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INOVAÇÃO NA BIOTECNOLOGIA MEDIANTE APLICAÇÃO DE PROTEASES COMERCIAIS E ENZIMA RECOMBINANTE

THAMARA THAIANE DA SILVA CROZATTI

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Tese apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá e ao Projeto de Doutorado em Inovação do CNPq, como parte dos requisitos para obtenção do título de doutor em Ciência de Alimentos.

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Prof. Dr. Eduardo César Meurer

Prof. Dr. Daniel Tait Vareschini

Prof. Dr. Marco Aurelio Schuler de Oliveira

Profa. Dra. Grasiele Scaramal Madrona

Profa. Dra. Graciette Matioli
Orientadora

Maringá – 2023

Orientadora

Profa. Dra. Graciette Matioli

BIOGRAFIA

Thamara Thaiane da Silva Crozzati, filha de Paulo Cleber da Silva e Olinda Aparecida dos Santos da Silva, nasceu em São Paulo - SP, no dia 06 de janeiro de 1994. Concluiu o ensino médio no Colégio Haya, em Osasco – SP. Possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá, campus Maringá (2017), e mestrado em Ciência de Alimentos pela mesma universidade (2019). Possui especialização em Ciência e Tecnologia de Alimentos pela Universidade Federal de Pelotas (2022).

Em 2014, ingressou ao Laboratório de Biotecnologia Enzimática, pertencente ao Departamento de Farmácia da Universidade Estadual de Maringá, onde participou de Projetos de Inicação Científica, sob orientação da Profa. Dra. Graciette Matioli. Têm experiência na área de biotecnologia, com ênfase em biotecnologia enzimática, corantes naturais, peptídeos bioativos e resíduos industriais. Atualmente, é aluna regular do Programa de Doutorado em Inovação (DAI) do CNPq junto ao Programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá também sob orientação da Profa. Dra. Graciette Matioli.

Dedico

À minha família, por sempre acreditar e ser a maior riqueza e força da minha vida...

"Acima de tudo se cumbram de amor,
que é o vínculo perfeito."
(Colossenses 3:14)

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Primeiramente gostaria de agradecer a Deus, por me mostrar que com amor e fé tudo é possível. Sua luz e preceitos são responsáveis por guiar todos os meus passos e fortelecer diariamente meu coração e minha esperança.

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Ao Prof. Dr. Eduardo César Meirer e todos os integrantes do Laboratório de Espectometria de Massas da Universidade Federal do Paraná, campus Jandaia do Sul, por abrir as portas do laboratório e me fazer sentir em casa, pela confiança e tantos ensinamentos. Que sorte a minha encontrar de pessoas de bem e bom coração nessa trajetória.

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Agradeço, também, aos órgãos financiadores CNPq, CAPES e Fundação Araucária pelo suporte financeiro durante toda minha caminhada científica.

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APRESENTAÇÃO

Esta pesquisa foi desenvolvida com o apoio de uma equipe multidisciplinar por meio de parcerias estabelecidas entre o Laboratório de Biotecnologia Enzimática (DFA-UEM), empresa BRF S.A., Laboratório de Espectometria de Massas (UFPR) e Laboratório de Bioquímica de Procariotos (DBQ -UEM).

A presente tese de doutorado está apresentada na forma de dois artigos científicos:

1. **AUTORES:** Thamara Thaiane da Silva Crozatti, Juliana Harumi Miyoshi, Angélica Priscila Parussolo Tonin, Larissa Fonseca Tomazini, Marco Aurélio Schuler Oliveira, Jose Uebi Maluf, Eduardo Cesar Meurer e Graciette Matioli.
   TÍTULO: Obtaining of bioactive di and tripeptides from enzymatic hydrolysis of Soybean meal and its protein isolate using Alcalase® and Neutrase®
   REVISTA: International Journal of Food Science and Technology.
   Artigo publicado (Fator de Impacto 3,612 – Qualis A2).

2. **AUTORES:** Thamara Thaiane da Silva Crozatti, Paula Vitória Larentis, Vanderson Carvalho Fenelon, Juliana Harumi Miyoshi, Júlia Rosa de Brito, Giovanna da Silva Salinas, Beatriz de Oliveira Mazzotti, Giovanni Cesar Teles, Quirino Alves de Lima Neto e Graciette Matioli
   TÍTULO: Challenges and alternatives for the production of cyclodextrins from the CGTase enzyme from recombinant *Bacillus subtilis* WB800.
   REVISTA: Food Science and Technology.
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RESUMO GERAL

Inovação esta aliada ao processo de transformação e criação de um novo produto, ideia ou serviço, de forma que o mesmo seja entregue a sociedade e legitimado pelo seu valor. Nesse contexto, o Programa de Doutorado Acadêmico em Inovação (DAI) refere-se a uma iniciativa do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) para que os Programas de Pós-Graduação possam fomentar projetos de interação com empresas por meio de suas teses. Considerando o exposto acima, esta tese teve como foco a inovação em biotecnologia enzimática e está apresentada na forma de dois artigos científicos.

ARTIGO 1 - Obtenção de di e tripeptídeos bioativos a partir da hidrólise enzimática do farelo de soja e seu isolado protéico utilizando Alcalase® e Neutrase®

INTRODUÇÃO. Peptídeos bioativos são cadeias curtas de aminoácidos unidas por ligações peptídicas. Possui especial interesse devido sua alta bioatividade e, principalmente, a suas propriedades funcionais. Portanto, trata-se de compostos que atendem as demandas do mercado moderno, o qual que está cada vez mais interessado na seleção de alimentos de maior qualidade e que possam estar aliados a promoção de saúde.

Peptídeos bioativos podem ser obtidos a partir de diferentes métodos, com destaque especial para os processos de hidrólises enzimáticas, que são vantajosos para o setor alimentício, especialmente por apresentar vantagens como maior controle do grau de quebra da proteína, baixo custo e por ser uma potente ferramenta de liberação de peptídeos biologicamente ativos. O maior número de peptídeos isolados é proveniente de fontes animais e vegetais e a proteína de soja tem se evidenciado uma fonte rica de peptídeos bioativos. Assim, o uso de subprodutos provenientes do processamento da soja também pode ser uma alternativa próspera para o desenvolvimento de alimentos ricos em propriedades bioativas tanto para consumo humano quanto animal.

OBJETIVOS. Considerando o interesse na obtenção de compostos bioativos com significativa bioatividade e propriedade funcionais, o presente trabalho objetivou aplicar proteases comerciais para obtenção de di e tripeptídeos bioativos provenientes de subprodutos da soja e, por consequência, agregar maior valor aos mesmos.

MATERIAL E MÉTODOS. O farelo de soja desengordurado (SM) foi fornecido pela empresa BRF S.A. Foi realizada a análise de composição centesimal do SM. Foi preparado o isolado de proteína de soja (SPI), o qual foi obtido por extração aquosa a partir do SM. As hidrólises enzimáticas do SM e do SPI foram realizadas isoladamente com as enzimas comerciais Alcalase® (pH 8 a 55 °C) (SMA e SPIA) e Neutrase® (pH 7 a 50 °C) (SMN e SPIN) com 10 mg para 1% substrato (m/m) por 4 h. O rendimento dos hidrolisados (%) foi calculado pela razão da massa do hidrolisado obtida e a massa inicial do SM (g) e SPI (g).

Foi determinado o teor de proteína bruta pelo método de Kjeldahl e foi mensurado o grau de hidrólise das reações utilizando reagente OPA a 340 nm. Foi realizado eletroforese de proteína em gel de poliacrilamida desnaturante (SDS-PAGE) e determinado a atividade antioxidante dos hidrolisados pelos métodos de atividade sequestrante de radicas DPPH e ABTS.

A identificação de peptídeos bioativos foi realizada pela técnica de rápida absorção por LC-MS/MS utilizando um sistema inovador, que consiste no rastreamento rápido de moléculas protonadas de di e tripeptídeos, baseado na perda neutra (NL) de 46 Da, sem necessidade de pré-tratamento ou separação cromatográfica demorada.

RESULTADOS E DISCUSSÃO. A análise da composição centesimal realizada no MS revelou um teor de proteína total bastante elevado (49,31 ± 0,06%), o que indica que o subproduto é uma fonte promissora de peptídeos bioativos. Os valores dos rendimentos obtidos na preparação do SPI e nas hidrólises foram: SPI 22,99 ± 0,23%; SMA 16,80 ± 0,10%; SMN 16,40 ± 0,30%; SPIA 22,10 ± 1,00% e SPIN 22,94 ± 0,23%, destacando-se maior eficiência nos hidrolisados SPI do que nos hidrolisados SM.

Os resultados do teor de proteína bruta determinados para SPI e hidrolisados foram: 102,6 ± 3,62% para SPI; 71,8 ± 1,43% para o hidrolisado de SMA; 74,5 ± 1,04% para SMN; 98,8 ± 0,33% para SPIA e 99,7 ± 0,34% para SPIN. Tais resultados se mostraram satisfatórios, pois um produto comercial de isolado de proteína de soja deve conter no mínimo 90% de proteína. Para a hidrólise realizada no SM, foi observado um teor de proteína bruta acima de 70%, o que sugere que a hidrólise também foi eficaz neste caso. Os resultados obtidos na análise de grau de hidrólise revelaram uso da enzima Alcalase® apresentou melhores resultados (16,72 ± 0,25% e 12,59 ± 0,80% para SMA e SPIA, respectivamente) em comparação com a enzima Neutrase® (8,45 ± 0,62% para SMN e 1,29 ± 0,28% para SPIN), o que indica que a enzima com alta atividade proteolítica pode ter influenciado o comprimento da cadeia peptídica e aumentado a quantidade de aminoácidos livres. O SDS-PAGE revelou que ambos os tratamentos enzimáticos resultaram em peptídeos com massas menores que 25 KDa. A atividade sequestrante de radicas DPPH e ABTS revelaram que a hidrólise realizada com Alcalase® e Neutrase® melhorou a atividade antioxidante de SM e SPI, mas o SPI se destacou por apresentar maior atividade antioxidante (406, 33 ± 0,22 µ mol de TE/mg de amostra) do que SM e seus hidrolisados.

A análise de LC-MS/MS exibiu que a aplicação do processo de hidrólise com a protease comercial Alcalase® no SM promoveu a obtenção de 19 peptídeos (16 di e 03 e tripeptídeos). O hidrolisado SPIA gerou 27 dipeptídeos e 24 tripeptídeos. O emprego das proteases comerciais gerou peptídeos com bioatividades que corresponderam principalmente à atividade inibitória da ECA, à atividade inibitória da dipeptidil peptidase IV (DPP-IV), atividade antioxidante, ação estimulante e atividade inibitória da renina.

CONCLUSÕES. O processo de hidrólise da proteína de soja com Alcalase® proporcionou a obtenção de proteínas com cadeias mais curtas, características de peptídeos com propriedades bioativas. A hidrólise de SM e SPI potencializou significativamente a capacidade antioxidante da proteína de soja, principalmente com o uso de Alcalase®. O sistema inovador utilizado para a identificação de di e tripeptídeos por LC-MS/MS foi eficiente para o estudo de subprodutos da soja e identificou um número significativo de di e tripeptídeos, que apresentaram seqüências de bioatividades antioxidantes, anti-hipertensivas e antidiabéticas. Assim, a aplicação de processos biotecnológicos realizados com enzimas comerciais pode ser uma alternativa promissora para melhorar as características tecnológicas e funcionais do subproduto do farelo de soja.

Palavras-chave: Farelo de soja, hidrólise enzimática, di-peptidos, tri-peptidos, bioatividades.

ARTIGO 2 - Desafios e alternativas para a produção de ciclodextrinas a partir da enzima CGTase de *Bacillus subtilis* WB800 recombinante.

INTRODUÇÃO. As ciclodextrinas (CDs) são oligossacarídeos cíclicos obtidas a partir da reação de transglicosilação do amido, a qual é catalisada pela enzima ciclomaltodextrina glucanotransferse (CGTase). Devido a característica apolar da cavidade as CDs possuem a capacidade de encapsular inúmeras moléculas. Embora apresente alta aplicabilidade o custo de produção das CDs é alto, e seu rendimento é baixo. Nesse contexto, é significativamente relevante avaliar novas alternativas biotecnológicas de otimização da produção de CDs.

OBJETIVOS. Avaliar estratégias de produção de CDs a partir da CGTase do *B. subtillis* WB800 recombinante utilizando sistema contínuo de produção associado a ultrafiltração para enzima semipurificada e purificada, e meios de produção alternativos para a enzima bruta.

MATERIAL E MÉTODOS. Para a produção da *B. subtili*s CGTase recombinante, foi utilizado o meio 2xYT, suplementado com canamicina (25 μg/mL) e incubado a 30 °C, a 100 rpm, por 5 dias. Os meios alternativos utilizados foram: meio para produção de moléculas de CDs apenas com adição da enzima (meio 1A) e apenas com a presença do microrganismo (meio 1B), e o meio 2xYT apenas com adição da enzima (meio 2A) e somente com a presença do microrganismo (meio 2B). Os meios foram suplementados com canamicina e adicionados de amido de milho e, posteriormente, incubados a 30 °C e 50 °C, 100 rpm, por 5 dias. Alíquotas de 5 mL foram coletadas a cada 24 horas para análise cromatográfica.

RESULTADOS E DISCUSSÃO. A enzima de CGTase de *B. subtilis* WB800 recombinante exibiu baixa atividade enzimática (1,60 μmol de β-CD/min/mL), possivelmente devido interferência na sua estabilidade estrutural durante o armazenamento. A 30 °C, o tempo de 48 h foi o que apresentou maior produção de CDs, principalmente β-CD, com destaque para o meio 2xYT, independente da presença do microrganismo ou apenas da enzima (13,26 e 15,06 mmol/L de β-CD, respectivamente). Contudo, foi a 50 °C que a produção de CDs foi mais competente, sendo a produção de β-CD a mais eficiente (média de 15 mmol/L). Em todos os ensaios foi possível observar que a concentração de β-CD começou a diminuir progressivamente, principalmente no meio contendo o microrganismo (meio 1A). Esse evento pode estar relacionado ao fato de o microrganismo estar produzindo outras enzimas que atuam na degradação da CGTase recombinante ou, ainda, devido à possibilidade do microrganismo consumir as CDs ao longo do tempo.

CONCLUSÕES. Meios alternativos acrescidos de amido de milho mostraram-se uma estratégia interessante para a produção de β-CD, que atualmente é a CD mais utilizada e disponível comercialmente. Além disso, a utilização da enzima sem purificação é uma alternativa promissora, pois contribui para a redução de custos e etapas na produção de CDs, podendo favorecer sua aplicação industrial.

Palavras-chave: Ciclodextrinas, CGTase recombinante, Sistema Contínuo, Ultrafiltração.

GENERAL ABSTRACT

Innovation is allied to the process of transformation and creation of a new product, idea or service, so that it is delivered to society and legitimized by its value. In this context, the Academic Doctorate Program in Innovation (DAI) refers to an initiative of the National Council for Scientific and Technological Development (CNPq) so that Graduate Programs can promote interaction projects with companies through their theses. Considering the above, this thesis focused on innovation in enzyme biotechnology and is presented in the form of two scientific articles.

ARTICLE 1 - Obtaining bioactive di and tripeptides from the enzymatic hydrolysis of soybean meal and its protein isolate using Alcalase® and Neutrase®

INTRODUCTION. Bioactive peptides are short chains of amino acids joined by peptide bonds. It is of special interest due to its high bioactivity and, mainly, its functional properties. Therefore, these are compounds that meet the demands of the modern market, which is increasingly interested in the selection of higher quality foods that may be allied to health promotion.
Bioactive peptides can be obtained from different methods, with special emphasis on enzymatic hydrolysis processes, which are advantageous for the food sector, especially because they have advantages such as greater control of the degree of protein breakdown, low cost and because it is a potent biologically active peptide release tool. The largest number of isolated peptides comes from animal and vegetable sources and soy protein has proven to be a rich source of bioactive peptides. Thus, the use of by-products from soybean processing can also be a prosperous alternative for the development of foods rich in bioactive properties for both human and animal consumption.

AIMS. Considering the interest in obtaining bioactive compounds with significant bioactivity and functional properties, this work aimed to apply commercial proteases to obtain bioactive di and tripeptides from soy by-products and, consequently, add greater value to them.

MATERIAL AND METHODS. The defatted soybean meal (SM) was provided by the company BRF S.A. SM centesimal composition analysis was performed. Soy protein isolate (SPI) was prepared, which was obtained by aqueous extraction from SM. The enzymatic hydrolysis of SM and SPI were performed separately with the commercial enzymes Alcalase® (pH 8 at 55 °C) (SMA and SPIA) and Neutrase® (pH 7 at 50 °C) (SMN and SPIN) with 10 mg for 1% substrate (w/w) for 4 h. The hydrolyzate yield (%) was calculated by the ratio of the hydrolyzate mass obtained and the initial mass of SM (g) and SPI (g).
The crude protein content was determined by the Kjeldahl method and the degree of hydrolysis of the reactions was measured using OPA reagent at 340 nm. Protein electrophoresis was performed on a denaturing polyacrylamide gel (SDS-PAGE) and the antioxidant activity of the hydrolysates was determined by the DPPH and ABTS radical scavenger activity methods.
The identification of bioactive peptides was carried out by the rapid absorption technique by LC-MS/MS using an innovative system, which consists of the rapid tracking of protonated molecules of di and tripeptides, based on the neutral loss (NL) of

46 Da, without the need for time-consuming pretreatment or chromatographic separation.

RESULTS AND DISCUSSION. Analysis of the centesimal composition performed on MS revealed a very high total protein content (49.31 ± 0.06%), which indicates that the by-product is a promising source of bioactive peptides. The yield values obtained in the preparation of the SPI and in the hydrolysis were: SPI 22.99 ± 0.23%; SMA 16.80 ± 0.10%; NMS 16.40 ± 0.30%; SPIA 22.10 ± 1.00% and SPIN 22.94 ± 0.23%, highlighting greater efficiency in SPI hydrolysates than in SM hydrolysates.

The results of crude protein content determined for SPI and hydrolysates were: 102.6 ± 3.62% for SPI; 71.8 ± 1.43% for SMA hydrolyzate; 74.5 ± 1.04% for NMS; 98.8 ± 0.33% for SPIA and 99.7 ± 0.34% for SPIN. Such results were satisfactory, as a commercial product of soy protein isolate must contain at least 90% protein. For the hydrolysis performed in the SM, a crude protein content above 70% was observed, which suggests that the hydrolysis was also effective in this case. The results obtained in the analysis of the degree of hydrolysis revealed that the use of the enzyme Alcalase® presented better results (16.72 ± 0.25% and 12.59 ± 0.80% for SMA and SPIA, respectively) in comparison with the enzyme Neutrase® (8.45 ± 0.62% for SMN and 1.29 ± 0.28% for SPIN), which indicates that the enzyme with high proteolytic activity may have influenced the length of the peptide chain and increased the amount of free amino acids. SDS-PAGE revealed that both enzymatic treatments resulted in peptides with masses less than 25 kDa. The scavenging activity of DPPH and ABTS radicals revealed that hydrolysis performed with Alcalase® and Neutrase® improved the antioxidant activity of SM and SPI, but SPI stood out for presenting greater antioxidant activity (406, 33 ± 0.22 µmol TE/mg of sample) than SM and its hydrolysates.

The LC-MS/MS analysis showed that the application of the hydrolysis process with the commercial protease Alcalase® in the SM promoted the obtaining of 19 peptides (16 di and 03 and tripeptides). SPIA hydrolyzate generated 27 dipeptides and 24 tripeptides. The use of commercial proteases generated peptides with bioactivities that corresponded mainly to ACE inhibitory activity, dipeptidyl peptidase IV (DPP-IV) inhibitory activity, antioxidant activity, stimulating action and renin inhibitory activity.

CONCLUSIONS. The hydrolysis process of soy protein with Alcalase® provided proteins with shorter chains, characteristics of peptides with bioactive properties. The hydrolysis of SM and SPI significantly potentiated the antioxidant capacity of soy protein, mainly with the use of Alcalase®. The innovative system used for the identification of di and tripeptides by LC-MS/MS was efficient for the study of soybean by-products and identified a significant number of di and tripeptides, which presented sequences of antioxidant, antihypertensive and antidiabetic bioactivities. Thus, the application of biotechnological processes carried out with commercial enzymes can be a promising alternative to improve the technological and functional characteristics of the soybean meal by-product.

Keywords: Soybean meal, enzymatic hydrolysis, dipeptides, tripeptides, bioactivities.

ARTICLE 2 - Challenges and alternatives for the production of cyclodextrins from the recombinant *Bacillus subtilis* WB800 CGTase enzyme.

INTRODUCTION. Cyclodextrins (CDs) are cyclic oligosaccharides obtained from the starch transglycosylation reaction, which is catalyzed by the enzyme cyclomaltodextrin glucanotransferse (CGTase). Due to the non-polar characteristic of the cavity, CDs have the capacity to encapsulate countless molecules. Although it has high applicability, the production cost of CDs is high, and its yield is low. In this context, it is significantly relevant to evaluate new biotechnological alternatives for optimizing CD production.

AIMS. Evaluate CD production strategies from recombinant *B. subtillis* WB800 CGTase using a continuous production system associated with ultrafiltration for semipurified and purified enzyme, and alternative production means for the crude enzyme.

MATERIAL AND METHODS. For the production of recombinant *B. subtilis* CGTase, 2xYT medium was used, supplemented with kanamycin (25 µg/mL) and incubated at 30 °C, at 100 rpm, for 5 days. The alternative medium used were: medium for the production of CD molecules with only the addition of the enzyme (medium 1A) and only with the presence of the microorganism (medium 1B), and the 2xYT medium with only the addition of the enzyme (medium 2A) and only with the presence of the microorganism (medium 2B). The media were supplemented with kanamycin and corn starch and subsequently incubated at 30 °C and 50 °C, 100 rpm, for 5 days. Aliquots of 5 mL were collected every 24 hours for chromatographic analysis.

RESULTS AND DISCUSSION. The recombinant *B. subtilis* WB800 CGTase enzyme exhibited low enzymatic activity (1.60 µmol β-CD/min/mL), possibly due to interference in its structural stability during storage. At 30 °C, the time of 48 h showed the highest production of CDs, mainly β-CD, with emphasis on the 2xYT medium, regardless of the presence of the microorganism or just the enzyme (13.26 and 15.06 mmol/ L of β-CD, respectively). However, it was at 50 °C that the production of CDs was more competent, with the production of β-CD being the most efficient (average of 15 mmol/L). In all assays, it was possible to observe that the concentration of β-CD began to progressively decrease, mainly in the medium containing the microorganism (medium 1A). This event may be related to the fact that the microorganism is producing other enzymes that act in the degradation of the recombinant CGTase or, even, due to the possibility of the microorganism consuming the CDs over time.

CONCLUSIONS. Alternative medium added with corn starch proved to be an interesting strategy for the production of β-CD, which is currently the most widely used and commercially available CD. In addition, the use of the enzyme without purification is a promising alternative, as it contributes to the reduction of costs and steps in the production of CDs, which may favor its industrial application.

Keywords: Cyclodextrins, CGTase recombinant, Continuous System, Ultrafiltration.

ARTICLE 1

Obtaining of bioactive di and tripeptides from enzymatic hydrolysis of Soybean meal and its protein isolate using Alcalase® and Neutrase®

Running title: Obtaining di-tripeptides from soybean meal

Thamara Thaiane da Silva Crozatti[a], Juliana Harumi Miyoshi[a], Angélica Priscila Parussolo Tonin[a], Larissa Fonseca Tomazini[a], Marco Aurélio Schuler Oliveira[a], Jose Uebi Maluf[b], Eduardo Cesar Meurer[c] e Graciette Matioli[a*]

[a] *State University of Maringá (UEM), Av. Colombo, 5790 - 87020-900, Maringá, PR, Brazil*

[b]*BRFoods, Av. Senador Atílio Fontana, 4040, 85902-160, Toledo-PR, Brazil*

[c]*Federal University of Paraná (UFPR), Advanced Campus Jandaia do Sul, 86900-000, Jandaia do Sul-PR, Brazil*

* Corresponding author: Graciette Matioli

E-mail address: gmatioli@uem.br

Orcid: https://orcid.org/0000-0002-2531-2567

Tel.: +55 44 3011-3868;

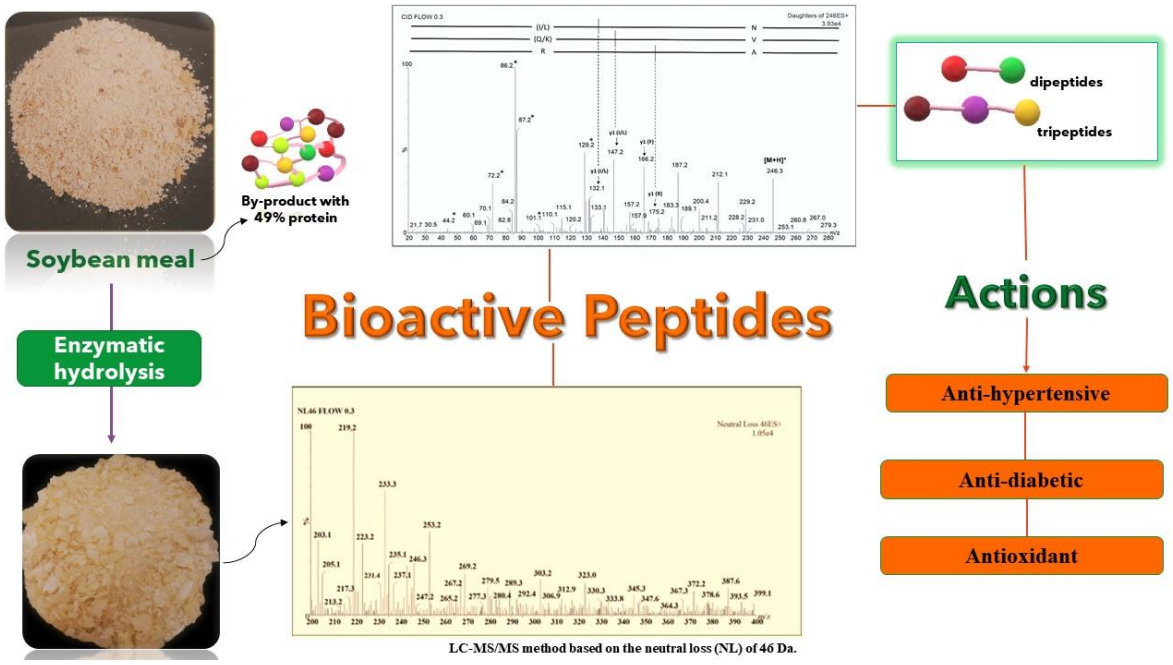
Fax: +55 44 3011-4119

Abstract

The obtaining of bioactive di and tripeptides using Alcalase® and Neutrase® enzymes in the hydrolysis of soybean meal (SM) and its protein isolate (SPI) was evaluated. An innovative system by fast LC-MS/MS neutral loss screening and *de novo* sequencing was used to identify bioactive peptides. Soy protein characterization, gel electrophoresis, and antioxidant activity of the obtained peptides were performed. Results achieved showed that the use of Alcalase® and SPI preparation potentiated the peptide breaking bonds and favored the obtainment of bioactive peptides. The antioxidant activity of tested samples was significantly improved with enzymatic hydrolysis. LC-MS/MS analyses identified 19 peptides in SM and 51 in SPI, all obtained after hydrolysis with Alcalase® and, according to BIOPEP, with relevant bioactivities and expressive functional potential. Therefore, it is suggested that bioactive peptides achieved in this study can enable the development of new ingredients and provide greater added value to soy by-products.

Keywords: Soybean meal, enzymatic hydrolysis, dipeptides, tripeptides, bioactivities.

Graphical Abstract



1. INTRODUCTION

The demand for higher nutritional and sensory quality food products has increased significantly and, based on this scenario, the valuation of the food with functional ingredients also increases, among which bioactive peptides stand out.

Bioactive peptides are short-chain amino acid fragments joined by peptide bonds, which can vary between two and twenty amino acid segments (Hartmann & Meisel, 2007; Li-Chain, 2015). Nowadays, more than 4372 bioactive peptides already identified are available in the literature, which may come from sources such as plants, and animals, among others (Minkiewicz, Iwaniak & Darewicz, 2019).

The heightened interest in peptides is associated with their high bioactivity and, above all, their functional properties and health promotion (Coscueta et al., 2016; Liu et al., 2020). Such organic compounds can be obtained by different chemical processes, such as hydrolysis processes, fermentation methods, among others, which consist of breaking the protein into smaller pieces to modify the protein structures and improve its functional characteristics (Shen et al., 2020).

Enzymatic hydrolysis processes are highly beneficial to the food industry, especially because it has advantages, such as greater control of protein breakdown degree, low cost, among others (Sight et al., 2014). In this context, the use of different proteases is highlighted, which are classified into endopeptidases and exopeptidases, such as Alcalase®, Neutrase®, papain, trypsin, etc. (Liu et al., 2020; Tascias-Pascacio et al., 2020).

The largest number of isolated peptides comes from animal sources such as milk protein and dairy products (Hartmann and Meisel, 2007; Sigh et al., 2014). However, some vegetable sources have been expanding and showing up in recent years, such as soy protein, that in addition to exhibits one of the most important production

chains in the world agribusiness, standing out mainly in soybeans, bran, and oil production, presents, together with its by-products, a rich source of bioactive peptides (Gorçuc et al., 2020; Coscueta et al., 2019).

Different analytical methods are used to evaluate hydrolyzed proteins containing three or more amino acid residues. However, the techniques commonly used to investigate the smallest peptides (di- or tri-) have the disadvantage of time-consuming sample preparation and the need for chromatographic fractionation. Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has been frequently selected for the identification of biopeptides in complex mixtures (Poliseli et al., 2021).

To the best of authors' knowledge, there is no previously published work presenting the process of enzymatic hydrolysis that can be used to obtain di and tripeptides directly from soybean meal without the previous need to obtain its protein isolate. Nor is the author's knowledge of published works that describe an LC-MS/MS system for rapid screening of protonated di and tripeptide molecules from soybean meal and its protein isolate based on the neutral loss (NL) of 46 Da, without the need for time-consuming pretreatment or chromatographic separation.

Thus, this work aimed to evaluate the use of commercial enzymes to obtain di and tripeptides directly from soybean meal and, in addition, to identify tri- and dipeptides in soybean meal and its protein isolate by fast LC-MS/MS neutral loss screening and *de novo* sequencing.

2. MATERIALS AND METHODS

2.1. Materials

The defatted soybean meal was supplied by the BRFoods Company (Brazil). Alcalase® 2.5 L and Neutrase® 0.8 L were purchased from Novozymes Latin America Ltda. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and HPLC-grade Acetonitrile were purchased from Sigma-Aldrich Brazil Ltda. Deionized water was obtained using a Milli-Q system (Millipore, Billerica, MA). All other reagents used were of degree of analytical purity.

2.2. Analysis of the centesimal composition of soybean meal (SM)

Proximate composition analysis of soybean meal (SM) was performed at the Food Analysis Laboratory of the State University of Londrina (Londrina - PR) according to the methods proposed by the AOAC (2016). Carbohydrates, lipids, proteins, ash, and moisture analyzes were performed.

2.3. Preparation of Soy Protein Isolate (SPI)

Soy protein isolate (SPI) was obtained by aqueous extraction from defatted soybean meal (SM), as proposed by Coscueta et al. (2016), with modifications. A solution containing soybean meal and distilled water in a 1:10 ratio, respectively, with an initial pH equal to 8.5 was added to a jacketed glass reactor. The solution was heated to 70 °C for 60 min. After heat treatment, the extract was centrifuged at 2379 xg for 45 min and the protein was precipitated by acidification with HCl (1 N) until reaching pH 4.5. The insoluble portion was centrifuged at 2379 xg for 30 min and the precipitate was lyophilized at -50°C for approximately 36-48 h and stored at -4 °C until use. The SPI yield obtained (%) was calculated by the ratio of the initial mass of the SM (g) and the final mass of the SPI (g).

2.4. Enzymatic hydrolysis of SM and SPI

The enzymatic hydrolysis of SM and SPI were carried out as proposed by Shen et al. (2020), with modifications (Figure S1). Initially, a complete hydration process was applied in the SM and SPI, in which 4% of the sample (m/m) was suspended in distilled water and kept under stirring at room temperature for 2 h. Afterward, the samples were stored at 4 °C for 12 h, followed by the hydrolysis process.

The hydrolysis was carried out in a jacketed glass reactor using two commercial enzymes separately: Alcalase® (pH 8 at 55 °C) and Neutrase® (pH 7 at 50 °C). The amount of applied enzyme in each assay was 10 mg for 1% substrate (w/w) and the reaction time was 4 h. The enzymes were inactivated by heat treatment at 100 °C for 10 min, and the supernatant was separated by centrifugation at 2379 xg for 45 min at 20 °C. The hydrolysates obtained were lyophilized and stored at -10 °C. The yield of hydrolysates (%) was calculated by the hydrolyzate mass ratio obtained and the initial mass of SM (g) and SPI (g).

2.5. Determination of crude protein

Crude protein content was determined by Kjeldahl method according to AOAC (2016), using a nitrogen analyzer (Luca Model -74, São Paulo, BR) and a conversion factor of nitrogen to protein equal to 6.25.

2.6. Determinations of degree of hydrolysis

The degree of hydrolysis (DH) of SM and SPI were determined as described by Nielsen, Petersen, and Dambmann (2001), with modifications. Each tube containing 400 µl of the standard, blank (distilled water) and sample was added with 3.0 mL of

OPA reagent and reacting solutions for 2 min. Absorbance was then read at 340 nm. The %DH was calculated following the equations reported by Adler-Nissen (1984).

2.7. Gel electrophoresis (SDS-PAGE)

Protein electrophoresis in denaturing polyacrylamide gel (SDS-PAGE) were performed on all hydrolysates, according to the protocol described by Laemmli (1970). The concentration of separating gel was 12% (m/v) and of the stacking gel was 4%. Electrophoresis were performed in a vertical system following the manufacturer's instructions (Bio-Rad). Samples were mixed with sample buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 0.0625M Tris-HCl pH 6.8, 5% β-mercaptoethanol) and boiled before the application. The running voltage ranged from 100 to 200 V using Laemmli buffer (3 g/L Tris-base, 14 g/L Glycine, and 1 g/L SDS). After electrophoresis, gel proteins were stained with Coomassie Blue R-250 dye and decolourised in a solution with 50% (v/v) methanol and 10% (v/v) acetic acid.

2.8. Antioxidant activity

The antioxidant activity of protein hydrolysates was evaluated by root scavenging activity DPPH and ABTS methods. The DPPH analysis was performed as described by Li, Du and Ma (2011), with modifications. A stock solution of DPPH 6,25,10-5 mol/L in methanol was prepared and, later, a working solution was prepared using water as a diluent, in which the absorbance of this solution at 517 nm was 0.700 ± 0, 02. A 25 µL sample was used at a concentration of 1 mg/mL and 2000 µL of the working solution, the pH of the sample was adjusted as appropriate to obtain complete dissolution of the sample. After standing for 30 minutes in the dark, readings were taken at 517 nm. The DPPH radical scavenging activity was performed in triplicate and then

compared to a standard curve made from the Trolox readings (200-2000 µmol L^{-1}). Results were expressed as µmol of Trolox protein equivalent (TE) per mg of sample.

The ABTS radical scavenging activity was performed as proposed by Rufino et al. (2007), with modifications. A 7 mmol/L ABTS solution and a 140 mmol/L potassium persulfate solution were prepared. Subsequently, the solution was prepared using 5 mL of ABTS stock solution with 88 µL of potassium persulfate solution and incubated for 16 h in the dark at room temperature. The resulting ABTS solution was diluted with water to an absorbance of 0.70 ± 0.02 at 734 nm. 30 µL of the sample were used (and it was diluted when necessary and the pH adjusted if it was necessary to obtain a complete dissolution of the sample) and 3 mL of the ABTS solution, which were left in the dark for 6 min. ABTS radical scavenging activity was determined in triplicate and then compared to a standard curve made from Trolox readings (200-2000 µmol L^{-1}). Results were expressed as µmol of Trolox protein equivalent (TE) in mg of sample.

2.9. Sequential Mass Spectrometry (LC-MS/MS)

The bioactive peptides of the hydrolysates were analyzed as described by Poliseli et al. (2021), using a Quattro Premier XE triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, The USA) equipped with an electrospray ionization source, a Waters 515 pump and an XBridge (Waters) C18 3.5 µm (4.6 x 50 mm) column. For sample preparation, 0.1 g of hydrolyzed was dissolved in 1 mL of 50 mM ammonium bicarbonate solution. The solution was mixed in vortex for 1 min and then the first dilution was carried out where 100 µL of this solution was mixed with 900 µL of mobile phase acetonitrile: water: formic acid (70:30:0.1) (v/v/v) and was centrifuged at 3 xg for 10 min. The sample remained refrigerated at 4 °C for 60 min. Then, the

second dilution was performed, in which 100 µL of the solution was mixed with 900 µL of mobile phase, followed by vortexing for 1 min. The diluted sample was injected into the reodyne valve of the LC-MS/MS system, the injection volume was 5 µL and the analysis run time was 1 minute for each sample.

The LC-MS/MS (full scan and fragmentation) experiments were conducted using a conventional electrospray ionization source (ESI). The desolvation and source gas temperatures were 350 °C and 110 °C, respectively. The electrospray source was operated in positive ionization mode (ESI +) at 4.0 kV. The cone voltage, collision energy, and collision gas pressure (argon) were 20 V, 15 V, and 3.0×10^{-3} Torr, respectively. The spectra obtained were interpreted as described by Cantú et al. (2008) and the amino acid sequence of the peptide's functionality contained in the fragmentation spectra were evaluated using the BIOPEP-UWM database.

2.10. Statistical analysis

The results obtained were evaluated by analysis of variance (ANOVA) and Tukey post-test ($p < 0.05$) for comparison between samples, using Sisvar version 5.7 (Build 91). All assays were performed in triplicate and results were reported as mean ± SD (standard deviation).

3. RESULTS AND DISCUSSION

3.1. Proximate composition of SM and protein yields in SPI and hydrolysates

The proximate composition analysis performed in the SM resulted in the following values: carbohydrates 35.71 ± 0.02%; lipids 0.56 ± 0.03%; proteins 49.31 ± 0.06%; ash 6.16 ± 0.04% and humidity 8.26 ± 0.05%.

Soybean meal represents a by-product of the oil extraction industry and stands out for being an interesting resource for the extraction of proteins of great applicability for the development of products aimed at human and animal food (Xing et al., 2018). In the present study, the total protein content observed was quite high (49.31%), which indicates that it is a promising source of bioactive peptides. Similar results of proximate composition were found by Orts et al. (2019), who studied the potential of soybean pulp (Okara) for extracting bioactive compounds and observed a protein content close to 41%, and by Peng et al. (2020), who studied the functional potential of soy protein and found a total protein content for soy flour close to 37% and carbohydrate and ash content equal to 34.5% and 6.7%, respectively.

The values of the yields obtained in the preparation of the SPI and in the hydrolysis carried out with the enzymes Alcalase® and Neutrase® were: SPI 22.99 ± 0.23%; SMA 16.80 ± 0.10%; SMN 16.40 ± 0.30%; SPIA 22.10 ± 1.00% and SPIN 22.94 ± 0.23%. As expected, there was greater efficiency in the SPI hydrolyzates than in the SM hydrolysates, which may be related to the fact that the process of obtaining the SPI results in a purer material, without the presence of residues from the processing of crude soybeans.

Crude protein content results determined by Kjeldahl's methods for SPI and hydrolysates were: 102.6 ± 3.62% for SPI; 71.8 ± 1.43% for the SMA hydrolyzate; 74.5 ± 1.04% for SMN; 98.8 ± 0.33% for SPIA and 99.7 ± 0.34% for SPIN. Such results proved to be satisfactory, since a commercial soy protein isolate product must contain at least 90% protein (dry basis) (Huang et al., 2020).

For the enzymatic hydrolysis carried out directly in the SM, a crude protein content above 70% was observed, which suggests that the hydrolysis was also effective in this case, with the crude protein value of the SM obtained after hydrolysis being approximately 20% greater than the initial (49.31%).

Protein hydrolysis can be measured in terms of the degree of hydrolysis (DH). DH is associated with amino acid composition and may imply the biological activity of

246 the peptides formed (Shahi et al., 2020). In the present study, the use of Alcalase®
247 enzyme showed better DH results (16.72 ± 0.25% and 12.59 ± 0.80% for SMA and
248 SPIA, respectively) compared to Neutrase® enzyme (8.45 ± 0.62% for SMN and 1.29 ±
249 0.28% for SPIN), which suggests that the enzyme with high proteolytic activity may
250 have influenced the peptide chain length and increased the amount of free amino acids.
251 The DH in Alcalase® hydrolysates are similar to those presented by Chiang et al.
252 (2006) for pepsin and trypsin, as well as those presented by Coscueta et al. (2016) for
253 Corollasse PP. Although the use of Neutrase® has shown low DH, the application of
254 hydrolysis directly in the SM can be an alternative for the food segment, as the acid
255 hydrolysis of 8% leads to the release of bitter peptides (Coscueta et al., 2016). New
256 studies must be carried out in order to improve the efficiency of the hydrolysis process
257 with Alcalase® and Neutrase®.

258 **3.2. SDS-PAGE**

259 The figure 1A shows the gel electrophoresis results obtained for the proteins
260 from each hydrolyzed material. For both SM and SPI, it was found that the hydrolysis
261 performed with the commercial enzyme Alcalase® presented more intense bands at the
262 end of the gel, which shows the presence of smaller protein fragments when compared
263 to the hydrolysis performed with the enzyme Neutrase®. Nevertheless, the SDS-PAGE
264 revealed that both enzymatic treatments resulted in peptides with masses smaller than
265 25 KDa, being that many peptides with a mass lower than 18 KDa, the smaller marker
266 used.
267

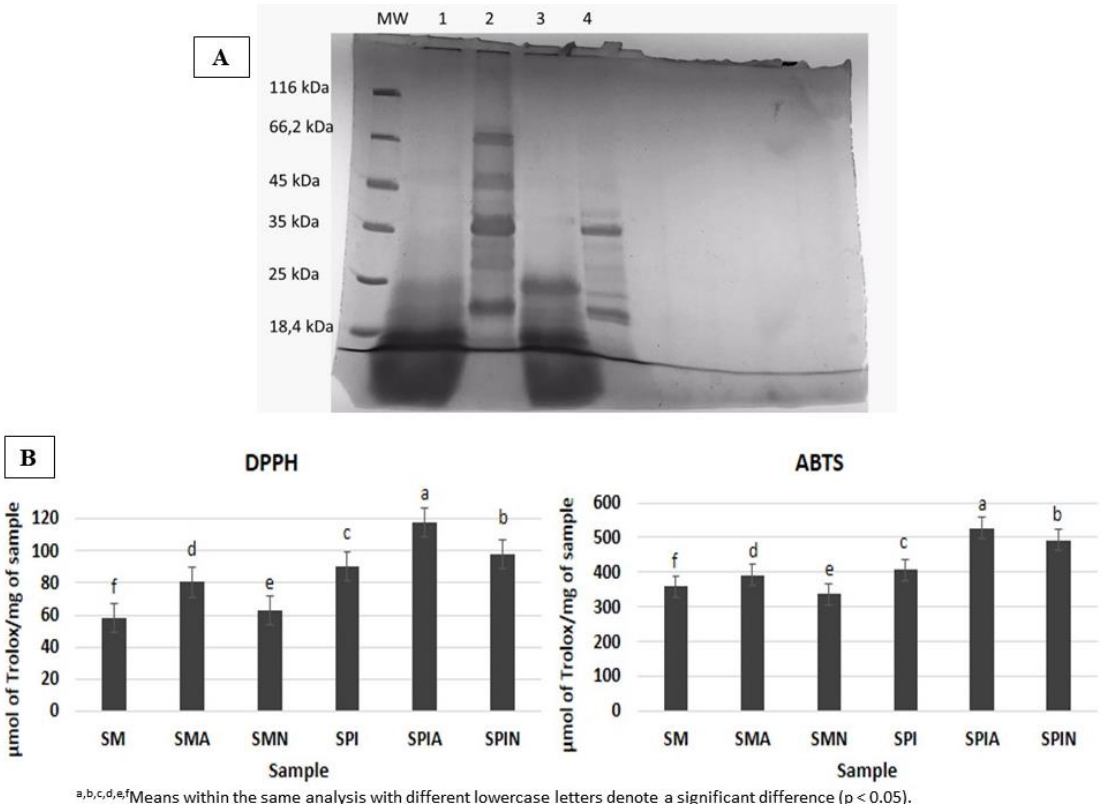


Figure 1: (A) Denaturing polyacrylamide gel (SDS-PAGE) obtained for samples hydrolyzed with the enzymes Alcalase® and Neutrase® in soybean meal (1 and 3, respectively) and protein isolate (2 and 4, respectively). (B): Antioxidant sequestration activities of ABTS (A) and DPPH (B), in soybean meal (SM), in protein isolate (SPI), and hydrolysates with the enzymes Alcalase® (SMA and SPIA) and Neutrase® (SMN and SPIN).

The path covered in the gel by the peptides obtained with the hydrolysis by Neutrase® showed well-distributed bands located in the region of higher molecular weight. The peptides obtained with the hydrolysis with Alcalase® flowed more easily in the gel and, therefore, the bands corresponded to the region of lower molecular weight. Similar to the present study, Ahmadifard et al. (2016) evaluated the behavior of commercial enzymes in soy protein and observed a greater hydrolysis potential of Alcalase® enzyme compared to other evaluated enzymes, such as papain. The authors

also observed a greater number of bands in samples hydrolyzed for 30 min with Alcalase®, however, after increasing the hydrolysis time, these denser bands disappeared, highlighting only fragments of lower molecular weight. Coscueta et al. (2016), who used the Colorase PP enzyme, also identified denser bands at the end of the gel, characterizing a greater presence of small peptides.

3.3. Antioxidant activity

Figure 1B reveals the results obtained by analyzing ABTS and DPPH radical scavenging in SM, SPI, and hydrolysates with commercial proteases from them. The results obtained revealed that the hydrolysis performed with Alcalase® and Neutrase® improved the antioxidant activity of SM and SPI, but SPI stood out as it exhibited greater antioxidant activity (406, 33 ± 0.22 µ mol of TE/mg of sample) than SM and its hydrolysates SMA and SMN (329.66 ± 0.19; 398.00 ± 0.22 and 351, 33 ± 0.20, respectively). Regarding the application of enzymes, it is possible to suggest that the use of Alcalase® potentiated the antioxidant characteristics of the samples, with SPIA showing a better result (569.66 ± 0.02 µmol of TE/mg of the sample). Similar behavior was evidenced for the DPPH radical, which also showed greater efficiency in the scavenging of free radicals for the SPIA sample (98 ± 0.01 µmol of TE/mg of the sample). These results suggest that Alcalase®, being more efficient in breaking peptide bonds, contributed to obtaining proteins with simple structure and lower molecular weight, which have greater antioxidant capacity.

Shen et al. (2020) evaluated the formation and characterization of soy protein nanoparticles by enzymatic hydrolysis with the proteases Flavorzyme, Alcalase, and Protamex and observed a more pronounced antioxidant effect in the hydrolyzate with

Alcalase, which according to the authors, can be attributed to the progressive generation of polypeptides. Thus, the formation of peptides with significant antioxidant capacity, in addition to bringing health benefits, shows an interesting alternative for replacing artificial antioxidants in foods.

Finally, it is noteworthy that the application of the enzymatic hydrolysis process contributed to the better scavenging of ABTS and DPPH radicals. In both, a similar antioxidant behavior was observed, but the antioxidant activity in µmol of Trolox/mg of sample was significantly higher for the ABTS radical. The greater scavenging capacity of the ABTS radical compared to the DPPH radical may be related to the difference in the solubility of the radicals, since the DPPH radical has a certain limitation to mimic the role of hydrophilic antioxidants, as it presents dissolution barriers in aqueous solution, on the contrary of the ABTS radical that offers affinity with aqueous and organic solutions (Aloglu and Oner, 2011). Similar results were observed by Mukia et al. (2021) who evaluated the generation of antioxidant peptides from SPI obtained by the action *of Chryseobacterium* sp.

3.4. Mass spectrometry

Liquid chromatography (LC) coupled with mass spectrometry (MS/MS) has proven to be a sensitive technique, commonly used for the determination of molecular weight and sequence of proteins and peptides, and for the identification of biopeptides in complex mixtures (Silveira et al., 2013). In the present study, the rapid LC-MS/MS method based on the neutral loss (NL) of 46 Da (CO and H_2O or formic acid) was used, with the main objective of identifying di and tripeptides obtained from soy protein from enzymatic hydrolysis. The system used consists of rapid tracking (5 min) of protonated di and tripeptide molecules, in which there is a selective neutral loss

(NL) of 46 Da, due to the carboxylic acid portion, and also comprises the search for dissociation fragments collision-induced (CID) by *de novo* sequencing (Poseli et al., 2021).

Previously, full scan analyzes were performed on all hydrolyzed samples (SMA, SMN, SPIA, and SPIN). The spectra obtained in the hydrolysates with the Alcalase® enzyme showed better results, as they presented well-formed peaks in a short run time. On the other hand, the hydrolysates with Neutrase® enzyme did not present regular peaks, and, based on this interpretation, only the SMA and SPIA samples were selected for the fragmentation step. The results obtained are in agreement with those described by Tascias-Pascacio et al. (2020), who points out that the enzyme Alcalase is more efficient in releasing peptides.

Figure S2 (A and B) shows one of the full scan spectra obtained for the SMA and SPIA samples, in which the possible combinations of di and tri-peptides with sufficient intensity to fragment (absolute intensity close to 1000) were identified. The identification using the *m/z* ions for the SMA was: 203, 205, 219, 223, 231, 233, 235, 237, 253, 263, 267, 279, 280, 281, 284, 303, 323, 331, 333, 345, 367, 372 and 387. Identification using the *m/z* ions for the SPIA was: 203, 215, 217, 219, 223, 231, 233, 235, 237, 243, 246, 247, 253, 254, 263, 267, 269, 272, 279, 281, 292, 295, 297, 323, 325, 328, 231, 344, 352, 355, 269, 371, 372, 382, 385, 388. Therefore, it is suggested that the SPIA sample was hydrolyzed in a more potent way than the SMA, due to the observation of a greater amount of *m/z* ions that characterize bioactive peptides for SPIA.

Subsequently, new complete Collision Induced Dissociation (CID) MS/MS were performed in triplicate by *de novo* sequencing and all fragmentation spectra of each selected ion were acquired, where the interpretation of the mass spectra was

carried out by identifying the ammonium ion, from y1 ions (protonated amino acids of 20 amino acid possibilities), from y2 ions (for tripeptides), from confirmatory amino acid residues, and b2 ions. Figure 2A shows one of the mass spectra obtained by the fragmentation step for the ion of *m/z* 246 in the SPIA hydrolyzate. For this ion, four different y1 values were identified (I/L (132), Q/K (147), F (166), and R (175)), with only the N(I/L) di-peptide sequences being confirmed), V(I/L) and AR. Figure 2B shows one of the tripeptide identification spectra in the SPIA sample with an ion of *m/z* 394, were y1 Threonine (T - 120), Leucine, and Isoleucine (I/L - 132), Phenylalanine (F - 166), and Tyrosine (Y - 182). After the interpretations, the sequences of tri-peptides (I/L)CT, TC(I/L), D(F/M)(I/L), D(I/L)F, and DPY were confirmed.

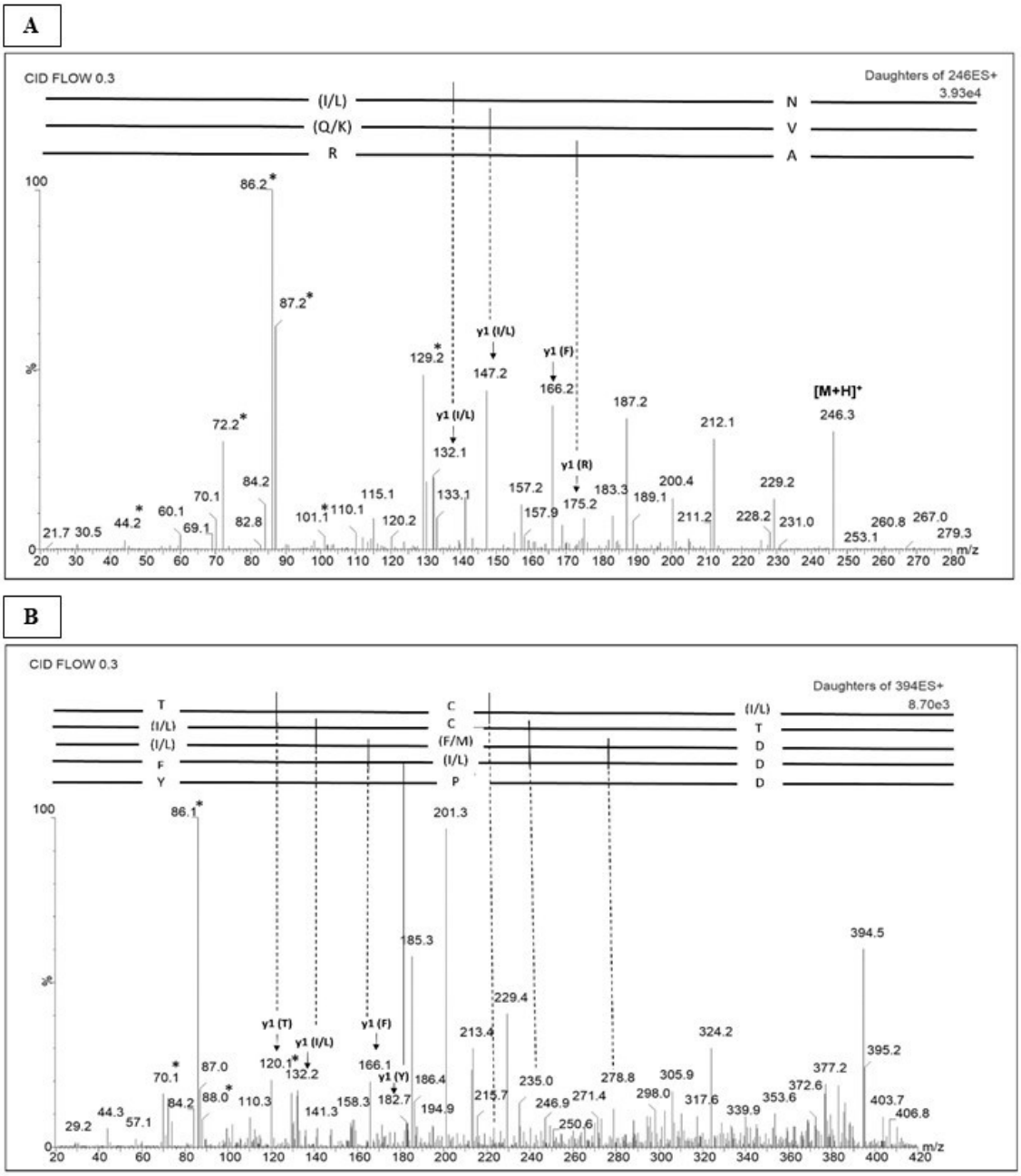


Figure 2: (A) Mass spectrum obtained and interpreted in the fragmentation step for *de novo* sequencing for the m/z 246 ions of the SPIA protein. (*) represents the confirming amino acid ammonium ions. (B) Mass spectrum obtained and interpreted in the fragmentation step for *de novo* sequencing for the m/z 394 ions of the SPIA protein. (*) represents the confirming amino acid ammonium ions.

Tables 1 and 2 present the fragmentation spectra interpretations performed for all *de novo* sequencing spectra, the di and tri-peptide identification results obtained for the SMA and SPIA samples, as well as their biological activities that were determined based on in the BIOPEP-UWM database (Minkiewicz, Iwaniak & Darewicz, 2019).

380 **Table 1:** Amino acid sequences of peptides identified by LC-MS/MS in SM hydrolyzate with Alcalase ®.

Sample	$(M+H)^+$	y^1	Ammonium Ion	b^2	y^2	Amino acid sequence	Biological activity*
SMA	203	132 (I/L)	44 (A); 86 (I/L)	185	-	A(I/L)	ACE inhibitor.
	205	118 (V)	60 (S); 72 (V)	187	-	SV	Dipeptidyl peptidase IV inhibitor.
	219	120 (T)	72 (V); 74 (T)	201	-	VT	Dipeptidyl peptidase IV inhibitor.
	219	132 (I/L)	60 (S); 86 (I/L)	201	-	S(I/L)	Dipeptidyl peptidase IV inhibitor.
	231	132 (I/L)	72 (V); 86 (I/L)	213	-	V(I/L)	Dipeptidyl peptidase IV inhibitor; Stimulating.
	233	132 (I/L)	74 (T); 86 (I/L)	215	-	T(I/L)	Dipeptidyl peptidase IV inhibitor.
	237	166 (F)	44 (A); 120 (F)	219	-	AF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	253	156 (H)	70 (P); 110 (H)	235	-	PH	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	253	166 (F)	60 (S); 120 (F)	235	-	SF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor; Renin inhibitor.
	263	116 (P)	70 (P); 120 (F)	-	-	FP	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	267	166 (F)	74 (T); 120 (F)	-	-	FT	Inibidor de renina.
	279	166 (F)	86 (I/L); 120 (F)	261	-	(I/L)F	ACE inhibitor.
	280	133 (N)	87 (N); 120 (F)	-	-	FN	Dipeptidyl peptidase IV inhibitor.
	281	166 (F)	120 (F)	263	-	DF	ACE inhibitor.
	281	182 (Y)	72 (V); 136 (Y)	-	-	YV	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	284	156 (H)	110 (H)	266	-	(Q/K)H	Dipeptidyl peptidase IV inhibitor.
	331	132 (I/L)	44 (A); 86 (I/L); 101 (Q/K)	200	203	(Q/K)A(I/L)	Antioxidant.
	372	120 (T)	88 (D); 74 (T); 110 (H)	253	235	HDT	-
	372	147 (Q/K)	70 (P); 101 (Q/K)	266	275	P(Q/K) (Q/K)	-

From the obtained results, it was observed that the application of the hydrolysis process with the commercial protease Alcalase® in the SM (Table 01) promoted the obtainment of 19 peptides (16 di and 03 and tri-peptides). The SPIA hydrolyzate (Table 02) generated 27 di-peptides and 24 tri-peptides, which confirms the hypothesis that, although the application of the hydrolysis process directly to the soybean meal is satisfactory, it was the SPI preparation that evidenced to be more efficient for obtaining bioactive peptides. In general, the results reveal the obtainment of a very significant number of di and tri-peptides, which is very promising, since shorter-chain peptides are characterized by exhibiting better biological activity and also by being absorbed with more ease and efficiency than free amino acids (Agrawal et al., 2016).

391 **Table 2:** Amino acid sequences of peptides identified by LC-MS/MS in SPI hydrolyzate with Alcalase ®.

Sample	$(M+H)^+$	y^1	Ammonium Ion	b^2	y^2	Amino acid sequence	Biological activity *
SPIA	203	132 (I/L)	44 (A); 86 (I/L)	185	-	A(I/L)	ACE inhibitor.
	217	118 (V)	72 (V)	199	-	VV	Dipeptidyl peptidase IV inhibitor.
	223	166 (F)	120 (F)	205	-	GF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	231	132 (I/L)	72 (V); 86 (I/L)	213	-	V(I/L)	Dipeptidyl peptidase IV inhibitor; Stimulating.
	233	120 (T)	74 (T); 86 (I/L)	-	-	(I/L)T	Dipeptidyl peptidase IV inhibitor.
	233	132 (I/L)	74 (T); 86 (I/L)	215	-	T(I/L)	Dipeptidyl peptidase IV inhibitor.
	235	120 (T)	74 (T); 102 (E)	-	-	ET	Dipeptidyl peptidase IV inhibitor.
	235	148 (E)	60 (S); 102 (3)	217	-	SE	Stimulating.
	237	166 (F)	44 (A); 102 (F)	219	-	AF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	243	156 (H)	60 (S); 110 (H)	225	-	SH	Dipeptidyl peptidase IV inhibitor.
	246	132 (I/L)	87 (N); 86 (I/L)	-	-	N(I/L)	Dipeptidyl peptidase IV inhibitor.
	246	147 (Q/K)	72 (V); 101 (Q/K)	228	-	V(Q/K)	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	246	175 (R)	44 (A); 110 (H)	228	-	AR	ACE inhibitor.
	253	156 (H)	70 (P); 110 (H)	235	-	PH	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	253	166 (F)	60 (S); 120 (F)	235	-	SF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor; Renin inhibitor.
	253	182 (Y)	44 (A); 136 (Y)	235	-	AY	ACE inhibitor; Dipeptidyl peptidase IV inhibitor; Antioxidant.
	263	150 (M)	86 (I/L); 104 (M)	245	-	(I/L)M	Dipeptidyl peptidase IV inhibitor.
	267	120 (I)	74(T); 120 (F)	-	-	FT	Renin inhibitor.
	267	166 (F)	74 (T); 120 (F)	249	-	TF	ACE inhibitor; Dipeptidyl peptidase IV inhibitor; Renin inhibitor.

	269	156 (H)	86 (I/L); 110 (H)	251	-	(I/L)H	ACE inhibitor.
	269	182 (Y)	60 (S); 136 (Y)	251	-	SY	ACE inhibitor; Dipeptidyl peptidase IV inhibitor.
	279	166 (F)	86 (I/L); 120 (F)	261	-	(I/L)F	ACE inhibitor.
	281	166 (F)	88 (D); 120 (F)	263	-	DF	ACE inhibitor.
	281	182 (Y)	72 (V); 136 (Y)	247	-	VY	ACE inhibitor; Dipeptidyl peptidase IV inhibitor; Antioxidant.
	292	205(W)	60 (S); 159 (W)	185	-	SW	Dipeptidyl peptidase IV inhibitor.
	295	166 (F)	102 (E); 120 (F)	277	-	EF	CaMPDE inhibitor; Renin inhibitor; Lipid-lowering.
	295	182 (Y)	86 (I/L); 136 (Y)	277	-	(I/L)Y	ACE inhibitor; Antioxidant.
SPIA	328	120 (T)	44 (A); 74 (T); 110 (H)	209	257	AHT	-
	328	120 (T)	44 (A); 74 (T); 110 (H)	209	191	HAT	-
	328	156 (H)	44 (A); 74 (T); 110 (H)	173	207	ATH	-
	328	156 (H)	44 (A); 74 (T); 101 (H)	173	227	TAH	-
	331	147 (Q/K)	86 (I/L); 44 (A); 101(Q/K)	185	218	(I/L)A(Q/K)	ACE inhibitor.
	344	132 (I/L)	70 (P); 88 (D); 86 (I/L)	213	229	DP(I/L)	-
	344	132 (I/L)	72 (V); 86 (I/L)	213	245	V(I/L)(I/L)	-
	344	132 (I/L)	72 (V); 86 (I/L)	213	231	(I/L)V(I/L)	ACE inhibitor.
	352	166 (F)	120 (F); 159 (W)	205	-	WF	Dipeptidyl peptidase IV inhibitor.
	352	120 (T)	72 (T)	233	221	MTT	-
	372	156 (H)	86 (I/L); 110 (H)	217	269	C(I/L)H	-
	372	156 (H)	86 (I/L); 110 (H)	217	259	(I/L)CH	-
	382	120 (T)	120 (F)	263	283	(F/M)DT	-
	382	166 (F)	60 (S); 102 (E); 120 (F)	217	253	ESF	-
	382	166 (F)	120 (F)	217	281	TDF	-
	385	120 (T)	110 (H)	266	257	H(Q/K)T	-
	388	147 (Q/K)	86 (I/L); 101 (Q/K)	242	260	(Q/K)(I/L)(Q/K)	-
	388	175 (R))	72 (V); 129 (R)	214	289	VNR	-

SPIA	388	175(R)	72 (V); 129 (R)	214	274	NVR	-
	394	120 (T)	86 (I/L)	275	223	(I/L)CT	-
	394	132 (I/L)	86 (I/L)	263	235	TC(I/L)	-
	394	132 (I/L)	86 (I/L); 88 (D); 120 (F/M)	263	279	D(F/M)(I/L)	-
	394	166 (F)	86 (I/L); 120 (F/M)	229	279	D(I/L)F	-
	394	182 (Y)	70 (P); 136 (V)	213	279	DPY	-

392 *Biological activity identified by the BIOPEP-UWM database (Minkiewicz, Iwaniak & Darewicz, 2019).

393 The use of commercial proteases is commonly related to the interest in
394 obtaining antioxidant peptides (Mukia et al., 2021). However, from the identification of
395 all peptide sequences obtained by the BIOPEP database, it was observed that the
396 bioactivities presented by the peptides in this study corresponded mainly to the
397 inhibitory activity of ACE, the inhibitory activity of dipeptidyl peptidase IV (DPP -IV),
398 in addition to antioxidant activity, stimulating action and renin inhibitory activity
399 (Table 2).

400 Other studies have also highlighted the effectiveness of soy peptides as ACE
401 and dipeptidyl peptidase IV inhibitors (Gu and Wu, 2013; Coscueta et al.; 2019;
402 Nongonierna et al., 2019). This property is very important for the production of high
403 added value foods, which are especially associated with health promotion since the
404 action of these peptides is directly related to the functionalities of antihypertensive and
405 antidiabetic activity.

406 According to Sight et al. (2014), ACE inhibitor peptides are responsible for
407 blocking the first step of the renin-angiotensin system and interrupting the negative
408 response effects of angiotensin II. Therefore, they are considered a useful therapy in the
409 treatment of hypertension, and the presence of di- and tripeptides can further enhance
410 their functionality, as the ACE active site is more suitable to accommodate short-chain
411 peptides. The DPP-IV inhibiting enzyme, on the other hand, acts to increase insulin
412 secretion, which enables better glycemic regulation and, consequently, proves to be
413 efficient to act in the prevention and treatment of diabetes (Nongonierna et al., 2019).

414 Correlating the results of antioxidant activity achieved in this research with the
415 bioactive peptides detected by LC-MS/MS, it is possible to suggest that the increase in
416 free radical scavenging capacity may be related to the greater presence of di-peptides
417 with antioxidant biological activity found at SPIA. In addition, the peptides formed

418 with antioxidant action presented in their structure the amino acid tyrosine ((I/L)Y,
419 VY, and AY), which, according to other studies, contributes to improving the
420 antioxidant activity of the peptide (Sompinit et al., 2020).
421

422 **4. CONCLUSION**

423 The hydrolysis processe of soy protein with Alcalase® provided the obtainment
424 of proteins with shorter chains, characteristics of peptides with enhanced bioactive
425 properties. SM and SPI hydrolysis significantly potentiated the antioxidant capacity of
426 soy protein, specially with the use of Alcalase®, and the SMA and SPIA samples were
427 hydrolyzed in a way more efficient. Therefore, the hypothesis that it is possible to obtain
428 bioactive peptides directly from SM was confirmed.
429 The innovative system used for the identification of di and tripeptides by LC-
430 MS/MS was efficient for the study of soybean by-products and identified a very
431 expressive number of di and tripeptides, which showed sequences of antioxidant,
432 antihypertensive and antidiabetic bioactivities.
433 That way, the application of biotechnological processes carried out with
434 commercial enzymes can be a promising alternative to improve technological and
435 functional characteristics of soybean meal protein and, consequently, enable its use in
436 the development of better quality products and greater added value.
437

438 **5. ACKNOWLEDGMENT**

439

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6. DECLARATION OF INTERESTS

All authors declare no conflicts of interest.

7. ETHICAL APPROVAL

Ethical approval was not required for this research.

8. DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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Supplementary Information - Figure Captions

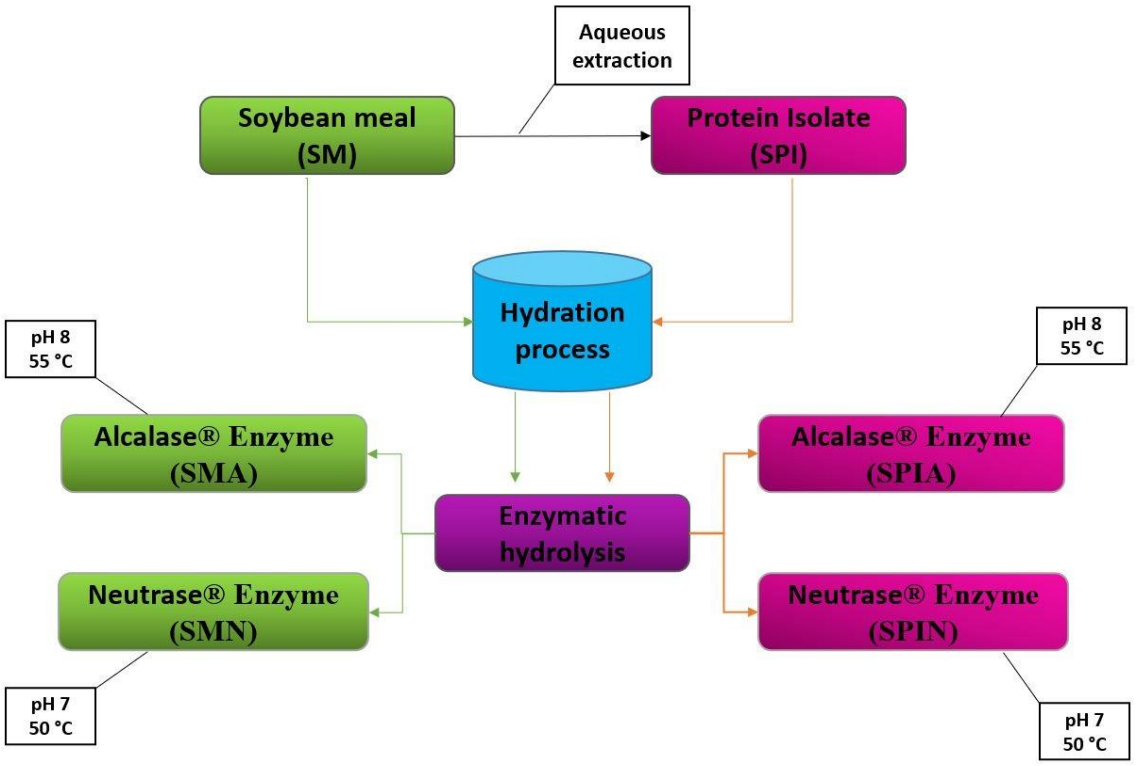


Figure S1: Flowchart of enzymatic hydrolysis processes performed in soybean meal (SM) and soybean protein isolate (SPI), using two commercial proteases, Alcalase® and Neutrase® separately.

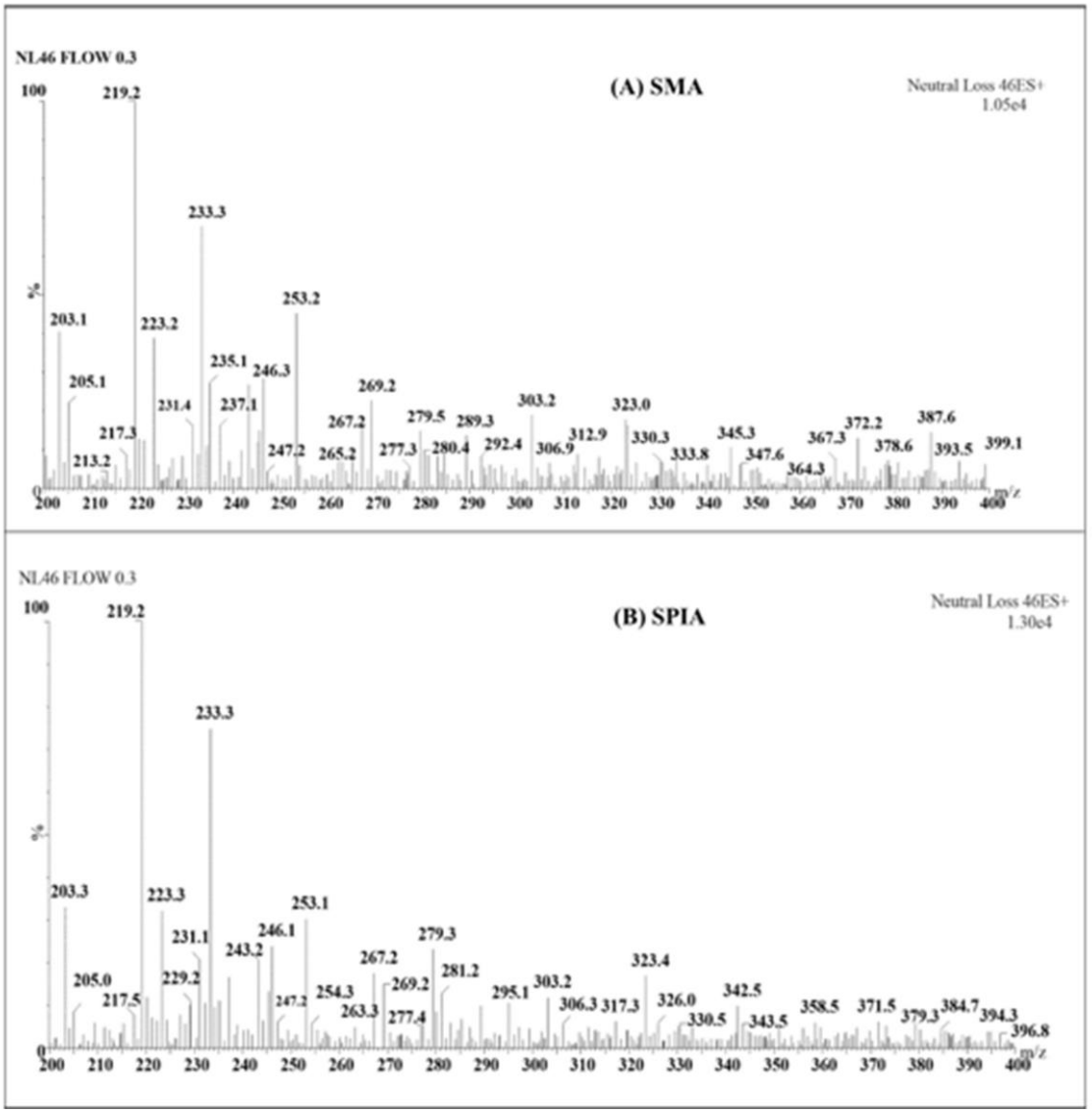


Figure S2: Liquid chromatography-mass spectrum (LC-MS/MS) for a 46 Da neutral lss of (A) SM hydrolyzed with Alcalase® and (B) SPI hydrolyzed with Alcalase®.

602 **ARTICLE 2**

603

604 **Challenges and alternatives for the production of cyclodextrins from the CGTase**
605 **enzyme from recombinant *Bacillus subtilis* WB800.**

606 Thamara Thaiane da Silva Crozatti[a], Paula Vitória Larentis[b], Vanderson Carvalho
607 Fenelon[c], Juliana Harumi Miyoshi[d], Júlia Rosa de Brito[e], Giovanna da Silva Salinas[f],
608 Beatriz de Oliveira Mazzotti[g], Giovanni Cesar Teles[h], Quirino Alves de Lima Neto[i] e
609 Graciette Matioli[j*]

610

611 [a] *Postgraduate Program in Food Science, State University of Maringá (UEM), Av. Colombo,*
612 *5790 - 87020-900, Maringá, PR, Brazil – thamarathaiane01@hotmail.com*

613 [b]*Department of Pharmacy, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-*
614 *900, Maringá, PR, Brazil - paula96vitoria@gmail.com*

615 [c]*Postgraduate Program in Pharmaceutical Sciences, State University of Maringá (UEM), Av.*
616 *Colombo, 5790 - 87020-900, Maringá, PR, Brazil - vanderson2912@gmail.com*

617 [d]*Postgraduate Program in Pharmaceutical Sciences, State University of Maringá (UEM), A*
618 *Colombo, 5790 - 87020-900, Maringá, PR, Brazil - jhm_1992@hotmail.com*

619 [e]*Department of Food Engineering, State University of Maringá (UEM), Av. Colombo, 5790 -*
620 *87020-900, Maringá, PR, Brazil - juliarosa.brito@outlook.com*

621 [f]*Department of Pharmacy, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-*
622 *900, Maringá, PR, Brazil – ra103148@uem.br*

623 [g]*Department of Pharmacy, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-*
624 *900, Maringá, PR, Brazil - ra112550@uem.br*

[h] *Postgraduate Program in Food Science, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-900, Maringá, PR, Brazil – giovanycesar@hotmail.com*

[i]*Postgraduate Program in Biosciences and Pathophysiology, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-900, Maringá, PR, Brazil - qalneto@uem.br*

[j]*Department of Pharmacy, Postgraduate Program in Food Science and Postgraduate Program in Pharmaceutical Sciences, State University of Maringá (UEM), Av. Colombo, 5790 - 87020-900, Maringá, PR, Brazil – gmatioli@uem.br**

* Corresponding author:

E-mail address: gmatioli@uem.br

Tel.: +55 44 3011-3868; fax: +55 44 3011-4119

Relevance of the work: This research presents strategies for obtaining cyclodextrins (CDs) from the recombinant CGTase enzyme, a continuous production system associated with ultrafiltration, and alternative means of production of CDs, aiming at the industrial application of the molecule.

Abstract

Cyclodextrins (CDs) have the ability to encapsulate numerous molecules and have applicability in several industrial areas, however, their cost has made their use difficult. To seek alternatives that may enable the use of CDs, the present study evaluated the efficiency of the ultrafiltration process in a continuous system to produce CDs from the enzyme cyclomaltodextrin glucanotransferase (CGTase) from recombinant *Bacillus subtilis* WB800. The possibility of using the crude enzyme as an alternative means of producing CDs was also evaluated. All strategies evaluated in this research proved to be promising for the production of CDs, with the production of β-CD being the most efficient (average of 15 mmol/L) using crude recombinant enzyme and a temperature of 50 °C. Therefore, the results obtained can contribute to the reduction of stages and costs of production of CDs, favoring their industrial application.

Keywords: Cyclodextrins, CGTase recombinant, Continuous System, Ultrafiltration.

Practical Application: This study presents innovative alternatives to produce cyclodextrins from the recombinant enzyme.

1. INTRODUCTION

*Cyclodextrins (CDs) are cyclic oligosaccharides with expressive applicability in several industrial segments, such as the food, pharmaceutical, cosmetics, chemical sectors, among others. CDs are formed by 6 (α-CD), 7 (β-CD), and 8 (γ-CD) glucose units, joined by α-1,4 glycosidic bonds, obtained from the transglycosylation reaction of starch, which is catalyzed by the enzyme cyclomaltodextrin glucanotransferase (CGTase) (*Ogunbadejo & Al-Zuhair, 2021; Samamed et al., 2022).

Due to the nonpolar characteristic of the CD cavity, inclusion complexes with a wide range of organic and inorganic molecules are capable of formation, modifying the physicochemical properties of the guest molecule, making it possible to increase its stability and solubility. (Del Valle, 2004; Brewster & Loftsson, 2007; Fenelon et al., 2015). Such skills explain the growing interest in the development of innovative biotechnological processes that can enable the industrial use of CDs (Astray et al., 2009; Cid-Samamed et al., 2022).

The research group of the present study obtained promising results when they evaluated the production of CDs from genetically modified bacteria and ultrafiltration systems. (Fenelon et al., 2018; Gimenez et al., 2019). Therefore, it is highly relevant to evaluate new biotechnological alternatives to optimize the production of CDs, especially in Brazil, which has substrate availability and still does not produce the molecule on an industrial scale.

In view of the above, the present study aimed to evaluate strategies for the production of CDs from the CGTase of the recombinant B. subtilis WB800, using a continuous production system associated with ultrafiltration for the semi-purified and purified enzyme, and alternative production means for the crude enzyme.

2. MATERIALS AND METHODS

2.1 Materials

The materials used were ethanol, soluble starch, commercial corn starch, tryptone, yeast extract, sodium carbonate, sodium chloride, and agar. β-cyclodextrin and HPLC grade acetonitrile were purchased from Sigma-Aldrich Ltda, São Paulo, Brazil. The antibiotics used were Kanamycin Sulfate and Hygromycin B., from *Streptomyces hygroscopicus*. All other reagents used were of analytical or chromatographic grade.

2.2 Methods

2.2.1 Cultivation and growth of the recombinant *B. subtilis WB800* bacterium

For the cultivation and growth of the bacteria, 2xYT medium (liquid and solid) was used, composed of 1.6% tryptone, 1% yeast extract and 0.5% NaCl and 1.5% agar for the solid medium, both supplemented with the antibiotics hygromycin (100 µg/mL) and kanamycin (25 µg/mL*). B. subtilis* WB80*0* containing the recombinant plasmid pWB980-CGTase was grown in a Petri dish containing 2xYT solid medium supplemented with kanamycin (25 µg/ml) and hygromycin (100 µg/ml) for 12 h at 37°C. Then, an isolated colony was added to 5 mL of 2xYT liquid medium supplemented with antibiotics and placed in a shaker at 37 °C overnight at 100 rpm. Subsequently, a pre-inoculum (50 mL) was prepared, also supplemented with both antibiotics. In this pre-inoculum, 0.5 mL of the previously activated enzyme was added to the liquid medium, and it was incubated in a shaker at 37 °C for 24 h at 100 rpm.

2.2.2 Production means and obtaining the crude extract of recombinant CGTase

For the production of recombinant *B. subtilis* CGTase, the methodology proposed by Fenelon et al. (2015) was used, with modifications. 2xYT medium supplemented with kanamycin (25 µg/mL) was used and 250 mL of liquid medium was prepared. 5 mL aliquots of the pre-inoculum were transferred to the production medium, which was incubated at 30 °C, at 100 rpm, for 5 days. 5 mL aliquots were collected every 24 hours to determine enzymatic activity. After the period of production of recombinant CGTase, the entire contents were centrifuged at 8,000 rpm, 4 °C for 10 min. The pellet composed of bacteria and insoluble compounds was discarded and a sample of the supernatant containing the enzyme was separated and named crude extract.

2.2.3 Obtaining semipurified and purified recombinant CGTase

The procedure for obtaining semi-purified recombinant CGTase was carried out through ultrafiltration processes, according to the methodology described by Fenelon et al. (2015). To obtain purified recombinant CGTase, the technique of biospecific affinity chromatography (CAB) was used, according to the methodology described by Moriwaki et al. (2009). An aliquot of each sample was used to determine the enzyme activity and protein concentration.

2.2.4 CDs production assays by CGTase of recombinant *B. subtilis* WB800

For the assays for the production of CDs by the CGTase of semi-purified and purified B. subtilis WB800, the reaction medium used was: substrate corn starch 5% (w/V), ethanol 10% (V/V), Tris-HCl 50 buffer mmol/L (pH 8.0) 20% (V/V), CaCl 2 solution 5 mmol/L 10% (V/V) and purified water q.s.p. 100% (Fenelon et al., 2015). The media were previously sterilized in an autoclave at 121 °C for 15 min.

2.2.4.1 Production of CDs in a continuous ultrafiltration system with CGTase from semipurified and purified recombinant *B. subtilis* WB800

Production was carried out continuously with 5% corn starch (w/V) substrate, in the presence of 10% ethanol (V/V), in a glass jacketed reactor coupled to a Hollow Fiber TE-0198 ultrafiltration module equipped with 50,000 NMWL exclusion threshold column. This system provided a constant separation of the CDs and other inhibitory products formed in the reaction medium and, at the same time, the retention of the recombinant CGTase, which returned to the reactor. The system was operated with a volume of 800 mL of the reaction medium and followed the parameters optimized by Matioli et al. (2001). The pH was controlled and maintained at 8.0 and the temperature at 50 °C. The concentration of semi-purified and purified recombinant CGTase was adjusted to obtain 0.1 U/mL of reaction medium (Fenelon et al., 2018). After the first 12 h of reaction, the continuous system was put into operation. Pump power was adjusted to 15%, resulting in an average flow of 4.5 mL/min, which was maintained until the drastic reduction of recombinant CGTase activity.

2.2.5 Production of CDs in alternative media

The production of CDs by the CGTase of *B. subtilis* WB800 was also studied using alternative media, which were evaluated to verify the efficiency of the recombinant CGTase in the production of CDs directly in the enzyme production step. The media used were: CD production media described in item 2.2.4 (medium 1A and 1B) and enzyme production media described in item 2.2.1, plus 5% corn starch substrate (medium 2A and 2B). All media were kanamycin supplements (25 µg/mL).

- **Medium 1A (with the presence of the microorganism):** 50 mL of the pre-inoculum **without centrifugation**, 5% corn starch substrate (w/V), 50 mmol/L Tris-HCl buffer (pH 8.0) 20% (V/ V), 5 mmol/L 10% CaCl2 solution (V/V) and purified water q.s.p 100%.

- **Medium 1B (only with the presence of the enzyme):** 50 mL of **centrifuged** pre-inoculum, 5% corn starch substrate (w/V), 50 mmol/L Tris-HCl buffer (pH 8.0) 20% (V/ V), 5 mmol/L 10% CaCl2 solution (V/V) and purified water q.s.p. 100%.

- **Medium 2A (with the presence of the microorganism):** 50 mL of pre-inoculum **without centrifugation**, 5% (w/v) corn starch substrate, 1.6% tryptone, 1% yeast extract, 0.5% NaCl and purified water q.s.p. 100%.

- **Meio 2B (only with the presence of the enzyme):** 50 mL of **centrifuged** pre-inoculum, 5% corn starch substrate (w/v), 1.6% tryptone, 1% yeast extract, 0.5% NaCl and purified water q.s.p. 100%.

For this evaluation, the tests were divided into two stages: 30 and 50 °C. All media were incubated in shakers at 100 rpm for 5 days. 5 mL aliquots were collected every 24 h for chromatographic analysis.

2.3 Analytical methods

2.3.1 Determination of enzyme activity, determination of protein concentration, and chromatographic determination of CDs

The protein concentration of CGTase from *B. subtilis* WB800 was determined by the method of Bradford (1976). Enzyme activity was determined according to the production of β-CD, quantified in a spectrophotometer at 550 nm (Matioli et al., 1998). The concentrations of α-CD, β-CD, and γ-CD were determined by HPLC using a Waters 2695 liquid chromatograph (Milford, MA, USA) equipped with a Waters 2414 refractive index detector and a Microsorb-MV 100 NH2 column. Acetonitrile and water solution (60:40) were used as mobile phase and flow rate of 1 mL/min at room temperature. Standard solutions and samples were filtered using 0.45 μm membrane. Analytical curves were constructed for α-CD, β-CD, and γ-CD in different concentration

778 ranges.

779 **2.4 Statistical analysis**

780 The assays were performed in triplicate and the results of enzymatic activity were
781 evaluated using analysis of variance (ANOVA) at a 5% significance level.

782

783 **3. RESULTS AND DISCUSSION**

784 **3.1 Enzymatic activity of CGTase from recombinant *B. subtilis* WB800**

785 After 5 days of production, the enzymatic activity of the crude extract was determined,
786 which resulted in 1.60 μmol of β-CD/min/mL. Results of activities and total protein of
787 semipurified and purified recombinant CGTases are described in Table 1.

788 **Table 1:** Enzyme activity, total protein, and specific activity of semipurified and
789 purified recombinant CGTase.

Fraction	Enzyme activity (μmol β-CD/min/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)
Semipurified	10,40 ± 0,02	4,31 ± 0,01	2,40 ± 0,04
Purified	8,90 ± 0,01	0,34 ± 0,03	25,62 ± 0,02

790

791 In the work carried out by Gimenez et al (2019), which used the same recombinant
792 CGTase of the present research, the value of the enzymatic activity for the purified
793 enzyme was 157.78 μmol of β-CD/min/mL, and the specific enzymatic activity of
794 114.92 U/mg. Comparing the results of Gimenez et al. (2019) with the present study, it
795 is possible to observe that the specific enzymatic activity, both for the semipurified and
796 the purified enzyme, was significantly lower. Therefore, to verify the role of the

recombinant bacterium in the production of the enzyme, new assays of activation and growth of the recombinant *B. subtilis* WB800 microorganism were carried out and the results obtained were similar to those shown in Table 1, inferring that some elements may have negatively influenced the activity or in the process of secretion of the enzyme in the production medium.

Thus, and according to Zhao et al. (2020), the plasmid pWB980, the same one used in the present study, is a promising expression vector in *Bacillus* due to its high copy number and high stability. However, the low rate of transformation of recombinant plasmids in wild-type cells may limit their application. Furthermore, the authors describe that plasmid stability consists of structural and segregation stability. Thus, it is hypothesized that the plasmid used for the cloning of the recombinant *B. subtilis* WB800 bacterium may have suffered some interference in its structural stability during the storage time, implying the segregation structure of the recombinant enzyme.

3.2 Production of CDs in continuous ultrafiltration system for 120 h

The production of CDs using a continuous system associated with the ultrafiltration process was carried out from the semi-purified and purified enzymes. Continuous production was maintained for 120 h (5 days). Aliquots of the ultrafiltrate were collected every 12 hours to determine the concentration of CDs produced. The production of β-CD in the first 12 h, without ultrafiltration, was 17.16 mmol/L. After this period, the continuous process with ultrafiltration was started and, after 24 h, it was possible to observe a decrease in the concentration of CDs produced (9.83 mmol/L of β-CD). The production of CDs was approximately constant for 120 h, which was terminated with 7.14 mmol/L of β-CD (Figure 1).

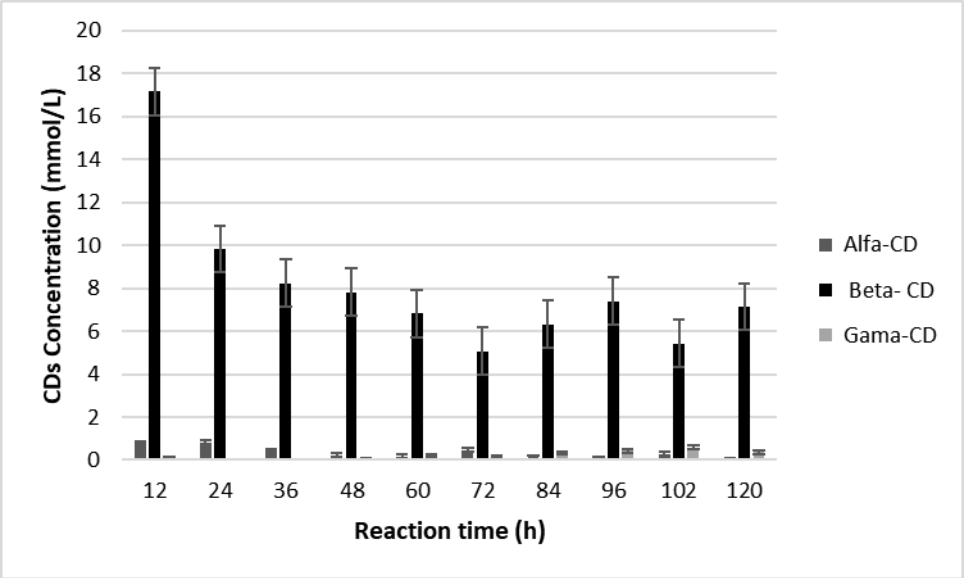


Figure 1: Production of CDs in a continuous ultrafiltration system for 120 h, using semi-purified recombinant CGTase, 5% (w/V) corn starch substrate, 10% (V/V) ethanol, pH 8.0.

The production of α-CD and γ-CD in the first 12 h was 0.78 mmol/L and 0.09 mmol/L, respectively, and decreased throughout the 120 h of the assay. With the results obtained, a greater selectivity was observed for the production of β-CD.

Figure 2 shows the production of CDs using the purified recombinant CGTase and, unlike the production from the semi-purified enzyme, the 12 h batch showed more significant production of α-CD and β-CD, that is, 12.35 mmol /L and 9.46 mmol/L, respectively. Similar behavior was verified in the other batches. It is possible to suggest that the purification of the enzyme eliminates compounds that prevent or inhibit the production of α-CD by binding to the active site of the enzyme responsible for the production of this CD. A reduction in the production of total CDs of around 30% was also observed, while the expectation was the opposite. Therefore, it is possible to suggest that compounds eliminated during purification may be important to maintain or

increase enzyme activity. Another viable possibility is to alter the protein structure of the enzyme during purification.

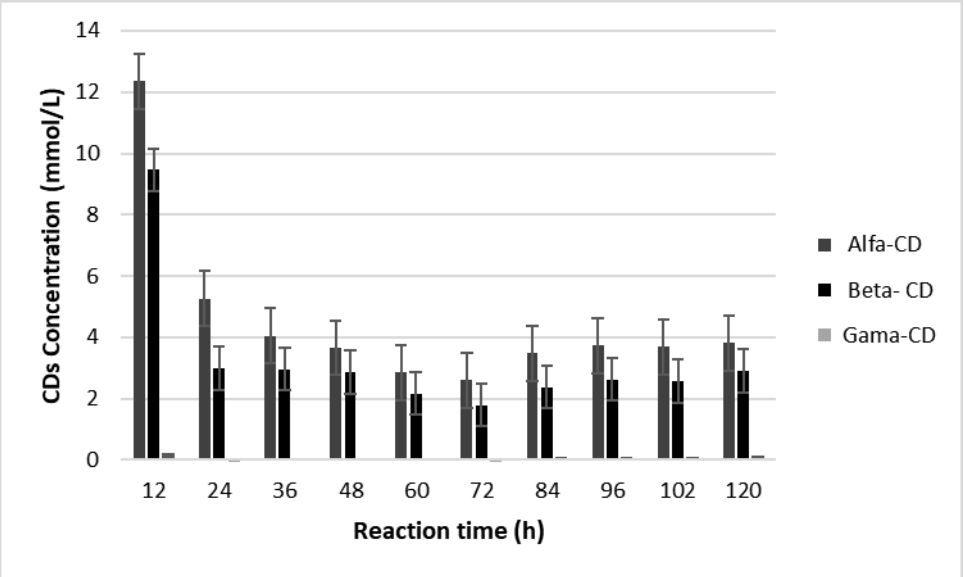


Figure 2: Production of CDs in a continuous ultrafiltration system for 120 h, using purified recombinant CGTase, 5% (w/V) corn starch substrate, 10% (V/V) ethanol, pH 8.0.

Koga et al. (2020) also used the ultrafiltration system with the commercial enzyme Toruzyme® and evaluated the production of CDs in eight batches of 72 h. The authors obtained a maximum production of α-, β- and γ-CD equal to 24.75 mmol/L, 20.59 mmol/L, and 1.66 mmol/L, in the first batch, and a production of 13.51 mmol/L of α-CD and 7.96 mmol/L of β-CD in the last batch. The Toruzyme® enzyme is marketed as an α, β-CGTase, that is, it produces similar amounts of α- and β-CD, requiring a subsequent process of separation of these CDs.

In view of the results obtained in the present study, it is possible to suggest that, even with the low enzymatic activity observed previously (item 3.1), the continuous process associated with ultrafiltration is a promising strategy for the production of CDs, since the production of α- and β-CD , although it decreased after 24 h of production, it was

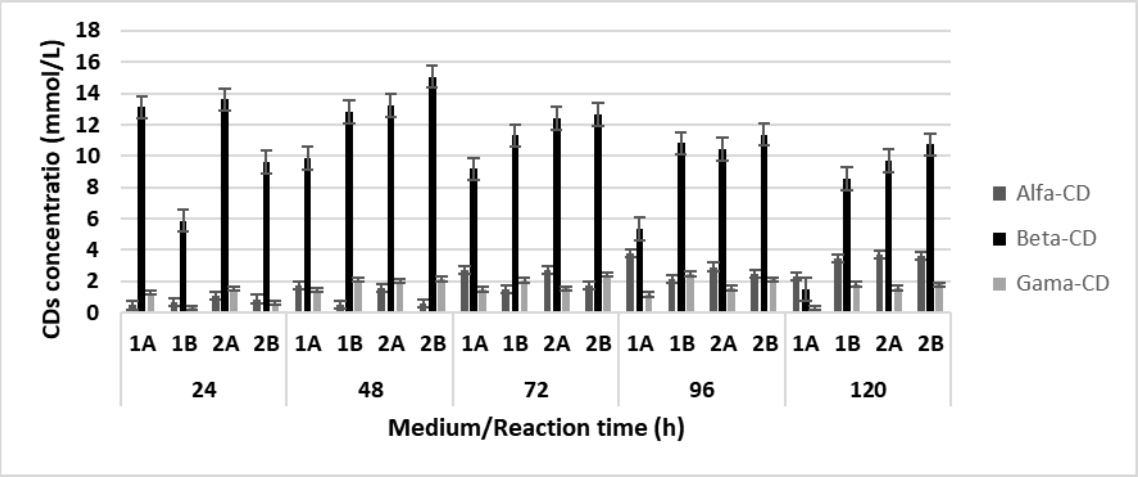
constant throughout the 120 h of the assay, without the need to add more enzyme to the reaction medium during the time of production of the CDs. It is also worth noting that the use of a semi-purified enzyme, in addition to resulting in a more economical process because it does not have expenses with the purification of the enzyme, produces much more β-CD in relation to other CDs, not requiring separation and purification.

3.3 Production of CDs in alternative media

A new challenge for this research was to obtain CDs directly from the production medium of the recombinant CGTase enzyme. The media used were selected based on previous studies, which showed good results in the production of CDs and growth of the recombinant CGTase enzyme (Fenelon et al., 2015; Hao et al., 2017).

Research has also shown that corn starch is one of the most efficient substrates for the production of CDs (Fenelon et al., 2015). Therefore, to analyze the behavior of the bacteria in the production of CDs simultaneously with the step of obtaining the recombinant CGTase enzyme, 5% corn starch (w/v) substrate was added to all tested media. In addition, all media were supplemented with the antibiotic kanamycin (25 μg/mL), to provide selectivity, since only the recombinant bacterium is resistant to kanamycin.

Figure 3 presents the results obtained in the first step, which used an incubation temperature equal to 30 °C, which is the ideal temperature for the growth of the recombinant CGTase enzyme.



875

876 **Figure 3 -** Chromatographic determination of CDs using CD production medium with
877 5% (w/v) corn starch substrate with microorganisms (1A medium) and enzyme only (1B
878 medium), and recombinant bacteria growth medium (2xYT) with the addition of 5% of
879 the starch substrate with microorganisms (2A medium) and only with enzyme (2B
880 medium). All media were incubated at 30°C, 100 rpm, for 5 days.

881 With the results obtained, it was verified that all the evaluated media showed
882 considerable production of CDs, even without the enzyme going through the semi-
883 purification and/or purification step. The 48 h time showed the highest production of
884 CDs, especially β-CD, especially in the 2xYT medium, regardless of the presence of the
885 microorganism or just the enzyme (13.26 and 15.06 mmol/L of β- CD, respectively).
886 Also, Gimenez and collaborators (2019) evaluated different means of production of
887 recombinant CGTase and observed that the 2xYT medium was the most efficient for the
888 growth of the bacteria and the production of the enzyme. Thus, the results obtained in
889 this research corroborate those obtained in previous research.

890 Furthermore, it was possible to observe that the concentration of β-CD began to
891 progressively decrease, especially in medium containing microorganisms (1A medium).
892 This event may be related to the fact that the microorganism is producing other enzymes
893 that act in the degradation of the recombinant CGTase or, still, due to the possibility of

the microorganism consuming the CDs over time. Similar behavior was observed by Fenelon et al (2018), who evaluated the strategy of producing CDs in 12 h repetitive batches with the semi-purified non-recombinant enzyme and observed a maximum production value equal to 12.6 mmol/L in The first batch and the following batches verified that the production of β-CD progressively decreased until reaching values below 50% of the initial capacity. The authors also evaluated the continuous production strategy with ultrafiltration and until 36 h the production of β-CDs remained high (15.3 mmol/L), however, the β-CD yield gradually decreased throughout the assay.

Figure 4 shows the results obtained when using an incubation temperature equal to 50 °C, which is the ideal temperature for the growth of the recombinant CGTase enzyme.

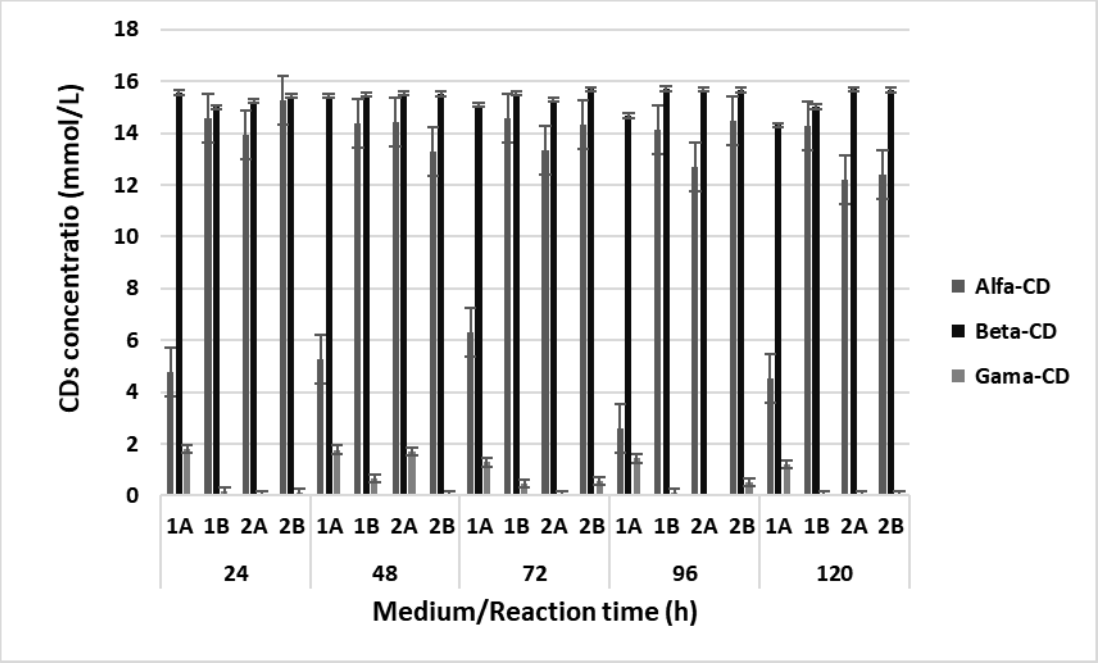


Figure 4 - Chromatographic determination of CDs using CD production medium with 5% (w/v) corn starch substrate with microorganisms (1A medium) and enzyme alone (1B medium), and recombinant bacteria growth medium (2xYT) with the addition of

5% of the starch substrate with microorganisms (2A medium) and only with enzyme (2B medium). All media were incubated at 50 °C, 100 rpm, for 5 days.

The temperature of 50 °C was more efficient for the production of CDs, especially for β-CD, which showed an average production of approximately 15 mmol/L throughout the entire assay. The production of α-CD was also more significant compared to the previous trial. The CD production medium (Medium 1) showed a slight drop in CD yield, while the 2xYT medium maintained a continuous yield. In addition, and similar to the production at 30 °C, medium 1A was the one that showed the highest production of β-CD at the initial time (24 h) and the one that showed the lowest yield in the final period (120 h), which it also suggests the production of other enzymes that may be degrading the produced CDs.

Gregolim et al (2019) evaluated optimal conditions for the production of CDs for CGTase from B. subtilis WB800 and compared it with the production of strain 37 of *B. firmus* and found that the catalytic properties of the recombinant CGTases were equivalent, that is, the yield of production was similar for the two lines. The authors showed a β-CD yield of approximately 13 mmol/L in 24 h of production, which is lower than that observed in the present study.

4. CONCLUSION

Although the recombinant B. subtilis WB800 CGTase enzyme showed low enzymatic activity, possibly due to interference in its structural stability during storage, it was possible to conclude that the use of the continuous production system associated with the ultrafiltration process proved to be a beneficial alternative to optimize CD production. Alternative media plus corn starch was an interesting strategy, especially for

the production of β-CD, which is currently the most used and commercially available. In addition, the use of the crude enzyme is a promising alternative, as it contributes to the reduction of costs and steps in the production of CDs and, consequently, can favor its industrial application.

5. ACKNOWLEDGMENT

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ANEXOS

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Original article

Obtaining of bioactive di- and tripeptides from enzymatic hydrolysis of soybean meal and its protein isolate using Alcalase® and Neutrase®

Thamara Thaiane da Silva Crozatti,[1] Juliana Harumi Miyoshi,[1] Angélica Priscila Parussolo Tonin,[1] Larissa Fonseca Tomazini,[1] Marco Aurélio Schuler Oliveira,[1] José Uebi Maluf,[2] Eduardo Cesar Meurer[3] & Graciette Matioli[1]*

1 State University of Maringá (UEM), Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil
2 BRFoods, Av. Senador Atílio Fontana, 4040, 85902-160, Toledo, PR, Brazil
3 Federal University of Paraná (UFPR), Advanced Campus Jandaia do Sul, 86900-000, Jandaia do Sul, PR, Brazil

(Received 24 January 2022; Accepted in revised form 24 May 2022)

Summary The obtaining of bioactive di- and tripeptides using Alcalase® and Neutrase® enzymes in the hydrolysis of soybean meal (SM) and its protein isolate (SPI) was evaluated. An innovative system by fast LC-MS/MS neutral loss screening and *de novo* sequencing was used to identify bioactive peptides. Soy protein characterisation, gel electrophoresis and antioxidant activity of the obtained peptides were performed. Results achieved showed that the use of Alcalase® and SPI preparation potentiated the peptide breaking bonds and favoured the obtainment of bioactive peptides. The antioxidant activity of tested samples was significantly improved with enzymatic hydrolysis. LC-MS/MS analyses identified nineteen peptides in SM and 51 in SPI, all obtained after hydrolysis with Alcalase® and, according to BIOPEP, with relevant bioactivities and expressive functional potential. Therefore, it is suggested that bioactive peptides achieved in this study can enable the development of new ingredients and provide greater added value to soy by-products.

Keywords Soybean meal, enzymatic hydrolysis, dipeptides, tripeptides, bioactivities.

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• Review your submission before sending to the Journal. Click the 'Submit' button when you are finished reviewing.

3.3. Blinded Review
All manuscripts submitted to the *International Journal of Food Science & Technology* will be reviewed by at least two experts in the field. The *International Journal of Food Science & Technology* uses single-blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

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After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by spam filtering software on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

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5 Cambridge Court
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London, W6 7NJ, UK

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(a) a concise Summary (fewer than 150 words) containing the main results and conclusions;
(b) up to ten keywords that accurately identify the paper's subject, purpose and focus;
(c) an Introduction giving essential background but no subheadings; objectives must be clearly stated;
(d) Materials and methods with sufficient full experimental detail (where possible by reference) to permit repetition; sources of material must be given and statistical methods must be specified by reference, unless non-standard;
(e) Results should be presented concisely, using well-designed tables and/or figures; the same data may not be used in both; appropriate statistical data should be given. All data must be obtained with attention to statistical detail in the planning stage. If a sufficiently large number of replicates are not organized before the experiment is undertaken, biological variation is not eliminated satisfactorily. As replicate design has been recognised to be important to biological experiments for a considerable time, the Editor has decided that any paper that appears not to have adequate mathematical treatment of the data will be returned un-refereed;
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(g) brief Acknowledgements;
(h) References as shown below.

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Letters to the Editor: These are brief comments on material published in previous issues; they are published at the discretion of the Editor. They are the only items not subject to multiple peer review.

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Authors should very carefully consider the preparation of papers to ensure that they communicate efficiently. Papers are much more likely to be accepted if they are carefully designed and laid out, have few or no errors, are concise, and conform to the style and instructions. They will also be published with much less delay than those that require much scientific and editorial correction.

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Graphical abstract: Please upload the Graphical Abstract as the first file in the Manuscript. Please ensure that it is clearly sub-titled 'Graphical Abstract.' The Graphical Abstract should be designed to be read on-line in conjunction with the text abstract, it should be approximately square, ideally in colour and should contain a high impact Figure, Graph or Photograph that summarises the key findings of your research.

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Cochran, W.G., Cox, G.M. (1992).
Experimental Designs, 2nd edn. New York: Wiley.
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Sokal, R.R., Rohlf, F.J. (1994) *Biometry*, 3rd edn. San Francisco: W.H. Freeman.
Steel, R.G.D., Torrie J.H., Dickey, D. (1996). *Principles and Procedures of Statis*tics. McGraw-Hill.
General Papers
Chatfield, C. (1985). The initial examination of data. *Journal of the Royal Statistical Society A*, **148**, 214-253
Preece, D.A. (1987). Good statistical practice. *The Statistician*, **36,** 397-408.

Repeated Measures
Kenward, M.G. (1987). A method for comparing profiles of repeated measurements. *Applied Statistics*, **36,** 296-308.

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References follow the Harvard system of referencing. References in the text should cite the authors' names followed by the date of their publication, unless there are three or more authors when only the first author's name is quoted followed by *et al*. e.g. Smith *et al.* (1999) or Jones and Smith (2000). Add a, b, c etc. to distinguish between two or more references with the same author name and year date (e.g. Jones 1999a,b). References at the end of the paper should be listed in alphabetical order with the title of the article or book and the title of the journal given in full, as shown:

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Stone, H. & Sidel, J. L. (1985). *Sensory Evaluation Practices*. Pp. 56-59. Orlando, USA: Academic Press.
Dubois, P. (1983). Volatile phenols in wines. In: *Flavour of Distilled Beverages* (edited by J. R. Piggott). Pp. 110-119. Chichester, UK: Ellis Horwood.

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We recommend the use of a tool such as Reference Manager for reference management and formatting.

Reference Manager reference styles can be searched for here:
www.refman.com/support/rmstyles.asp

5.5. Tables, Figures and Figure Legends

Tables: Tables should be few in number, carefully designed, uncrowded, and include only essential data. Each must have an Arabic number, e.g. Table 3, a self-explanatory caption and be on a separate sheet. Vertical lines must not be used.

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ANEXO 3: Comprovante de aceite/publicação

(Food Science and Technology)

De: **Adriano Cruz** <onbehalfof@manuscriptcentral.com>
Date: ter., 6 de dez. de 2022 às 23:42
Subject: Food Science and Technology - Decision on Manuscript ID CTA-2022-1041.R1
To: <gmatioli@uem.br>

07-Dec-2022

Dear Dr. Matioli:

It is a pleasure to accept your manuscript entitled "Challenges and alternatives for the production of cyclodextrins from the CGTase enzyme from recombinant Bacillus subtilis WB800" in its current form for publication in the Food Science and Technology. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Food Science and Technology, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Adriano Cruz
Editor-in-Chief, Food Science and Technology
adriano.cruz@ifrj.edu.br

Original Article
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Challenges and alternatives for the production of cyclodextrins from the CGTase enzyme from recombinant *Bacillus subtilis* WB800

Thamara Thaiane da Silva CROZATTI[1], Paula Vitória LARENTIS[2], Vanderson Carvalho FENELON[3], Juliana Harumi MIYOSHI[3], Júlia Rosa de BRITO[4], Giovanna da Silva SALINAS[2], Beatriz de Oliveira MAZZOTTI[2], Giovanni Cesar TELES[1], Quirino Alves de LIMA NETO[5], Graciette MATIOLI[1,2,3*]

Abstract

Cyclodextrins (CDs) have the ability to encapsulate numerous molecules and have applicability in several industrial areas, however, their cost has made their use difficult. To seek alternatives that may enable the use of DCs, the present study evaluated the efficiency of the ultrafiltration process in a continuous system to produce CDs from the enzyme cyclomaltodextrin glucanotransferase (CGTase) from recombinant *Bacillus subtilis* WB800. The possibility of using the crude enzyme as an alternative means of producing CDs was also evaluated. All strategies evaluated in this research proved to be promising for the production of CDs, with the production of β-CD being the most efficient (average of 15 mmol/L) using crude recombinant enzyme and a temperature of 50 °C. Therefore, the results obtained can contribute to the reduction of stages and costs of production of CDs, favoring their industrial application.

Keywords: cyclodextrins; CGTase recombinant; continuous system; ultrafiltration.

Practical Application: This study presents innovative alternatives to produce cyclodextrins from the recombinant enzyme.

ANEXO 4: Regras da Revista

(Food Science and Technology)

1. Sobre o jornal

Informação básica

Ciência e Tecnologia de Alimentos, de periodicidade de fluxo contínuo, Sociedade Brasileira de Ciência e Tecnologia de Alimentos - SBCTA, com o objetivo de publicar artigos científicos e comunicações na área de ciência de alimentos.

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Fator de Impacto 2022 JCR 2.6

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Estrutura de Papel

A revisão da estrutura do manuscrito e das informações fornecidas é de responsabilidade dos autores. Os manuscritos originais não devem exceder 16 páginas (excluindo as referências).
O texto deve ser apresentado com espaçamento duplo entre linhas no formato de uma coluna. Todas as linhas devem estar niveladas com a margem esquerda da coluna, deixando uma margem de 2,5 cm à direita e à esquerda. As linhas de texto devem ser numeradas sequencialmente ao longo do texto. Todas as páginas devem ser numeradas sequencialmente (ver item "Formato dos Arquivos" no final deste guia).

Carta de apresentação

A carta de apresentação do manuscrito deve incluir o seguinte:

- Declaração de relevância e importância do trabalho : um breve texto com no máximo 100 palavras descrevendo a relevância do trabalho de forma concisa;
- Títulos :
- Título em inglês;
- Cabeçalho da página (não mais que 6 palavras).

Folha de rosto

A página de título do manuscrito deve incluir o seguinte:

- Nome completo e e-mail dos autores;
- Nomes abreviados dos autores para citação (Ex.: nome completo: José Antonio da Silva; nome abreviado: Silva, JA);
- Filiação dos autores: nome da instituição a que pertence cada autor (nome completo e siglas, endereço postal completo, CEP, cidade, estado e país). Por favor, correlacione cada autor à sua instituição correspondente;
- Informações de correspondência dos autores (nome completo, endereço postal completo, números de telefone e fax e endereço de e-mail do autor correspondente).

Resumo, Aplicação prática e página de palavras-chave

Abstrato

O resumo deve:

- Estar somente em inglês;
- Ser um único parágrafo contendo menos de 200 palavras;
- Declarar claramente o objetivo principal e a justificativa do artigo;
- Apresentar resumidamente as principais conclusões;
- Se aplicável, descreva os métodos e resultados dos materiais;
- Resuma as conclusões;
- Seja parcimonioso com abreviaturas e acrônimos.

O resumo não deve incluir:

- notas de rodapé;
- Dados significativos e valores estatísticos;
- Referências.

Aplicação prática

Texto curto com no máximo 85 caracteres, indicando inovações e características importantes do estudo. A “Aplicação Prática” será publicada.

Palavras-chave

O manuscrito deve ter no mínimo três (3) e no máximo seis (6) Palavras-chave. As palavras-chave devem estar apenas em inglês. Evite usar termos incluídos no texto principal do manuscrito nas palavras-chave.

páginas de texto

O manuscrito deve ser organizado da seguinte forma:

- Introdução;
- Materiais e métodos; deve incluir delineamento experimental e análise estatística de dados;
- Resultados e Discussão (também podem ser separados);
- Conclusões;
- Referências;
- Agradecimentos (opcional).

No texto principal:

- Abreviaturas, acrônimos e símbolos devem ser claramente definidos na primeira utilização;
- Notas de rodapé não são permitidas;
- O uso de títulos e subtítulos é encorajado quando necessário, mas faça uso deles sem comprometer a clareza do texto. Devem ser numeradas na ordem em que aparecem no texto;
- As equações devem ser geradas por computador e numeradas sequencialmente com algarismos arábicos entre parênteses na ordem em que são referidas no texto. As equações devem ser referenciadas no texto e no local indicado pelo autor. Por favor, não envie imagens de equações. Não serão aceitas equações fornecidas separadamente; serão aceitos apenas aqueles inseridos no texto.

Tabelas, Figuras e Gráficos

Forneça no máximo sete (7) Tabelas, Figuras e Gráficos. Devem ser numeradas em algarismos arábicos na ordem em que são destacadas no texto. No *Manuscrito.pdf* – versão para avaliação dos revisores e no *Manuscrito.doc* – versão para produção, tabelas, equações, figuras, quadros e suas respectivas legendas devem constar no corpo do texto nos locais indicados pelos autores. Veja abaixo as instruções da versão para produção.

Figuras e gráficos (versão para produção)

Figuras e quadros devem ser fornecidos no texto principal e numerados consecutivamente com algarismos arábicos e suas respectivas legendas devem ser incluídas no texto principal no local indicado pelos autores. Ao fornecer figuras contendo fotografias ou micrografias, certifique-se de que sejam digitalizadas em alta resolução para que cada foto tenha pelo menos 1.000 pixels de largura. Todas as fotografias devem conter o nome do autor. Os gráficos devem ser usados para apresentar arquivos, esquemas e fluxogramas.

Tabelas (versão para produção)

As tabelas devem ser fornecidas no texto principal e numeradas com algarismos arábicos. Devem ser inseridos no texto no local indicado pelo autor. As tabelas devem ser preparadas usando o Microsoft Word® 2007 ou posterior; não devem ser importados do Excel® ou Powerpoint® e devem:

- Ter uma legenda e um título;
- Seja auto-explicável;
- Ter os algarismos significativos definidos segundo critério estatístico considerando os algarismos significativos no desvio padrão;
- Ser usado com parcimônia para garantir consistência visual e que o texto seja fácil de ler;
- Mostrar dados que não são mostrados nos gráficos;
- Ter o formato mais simples possível; não é permitido o uso de sombras, cores ou linhas verticais e diagonais;
- Ter apenas letras minúsculas sobrescritas indicando notas de rodapé (abreviaturas, unidades, etc). As colunas devem ser indicadas primeiro, depois as linhas, e esta mesma ordem deve ser seguida para as notas de rodapé.

Nomes proprietários

Devem ser especificadas as matérias-primas, equipamentos para fins especiais e softwares de computador utilizados na pesquisa (marca-fabricante, modelo, cidade e país de origem).

Unidades de medida

- Utilizar unidades do SI (Sistema Internacional de Unidades);
- As temperaturas devem ser expressas em graus Celsius (°C).

Referências

Citações no texto

As referências bibliográficas inseridas no texto devem ser feitas de acordo com o sistema "Autor/Data". Por exemplo, citação contendo um autor: Sayers (1970) ou (Sayers, 1970); com dois autores: Moraes & Furuie (2010) ou (Moraes & Furuie, 2010); citações com mais de dois autores devem apresentar a menção do primeiro autor seguida da expressão “et al.”. Quando a citação se referir a uma instituição, seu

nome deve ser apresentado por extenso.

Lista de referência

A Revista Food Science and Technology (CTA) adota o estilo de citações e referências bibliográficas da *American Psychological Association* - APA. A política completa e os tutoriais podem ser verificados em http://www.apastyle.org .

A lista de referências deve ser preparada primeiro alfabeticamente e, se necessário, cronologicamente. Referências múltiplas do mesmo autor no mesmo ano devem ser identificadas pelas letras 'a', 'b', 'c', etc. colocadas após o ano de publicação.

Artigos em preparação ou submetidos para revisão não devem ser incluídos nas referências. Os nomes de todos os autores devem ser listados nas referências; portanto, o uso da expressão 'et al.' não é permitido. Conforme determinação da sbCTA, artigos aceitos cujas referências bibliográficas não estejam de acordo com as normas da Revista NÃO SERÃO PUBLICADOS até que as normas sejam atendidas.

Exemplos de estilo para referências:

Livros
Baccan, N., Aleixo, LM, Stein, E., & Godinho, OES (1995). *Introdução à semimicroanálise qualitativa* (6. ed.). Campinas: EduCamp.
Universidade Estadual de Campinas - UNICAMP. (2006). *Tabela brasileira de composição de alimentos - TACO* (versão 2, 2. ed.). Campinas: UNICAMP/NEPA.

Capítulo de livro
Sgarbieri, VC (1987). Composição e valor nutritivo do feijão Phaseolus vulgaris L. In EA Bulisani (Ed.), *Feijão: fatores de produção e qualidade* (cap. 5; pp. 257-326). Campinas: Fundação Cargill.

Artigos de periódicos
Versantvoort, CH, Oomen, AG, Van de Kamp, E., Rompelberg, CJ e Sips, AJ (2005). Aplicabilidade de um modelo de digestão in vitro na avaliação da bioacessibilidade de micotoxinas de alimentos. *Food and Chemical Toxicology, 43* (1), 31-40.

Sillick, TJ, & Schutte, NS (2006). A inteligência emocional e a auto-estima medeiam entre o amor parental precoce percebido e a felicidade adulta. *E-Journal of Applied Psychology, 2* (2), 38-48. Recuperado de http://ojs.lib.swin.edu.au/index.php/ejap

Trabalho eletrônico (e-work)
Richardson, ML (2000). *Abordagens para diagnóstico diferencial em imagem musculoesquelética* (versão 2.0). Seattle: Escola de Medicina da Universidade de Washington. Obtido em http://www.rad.washington.edu/mskbook/index.html

Legislação
Brasil, Ministério da Educação e Cultura. (2010). *Institui a Política Nacional de Resíduos Sólidos; altera a Lei nº 9.605, de 12 de fevereiro de 1998; e dá outras providências* (Lei nº 12.305, de 2 de agosto de 2010). Diário Oficial da República Federativa do Brasil.

Teses e Dissertações
Fazio, MLS (2006). *Qualidade microbiológica e ocorrência de leveduras em polpas congeladas de frutas* (Dissertação de mestrado). Universidade Estadual Paulista, São José do Rio Preto.

Artigos previamente apresentados em conferências científicas
Sutopo, W., Nur Bahagia, S., Cakravastia, A., & Arisamadhi, TMA (2008). Um Modelo de Estoque Regulador para Estabilização de Preços de Commodities em Tempo Limitado de Fornecimento e Consumo Contínuo. Nos *Anais da 9ª Conferência de Sistemas de Gerenciamento e Engenharia Industrial da Ásia-Pacífico* (APIEMS), Bali, Indonésia.

Formato de arquivos

O texto principal do manuscrito deve ser submetido da seguinte forma:

Manuscrito.doc:

- formato Microsoft Word® 2007 ou posterior;
- Fonte: Times New Roman, Arial ou Tahoma tamanho 12;
- Espaçamento duplo entre linhas;
- Figuras, tabelas, tabelas, equações e respectivas legendas devem ser incorporadas ao texto na posição preferida pelo autor;
- O arquivo não pode exceder 16 páginas, além da lista de referências bibliográficas
- As linhas e páginas devem ser numeradas sequencialmente;
- A folha de rosto com o nome dos autores e instituições deve ser apresentada em arquivo separado
- O manuscrito deve ser nomeado.

Depois de verificar o estilo do formato e criar os arquivos de acordo com as c prossiga para a submissão on-line usando o On-line (consulte abaixo).

Link: http://mc04.manuscriptcentral.com/cta-scielo

Taxa de publicação

A Food Science and Technology (CTA) cobrará uma taxa de publicação para os artigos aceitos de acordo com os seguintes critérios:

- USD 300,00 - De autores não associados ao sbCTA;
- USD 250,00 - Se pelo menos um autor for membro do sbCTA e for remunerado com anuidade;
- USD 200,00 - Se pelo menos dois autores forem sócios da sbCTA e estiverem empatados com a anuidade;
- **USD 750,00 - Autores de outros países**

O processo de criação do artigo aceito inicia-se somente após o pagamento da taxa de publicação.

Revisão da Língua Inglesa

Os trabalhos devem ser enviados em inglês, acompanhados de carta atestando sua edição, assinada por especialista na língua inglesa (nativo ou não nativo). Toda edição em inglês deve ser acompanhada de uma carta detalhando os ajustes feitos no documento original.

Antes da submissão online, o autor correspondente deverá preencher e assinar o Termo de Adesão e Cessão de Direitos de Reprodução Gráfica.

https://www.sbcta.org.br/downloads/Terms-of-Agreement-and-Submitting-Rights.pdf

Envie este formulário por e-mail para o Conselho Editorial da sbCTA para publicacoes@sbcta.org.br ou +55 19 32410527. O processo de avaliação não terá início até que o Termo de Acordo e Cessão de Direitos de Reprodução Gráfica seja enviado e recebido.

Contato

Sociedade Brasileira de Ciência e Tecnologia de Alimentos / SBCTA
Av. Prof. Brasil 2880 – 13001-970 Campinas - SP, Brasil - Caixa Postal: 271
Fone/Fax: +55 (19) 3241-0527 – Fone: +55 (19) 3241-5793
e-mail: publicacoes@sbcta.org.br