941
12	45	10.5	4000	30.589	23.123	77.440	0.247	12.565	18.413	5.412

13	45	8.0	100	26.085	20.369	71.042	0.290	46.403	35.836	12.012
14	45	8.0	8000	46.037	42.832	64.869	0.465	10.458	65.491	5.132
15	45	8.0	4000	21.932	21.915	74.532	0.378	29.347	31.254	6.254
16	45	8.0	4000	22.039	23.316	71.581	0.262	37.342	33.275	5.283
17	45	8.0	4000	23.987	18.672	74.573	0.210	21.621	18.412	5.132

Table 3 Analysis of variance for the model and the regression coefficients for soluble protein concentration.

Source	**Sum of square**	**Degree of freedom**	**Mean square**	***F*-value**	***P*-value**
T (L)	2559.80	1	2559.79	1916.25	0.0005[a]
T (Q)	1465.39	1	1465.39	1096.99	0.0009[a]
pH (L)	257.14	1	257.14	192.50	0.0052[a]
pH (Q)	94.27	1	94.27	70.57	0.0139[a]
E/S (L)	878.20	1	878.20	657.41	0.0015[a]
E/S (Q)	790.92	1	790.92	592.08	0.0017[a]
T (L) by pH (L)	22.61	1	22.61	16.93	0.0543
T (L) by E/S (L)	1052.26	1	1052.26	787.72	0.0013[a]
pH (L) by E/S (L)	86.13	1	86.13	64.48	0.0152[a]
Lack of Fit	974.69	5	194.94	145.93	0.0068[a]
Pure Error	2.67	2	1.34		
Total sum of square	7643.51	16			

[a] Statistically significant at 95% of confidence level.

Table 4 Analysis of variance for the model and the regression coefficients for ABTS

Source	**Sum of square**	**Degree of freedom**	**Mean square**	***F*-value**	***P*-value**
T (L)	328.64	1	328.6364	58.01649	0.0168[a]
T (Q)	91.30	1	91.2983	16.11753	0.0568
pH (L)	87.76	1	87.7618	15.49321	0.0589
pH (Q)	11.97	1	11.9671	2.11264	0.2832
E/S (L)	424.53	1	424.53	74.96	0.0131[a]
E/S (Q)	145.05	1	145.05	25.61	0.0369[a]
T (L) by pH (L)	11.38	1	11.38	2.01	0.2921
T (L) by E/S (L)	164.17	1	164.17	28.98	0.0328[a]
pH (L) by E/S (L)	8.41	1	8.41	1.48	0.3474
Lack of Fit	42.05	5	8.41	1.48	0.4492
Pure Error	11.33	2	5.66		
Total sum of square	1343.76	16			

[a] Statistically significant at 95% of confidence level.

5 .Conclusão

Este estudo foi realizado para avaliar propriedades funcionais e biológicas dos hidrolisados protéicos de soja, sua capacidade de evitar a oxidação em carne de porco e peixe e ainda verificar a influência do pH, temperatura e relação enzima/substrato na obtenção de hidrolisados protéicos com elevada atividade antioxidante e propriedades funcionais.

Assim, foi possível notar que os hidrolisados protéicos de soja possuem uma considerável atividade antioxidante observada pela capacidade de seqüestro dos radicais DPPH e ABTS. E ainda a aplicação desses hidrolisados em diferentes tipos de carne se mostraram capazes de evitar a oxidação.

Quando analisadas a influência do pH, temperatura e relação enzima substrato na obtenção dos hidrolisados protéicos com diferentes características foi possível observar que algumas variáveis afetam de maneira significativa uma determinada resposta, enquanto outras não. Como por exemplo, a temperatura e a concentração de enzima/substrato (nas faixas estudadas), assim como a interação entre essas variáveis, possuem um efeito significativo para a obtenção de hidrolisados com capacidade de sequestro do radical ABTS. Enquanto que para a obtenção de hidrolisados com capacidade de sequestro do radical DPPH nenhuma das variáveis possuiu efeito significativo. Ainda, observou-se que dependendo da função que será dada ao hidrolisado é necessário utilizar uma temperatura, pH e concentração enzima/substrato adequado. Os resultados mostraram que a hidrólise enzimática da proteína de soja pode ser uma alternativa promissora na obtenção de antioxidantes naturais, além de conferir propriedades funcionais interessante

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