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APPLICATION OF ENZYMATIC PRE-TREATMENTS BY CARBOHYDRASES AND BY PROTEASE DURING THE SOYBASE PROCESSING

APLICAÇÃO DE PRÉ-TRATAMENTOS ENZIMÁTICOS POR CARBOIDRASES E PROTEASE DURANTE O PROCESSAMENTO DE BASE DE SOJA

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RESUMO

Extrato de soja é um produto que vem apresentando aumento no consumo na última década devido às alegações benéficas à saúde e por ser substituto de leite de vaca às pessoas intolerantes ou alérgicas. Durante o seu processamento, na etapa de filtragem, é gerada uma grande quantidade do resíduo okara, o qual é rico em proteínas e isoflavonas, demonstrando que esses compostos não são extraídos ao produto principal. A utilização de processos enzimáticos durante a fabricação poderia extrair esses compostos intracelulares da matriz sólida, aumentando a extração para a matriz líquida. Os objetivos do trabalho foram aplicar dois tipos de enzimas, carboidrases e protease, durante processamento do extrato de soja, e avaliar os efeitos na recuperação de proteínas e na recuperação e perfil de isoflavonas na matriz líquida. Para os experimentos com carboidrases, foram estudadas as concentrações de 2, 4 e 6%, e para protease, 1, 2 e 4% de enzima. Foram aplicados pH e temperatura ótima de ação enzimática, e a avaliação dos resultados foi realizada em relação a um experimento controle, sem adição da enzima, e ao processamento padrão, sem adição de enzima, ajuste de pH e temperatura. O uso do complexo multienzimático de carboidrases resultou em maior rendimento de processo, gerando menor quantidade de okara, consequentemente uma maior quantidade de extrato de soja. A recuperação proteica foi maior para todos os tratamentos estudados, alcançando valor máximo com 6% de enzima. A recuperação de isoflavonas totais aumentou de acordo com o aumento da quantidade de enzima. O emprego do complexo de carboidrases também resultou em um extrato de soja com maior quantidade de agliconas em relação às outras formas de isoflavonas. A utilização de enzima protease aumentou a recuperação de proteína, em relação à amostra padrão, somente no tratamento com 2% enzima e 15 min de hidrólise. As isoflavonas foram degradadas devido aos parâmetros de pH e temperatura usados, e a hidrólise da proteína contribuiu para uma maior degradação. Uma recuperação significativa das isoflavonas não degradadas em relação à amostra padrão foi alcançada, mas nenhuma influência da proteólise foi observada. Os parâmetros de processamento e o uso da enzima influenciaram na interconversão entre as formas de isoflavonas, com diminuição das formas de malonilglicosídeos, predomínio das formas de β-glicosídeos e aumento das formas de agliconas, em relação ao processo padrão.

Palavras-chave: Extrato de soja, enzimas, hidrólise, isoflavonas, proteínas.

ABSTRACT

Soy extract is a product that has been increasing in consumption in the last decade due to its beneficial health claims and because it is a substitute for cow's milk for people who are intolerant or allergic. During processing, in the filtration stage, a large amount of okara residue is generated, which is rich in proteins and isoflavones, demonstrating that these compounds are not extracted to the main product. The use of enzymatic processes during manufacture could extract these intracellular compounds from the solid matrix, increasing the extraction to the liquid matrix. The objectives of the work were the application of two types of enzymes, carboidrases and protease, during the processing of the soy extract, and evaluation of the effects on protein recovery, recovery and isoflavone profile in the liquid matrix. For the experiments with carboidrases, the concentrations of 2, 4 and 6% were studied, and for protease, 1, 2 and 4% of enzyme. pH and optimum temperature of enzymatic action were used, and the evaluation of the results was carried out in relation to a control experiment, without enzyme addition, and standard processing, without adding enzyme, pH and temperature adjustment. The use of the carbohydrases multi-enzymatic complex resulted in a higher process yield, generating a lower amount of okara, consequently a greater amount of soy extract. Protein recovery was increased for all treatments studied, reaching a maximum value with 6% enzyme. The recovery of total isoflavones increased with increasing amount of enzyme. The use of the complex of carbohydrases also resulted in a soy extract with a greater amount of aglycone compared to other isoflavone forms. The use of protease enzyme increased the protein recovery, in relation to the standard sample, only in the treatment with 2% enzyme and 15 min of hydrolysis. Isoflavones were degraded due to the pH and temperature parameters used, and the protein hydrolysis contributed to further degradation. A reduced recovery of the non-degraded isoflavone was achieved, but no influence of proteolysis was observed, in relation to standard sample. The processing parameters and protease use influence the interconversion among the isoflavone forms, with a decrease in the malonylglucosides, a predominance of β-glucosides and an increase in aglycones, in relation to the standard process.

Keywords: Soy extract, enzymes, hydrolysis, isoflavones, proteins.

SUMÁRIO

CAPÍTULO 1. INTRODUÇÃO GERAL, OBJETIVOS E ESTRUTURA DO TRABALHO

1.1.Introdução Geral

A soja é a principal cultura de oleaginosas e considerada uma das fontes mais econômicas de proteína para mitigar a desnutrição e a subnutrição nos países em desenvolvimento (KUMAR; RANI; HUSSAIN, 2016). Uma ampla variedade de produtos à base de soja pode ser encontrada no mercado (KUMAR; RANI; HUSSAIN, 2016). Entre esses produtos, estão os fermentados (missô e shoyu) e não fermentados, como o extrato de soja, tofu, farinha (kinako), proteína texturizada (PTS), entre outros (CAPRIOTTI et al., 2014). O extrato de soja apresentou um notável aumento de vendas na última década. Esse crescimento pode ser devido a várias razões, entre elas, os benefícios da soja para a saúde, além do uso desse produto em alternativa ao leite de vaca para pessoas vegetarianas, alérgicas à proteína do leite e/ou intolerantes à lactose (LAWRENCE; LOPETCHARAT; DRAKE, 2016; XU; CHANG, 2009). O extrato de soja contém altas quantidades de proteína, ferro e niacina, e, em comparação ao leite de vaca, baixas concentrações de gordura, carboidratos e cálcio, além de ser livre de colesterol (JINAPONG; SUPHANTHARIKA; JAMNONG, 2008).

O extrato de soja é o produto obtido através da maceração dos grãos, homogeneização em água, filtragem e pasteurização. Após a etapa de filtragem, o resíduo insolúvel retido é denominado okara (O'TOOLE, 1999). O filtrado é uma solução coloidal que contém 3,6% de proteínas, sendo 80% desse conteúdo representado por β-conglicinina (7S) e glicinina (11S), além de também ser uma excelente fonte de isoflavonas (HSIAO et al., 2016).

Nos derivados de soja, as isoflavonas são encontradas em doze componentes, ocorrendo em quatro estruturas químicas distintas, sendo agliconas (daidzeína, genisteína e gliciteína), β-glicosídeos (daidzina, genistina e glicitina), acetilglicosídeos (acetildaidzina, acetilgenistina e acetilglicitina) e malonilglicosídeos (malonildaidzina, malonilgenistina e malonilglicitina) (JACKSON et al., 2002). Esses compostos têm sido amplamente estudados devido aos seus benefícios à saúde humana, como prevenção e tratamento de alguns tipos de câncer e doenças cardiovasculares, aumento da qualidade óssea e controle dos sintomas da menopausa (AHN; PARK, 2017; BAÚ; IDA, 2015). Estudos com extrato de soja demonstraram benefícios para a saúde de isoflavonas de soja (fitoestrógenos) para redução ou

prevenção de vários tipos de câncer, diabetes, hipertensão, doença renal e osteoporose (AHN; PARK, 2017; MESSINA, 2014; MESSINA; BENNINK, 1998; ZHANG et al., 2016). As isoflavonas também são evidenciadas quanto à capacidade antioxidante (DEVI et al., 2009; RODRÍGUEZ-ROQUE et al., 2013). Um estudo realizado por Dixit et al. (2012) demonstrou a capacidade antioxidante da isoflavona de soja ao inibir danos celulares, através da neutralização do estresse oxidativo induzido por radiação gama.

O conteúdo e a distribuição de isoflavonas nos produtos à base de soja podem ser afetados pela variedade da matéria-prima, condições de armazenamento e parâmetros de processamento (ROSTAGNO; PALMA; BARROSO, 2003; XU; CHANG, 2008; ZHANG; CHANG; LIU, 2015). Wang e Murphy (1996) demonstraram que, devido à susceptibilidade ao calor, as formas malonil- e acetilglicosídeos de isoflavonas podem ser convertidas a β-glicosídeos, que por sua vez podem ser hidrolisadas pela ação das β-glicosidases endógenas da soja para as formas agliconas, evidenciando que as condições de processamento têm efeito no perfil de isoflavonas nos produtos de soja. As formas de aglicona são absorvidas mais facilmente do que as conjugadas glicosiladas devido ao seu baixo peso molecular (KANO et al., 2006; XU et al., 2000). Baú & Ida (2015) confirmaram que a conversão de isoflavonas β-glicosiladas em agliconas e também sua estabilidade térmica são influenciadas pelos efeitos do tempo e temperatura de processamento do extrato de soja.

A composição química do resíduo okara composta por macronutrientes, micronutrientes e fitoquímicos indica que estes compostos não são totalmente extraídos para o extrato de soja (LI; QIAO; LU, 2012; VONG; LIU, 2016). As proteínas compõem entre 15,2 e 33,4% do resíduo (base seca), sendo as principais 7S e 11S, e a concentração de isoflavonas varia entre 12% a 30% do conteúdo presente inicialmente nos grãos de soja, o que resulta em uma concentração entre 0,02% a 0,12% em base seca (JACKSON et al., 2002; VONG; LIU, 2016).

A proteína presente no okara tem alta qualidade nutricional, conteúdo de aminoácidos essenciais semelhante a um isolado de proteína de soja comercial e outros produtos de soja e apresenta alta digestibilidade *in vitro* (LI; QIAO; LU, 2012; MA et al., 1996; VONG; LIU, 2016). As isoflavonas são retidas em maior proporção pelos glicosídeos (JACKSON et al., 2002). Entretanto, o aproveitamento desse resíduo é dificultado devido à sua alta umidade, o que implica na sua rápida degradação (VONG; LIU, 2016). Essa alta

perecibilidade do okara faz com que maior parte desse resíduo seja subaproveitada, podendo ser descartada, usada como alimentação animal ou queimada (LI; QIAO; LU, 2012).

Dessa forma, torna-se desejável o estudo da aplicação de processos que busquem melhorar a retenção desses compostos no extrato de soja, diminuindo a perda para o resíduo okara.

1.2.Objetivos

1.2.1. Objetivo Geral

Aplicar pré-tratamentos enzimáticos com carboidrases e protease durante a fabricação de extrato de soja, e avaliar os efeitos sobre a recuperação de proteínas, recuperação e perfil de isoflavonas nesse produto de interesse comercial.

1.2.2. Objetivos Específicos

- Avaliar o efeito do pré-tratamento na soja com complexo multienzimático composto por carboidrases na recuperação de isoflavonas e proteínas no extrato de soja;
- Avaliar o efeito do pré-tratamento na soja com protease composto na recuperação de isoflavonas e proteínas no extrato de soja;
- Avaliar a extração, degradação e interconversão das formas de isoflavonas ocorridas devido aos pré-tratamentos enzimáticos.

1.3.Estrutura do Trabalho

Essa tese de doutorado está estruturada da seguinte forma:

Capítulo 2 – Plant-based beverages: Ecofriendly technologies in the production process: aborda uma revisão bibliográfica sobre aplicação de tecnologias ecológicas como ultrassom, homogeneização à alta pressão, extração enzimática e

fermentação em bebidas à base de plantas, e os efeitos desses processos no aproveitamento dessas matrizes alimentares.

Capítulo 3 – Enzymatic pretreatment in the extraction process of soybean to improve protein and isoflavone recovery and to favor aglycone formation: corresponde ao artigo referente à primeira etapa da pesquisa. Reporta a aplicação do complexo enzimático de carboidrases Viscozyme L® na pasta de soja, e seus efeitos quanto à recuperação de proteínas e isoflavonas, bem como os fenômenos de degradação e interconversão entre as suas formas, no extrato de soja ("soybase").

Capítulo 4 – Application of hydrolysis by protease during the soybase preparation and its effects on the protein recovery, isoflavone recovery and profile: corresponde ao artigo resultante da segunda etapa da pesquisa. Reporta a aplicação da enzima protease Protamex® na pasta de soja, e os resultados de recuperação de proteínas, recuperação, degradação e interconversão de isoflavonas no extrato de soja ("soybase").

Capítulo 5 – Discussão geral: Relevância aos principais resultados obtidos.

Capítulo 6 – Conclusão geral: Contém as principais conclusões do trabalho.

CAPÍTULO 2. PLANT-BASED BEVERAGES: ECOFRIENDLY TECHNOLOGIES IN THE PRODUCTION PROCESS

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PLANT-BASED BEVERAGES: ECOFRIENDLY TECHNOLOGIES IN THE PRODUCTION PROCESS

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ABSTRACT

Plant-based beverages are the main substitutes for cow's milk and for people who have dietary restrictions; either for health reasons or preference for plant foods. Soymilk has been the most consumed milk substitute; however, production from other plant sources such as rice, almonds, nuts, grains, and seeds are expected to increase in the coming years. Due to the increasing popularity of these products, their global market share has seen arise in growth and development. However, the use of these plant sources for the production of milk substitutes still has a number of limitations. This review provides an overview of some of the ecofriendly technologies reported in the literature, such as ultrasound, ultra-high-pressure homogenization, enzymatic processes, and fermentation; and how they may be applied to the traditional processing of plant-based beverages. These technologies are evaluated for their potential to improve the process performance and yield, as well increase the nutritional, sensory, and functional properties of these beverages.

Keywords: Plant-based beverage; ultra-high-pressure homogenization, ultrasound, carbohydrase, protease, fermentation.

2.1. Introduction

The rise in popularity and demand for cow's milk substitutes has increased worldwide in recent years. Plant-based beverages are the main substitute for cow´s milk; and are defined as aqueous extracts obtained through vegetable matter breakdown of cereals, pseudo-cereals, oilseeds of legumes, and nuts (Sethi, Tyagi, & Anurag, 2016). They are consumed by people with food restrictions caused by allergies, lactose intolerance or a specific diet; and by the growing of followers of vegetarianism, veganism and flexitarianism (Rosenfeld, 2018; Sethi et al., 2016). In addition, a growing number of people concerned about the environment are also adapting to plant-based diets. The main reason being, animal based food production has a larger share in environmental pressure, as its production requires approximately 100-fold more water compared to the production of an equivalent amount of plant protein (Jeske, Zannini, & Arendt, 2018).

As a reflection of the growth in demand for plant-based beverages, the global market of these products has been increasing in recent years. According to Research and Markets (2019), values are expected to reach above US$ 12.1 billion by 2024, with an increase of 4.91% per year. In 2018, this market grew by 51.5% owing to the large share of rice, oat, coconut, and almond beverages. Even though soybean-based beverage is the major product, corresponding to about 90% of the market in 2018, beverages produced from other plant sources are expected to increase in the following years. For plant-based beverages that are not soybean-based, such as rice, almonds, grains, and seeds; it is estimated that their total consumption will jump from 10.5 million liters to 30.1 million liters by 2022 (Tetrapak, 2019).

The plant-based beverage market is relatively new, and for it to continue growing and developing, it is necessary to improve some production steps. This review focuses on some of technologies reported in the literature; such as ultra high-pressure homogenization (UHPH), ultrasound, use of enzymes, and fermentation, which could be applied to traditional plant-based beverage processing. The aim of such applications is to improve process yield and its nutritional, sensory, and functional properties.

2.2. Nutritional and bioactive composition of plants

Plant composition is an important parameter for the manufacture of plant-based beverages, since they influence cell disruption and the release of proteins, carbohydrates, and bioactive compounds into the aqueous media.

The plant cell wall is organized into three main layers: the primary cell wall, the middle lamella, and the secondary cell wall. The main constituents are cellulose microfibrils, hemicellulose, pectin, lignin, and soluble protein. Cellulose corresponds to between 15 and 30% (dry mass) of the primary cell wall and up to 40% of the secondary cell wall; where it is found in the form of glucose polymers (microfibrils). These microfibrils are coated with polysaccharides, such as hemicellulose or xyloglucans. Pectin is present in approximately 35% (dry matter) of the dicotyledonous plant, and is composed of a group of branched polysaccharides; mainly homogalacturonan, rhamnogalacturonan, arabinans, galactans, and arabinogalactans. Lignin is the main constituent of the secondary cell wall, representing about 10–25% of the total dry matter of the plant; it is composed of aromatic compound complexes, and linked in a network to cellulose and xylose (Sticklen, 2008).

The globulins and albumins are the main protein forms found in leguminous plants. Globulins represent about 70% of legume seed proteins and consist mainly of 7S and 11S proteins; with molecular weights ranging from 8k to 600 kDa (Freitas, Ferreira, & Teixeira, 2000). These proteins are known as glycine and β-conglycinin in soybean, for example, while in peas and lentils they are legumin and viciline, respectively. In cereals, proteins represent about 6 to 15% of the grains; represented mainly by prolamins and globulins. In wheat, rye, barley and maize, prolamins make up 30–50% of total proteins and their content is less in oats and rice, representing 5–15% (Esfandi, Walters, & Tsopmo, 2019; Giuberti, Gallo, & Masoero, 2011). These plant proteins generally have minimal solubility at pH values close to the isoelectric point, between 4 and 5. Soybean protein, for instance, has a solubility lower than 10% at pH 4 (Spada, Marczak, Tessaro, & Cardozo, 2015).

Plants are also capable of producing several bioactive compounds, such as vitamins A, C and E, phenolic compounds, tannins, and lignins (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). These components result from secondary metabolism and their levels depend on factors such as the cultivar, cultivation conditions, storage, and transport. However, access to these compounds is hindered by the complexity of the plant cell wall polysaccharides (Bennett et al., 2011; Ghandahari Yazdi, Barzegar, Sahari, & Ahmadi Gavlighi, 2019), which has a negative impact on the beverage production, resulting in low component recovery into the aqueous medium.

2.3. Plant-based beverage production and waste generation

The plant-based beverage production process may vary according to the initial raw material and the purpose of the final product; however, some steps are common and frequently used by industries. Aydar et al. (2020) reported the common procedural steps, and the differences that occur among the production processes of plant-based beverages; depending on the raw material. Figure 1 simplifies the common steps involved in the production of plant-based beverages.

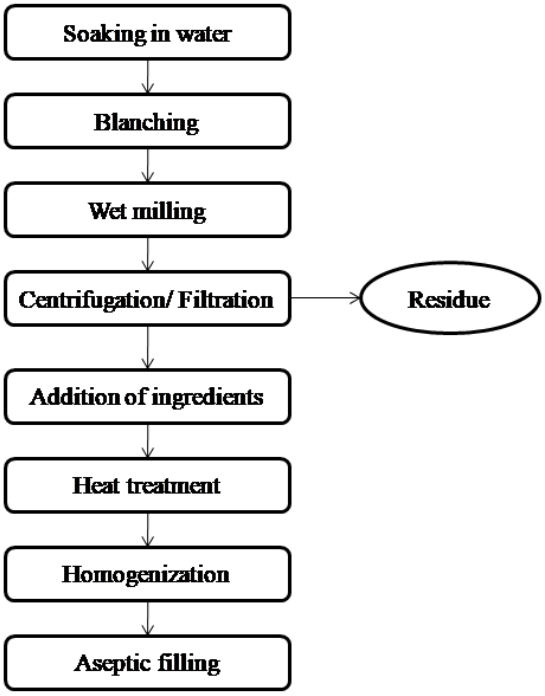

Figure 1. Common steps in the production of plant-based beverages (Aydar, Tutuncu, & Ozcelik, 2020).

The water soaking stage can be used for soybeans, hazelnuts, rice, almonds, tiger nuts, grains, sesame seeds, and peanuts (Aydar et al., 2020). In this process step, the swelling and softening of cereals and nuts happen; and, after being submerged, the apparent amylose content decreases (Padma, Jagannadarao, Edukondalu, Ravibabu, & Aparna, 2018). For example, when tiger nuts are immersed in water, the tiger nut milk extraction yield is higher (Kizzie-Hayford, Jaros, Zahn, & Rohm, 2016). Blanching can be applied to soybeans, almonds, coconuts, sesame, peanuts, rice, and quinoa (Aydar et al., 2020). This step has

several advantages, including decreased microbial load and inactivation of enzymes such as lipases (Pardeshi, Murumkar, & Tayade, 2014; Seow & Gwee, 1997; Sethi et al., 2016).

Wet milling can be carried out on tiger nuts, soy, coconut, cashew, hazelnuts, hemp seeds, cowpeas, almonds, walnuts, and peanuts (Aydar et al., 2020). During this step, water is added to the raw material and then grinding is carried out. Another important step observed during the production process is filtration; which is responsible for separating the beverage from the residues. Ultrafiltration can be used in the production of hazelnut, sesame, and corn beverages (Aydar et al., 2020). The amount of residue generated from plant-based beverages varies according to the initial raw material. Moreover, the residues resulting from the filtration stage are usually composed of insoluble solids and macronutrients that have not been fully leached into the beverages (B. Li et al., 2012; Ostermann-Porcel, Rinaldoni, Rodriguez-Furlán, & Campderrós, 2017; Vong & Liu, 2016). For instance, it is estimated that 1 kg of soybean used for soymilk production results in 1.1 kg of fresh residue named okara; which contains high amounts of dietary fiber and protein, as well as minerals, vitamins and isoflavones (Khare, Jha, & Gandhi, 1995; B. Li et al., 2012; O'Toole, 1999b). The okara generated worldwide is around 14 million tonnes annually, and is destined for animal feed or dumped and burned as waste, due to its quick degradation (B. Li et al., 2012; Nguyen et al., 2013). Annual processing of sesame seeds results in an estimated 360,000 tons of bran. This bran is underutilized or has limited use as animal feed, and corresponds to a loss in total weight of the sesame seed of 15 to 18%; with an additional waste of about 15% of protein content (Görgüç, Bircan, & Yılmaz, 2019). The rice grain processing results in approximately 20% of husk and 10% of bran, which are by-products rich in fibers and high quality protein, respectively (Phongthai, Homthawornchoo, & Rawdkuen, 2017).

Therefore, the reduction of residues generated from the production of plant-based beverages means lower damage to the environment, since a smaller amount of this material will be discarded. Moreover, the most relevant issue for the industry is to improve the recovery of macro (fibers, proteins, and sugars), and micronutrients (for instance vitamins and phenolic compounds) that are not fully extracted in the conventional processes; and end up being discarded after filtration or centrifugation processes.

Currently, techniques such as high pressure, ultrasound, enzymatic processes, fermentation, and use of additives have been studied and applied together with the traditional processes of plant-based beverages; in order to improve the extraction yield and decrease the amount of residue generated in production. Moreover, studies reported that such techniques

enhance nutritional and sensorial properties of plant-based beverages. These technologies will be addressed in the following section of this review.

2.4. Ecofriendly technologies applied in the production of plant-based beverages

During the production of plant-based beverages, it is crucial to apply a method that, in addition to having good performance, results in a final product possessing good physical stability and minimizes the need for additives, such as hydrocolloids and emulsifiers (Bernat, Cháfer, Chiralt, & González-Martínez, 2014). Moreover, ongoing efforts are underway to improve the sensorial, nutritional, and functional properties of plant-based beverages. The main challenges to be overcome include the removal of strange flavors, inactivation/removal of inhibitors, and increased of shelf life (Paul, Kumar, Kumar, & Sharma, 2020). In this sense, certain technologies can be used for the development of these products, since conventional processes often do not achieve the expected results. Some techniques have shown good results in this regard and are presented below.

2.4.1. Ultrasound-assisted technology

Ultrasound-assisted technology in food processing is considered a green technology that stands out to researchers due various advantages; these include, efficient use of energy, reduced temperatures, and cost savings, among others (Hu et al., 2013; Soria & Villamiel, 2010). This technology uses physical and chemical phenomena that fundamentally differ from conventional processing applications, presenting advantages in terms of extraction productivity, yield and selectivity, better processing time, improved quality, and with a reduction in chemical and physical risks (Chemat, Zill-E-Huma, & Khan, 2011).

The cavitation phenomenon produced by ultrasound in the solvent leads to the increase in extraction efficiency of organic compounds. This phenomenon occurs through the passage of an ultrasonic wave, where cavitation microbubbles are generated. Violent collapse of these microbubbles results in the formation ofregions of high pressure and temperature. As a consequence, sono-physical and sono-chemical effects of ultrasound on food can occur. For instance, some authors reported enzymatic inactivation and alterations in the secondary

structure of proteins, changing their functional properties and nutritional value (Hu et al., 2013; Islam, Zhang, & Adhikari, 2014; Soria & Villamiel, 2010).

It has been shown that ultrasound treatment can lead to a reduction in the size of different plant proteins, such as soybeans (Hu et al., 2013) and peas (O'Sullivan, Murray, Flynn, & Norton, 2016); however, without the complete breakdown of the primary protein structure. This is due to the fact that ultrasonic cavitation induces high levels of hydrodynamic shear, breaking down hydrophobic, electrostatic interactions and hydrogen bonds, or non-covalent forces of proteins; decreasing their size and keeping their aggregates in solution (O'Sullivan et al., 2016). The decrease in the size of proteins implies a greater protein-water interaction, and increased solubilization of plant protein, which normally presents low solubility. An increase of approximately 40% in the solubility of isolated soy protein was verified by the decrease in protein size after ultrasound treatment (Arzeni et al., 2012). The increase in the plant protein solubility is a challenge in the production of plant-based beverages, since they are often consumed as a substitute for protein-rich cow's milk products (Sethi et al., 2016).

On the contrary, the ultrasound effect was not observed on the protein structure and denaturation for rice (O'Sullivan et al., 2016) and almond proteins (Vanga, Wang, Orsat, & Raghavan, 2020). Walnut protein structures also showed stability, even under extreme ultrasound conditions (600W and 30min)(Zhu et al., 2018); as well as soy protein for 5 min at 600 W and 20kHz (Huang, Ding, Dai, & Ma, 2017), pea protein isolate at 20 kHz and varying amplitudes 30%, 60%, 90% for 30 min (Xiong et al., 2018) and peanut protein for various time combinations (15, 20, and 25 min), power (600, 660, and 720 W) and temperatures (20, 25, and 30 °C) (Q. T. Zhang et al., 2014).

High-intensity ultrasound treatment can increase surface hydrophobicity which can improve the functional properties of protein. Xiong et al. (2018) reported an improvement infoaming capacity of 1.38 times, with high interfacial stability. High degrees of hydrophobicity were also observed in the pea and soybean protein isolates due to the significant reduction in intrinsic viscosity induced by ultrasound treatment (Arzeni et al., 2012; O'Sullivan et al., 2016). The intrinsic viscosity of a protein solution is associated with its conformation and degree of hydration; which are related to the amounts of hydrophobic residues that are within the associated proteins. A decrease in the intrinsic viscosity also leads to the dehydration of amphiphilic biopolymers, increasing the hydrophobicity of the biopolymer and thus reducing the energy required for amphiphilic biopolymer adsorption to the oil-water interface (Khan et al., 2012; O'Sullivan et al., 2016). Higher emulsion activity

index (EAI) and stability (ESI) values were reported for systems stabilized with ultrasound-pretreated walnut protein; when higher power levels and times were used. The authors attributed this increase in EAI to the larger fraction of small soluble proteins available to adsorb to the oil-water interface, or to some change in the surface chemistry of proteins induced by sonication that increased their surface activity. The increase in ESI may be attributed to the formation of smaller droplets in the sonicated emulsions, or because of some change in the surface chemistry of the lipid droplet surfaces that altered the attractive or repulsive interactions between the droplets (Zhu et al., 2018).

The mechanical effect of solvent penetration into the sample matrix is also attributed to ultrasound technology. In this way, the contact area between the liquid and solid phases is increased, improving mass transfer; resulting in a greater release of intracellular substances to the liquid phase (Rostagno et al., 2003). This is of great interest, since a large amount of intracellular bioactive compounds (substances that have actions in the body, promoting good health) is not totally leached out to the aqueous solution after the filtration step. Thus, ultrasound application can favor the extraction of such substances to the liquid phase, enhancing the nutritional and biological values of plant-based beverages. Tabaraki & Nateghi (2011) applied ultrasound to polyphenol and antioxidant compound extractions from rice bran and reported the following optimal conditions: 35 kHz, 140 W, temperatures of 51–54 °C, extraction time of 40–45 min and concentrations of ethanol solvent of 65–67%. Under these conditions, the authors observed an improvement in the extraction of polyphenols by up to167% when compared to the control process. Thus, these results justify the use of ultrasound for the enhancement of this by-product; which could be used as raw material in the manufacture of plant-based beverages.

On contrary, Morales-de la Peña et al. (2018) reported no significant effect of ultrasound on the extraction of isoflavone compounds from soybean grains to the soymilk. Despite this result, the authors observed the interconversion among the different forms of isoflavones due to the application of ultrasound. The formation of the aglycone isoflavone form was observed, in which its levels in the ultrasound pretreated samples were 20% higher than the sample obtained by the control process. In addition, the ultrasound pretreatment also resulted in a lower soaking time for the soybeans, higher moisture content, and lower hardness; which are important characteristics for the production of soymilk (Falcão et al., 2018).

Ultrasound also demonstrated efficiency in inhibiting microbial activity. For almond milk processing, for example, 130 W and 80% amplitude level with 6 and 8 min

treatment times inhibited the activity of *E. coli* and *L. monocytogenes*; reducing the viable count of both pathogens to 1–1.3 log CFU/mL (Iorio et al., 2019). Other research related to ultrasound technology in plant matrices, and their main results, are compiled in Table 1.

Table 1. Ultrasound treatment parameters used in plant matrices, and their corresponding improvements.

Vegetable Matrice	Evaluated parameters	Improvements	Reference
Pea and soy proteinisolates	20 kHz 95% Amplitude 2 min	Reduction in the protein size Reduction in the intrinsic viscosity	(O'Sullivan et al., 2016)
Peanut protein isolate	0, 120, 300, 480, 660, 840, 1020 W 0, 15, 25, 35, 45, 55, 65, 75 °C 0, 1, 3, 5, 10, 20, 30 min	Increase in the interfacial area stabilized Improvement in emulsifying properties	(Q. T. Zhang et al., 2014)
Rice bran	35 kHZ 140 W 40, 50, 60 °C 15, 30, 45 min	Improvement in the polyphenol extraction and antioxidants	(Tabaraki & Nateghi, 2011)
Soy protein isolate	20 kHz 750 W 20% Amplitude 20 min	Particle size reduction Increase in solubility Decreased viscosity	(Arzeni et al., 2012)

Soybeans	24 kHz 200 W 100% Amplitude 10 min	Improvement in isoflavone extraction	(Rostagno et al., 2003)
Soybeans	20 kHz 6, 15, 24 W/cm² 35, 45, 55 °C 5, 15, 25 min	Increase in content of isoflavones aglycones Lower soaking time	(Falcão et al., 2018)
Soymilk	24 kHz 400 W 50, 75, 100% Amplitude 30, 45, 60 °C 20, 40, 60 min	Increase in isoflavone content	(Morales-de la Peña et al., 2018)
Walnut protein isolate	25 kHz 1200 W 200, 400, 600 W 15, 30 min	Increase in water-solubility Improvement in emulsion formation and emulsion stability	(Zhu et al., 2018)

2.4.2.Ultra-High-Pressure Homogenization

The ultra-high-pressure homogenization (UHPH) is a non-thermal technique in which a material is submitted to high pressures, ranging from 100 to 600 MPa. This technology is a continuous process that combines the effects of homogenization and pressure during the passage of food through the valve; promoting physical phenomena such as, shear forces, cavitation and turbulence (Donsì, Annunziata, & Ferrari, 2013; Zamora & Guamis, 2015). It has been widely applied to liquid or colloidal foods in order to improve their stability and physico-chemical properties, with minimal effect on nutritional properties (Bernat et al., 2014; H. H. Liu, Chien, & Kuo, 2013; Valencia-Flores, Hernández-Herrero, Guamis, & Ferragut, 2013).

Many advantages have been attributed to the application of UHPH to plant-based beverages, such as: deflocculation of primary fat globule clusters and uniform dispersion of clusters; changes in protein conformation; increased viscosity and stability of emulsions; inactivation of enzymes and microorganisms; and improved sensory and nutritional profile (Bernat et al., 2014; Cruz et al., 2007; Munekata et al., 2020; Wuytack, Diels, & Michiels, 2002). The impact of UHPH technology on plant-based beverage properties is shown in Table 2.

Some authors have reported effects of UHPH on the physical stability of plant-based beverages. In soybean beverages, for instance, the application of 200 and 300 MPa pressures, in combination with different inlet temperatures, improved beverage stability. It reduced colloidal particle size and decreased solid sedimentation, when compared to conventional heat treatments (pasteurization at 95 °C for 30 s and ultra-high temperature (UHT) to 142 °C for 6 s)(Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012). In addition, these same authors evaluated that UHPH treatment at 300 MPa and 80 °C increased colloidal stability with low sedimentation during a six-month storage period for soymilk, in relation to the product treated with UHT (Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014). This higher beverage physical stability can be attributed to the fact that UHPH treated samples presented smaller colloidal particles.

Moreover, the combination of pressure and inlet temperature in UHPH has also been reported as a promising technique for reducing the microbial load of plant-based beverages; due to the physical forces caused by the treatments (Donsì et al., 2013). Soymilk

treated at 200 MPa and 40 °C resulted in a decrease of approximately 50% of the total bacteria count, and 85% of the spore count; in relation to the base product without heat treatment. When the pressure was increased to 300 MPa, the total bacterial count decreased by 88%, while for spores it was 90%. Both pressures were enough to reduce the enterobacteria count below the detection level (Cruz et al., 2007). Soymilk and almond beverages, both treated under the same conditions of inlet temperatures and pressures, showed similar results of microbiological inactivation. At an inlet temperature of 75 °C, the treatments at 200 MPa were able to decrease the count of *B. cereus*, mesophilic bacteria and spores, below the detection level. This same result was observed for the homogenization pressure of 300 MPa and inlet temperatures of 55 °C, 65 °C, and 75 °C (Poliseli-Scopel et al., 2012; Valencia-Flores et al., 2013). Furthermore, UHPH processes also present a potential alternative treatment to conventional pasteurization for obtaining tiger nut milk and soymilk. The application of the UHPH conditions of 300 MPa and 80 °C was able to produce aseptically packed soymilk that is microbiologically stable for 6 months of storage at room temperature; commonly achieved for conventional UHT treatments (Poliseli-Scopel et al., 2014). The UHPH treatment carried out at 300 MPa and 40 °C achieved better microbial inactivation of tiger nut milk in comparison with conventional homogenization-pasteurization, for the control of mesophilic aerobic and psychrotrophic microorganisms; increasing the microbiological shelf-life from approximately 25 to 57 days (Codina-Torrella, Guamis, Zamora, Quevedo, & Trujillo, 2018). Therefore, with regard to microbiological inactivation, the combination of the inlet temperature and pressure must be considered. In UHPH processing, the rate of microorganism inactivation depends mainly on the working pressure and inlet temperature of the products, showing better results with the increase of both parameters.

In addition to improving microbiological stability, UHPH treatments also preserve the sensory qualities of plant-based beverages. Tiger nut milk treated under pressures of 200 MPa and 300 MPa were evaluated for sensorial attributes by trained panelists. Results indicated no differences in aroma, flavor and thicknesses when compared to samples treated by homogenization-pasteurization. Regarding color, all samples showed differences between them, in which the sample subjected to the highest pressure was evaluated as the whitest. The authors associated this result with the increase in light diffraction defined by the greater number of oil droplets dispersed in the continuous phase after homogenization (Codina-Torrella et al., 2018). Soymilk treated with 300 MPa by UHPH was also evaluated sensorially and compared to conventional UHT soymilk, during 180 days of storage. The judges did not

observe significant differences for the attributes of thickness, beany, grassy and oxidized flavors during the storage period. The panel evaluation on days 1 and 90 classified the UHT soymilk as having greater astringency mouthfeel than the UHPH soymilks, while no significant difference was observed in the last day of shelf life. Regarding color, the judges rated UHPH soymilk as slightly darker than UHT soymilk on all days of analysis, but with a significant difference ($p \geq 0.05$) only on day 90 (Poliseli-Scopel et al., 2014).

Treatment with UHPH may also affect bioactive compound extraction, as well as the nutritional and toxicological properties. The UHPH treatment of soymilk increased the content of total phytosterols and isoflavones; when the process was carried out at higher pressure (300 MPa) and temperature (75°C), with a residence time of 3 s. These results can be attributed to the effect of mechanical forces on the release of these compounds from soymilk fat globules, increasing the extraction capacity; however, in this study, there was also losses of compounds of interest, such as tocopherols, as the temperature and pressure increased (Toro-Funes, Bosch-Fusté, Veciana-Nogués, & Vidal-Carou, 2014). The UHPH treatment of raw almond milk at 350 MPa, 85 °C and 15 s of a residence time induced a chemical or physico-chemical modification of epitopes in almond proteins; with an almost complete loss of antigenicity, while the exposed free sulfhydryl groups decreased. This means that UHPH treatment of almond milk can reduce its allergenic potential. On the other hand, the vitamin B1 and B2 content remained similar to that of the untreated sample (Briviba, Gräf, Walz, Guamis, & Butz, 2016).

Table 2. UHPH treatments applied to plant-based beverages and their improvements.

Vegetable Matrice	UHPH conditions	Improvements	Reference
Almond beverage	200 MPa and 300 MPa 55 °C, 65°C and 75 °C inlet temperatures	Decrease in particle size High physical stability Reduction in microbiological population	(Valencia-Flores et al., 2013)
Soymilk	100, 200, 300, 400, 500 and 600 MPa 10, 20 or 30 min	Increase in soymilk viscosity Exposes of hydrophobic regions of the protein	(H. Zhang, Li, Tatsumi, & Isobe, 2005)
Soymilk	200 MPa and 300 MPa 55 °C, 65°C and 75 °C inlet temperatures	Increase in colloidal stability Low solid sedimentation during storage Reduction in microbiological population	(Poliseli-Scopel et al., 2012)
Soymilk	200 MPa and 300 MPa 55 °C, 65 °C and 75 °C inlet temperatures	Increase in total phytosterols and isoflavone content	(Toro-Funes et al., 2014)
Soymilk	300 MPa 80 °C inlet temperature	Higher colloidal stability Low solid sedimentation during storage Microbiological stability	(Poliseli-Scopel et al., 2014)
Soymilk	207 and 276 MPa 121 °C and 145 °C internal temperatures	Significant reduction in total microbial counts compared to untreated control sample No difference in sensory characteristics compared to commercial samples	(Sidhu & Singh, 2016)

Tiger nut milk beverage	200 and 300 MPa 40 °C inlet temperature	Higher colloidal stability than homogenized-pasteurized treatment Reduction in peroxidase activity Improved enzymatic stability	(Codina-Torrella, Guamis, Ferragut, & Trujillo, 2017)
Tiger nut milk beverage	200 and 300 MPa 40 °C inlet temperature	Higher microbial inactivation than homogenized-pasteurized treatment Increase of shelf life (up to 32 days) UHPH treated sample showed greater luminosity and whiteness than raw product and homogenized-pasteurized samples	(Codina-Torrella et al., 2018)

2.4.3. Enzyme-assisted extraction

The use of enzymes, such as carbohydrases and proteases, has been extensively studied in plant foods for different purposes; such as, to improve extraction of compounds; to remove antinutritional factors; to inactivate/remove allergens; and to enhance sensorial, nutritional, and functional properties (De Moura, Campbell, De Almeida, Glatz, & Johnson, 2011; Rosset, Acquaro, & Beléia, 2014a; Rosset, Prudencio, & Beléia, 2012; Sari, Bruins, & Sanders, 2013; Wei et al., 2018). However, specifically in plant-based non-alcoholic beverages, few studies have been conducted on using enzymes.

Carbohydrase enzymes catalyze the hydrolysis of polysaccharide bonds in plant cell wall layers, disintegrating the structure into low molecular weight sugars, and releasing proteins and other intracellular compounds. The primary cell wall is hydrolyzed by the action of cellulases, while pectinases digest the secondary cell wall (Kasai & Ikehara, 2005a; Kasai, Imashiro, & Morita, 2003; Kasai, Murata, Inui, Sakamoto, & Kahn, 2004). When the complex carbohydrates of the cell wall are hydrolyzed, simple sugars are released, and insoluble fibers are solubilized (Vong & Liu, 2016). Increases in the extraction of intracellular protein using carbohydrases has been reported by several authors for different plant matrices; such as rice bran, sesame bran, oat bran, soybean flour, and peanut (Table 3). The combination of the enzymes xylanase and phytase produced up to 92% extraction of proteins from rice flour, 17.5% more than the control process (M. Wang, Hettiarachchy, Qi, Burks, & Siebenmorgen, 1999). For pectinase mixtures, Jung et al (2006) reported a significant increase in the extraction yield of soybean protein compared to the pH 4 control sample; with improved foaming stability and lower viscosity.

One of the most commonly used enzyme products in the literature is Viscozyme L, a fungal multicomponent carbohydrase containing a wide range of enzymes; including arabanase, cellulase, hemicellulase, and xylanase. Thus, it is able to effectively hydrolyze polysaccharides of the plant cell wall. Its use increased process yields and soluble sugar contents of soybase (soybean extract). In addition, a positive influence on the conversion of conjugated isoflavones to aglycone in different soybean matrices was also observed; such as soybase and a beverage from okara residue (Penha, Falcão, Ida, Speranza, & Kurozawa, 2020; Vong & Liu, 2019a). The Viscozyme L action on cell wall degradation was also observed in

coconut kernel, with a consequent increase in the yield of coconut milk processes (Agarwal & Bosco, 2015).

In addition, carbohydrases can be potentially used to influence the sensory characteristics of plant-based beverages. For instance, enzymatic treatment for 3 h using cellulase (1.2% v/v) resulted in a soymilk with lower chalkiness, less beany flavor, higher body (related to thin or thick beverage) and overall quality; when compared with the non-enzymatic treated sample. Moreover, viscosity and physical stability characteristics were also higher in enzymatically treated samples (Rosenthal et al., 2003).

To increase protein extraction yield, the application of protease enzymes has also been evaluated in the literature. Proteolysis, or cleavage of protein chains, is an enzymatic process that occurs under moderate conditions; does not decrease the nutritional value of the protein source; and is easily controlled, in relation to chemical processes.

The release of low molecular weight peptides after enzymatic hydrolysis increases the ionizable amino and carboxyl groups; as a consequence, there is an increase in protein solubility (Panyam, Dinakar and Kilara, 1996; Tavano, 2013; G. Zhao, Liu, Zhao, Ren, & Yang, 2011). This property is the most notable effect of hydrolysis reactions on the functional properties of protein. The manipulation of protein solubility can improve extraction yield, as well the purity and functionality of resultant protein concentrates and isolates. Protein solubility also influences the texture and sensory characteristics of foods, which are essential parameters to be considered in the manufacture of beverages (Boye et al., 2010; Chang, Tu, Ghosh, & Nickerson, 2015; Mamauag et al., 2011). The action of the subtilisin enzyme in okara samples at pH 4.0, 6.0, 8.0 and 10.0 doubled the amount of proteins extracted, in relation to their respective controls (Orts et al., 2019). In this same work, the increase in pH resulted in higher protein solubility; which can be an important valorization aspect of this residue, and something to consider in the formulation of plant-based beverages. Enzymatic protein hydrolysis of quinoa-based milk through different commercial proteases increased protein solubility and surface hydrophobicity in all evaluated process conditions; where increases of 27.8% and 36.74% were observed, respectively, in relation to the non-enzymatically treated sample. On the contrary, foaming and emulsification properties were not significantly improved by enzymatic treatments using proteases (Jeske, Zannini, Cronin, & Arendt, 2018).

The application of proteases in soybeans, rapeseed and denatured heat-defatted peanut flour proved to be efficient in extracting proteins; probably due to the improvement on protein solubility. In addition, the protein hydrolysates released from enzymatic reactions

presented good functional properties; such as emulsifying and foaming properties, owing to the tendency of the peptides to form aggregates through hydrophobic interaction (G. Zhao, Liu, Ren, Zhao, & Yang, 2013).

Thus, the use of proteases may also represent a technological advancement in the production of enriched plant-based beverages, resulting from bioactive peptides released by protease action (Esfandi et al., 2019; Yamashita, Vanzela, Kurozawa, de Figueiredo, & Ida, 2018). Soybean protein hydrolysates, for example, are physiologically better than intact proteins due to their better intestinal absorption (Ziegler et al., 1998). A series of biological activities, such as antioxidant, anti-inflammatory, insulin resistance, anti-obesity, and immunomodulating were related to soybean peptides obtained by protein hydrolysis through proteases (Gibbs, Zougman, Masse, & Mulligan, 2004; Kong, Guo, Hua, Cao, & Zhang, 2008; B. P. Singh, Vij, & Hati, 2014). The modification of rice protein by alcalase and flavourzyme proteases improved the antioxidant capacity in relation to the intact protein (Gomes & Kurozawa, 2020). In okara, enzymatic treatment with subtilisin at pH 4.0 increased the antioxidant capacity by 3.2 times, when compared to the sample without enzymatic treatment (Orts et al., 2019). In sesame bran, peptides obtained through proteolitic hydrolysis also resulted in an improved antioxidant capacity, in addition to increasing the recovery of total phenolic content (Görgüç et al., 2019). Given that phenolic compounds may be conjugated with the hydrophobic interior of globular proteins, protein hydrolysis can disrupt such interactions; resulting in higher recovery of phenolic compounds from the food matrix into aqueous solution (Malaypally & Ismail, 2010).

Regarding sensory characteristics, little research on plant-based beverages and protease treatment have been carried out. Two non-commercial proteases from residual sunflower seeds were immobilized in calcium alginate spheres and incorporated into soymilk for enzymatic treatment at 30 °C for 1 h; this resulted in a product with reduced bean aroma and an increase in pleasant smell, in addition to an increased shelf life of 15 days compared to the 10 days of the non-treated sample. This was most likely due to the antimicrobial peptides released by enzymatic hydrolysis (Sahoo, Gaikwad, Ranveer, Dandge, & Waghmare, 2016). On the other hand, studies have demonstrated that extensive hydrolysis processes induce the formation of a bitter taste; one of the limitations of using protein hydrolysates in food systems. Hydrolyzed isolated soy protein, for example, may taste bitter, depending on the type of protease and the degree of hydrolysis (Seo, Lee, & Baek, 2008). Bitter taste is related to the release of low molecular weight peptides, composed mainly of hydrophobic amino acids (Matoba & Hata, 1972). Other studies have reported that in addition to hydrophobicity,

other factors, such as primary sequence, spatial structure, peptide length, and volume of the molecule are important for the perception of bitter taste (M. R. Kim, Kawamura, & Lee, 2003; Mi Ryung Kim, Yukio, Ki, & Lee, 2008; Kouge, Shinoda, Kanehisa, Okai, & Ishibashi, 1988). Thus, sensory analyses must be conducted in the development of hydrolyzed plant-based beverages using proteases to avoid this drawback.

Overall, several works reported in the literature demonstrated that enzymatic processes are promising for the extraction of bioactive compounds from plant matrices (Table 3). These studies have shown that the application of carbohydrase enzymes can improve bioactive compound extraction and increase the solubility of fibers, by breaking the polysaccharide structure of plant cell wall. Proteases, in turn, promote the hydrolysis of protein chains into peptides; releasing bioactive peptides, increasing protein solubility and, as consequence, their extraction to an aqueous medium. Thus, the application of enzymatic processes could be applied for the production of plant-based beverages, resulting in more nutritionally valued products and improving process yield.

Table 3. Carbohydrases and proteases enzymes used in plant-based beverages and potential raw vegetable materials, and their improvements.

	Vegetable Matrice	Enzymes	Improvement	Reference
Carbohydrases	Oat Bran	Viscozyme L	41.4% increase in protein extraction	(Guan & Yao, 2008)
	Okara	Viscozyme L and Celluclast	Increase in aglycone content Increase in soluble fiber content Decreased in the insoluble fiber content	(Vong, Lim, & Liu, 2017)
	Peanut	Viscozyme® L Cellulase Hemicellulose Pectinase	8.2% increase in protein extraction 10.2% increase in protein extraction 12.2% increase in protein extraction 9.9% increase in protein extraction	(C. Liu, Hao, Chen, & Yang, 2020)
	Rice bran	Viscozyme L Celluclast	Up to 41.77% increase in protein extraction	(Hourigan & Chesterman, 1997)
	Soybase	Viscozyme L	Increased yield and soluble sugar content of soybase Influences the conversion of conjugated isoflavones into aglycone	(Penha et al., 2020)
	Soy flour	Viscozyme L	23.23% increase in protein extraction	(Rosset, Acquaro, & Beléia, 2014)
	Soy grit	Enzyme complex of cellulase, xylanase, pectinase, arabinanase	21% increase in protein yield	(Perović, Knežević Jugović, & Antov, 2020)

		and β-glucanase		
		Combination of cellulase, xylanase and pectinase	13% increase in protein yield	
	Rice Bran	Combination of xylanase and phytase	Up to 40.4% increase in protein yield	(M. Wang et al., 1999)
	Sesame bran	Viscozyme L	17.2% increase in protein yield	(Görgüç et al., 2019)
	Rice Bran	Amylase Viscozyme L Celluclast	Up to 35.1% increase in protein extraction Up to 14.4% increase in protein extraction Up to 1.8% increase in protein extraction	(Tang, Hettiarachchy, Eswaranandam, & Crandall, 2003)
Proteases	Okara	Subtilisin	Doubled protein extraction 9.1-fold increase in the total amount of aglycone extracted	(Orts et al., 2019)
	Peanut flour	Alcalase Papain Protamex	Up to 30.8% increase in protein extraction Up to 22.0% increase in protein extraction Up to 11.5% increase in protein extraction	(G. Zhao et al., 2013)
	Rice flour	Protemax®	Maximum yield of 63.4% in protein extraction	(Vieira, Lopes Jr., Ramos, Capobiango, & Silvestre, 2008)
	Sesame bran	Alcalase	54.8% increase in protein yield	(Görgüç et al., 2019)
	Soybean	Protex 26L	Increase in protein recovery	(Sari et al., 2013)

	Protex 50FP Protex 5L Protex P Protex 40XL		
Soybean slurry	Protex 6L	Up to 30.0% increase in protein yield	(De Moura, Campbell, et al., 2011)

2.4.4. Fermentation

Fermentation is a widely studied technique that can bring numerous nutritional benefits to products derived from plants; in addition to improve the sensory and functional properties that may be desirable to consumers looking for milk substitute products. Fermentation parameters, including choice of substrate and microbiological culture, time, and temperature must be carefully controlled to obtain a product that is safe for consumption, with stability and sensory standards. Normally, yeasts, bacteria, and fungi can be used for fermentation, alone or in mixed cultures (Marsh, Hill, Ross, & Cotter, 2014). The lactic acid bacteria, bacilli and yeasts (e.g. *Saccharomyces*) are the most widely used microbes for plant based beverage fermentation; and the mixed cultures appear particularly promising due to the potential synergistic effects within the microbial consortia, helping to improve the quite diverse quality criteria with only one process (Jeske, Zannini, & Arendt, 2018; Tangyu, Muller, Bolten, & Wittmann, 2019). The main findings reported in the literature for fermentation are shown in Table 4.

Fermentation can enhance the nutritional quality of plant-based beverages by increasing the protein content, due to the growth of food-grade microbes during fermentation. It can also improve the solubility of plant protein and enrich the pool of available amino acids, vitamins, and minerals (Tangyu et al., 2019). The crude protein content increased significantly from 2.90% to 3.70% and 2.90% to 3.34% in soymilk after 48 h of fermentation with *B. infantil* and *B. longum*, respectively; in relation to the sample without fermentation. In addition, this fermentation treatment resulted in an increase in the content of the B complex vitamins, riboflavin, and thiamine (Hou, Yu, & Chou, 2000).

The increase in bioactive compound concentration was reported in plant-based fermented products. In soymilk, cultures of lactic acid bacteria producing the enzyme β-glucosidase are used to convert conjugated isoflavones into aglycone forms. These forms are more easily absorbed, therefore presenting better bioavailability. After 48 h of fermentation with the starter cultures of *L. plantarum, L. delbrueckii, B. breve*, and *B. thermophilum*, total conversion of the conjugated isoflavones into their aglycone forms was achieved (Pyo, Lee, & Lee, 2005). The glycosylated isoflavones were totally converted to aglycone after 24 h of fermentation with *L. rhamnosus* at 37 °C, and the total isoflavone content was also increased. In addition, the high amount of aglycone isoflavone was responsible for increasing the

inhibition of DNA oxidation induced by Fenton's agent (Marazza, Nazareno, de Giori, & Garro, 2012). Fermented soymilk with *S. thermophilus*, at 37 °C for 24 h, showed an increase of more than 200% in aglycone content (H. L. Chien, Huang, & Chou, 2006).

Lactic acid bacteria have also been used in fermentation to increase the antioxidant capacity of the plant-based beverages. For almond milk, three isolates of lactic acid bacteria were incubated at 37 °C for 24 h, and all fermented almond milks had a higher antioxidant capacity, compared to unfermented almond milk; due to the higher phenolic compound content in fermented samples (Wansutha, Yuenyaow, Jantama, & Jantama, 2018). Similarly, fermentation of soymilk for 24 h by *L. rhamnosus* increased the antioxidant capacity, owing to the formation of aglycone by fermentation (Marazza et al., 2012). Final pH values influenced soymilk fermentation by lactobacillus *L. acidophilus*, *L. paracasei*, *L. zeae* and *L. rhamnosus*. When the pH reached the lowest value (3.85), higher levels of aglycone isoflavone, phenolic compounds and antioxidant capacity were achieved; due to the increase in the viable cell count of these microorganisms at this pH (D. Zhao & Shah, 2014).

The almond milk fermented by probiotic microorganisms suggested an improvement in its bioactivity, due to the positive immunomodulatory effects on macrophages, and also in the increase in the bioavailability of dietary iron (Bernat, Cháfer, Chiralt, Laparra, & González-Martínez, 2015). In addition, almond milk fermentation by *Lactobacillus reuteri* and *Streptococcus thermophilus* allowed for the survival of probiotics after *in vitro* digestion with gastrointestinal enzymes above the minimum levels suggested; guaranteeing health benefits during the 28-day shelf life period, thus resulting in a functional food product. However, the acceptability of this product was reasonable, reinforcing that strategies should be taken in order to improve acceptance by consumers (Bernat, Cháfer, Chiralt, & González-Martínez, 2015).

Fermentation is also capable of reducing the level of antinutrients, such as tannins, phytates, and trypsin inhibitors in plant-based food (Tangyu et al., 2019). For instance, when soymilk was fermented simultaneously with *S. thermophilus* and *B. infantis*, the content of compounds with antinutritional activity, such as saponins and phytates, were significantly reduced; and the total phenolic content as well as antitumor cell proliferation effect of soymilk against HT-29 and Caco-2 cells were enhanced (Lai, Hsieh, Huang, & Chou, 2013).

Table 4. Microorganisms used in the fermentation of plant-based beverages and their improvements.

Vegetable Matrice	Cultures	Fermentation Conditions	Improvement	Reference
Almond Milk	*Lactobacillus rhamnosus* *Lactobacillus plantarum* *Bifidobacterium bifidum* *Bifidobacterium longum* *Streptococcus thermophilus* *Lactobacillus delbrueckii subs. Bulgaricus C*	37°C	Beneficial effects on human gut health Increased the bioavailability of dietary iron	(Bernat, Cháfer, Chiralt, Laparra, et al., 2015)
Almond Milk	*Lactobacillus reuteri* *Streptococcus thermophilus*	37°C/24h 42°C/24h	High viability of the probiotic bacteria	(Bernat, Cháfer, Chiralt, & González-Martínez, 2015)
Almond Milk	Three isolates of lactic acid bacteria (F3, M47, A62)	37°C/ 24h	Increase of antioxidant capacity	(Wansutha et al., 2018)
Soymilk	*L. plantarum* *L. delbrueckii* *B. breve* *B. thermophilum*	37°C 48h	Conversion of conjugated isoflavones into aglycones	(Pyo et al., 2005)
Soymilk	*Streptococcus thermophilus* *Lactobacillus acidophilus* *Bifidobacterium infantis* *Bifidobacteriumlongum*	37°C/ 24h 37°C/ 32h	Conversion of conjugated isoflavones into aglycones	(H. L. Chien et al., 2006)

Soymilk	*B. infantis* *B. longum*	37°C/ 48h	Increase of crude protein content Increase of riboflavin and thiamine content	(Hou et al., 2000)
Soymilk	*L. acidophilus* *L.paracasei* *L. zeae* *L. rhamnosus*	37°C/ 18h	Increase of aglycones and phenolic compound contents Increase in antioxidant capacity	(D. Zhao & Shah, 2014)
Soymilk	*L. rhamnosus*	37°C/ 24h	Conversion of conjugated isoflavones into aglycones Increase in antioxidant capacity Increase in ability to protect DNA oxidation	(Marazza et al., 2012)

2.5. Conclusions

The plant-based beverage market is on the rise, due to increased consumption by people with diets restrictive to cow's milk, preferences for foods of plant origin, and also by people concerned with the environment. This work reviewed several published studies that demonstrated the potential of applying technologies considered ecofriendly, such as ultrasound, ultra high pressure homogenization (UHPH), enzyme use and fermentation for the production of plant-based beverages.

The improvements promoted by ultrasound include: better extraction of intracellular compounds; reduction in the particle size of the protein, which can increase the interaction with water, promoting improvements in solubility; and technological properties such as viscosity, foaming and emulsifying capacity. The UHPH technology favors a reduction of colloidal particles, which causes a reduction of sedimentation, increases the physical stability of plant-based beverages, besides being a promising technique for the reduction of bacterial count. In addition it also preserves the sensory characteristics of beverages. With enzymatic hydrolysis using carbohydrases, the disintegration of the plant cell wall is possible, with a higher release of intracellular compounds. Carbohydrases can also act on insoluble fibers, increasing the soluble solids and process yield. Protease enzymes can increase the solubility and yield of protein extraction, also promoting the release of bioactive peptides with increased antioxidant capacity. Fermentation is a more commonly applied technique, resulting in an improvement in the nutritional quality of plant-based beverages, in addition to increasing protein content, bioactive compounds and antioxidant capacity.

Therefore, these techniques can be studied and incorporated into the production processes of plant-based beverages, mainly those produced using agro-industrial by-products; in order to improve the production yield, reduce the loss of compounds of interest, as well as product quality.

Declaration of interest

The authors report no conflicts of interest.

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CAPÍTULO 3. ENZYMATIC PRETREATMENT IN THE EXTRACTION PROCESS OF SOYBEAN TO IMPROVE PROTEIN AND ISOFLAVONE RECOVERY AND TO FAVOR AGLYCONE FORMATION

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ENZYMATIC PRETREATMENT IN THE EXTRACTION PROCESS OF SOYBEAN TO IMPROVE PROTEIN AND ISOFLAVONE RECOVERY AND TO FAVOR AGLYCONE FORMATION

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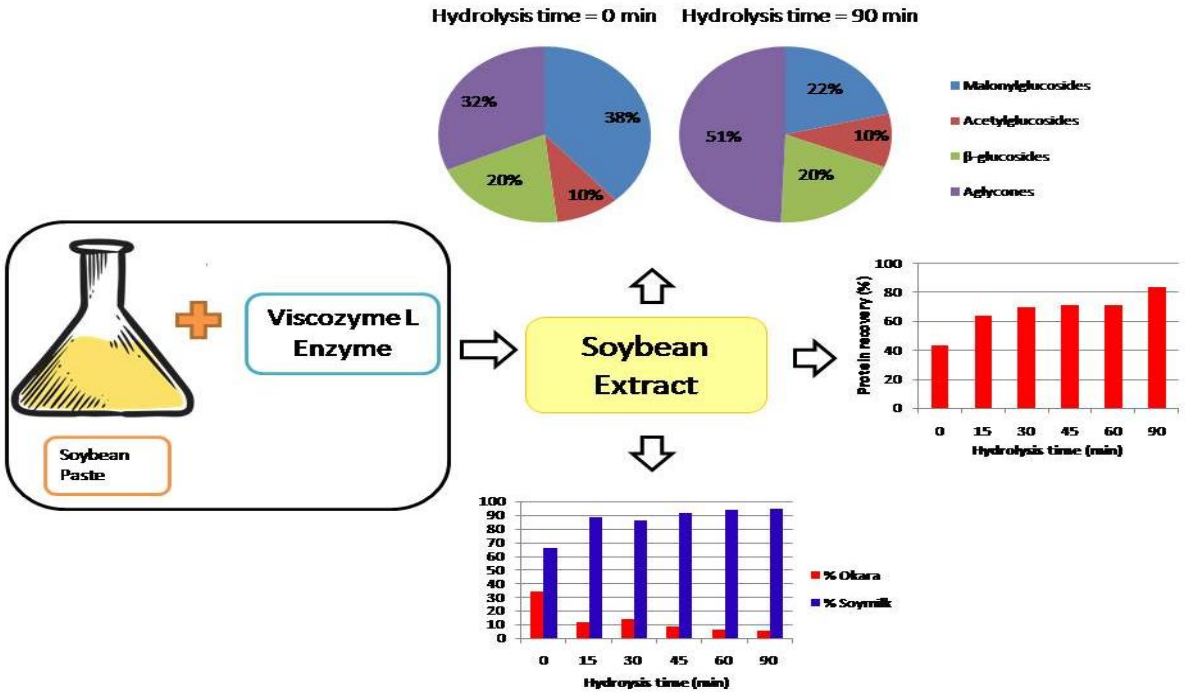
GRAPHICAL ABSTRACT



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ABSTRACT

This research aimed to evaluate the pretreatment of soybean with the carbohydrase multi-enzyme complex “Viscozyme L”, during the extraction process; in order to improve the recovery of proteins and isoflavones in soybase, and reduce the loss of these compounds through the okara residue. Three concentrations of enzyme were studied at 50°C, along with an experiment carried out without enzyme addition (control experiment). The results were also evaluated in relation to standard soybase processing. In comparison to the standard and control processes, the enzymatic pretreatment reduced up to 85% the total amount of okara residue. Due to the action of the multi-enzyme complex, protein and total isoflavone recovery increased from 42% to 83% and from 59% to 93%, respectively. The application of Viscozyme L also favored the conversion of conjugated forms of isoflavone to aglycone in the soybase, representing up to 50% of the total isoflavones. The enzymatic pretreatment of soybean with carbohydrase improved the nutritional quality of the soybase, while at the same time reducing residue generation; showing that the proposed food process can be considered environmentally friendly method.

Keywords: carbohydrase, okara, isoflavone conversion, protein recovery, process yield, enzyme-assisted extraction.

3.1. Introduction

Soybean protein has attracted the attention of consumers due to its high nutritional value; as it contains all the essential amino acids found in animal based proteins (Capriotti et al., 2014). It is an important ingredient used in several formulated products like sausages, cakes, bread and soups, and is a potential source of bioactive peptides (Singh, Vij, & Hati, 2014; Singh, Kumar, Sabapathy, & Bawa, 2008; Yimit, Hoxur, Amat, Uchikawa, & Yamaguchi, 2012). Additionally, soybeans are a rich source of bioactive phenolic acids and flavonoid compounds, mostly represented by isoflavones (Verardo et al., 2015). Several studies have demonstrated the benefits of these compounds in reducing or preventing various types of cancers, diabetes, hypertension, kidney diseases, and osteoporosis (Ahn & Park, 2017; M. Messina & Bennink, 1998; Mark Messina, 2014; Zhang et al., 2016).

Twelve isoflavone forms have been isolated from soybeans, including aglycones; represented by daidzein, genistein, and glycitein, and their respective nine glucosidic conjugates (malonyldaidzin, malonylgenistin, malonylglycitin; acetyldaidzin, acetylgenistin, aceylglycitin; daidzin, genistin and glycitin) (Jackson et al., 2002). The distribution and content of these isoflavone forms may be influenced by several factors; such as, the method of raw material storage and product processing conditions (Jung; Murphy; Sala, 2008; Rostagno; Palma; Barroso, 2003; Zhang; Chang; Liu, 2015). Conversions between isoflavones significantly affect their bioavailability (Xu, Wang, Murphy, & Hendrich, 2000). Some glycosylated forms are actively transported through the small intestinal epithelium by a glucose transporter (Cermak; Landgraf; Wolffram, 2018). However, other conjugated isoflavones must be converted into their respective aglycone forms by hydrolysis of the sugar moiety to facilitate absorption by the organism due to their low molecular weight (Barnes et al., 2011; Monteiro et al., 2018; Németh et al., 2003). The conversion of conjugated isoflavones to aglycones may occur through the action of enzymes produced by the gut microbiota; nevertheless, some people do not have a favorable gut microbiome to promote this mechanism (Turner; Thomson; Shaw, 2003). Various techniques can be employed for the conversion of the conjugated isoflavones to aglycone forms, such as ultrasound, the use of acid, and enzymatic and microbial hydrolysis (Vong; Lim; Liu, 2017; Yeom et al., 2012; Yu; Yang, 2019).

In the food industry, several products are derived from soybean, among them are soymilk and tofu; which have been used as an alternative for cow's milk by people with diet

restrictions, as well as for health benefits (Durazzo; Gabrielli; Manzi, 2015). The processing of these products basically consists of the homogenization of soybeans in water, filtering, and pasteurization of resultant soybase (Kamizake; Silva; Prudencio, 2016). In the filtering step, a large amount of insoluble residue is generated, which is called okara. This residue contains macro- and micronutrients and phytochemicals, indicating that there is a deficit of these compounds in the final product (Li; Qiao; Lu, 2012; O'toole, 1999a). For example, from the initial protein and isoflavone present in soybean, 15.2-33.4% of protein and about 30% of total isoflavone are retained in the okara residue (Vong; Liu, 2016; Wang; Murphy, 1996).

One alternative to improve the extractive capacity of bioactive compounds from vegetable matrices may be the use of carbohydrases; because they generally assist to disintegrate the cell wall, thereby, releasing compounds bound to the cellular matrix (Rosset; Acquaro; Beléia, 2014b). Because isoflavones are associated with the hydrophobic interiors of the globular soybean proteins, the increase in recovery of protein can improve isoflavone leaching in soybase (Rickert et al., 2004). Viscozyme L is a multi-component carbohydrase containing a wide range of enzymes, including arabanase, cellulase, β-gluconase, hemicellulase, and xylanase; which can effectively hydrolyze plant cell wall polysaccharides (Novozymes, 2001). Enzymatic treatment with this enzyme showed promising improvement in the extraction of plant proteins from defatted soybean flour, oat bran meals, and sesame bran (Görgüç; Bircan; Yilmaz, 2019; Guan; Yao, 2008; Liu et al., 2008; Rosset; Acquaro; Beléia, 2014b). In addition, as this enzyme has β-glucanase in its composition, it may be possible to convert the conjugated isoflavones into their aglycone forms; which can be nutritionally advantageous (Ahmad et al., 2014).

To our knowledge, no research has evaluated the isoflavone profile changes attributed to Viscozyme L activity in soybean paste during the production of soy-based products. A study developed by Vong & Liu (2019) showed that the application of Viscozyme L in okara was efficient for the conversion of glycosidic isoflavones to aglycone; probably due to catalysis by endo-β-glycosidases present in the carbohydrase complex. Thus, the evaluation of an enzyme assisted-extraction method to increase protein and isoflavone recovery, as well the conversion of glucosidic forms to aglycone isoflavone is promising; potentially adding value to soy-based products, like soymilk and tofu. In this context, the aim of this study was to assess enzymatic pretreatment with the multi-enzyme carbohydrase complex Viscozyme L in soybase processing to improve isoflavone and protein extraction and favor the formation of aglycones.

3.2. Materials and Methods

3.2.1. Materials

Soybean grains (cultivar BRS 257, lipoxygenase enzyme free, crop 2015-2016) were provided by SL Cereais e Alimentos (Mauá da Serra, Brazil).

For enzymatic pretreatment, the multi-enzyme carbohydrase complex Viscozyme® L (Novozymes Inc., Bagsvaerd, Denmark); composed of cellulase, hemicellulase, arabanase, β-gluconase, and xylanase, was used. This enzyme is derived from *Aspergillus aculeatus* and has a declared activity of 100 FBG/g (Fungal Beta-Glucanase units).

Acetyldaidzin (6”-O-acetyldaidzin), acetylgenistin (6”-O-acetylgenistin), acetylglycitin (6”-O-acetylglycitin), malonyldaidzin (6”-O-malonyldaidzin), malonylgenistin (6”-O-malonylgenistin), and malonylglycitin (6”-O-malonylglycitin) were purchased from Wako Pure (Osaka, Japan). Daidzin, genistin, glycitin, daidzein, genistein, and glycitein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used were of analytical grade.

3.2.2. Sample preparation

Soybean paste processing was performed as described by Baú and Ida (2015), with modifications. The soybean grains were manually selected, washed in running water, and kept in distilled water for maceration (1:3, w/v) for 14h at 4°C. This mixture was drained, washed in running water, and homogenized with distilled water (1:8, w/v) in a domestic blender (Britania, Brazil) for 40s at 25°C to obtain soybean paste.

3.2.3. Enzymatic pretreatment of soybean paste

In the current work, three extraction processes of soybean were evaluated: (i) enzyme-assisted extraction; (ii) control experiment (same procedure with thermal treatment step and no enzyme addition); (iii) standard procedure (no enzyme addition and no thermal

treatment step). A detailed schematic of these processes, for soybase and okara production, are shown in Fig. 2.

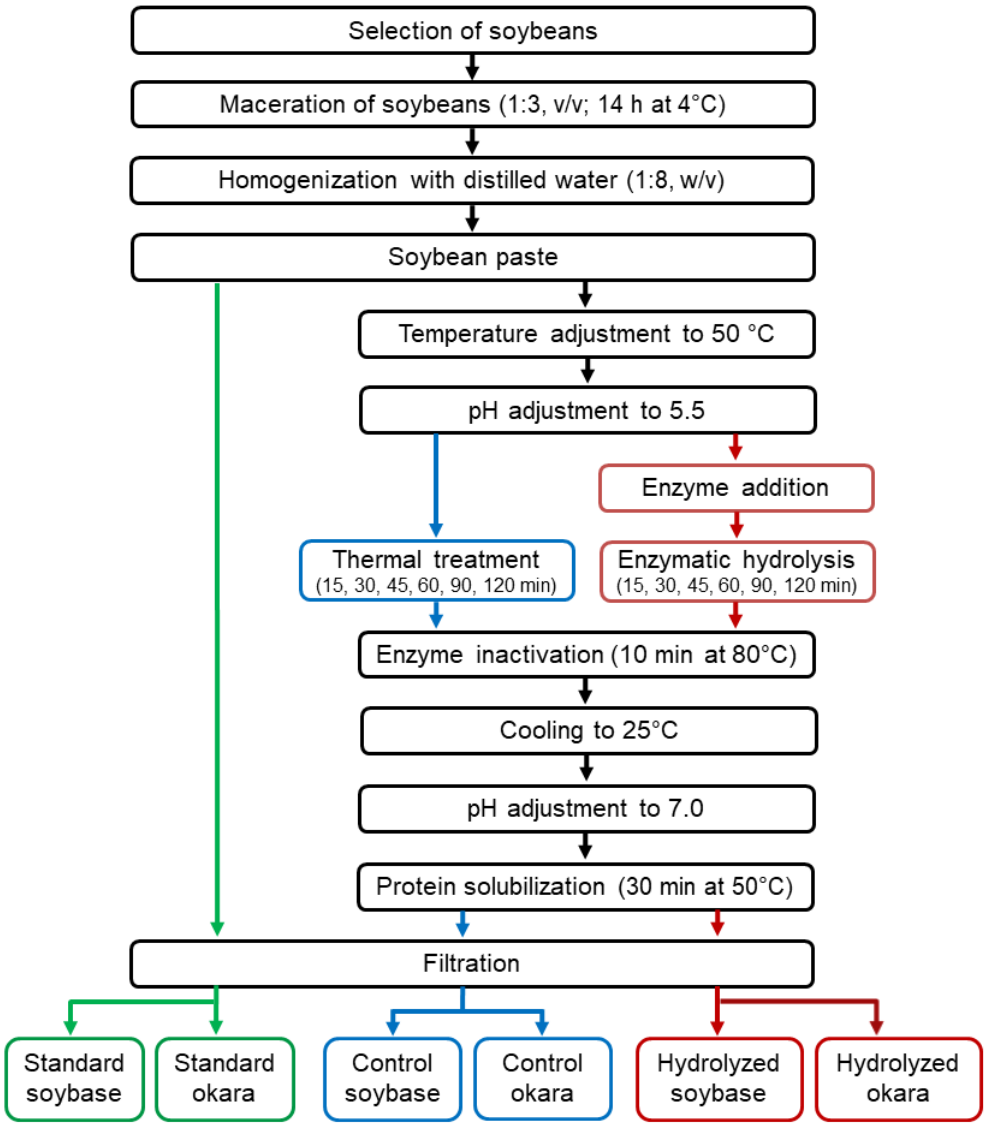

Fig. 2. Schematic of soybean extraction processes. Black steps are common to all processes. Red = enzyme-assisted extraction. Blue = control. Green = standard.

The enzymatic pretreatment of soybean paste was carried out in triplicate using a Dubnoff incubator-shaker (TE-053, Tecnal, Piracicaba city, Brazil). Approximately 50 g of soybean paste was placed in a 250 mL Erlenmeyer flask. The enzymatic hydrolysis conditions were conducted according to enzyme manufacturer's protocol (Novozymes): Temperature of 50°C and pH of 5.5 adjusted with 50% phosphoric acid solution. The enzyme was added as

soon as the desired hydrolysis conditions were reached. Enzyme concentrations used were 2, 4, and 6% (v/v, in relation to soybean paste). An experiment was carried out without the addition of enzyme under the same conditions as described above, denoted as control. The treatment times were 15, 30, 45, 60, 90, and 120 min.

After each experiment, the enzyme was inactivated through heating, where the soybean paste was kept at 80°C for 10 min in a water bath (Cole-Parmer, Brazil) and immediately cooled in an ice bath until reaching room temperature. The pH of the soybean paste was subsequently adjusted to 7.0 with 1 M sodium bicarbonate solution and the samples were placed in an incubator-shaker at a controlled temperature of 50°C for 30 min for protein solubilization. The soybean paste was filtered through a 100% cotton cloth to obtain soybase (aqueous extract) and okara (solid residue), which were subsequently weighed (Fig. 1).

A standard procedure was also performed according to the usual process used by soymilk manufacturers. After its preparation, the soybean paste was subjected only to the filtration step, obtaining the soybase and okara (Fig. 1).

The mass of okara in relation to the mass of soybean paste ($M_{okara}/M_{soybean\ paste}$, %) was calculated to evaluate the process yield. All soybase samples were analyzed for soluble solid content (SS) using a portable analog refractometer (Kasvi, Brazil) to observe the enzymatic hydrolysis catalyzed by the carbohydrase. Later, the soybase samples were freeze-dried (Liotop LP820, Liobras, São Carlos, Brazil) and analyzed for their protein and isoflavone contents. Protein recovery in soybase (PR, %) was calculated as an index of the protein extraction efficiency (Eq. 1).

$$PR = \frac{PC_{soybase} \times M_{soybase}}{PC_{paste} \times M_{paste}} \times 100\% \quad (1)$$

Where $PC_{soybase}$ and PC_{paste} are the protein contents of soybase and paste, respectively; and $M_{soybase}$ and M_{paste} are the masses of soybase and soybean paste, respectively.

The isoflavone content was also evaluated in the freeze-dried okara by conducting a mass balance of isoflavones to verify degradation during pretreatment (Eq. 2).

$$TI_{soybase\ +okara\ } = \frac{TI_{soybase\ } \times M_{soybase\ } + TI_{okara\ } \times M_{okara}}{M_{soybase\ } + M_{okara}} \quad (2)$$

where $TI_{soybase+okara}$ is the sum of total isoflavone concentration in soybase and okara (µmol/g soybase + okara); $TI_{soybase}$ and TI_{okara} are the total isoflavone concentrations in soybase and okara (µmol/g), respectively; and $M_{soybase}$ and M_{okara} are the masses of soybase and okara, respectively, obtained after the filtration step.

From Equation 2, total isoflavone recovery in soybase (TIR, %) was also calculated as an index of the isoflavone extraction efficiency (Eq. 3).

$$TIR = \frac{TI_{soybase\ } \times M_{soybase}}{TI_{soybase\ +okara\ } \times (M_{soybase\ } + M_{okara\ })} \times 100\% \quad (3)$$

3.2.4. Protein content

Total nitrogen was determined using the combustion method for freeze-dried soybase protein content, using the NDA 701 equipment (VelpScientifica, Italy). The software used for data acquisition was DUMASoft™, version 2.2.9. The results were expressed as g protein/100g of soybase, using a nitrogen conversion factor of 5.46 (Mosse, 1990).

3.2.5. Identification and quantification of isoflavones by UPLC

Freeze-dried soybase and okara samples were defatted with hexane (1:10, w/v) for 1h under constant agitation (300 rpm) using a water bath shaker (Tecnal, Brazil) at 25°C.

The identification and quantification of isoflavones were performed according to Falcão et al. (2018). Isoflavone extraction was carried out in duplicate using a solvent consisting of ultra-pure water, ethanol, and acetone (1:1:1, v/v/v). These samples were filtered using 0.22 µm Millex filters and were automatically injected into the Acquity UPLC® (Waters, Milford, MA, USA). The flow rate of the mobile phase in a non-linear gradient was adjusted to 0.3 ml $min^{-1}$and the compounds were detected at 260nm by a diode array detector.

Quantification of isoflavones was performed through calibration curves and the results were expressed as μmol isoflavones/100 g.

3.2.6. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were used to estimate the statistical parameters. Variables with a level of significance above 95% ($p<0.05$) were considered significant.

3.3. Results and Discussion

3.3.1. Process yield

In this study, the extraction process yield was evaluated as the percentage of okara mass in relation to soybean paste mass; therefore, higher okara mass percentage indicates less yield (Table 5).

Table 5. Mass of okara in relation to the mass of soybean paste (%) in enzymatically pretreated and control samples.

Treatment	Time (min)	Enzyme concentration			
		Control	**2%**	**4%**	**6%**
Hydrolysis	15	28.3 ± 1.6^{Ba}	20.5 ± 0.4^{Cb}	16.0 ± 1.0^{Bbc}	11.7 ± 0.0^{Bb}
	30	28.6 ± 1.8^{Ba}	19.9 ± 0.0^{Cb}	15.4 ± 2.7^{Bb}	13.6 ± 0.5^{Bb}
	45	28.7 ± 0.8^{Ba}	19.4 ± 0.1^{Cb}	17.9 ± 2.1^{Bb}	11.7 ± 1.4^{Bc}
	60	28.6 ± 1.9^{Ba}	23.4 ± 1.6^{BCb}	12.5 ± 0.7^{Bc}	5.8 ± 0.6^{Cd}
	90	32.7 ± 0.7^{ABa}	26.8 ± 1.4^{Bb}	12.5 ± 0.7^{Bc}	5.2 ± 0.4^{Cd}
	120	32.9 ± 2.3^{ABa}	29.0 ± 2.5^{Ba}	15.2 ± 0.3^{Bb}	14.1 ± 0.3^{Bb}
Standard		34.2 ± 1.2^{A}	34.2 ± 1.2^{A}	34.2 ± 1.2^{A}	34.2 ± 1.2^{A}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscript in capital letters are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscript in lowercase letters are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

All treatments significantly reduced ($p<0.05$) the amount of okara when compared with the standard process; however, lower values were observed for enzymatic treatments than the control process (Table 5). In general, an increase in enzyme concentration favored the reduction of okara mass. The lowest residue generation was observed for the 6% enzyme (w/w) treatments, especially after 60 and 90 min of enzymatic reaction. Under these conditions, only about 0.05 kg okara was obtained from 1 kg of soybean paste, indicating a reduction of 85% in okara generated from the standard process.

At 6% enzyme concentration, a decrease in the amount of okara was observed throughout all reaction times up to 90 min; however, there was a slight increase when the enzyme reaction time was 120 min. This result is probably due to the interaction of hydrolyzed carbohydrates with other compounds released from soybean cells; such as proteins, after the action of carbohydrase in the medium, forming insoluble compounds and, as consequence, increasing the amount of okara (Kasai; Ikehara, 2005b).

The decrease in okara generation obtained in this study is quite significant for the industrial scale production of soymilk and tofu. In general, okara contains about 42.4–58.1% of dietary fiber, which is mainly composed of insoluble fiber (IDF) (Vong; Liu, 2016) in the form of cellulose, hemicellulose and lignin (Guermani et al., 1992). Viscozyme L is formed by a complex of carbohydrases, such as cellulase and hemicellulase. These enzymes can act on the okara IDF, releasing larger amounts of soluble fiber or simple sugars in the soybase (Vong; Liu, 2019b). This can be verified through an increase in the soluble solids content (SS, °Brix) of the soybase (Table 6). Furthermore, a strong negative correlation between SS and $M_{okara}/M_{soybean\ paste}$ was observed under Pearson's correlation coefficient of $r = -0.93$ (data not shown). In the control group, SS increased by approximately 5% in relation to the standard sample, possibly due to the internal mass transfer of soluble solids during the heat treatment. SS increased up to 55%, 65%, and 105% in relation to the standard sample, when 2%, 4%, and 6% of enzyme concentration was used during pretreatment, respectively.

Table 6. Soluble solids content (°Brix), total proteins (g/100 g of soybase), and total isoflavone (µmol/100 g of soybase) in enzymatically pretreated soybase and control samples.

Treatment	Time (min)	Enzyme concentration			
		Control	**2%**	**4%**	**6%**
		Content of soluble solids (°Brix)			
Hydrolysis	15	4.4 ± 0.0^{Ad}	6.1 ± 0.1^{Bb}	5.7 ± 0.1^{Dc}	7.4 ± 0.0^{Ca}
	30	4.4 ± 0.0^{Ac}	6.2 ± 0.0^{Bb}	6.2 ± 0.0^{Cb}	7.4 ± 0.0^{Ca}
	45	4.4 ± 0.0^{Ac}	6.5 ± 0.1^{Ab}	6.7 ± 0.1^{Bb}	7.4 ± 0.0^{Ca}
	60	4.4 ± 0.0^{Ad}	5.4 ± 0.0^{Cc}	6.9 ± 0.1^{Ab}	7.6 ± 0.0^{Ca}
	90	4.2 ± 0.0^{Bd}	5.4 ± 0.0^{Cc}	6.6 ± 0.0^{Bb}	8.3 ± 0.1^{Ba}
	120	4.4 ± 0.0^{Ad}	5.4 ± 0.0^{Cc}	6.7 ± 0.1^{Bb}	8.6 ± 0.0^{Aa}
Standard		4.2 ± 0.0^{B}	4.2 ± 0.0^{D}	4.2 ± 0.0^{E}	4.2 ± 0.0^{D}
		Content of proteins (g/100 g of soybase)			
Hydrolysis	15	2.76 ± 0.04^{Aa}	2.72 ± 0.25^{ABa}	2.14 ± 0.05^{Ab}	2.37 ± 0.03^{ABCab}
	30	2.62 ± 0.19^{ABa}	2.82 ± 0.08^{Aa}	2.27 ± 0.19^{Aa}	2.53 ± 0.10^{ABa}
	45	2.78 ± 0.25^{Aa}	2.66 ± 0.11^{ABCa}	2.38 ± 0.04^{Aa}	2.48 ± 0.19^{ABCa}
	60	2.74 ± 0.04^{Aa}	2.28 ± 0.09^{BCb}	2.30 ± 0.07^{Ab}	2.33 ± 0.05^{BCb}
	90	2.76 ± 0.02^{Aa}	2.20 ± 0.17^{BCb}	2.41 ± 0.05^{Aab}	2.62 ± 0.00^{ABa}
	120	3.08 ± 0.16^{Aa}	2.27 ± 0.03^{BCb}	2.35 ± 0.07^{Ab}	2.72 ± 0.04^{Aa}
Standard		2.14 ± 0.12^{B}	2.14 ± 0.12^{C}	2.14 ± 0.12^{A}	2.14 ± 0.12^{C}
		Content of total isoflavone (µmol/100g of soybase)			
Hydrolysis	15	33.58 ± 2.16^{ABa}	31.96 ± 3.25^{Aab}	27.12 ± 0.88^{Cbc}	24.43 ± 1.31^{Bc}
	30	31.69 ± 2.34^{ABab}	34.47 ± 0.00^{Aa}	28.34 ± 0.67^{BCb}	27.27 ± 0.06^{ABb}
	45	35.09 ± 0.57^{ABa}	33.06 ± 0.39^{Aa}	33.90 ± 2.69^{ABa}	27.85 ± 2.71^{ABa}
	60	36.54 ± 0.75^{ABa}	29.85 ± 0.03^{Ac}	32.42 ± 0.06^{ABCb}	27.50 ± 0.89^{ABc}
	90	38.43 ± 3.32^{Aa}	29.01 ± 0.14^{Ab}	28.13 ± 0.92^{BCb}	28.60 ± 1.91^{ABb}
	120	38.80 ± 1.62^{Aa}	30.58 ± 0.02^{Ab}	34.94 ± 1.73^{Ab}	32.12 ± 0.38^{Ab}
Standard		29.16 ± 1.7^{B}	29.16 ± 1.70^{A}	29.16 ± 1.70^{ABC}	29.16 ± 1.70^{AB}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscript in capital letters are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscript in lowercase letters are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

The reduction in the amount of okara generated is desirable for the soymilk and tofu industries, since the effective use of this residue is difficult due to its rapid deterioration (Muliterno et al., 2017); representing an industrial problem due to the necessary expensive treatment. In addition, greater amounts of soymilk and tofu can be obtained, resulting in greater profit for these industries. Following the method described in this study, a higher

productivity and consequently lesser residue generation can be obtained, showcasing an environmentally friendly food processing technique (Rosenthal et al., 2003).

Despite the high cost of enzymes, their application in industrial processes has become possible due to intensified enzyme production and development of genetic engineering techniques (Cipolatti et al., 2016). In the last years, different and/or combinations of materials have emerged to increase enzyme activity and productivity in the final reaction (Bilal et al., 2019; Cipolatti et al., 2016). In addition, from the results presented in the current work, further studies can be deepened by evaluating the use of immobilized enzymes; as an economically feasible alternative in industrial usage, as the enzymes can be recovered for posterior reuse.

3.3.2. Protein content and protein recovery in soybase

Protein content in soybase samples are shown in Table 6. The control group had significantly higher protein values ($p < 0.05$) than the standard. This fact is due to the greater transfer mass of protein during the extraction, since in the standard procedure only the filtration of the soybean paste occurs.

For 2% enzyme concentration with hydrolysis times of 15 and 30 min, there was a significant increase ($p<0.05$) in protein content compared to the standard sample. However, these enzymatically pretreated samples did not differ from the control sample. Furthermore, an unexpected result was observed after 60 min of enzymatic hydrolysis, in which there was a significant decrease ($p<0.05$) in the protein content of soybase. For the enzyme concentration of 4%, no significant effect ($p>0.05$) of hydrolysis time was observed. At 6% enzyme concentration, there was a significant increase ($p<0.05$) in protein content, but overall no significant difference ($p>0.05$) was observed when compared to the control sample.

These results seem to show that cell wall hydrolysis by the carbohydrase complex did not have a positive effect on protein extraction in soybase. However, Table 5 indicates that the increase in enzyme concentration caused a reduction in the amount of okara, and, consequently, an increase in soybase mass. Due to this, the extracted protein became more diluted in the enzymatically pretreated soybase. To overcome this dilution effect, protein recovery (PR) was calculated.

After a 90 min reaction, the enzymatic treatments with 6% enzyme concentration resulted in PR values significantly ($p<0.05$) higher than the control process (Table 7). When the hydrolysis reaction was carried out with 6% enzyme concentration for 15 min to 90 min; the PR significantly ($p<0.05$) increased from 64% to 83%, reaching a value of approximately 2-fold higher than the standard procedure (42%). Thus, in addition to the improvement in process yield, the Viscozyme L was also able to enhance protein yield during extraction. On the other hand, for enzymatic hydrolysis using 2% enzyme concentration, an unexpected behavior was observed; throughout the enzymatic process times, there was a reduction in PR. This behavior can probably be due to the fact that the released proteins from soybean cells may have formed a complex with other compounds, becoming insoluble; or this amount of enzyme was not sufficient to act on protein extraction during and up to 90 min of hydrolysis, because the results did not differ ($p>0.05$) from the control samples.

This improvement on protein extraction for hydrolysis with 6% enzyme is attributed to the action of Viscozyme L on the polysaccharides of the cotyledons that remained, even after grinding the soybean to obtain the paste. Since some cotyledons contain intact protein bodies inside the plant's cell wall (Preece et al., 2015), protein molecules were released after structural hydrolysis by enzymes. The main polysaccharides of this structure are cellulose and arabinoxylans, which are disintegrated by the Viscozyme L action (Kasai et al., 2003). In addition, soybean hulls contain a large amount of hemicellulose, a polysaccharide that is also hydrolyzed by the carbohydrases enzymatic complex (Ouhida, Pérez, & Gasa, 2002). Thus, when hydrolyzing the cell wall polysaccharides, there is a greater release of intracellular compounds; as consequence, a higher protein extraction is achieved for soybase.

Our findings corroborate recent studies that reported the increase of protein extraction yield by Viscozyme L from vegetable matrices. Rosset et al. (2014) reported an increase of 70.3% of extracted protein from defatted soy flour pretreated with Viscozyme L when compared to sample obtained for alkaline method. An improvement on protein extraction yields from 31 to 44.8% was reported from sesame bran from a 93 min enzymatic reaction at 91.2 FBG enzyme/100g sample (Görgüç et al., 2019). In a recent study developed by Perović et al. (2020), the enzymatic extraction using a mix of carbohydrases (pectinase, xylanase, and cellullase) in soybean matrix proved to be more efficient than for each enzyme acting alone, when performed under alkaline conditions, reaching almost 50% in protein recovery.

Table 7. Protein Recovery (%) and Isoflavone Recovery (%) of enzymatically pretreated soybase and control samples.

Treatment	Time (min)	Enzyme concentration			
		Control	2%	4%	6%
		Protein recovery (%)			
Hydrolysis	15	61.6 ± 0.8^{Aa}	67.3 ± 7.4^{ABa}	56.0 ± 3.5^{ABa}	64.2 ± 4.6^{Ba}
	30	59.3 ± 4.9^{Aa}	73.4 ± 4.9^{Aa}	62.7 ± 6.0^{Aa}	69.4 ± 0.8^{Ba}
	45	61.2 ± 7.5^{Aa}	66.5 ± 4.4^{ABa}	59.3 ± 0.1^{Aa}	68.9 ± 4.3^{Ba}
	60	62.8 ± 2.5^{Aab}	54.8 ± 2.0^{BCb}	65.4 ± 4.3^{Aab}	70.5 ± 2.7^{ABa}
	90	60.0 ± 1.6^{Abc}	51.4 ± 3.2^{BCc}	68.5 ± 3.4^{Ab}	83.3 ± 5.2^{Aa}
	120	64.7 ± 2.7^{Ab}	49.3 ± 1.6^{Cc}	62.8 ± 2.7^{Ab}	73.5 ± 0.6^{ABa}
Standard		42.1 ± 2.7^{B}	42.1 ± 2.7^{C}	42.1 ± 2.7^{B}	42.1 ± 2.7^{C}
		Isoflavone recovery (%)			
	15	65.9 ± 0.0^{Ac}	76.6 ± 0.1^{ABab}	73.7 ± 1.4^{Cbc}	81.6 ± 3.5^{Aa}
	30	67.7 ± 0.3^{Ab}	78.8 ± 0.9^{Aab}	75.6 ± 2.1^{BCab}	80.7 ± 5.2^{Aa}
	45	67.3 ± 0.5^{Ac}	72.8 ± 2.5^{BCbc}	77.3 ± 2.0^{ABCb}	93.3 ± 1.7^{Aa}
	60	66.9 ± 1.4^{Ac}	70.7 ± 0.4^{CDc}	81.0 ± 1.1^{ABb}	88.4 ± 1.6^{Aa}
	90	66.3 ± 1.0^{Ac}	67.7 ± 1.8^{CDc}	77.3 ± 0.5^{ABCb}	90.7 ± 0.6^{Aa}
	120	66.4 ± 3.1^{Ab}	67.0 ± 0.8^{Db}	81.4 ± 1.1^{Aa}	80.6 ± 5.2^{Aa}
Standard		59.1 ± 1.0^{B}	59.1 ± 1.0^{E}	59.1 ± 1.0^{D}	59.1 ± 1.0^{B}

Values are expressed as mean (n = 2) ± standard deviation. Means followed by different superscript in capital letters are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscript in lowercase letters are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

Other types of enzymes, such as proteases, showed satisfactory results in protein extraction from soybean matrix. A study conducted by De Moura et al. (2011) indicated that the aqueous extraction from soybean slurry by protease reached approximately 96.5% of protein recovery. Sari et al. (2013) demonstrated that the use of various types of proteases increased protein recovery in soybean meal; reaching protein recovery levels of 90% with enzymes that act at basic pH, whereas in acidic pH the recovery was around 50%. Alkaline treatment with endoprotease also showed an increase in protein recovery, up to 30% in extruded soy flakes (De Moura, Campbell, et al., 2011). Therefore, the effectiveness of

enzyme application is dependent on the plant matrix and extraction conditions, as well as the type of enzyme applied.

3.3.3. Extraction, degradation, and interconversion of isoflavones in soybase

Isoflavone extraction, degradation, and interconversion to their different forms can occur simultaneously during the treatments. To verify if isoflavone degradation had occurred throughout the treatment, the mass balance of total isoflavone content in soybase and okara was estimated (Table 8).

Overall, hydrolysis time did not significantly ($p>0.05$) influence the sum of total isoflavone content in soybase and okara, indicating that isoflavone degradation did not occur during the applied treatments (Table 8).

Table 8. Mass balance of total isoflavone concentrations in soybase and okara (μmol total isoflavone/100g of soybase + okara).

Treatment	Time (min)	Enzyme concentration			
		Control	2%	4%	6%
Hydrolysis	15	36.08 ± 1.82^{A}	34.05 ± 2.94^{A}	30.92 ± 1.25^{A}	26.51 ± 2.54^{A}
	30	33.41 ± 2.32^{A}	35.30 ± 0.29^{A}	31.73 ± 0.91^{A}	29.26 ± 2.01^{A}
	45	37.18 ± 0.31^{A}	36.63 ± 0.85^{A}	36.01 ± 2.88^{A}	27.86 ± 2.94^{A}
	60	38.94 ± 1.64^{A}	32.23 ± 0.11^{A}	35.05 ± 0.71^{A}	29.29 ± 0.62^{A}
	90	39.37 ± 3.45^{A}	31.30 ± 1.03^{A}	31.81 ± 0.54^{A}	29.36 ± 1.22^{A}
	120	39.88 ± 0.28^{A}	32.23 ± 0.69^{A}	36.39 ± 2.39^{A}	33.55 ± 0.77^{A}
Standard		32.47 ± 1.95^{A}	32.47 ± 1.95^{A}	32.47 ± 1.95^{A}	32.47 ± 1.95^{A}

Values are expressed as mean (n = 2) ± standard deviation. Means followed by different superscript in capital letters are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscript in lowercase letters are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

Analyzing the total isoflavone content in the control sample (Table 6), in comparison to the standard sample, a significant increase ($p<0.05$) was observed only after 90 min of hydrolysis. A maximum value of total isoflavone content in the control sample was

obtained after 120 min of hydrolysis, corresponding to about 30% higher than that in the standard sample. Since the enzyme was not added in the control sample, the isoflavone extraction was only due to the influence of thermal treatment, which favored compounds leaching into soybase.

Overall, for enzyme-assisted extraction, total isoflavone content in soybase did not show a significant difference with respect to the standard sample ($p>0.05$). Similar to the protein content results in soybase (Table 6), these results could be due to the large amount of soybase generated after enzymatic treatment, which diluted the intracellular compounds in solution, and masked the improvement in isoflavone extraction. The recovery was calculated for total isoflavones (TIR, Eq. 3), and the results demonstrated that, in general, a higher TIR in soybase was achieved ($p<0.05$) by increasing the enzyme concentration (Table 7). Overall, the hydrolysis carried out with 6% enzyme concentration, significantly ($p<0.05$) achieved the highest TIR. The recovery was up to 90% of total isoflavones from soybean paste; since it was the treatment that generated the lowest amount of okara. There are several studies in literature that have modified or introduced steps in soy-based food processing, aiming to improve the extraction of isoflavones. For instance, Morales-de la Peña et al. (2019) reported that ultrasound pretreatment in hydrated-soybeans or soybean paste increased up to 62% total isoflavone content in soymilk over the control soymilk. The pretreatment of soy slurry with Viscozyme L (2.5ml/ 1000ml) was carried out in tofu production; however, under this condition the action of the carbohydrase complex did not result in a difference in total isoflavone content in relation to the standard procedure (Rosset & Del Pino Beléia, 2014).

A strong positive correlation between protein and isoflavone recoveries was observed under Pearson's correlation coefficient of $r = 0.82$ (data not shown), because isoflavones are able to interact with the hydrophobic interior of globular proteins due to their phenolic nature (Malaypally & Ismail, 2010). The higher the enzyme concentration, the greater is the cell wall breakdown. Consequently, more globular proteins were leached into soybase, carrying along with them the associated isoflavones.

In addition to degradation and leaching, isoflavone profile might be altered during enzyme-assisted extraction due to interconversion of isoflavones into different forms. This is influenced by processing parameters, such as pH, heat, and enzyme (J. T. Chien, Hsieh, Kao, & Chen, 2005; Lee et al., 2003). All isoflavone forms were found in soybase samples, with the exception of acetylgenistin (Table 9). The standard sample represents the isoflavone

profile of raw soybeans, in which the main isoflavone form was malonylglucosides, as reported by Andrade et al. (2016), while low acetylglucosides amounts were found.

To verify the effect of interconversions on the isoflavone composition in soybase, the percentage of individual isoflavone classes (malonylglucoside, acetylglucoside, β-glucoside, and aglycone) in relation to the total isoflavone content was calculated (Table 10). This was done to remove the influence of the isoflavone extraction on the interconversion of isoflavones.

The interconversions among the isoflavones classes occur due to the loss of radicals through decarboxylation reactions of malonylglucosides in acetylglucosides; de-esterification of malonylglucoside forms in β-glucosides; or hydrolysis of any glucosidic forms in aglycone (Niamnuy, Nachaisin, Poomsa-Ad, & Devahastin, 2012). Because all treatments, with exception of the standard procedure, were performed under the same conditions of temperature and pH, the differences in isoflavone profile may be due to the action of the carbohydrases enzyme complex promoting interconversion of isoflavones.

Table 9. Different forms of isoflavones in enzymatically pretreated soybase, control and standard samples (μmol/g).

[E] (%)	t (min)	Malonylglucosides			Acetylglucosides		β-glucosides			Aglycones		
		MDAI	MGLI	MGEN	ACDAI	ACGLI	DAI	GLI	GEN	ADAI	AGLI	AGEN
Control	15	0.66 ± 0.06	0.40 ± 0.05	0.87 ± 0.02	0.40 ± 0.18	0.20 ± 0.00	0.49 ± 0.04	0.34 ± 0.04	0.27 ± 0.01	0.71 ± 0.00	0.33 ± 0.02	0.70 ± 0.02
	30	0.55 ± 0.01	0.33 ± 0.01	0.67 ± 0.04	0.38 ± 0.06	0.17 ± 0.02	0.54 ± 0.03	0.34 ± 0.01	0.22 ± 0.02	0.69 ± 0.06	0.33 ± 0.03	0.69 ± 0.07
	45	0.64 ± 0.11	0.39 ± 0.03	0.87 ± 0.18	0.44 ± 0.15	0.18 ± 0.02	0.43 ± 0.03	0.27 ± 0.03	0.25 ± 0.03	0.80 ± 0.07	0.35 ± 0.02	0.87 ± 0.06
	60	0.62 ± 0.00	0.36 ± 0.01	0.88 ± 0.04	0.33 ± 0.07	0.17 ± 0.00	0.45 ± 0.01	0.28 ± 0.01	0.24 ± 0.02	0.88 ± 0.02	0.40 ± 0.01	0.94 ± 0.02
	90	0.66 ± 0.06	0.38 ± 0.03	0.75 ± 0.01	0.43 ± 0.22	0.19 ± 0.01	0.41 ± 0.02	0.34 ± 0.01	0.32 ± 0.03	0.92 ± 0.01	0.42 ± 0.03	0.95 ± 0.06
	120	0.70 ± 0.11	0.38 ± 0.03	0.78 ± 0.03	0.34 ± 0.02	0.18 ± 0.00	0.36 ± 0.01	0.29 ± 0.04	0.27 ± 0.04	0.93 ± 0.01	0.44 ± 0.00	0.98 ± 0.04
2	15	0.58 ± 0.09	0.37 ± 0.05	0.80 ± 0.04	0.28 ± 0.05	0.17 ± 0.02	0.34 ± 0.06	0.33 ± 0.08	0.14 ± 0.01	0.72 ± 0.07	0.31 ± 0.04	0.76 ± 0.06
	30	0.61 ± 0.19	1.02 ± 0.79	0.74 ± 0.55	0.24 ± 0.01	0.10 ± 0.01	0.26 ± 0.00	0.32 ± 0.01	0.23 ± 0.32	0.66 ± 0.04	0.29 ± 0.07	0.67 ± 0.03
	45	0.66 ± 0.02	0.41 ± 0.02	1.04 ± 0.02	0.24 ± 0.01	0.13 ± 0.02	0.16 ± 0.00	0.22 ± 0.01	0.04 ± 0.02	0.74 ± 0.01	0.33 ± 0.01	0.78 ± 0.01
	60	0.37 ± 0.01	0.26 ± 0.00	0.53 ± 0.01	0.27 ± 0.06	0.15 ± 0.01	0.44 ± 0.00	0.35 ± 0.05	0.15 ± 0.00	0.80 ± 0.00	0.38 ± 0.00	0.83 ± 0.00
	90	0.40 ± 0.02	0.24 ± 0.01	0.49 ± 0.04	0.23 ± 0.02	0.13 ± 0.01	0.45 ± 0.03	0.38 ± 0.02	0.16 ± 0.04	0.82 ± 0.00	0.41 ± 0.00	0.85 ± 0.01
	120	0.39 ± 0.00	0.23 ± 0.00	0.47 ± 0.01	0.23 ± 0.00	0.12 ± 0.00	0.45 ± 0.01	0.39 ± 0.01	0.20 ± 0.01	0.85 ± 0.00	0.43 ± 0.02	0.94 ± 0.00
4	15	0.47 ± 0.05	0.28 ± 0.01	0.59 ± 0.05	0.21 ± 0.00	0.14 ± 0.00	0.38 ± 0.03	0.35 ± 0.02	0.10 ± 0.01	0.63 ± 0.01	0.27 ± 0.01	0.62 ± 0.03
	30	0.39 ± 0.00	0.26 ± 0.00	0.57 ± 0.03	0.21 ± 0.00	0.13 ± 0.00	0.32 ± 0.00	0.29 ± 0.04	0.20 ± 0.00	0.67 ± 0.00	0.30 ± 0.00	0.69 ± 0.01
	45	0.47 ± 0.04	0.29 ± 0.02	0.63 ± 0.05	0.23 ± 0.01	0.14 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.16 ± 0.03	0.84 ± 0.04	0.37 ± 0.02	0.86 ± 0.05
	60	0.36 ± 0.01	0.27 ± 0.01	0.48 ± 0.01	0.43 ± 0.02	0.15 ± 0.01	0.34 ± 0.01	0.26 ± 0.00	0.19 ± 0.01	0.78 ± 0.00	0.36 ± 0.00	0.83 ± 0.00
	90	0.31 ± 0.02	0.20 ± 0.02	0.43 ± 0.01	0.21 ± 0.02	0.12 ± 0.01	0.29 ± 0.01	0.28 ± 0.04	0.16 ± 0.08	0.78 ± 0.01	0.38 ± 0.02	0.80 ± 0.02
	120	0.42 ± 0.01	0.28 ± 0.01	0.54 ± 0.03	0.41 ± 0.07	0.15 ± 0.00	0.29 ± 0.01	0.35 ± 0.01	0.24 ± 0.01	0.88 ± 0.02	0.45 ± 0.03	0.91 ± 0.02
6	15	0.31 ± 0.00	0.20 ± 0.00	0.45 ± 0.01	0.17 ± 0.02	0.10 ± 0.00	0.31 ± 0.02	0.25 ± 0.01	0.05 ± 0.01	0.57 ± 0.02	0.22 ± 0.01	0.60 ± 0.01
	30	0.33 ± 0.04	0.22 ± 0.02	0.46 ± 0.07	0.23 ± 0.00	0.12 ± 0.00	0.26 ± 0.08	0.22 ± 0.05	0.07 ± 0.02	0.62 ± 0.01	0.24 ± 0.01	0.67 ± 0.00
	45	0.35 ± 0.06	0.21 ± 0.02	0.46 ± 0.01	0.18 ± 0.01	0.11 ± 0.00	0.28 ± 0.03	0.26 ± 0.05	0.22 ± 0.20	0.69 ± 0.00	0.30 ± 0.02	0.71 ± 0.05
	60	0.28 ± 0.01	0.19 ± 0.00	0.34 ± 0.03	0.18 ± 0.00	0.09 ± 0.00	0.35 ± 0.01	0.31 ± 0.00	0.09 ± 0.02	0.70 ± 0.02	0.33 ± 0.01	0.71 ± 0.02
	90	0.25 ± 0.05	0.16 ± 0.02	0.34 ± 0.04	0.25 ± 0.03	0.10 ± 0.00	0.25 ± 0.04	0.23 ± 0.08	0.09 ± 0.03	0.69 ± 0.03	0.31 ± 0.03	0.73 ± 0.01
	120	0.34 ± 0.02	0.21 ± 0.01	0.42 ± 0.03	0.20 ± 0.01	0.10 ± 0.00	0.26 ± 0.01	0.27 ± 0.00	0.16 ± 0.00	0.78 ± 0.03	0.38 ± 0.02	0.80 ± 0.03
Standard		0.98 ± 0.00	0.58 ± 0.04	0.66 ± 0.05	0.35 ± 0.00	0.21 ± 0.00	0.47 ± 0.05	0.46 ± 0.00	0.24 ± 0.07	0.79 ± 0.02	0.30 ± 0.02	0.74 ± 0.02

MDAI, MGLI, and MGEN are the content of malonyldaidzin, malonylglycitin, and malonylgenistin, respectively. ACDAI and ACGLI are the content of acetyldaidzin, and acetyglycitin, respectively. DAI, GLI, and GEN are the content of daidizin, glycitin, and genistin, respectively. ADAI, AGLI, and AGEN are the content of daidzein, glycitein, and genistein. [E] is enzyme concentration (%) and t is hydrolysis time (min). Data represent mean values for each sample ± standard deviation (n = 2).

When hydrolysis time increased, in general, there was a significant decrease ($p<0.05$) in the concentration of the malonylglucoside class (Table 10), indicating its interconversion to other forms, as will soon be discussed. Moreover, a higher reduction of malonyglucosides was observed for samples pretreated enzymatically.

Table 10. Different isoflavone forms (%) in relation to the total isoflavones presents in enzymatically pretreated soybase and control samples.

Treatment	Time (min)	Enzyme concentration			
		Control	**2%**	**4%**	**6%**
		Malonylglucosides			
Hydrolysis	15	36.0 ± 0.7^{ABa}	36.6 ± 0.9^{Ba}	31.7 ± 0.0^{Bb}	30.0 ± 0.2^{Bb}
	30	31.4 ± 0.4^{Bb}	46.5 ± 1.5^{Aa}	30.4 ± 0.0^{Bb}	29.2 ± 3.6^{BCb}
	45	33.0 ± 2.4^{Bb}	44.4 ± 0.6^{Aa}	29.6 ± 0.8^{Bb}	27.1 ± 0.5^{BCDb}
	60	33.4 ± 0.1^{ABa}	25.5 ± 0.1^{Cb}	25.0 ± 0.0^{Cb}	22.8 ± 0.1^{CDb}
	90	30.8 ± 0.1^{Ba}	24.6 ± 1.0^{Cb}	23.7 ± 0.4^{Cb}	22.0 ± 1.3^{Db}
	120	32.6 ± 1.2^{Ba}	23.2 ± 0.0^{Cb}	25.3 ± 0.3^{Cb}	24.8 ± 1.7^{BCDb}
Standard		38.4 ± 2.2^{A}	38.4 ± 2.2^{B}	38.4 ± 2.2^{A}	38.4 ± 2.2^{A}
		Acetylglucosides			
Hydrolysis	15	11.1 ± 2.7^{Aa}	9.5 ± 0.1^{Aa}	8.6 ± 0.0^{BCb}	8.3 ± 0.7^{Ab}
	30	10.8 ± 1.6^{Aa}	7.1 ± 0.3^{Ac}	8.4 ± 0.0^{BCb}	10.3 ± 0.1^{Aa}
	45	11.5 ± 2.6^{Aa}	7.8 ± 0.4^{Bb}	8.1 ± 0.1^{Cb}	7.6 ± 0.6^{Ab}
	60	9.2 ± 1.3^{Aa}	9.2 ± 1.0^{ABa}	13.4 ± 0.0^{Aa}	7.5 ± 0.3^{Aa}
	90	10.2 ± 2.4^{Aa}	8.1 ± 0.9^{ABa}	8.9 ± 1.3^{BCa}	10.2 ± 1.6^{Aa}
	120	9.2 ± 0.1^{Ab}	7.4 ± 0.0^{Bc}	11.0 ± 1.4^{ABa}	7.5 ± 0.2^{Ac}
Standard		9.7 ± 0.0^{A}	9.7 ± 0.0^{A}	9.7 ± 0.0^{BC}	9.7 ± 0.0^{A}
		β-glucosides			
Hydrolysis	15	20.5 ± 0.2^{ABa}	16.5 ± 1.7^{BCa}	22.1 ± 0.0^{Aa}	18.8 ± 0.5^{Aa}
	30	22.4 ± 0.3^{Aa}	12.3 ± 0.1^{CDa}	20.1 ± 0.0^{Aa}	15.9 ± 3.4^{Aa}
	45	17.8 ± 2.4^{ABa}	8.9 ± 0.1^{Db}	18.2 ± 0.3^{Aa}	19.8 ± 7.1^{Aa}
	60	17.4 ± 0.5^{ABb}	20.8 ± 1.1^{ABa}	17.7 ± 0.0^{Ab}	20.9 ± 0.0^{Aa}
	90	19.8 ± 0.4^{ABa}	21.5 ± 0.1^{Aa}	17.5 ± 3.2^{Aa}	20.3 ± 2.2^{Aa}
	120	17.2 ± 0.4^{Bb}	22.2 ± 0.0^{Aa}	18.1 ± 0.8^{Ab}	17.7 ± 0.1^{Ab}
Standard		20.3 ± 2.2^{AB}	20.3 ± 2.2^{AB}	20.3 ± 2.2^{A}	20.3 ± 2.2^{A}
		Aglycones			

Hydrolysis	15	32.4 ± 2.2^{BCc}	37.4 ± 0.8^{ABb}	37.6 ± 0.0^{BCb}	41.7 ± 2.1^{ABa}
	30	35.8 ± 3.1^{ABCc}	40.0 ± 6.4^{ABb}	41.2 ± 0.0^{Bb}	46.5 ± 0.1^{Aa}
	45	37.6 ± 2.6^{ABCa}	40.1 ± 2.0^{ABa}	43.5 ± 0.3^{ABa}	45.6 ± 6.0^{Aa}
	60	40.0 ± 0.6^{Ac}	44.3 ± 0.2^{Ab}	43.9 ± 0.0^{ABb}	48.8 ± 0.2^{Aa}
	90	39.3 ± 1.7^{ABb}	45.0 ± 0.1^{Aab}	51.7 ± 4.6^{Aa}	51.1 ± 2.6^{Aa}
	120	39.9 ± 0.1^{Ab}	47.2 ± 0.0^{Aa}	42.9 ± 3.3^{Bb}	50.0 ± 1.8^{Aa}
Standard		31.6 ± 0.1^{C}	31.6 ± 0.1^{B}	31.6 ± 0.1^{C}	31.6 ± 0.1^{B}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscript in capital letters are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscript in lowercase letters are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

Overall, the addition of the carbohydrase complex had no significant ($p>0.05$) effect on the concentration of acetylglucosides (Table 10); therefore, we can suppose that Viscozyme L did not induce a decarboxylation reaction of malonylglucosides in acetylglucosides. According to Chien et al. (2005) and Kao et al. (2004), acetylglucosides are generally formed by dry heat at 100°C; thus the interconversion of the malonylglucosides into acetylglucosides was not expected.

With the exception of the sample pretreated with 2% enzyme, Viscozyme L did not significantly ($p>0.05$) influence the β-glucoside profile (Table 10), because its concentration remained constant over hydrolysis time. For samples enzymatically pretreated with 2%, there was an increase in the percentage of β-glucoside after 60 min of hydrolysis, along with a simultaneous decrease in the percentage of malonylglucosides (Table 10). A strong negative Pearson's correlation ($r = -0.96$; data not shown) was observed between the β-glucoside and malonylglucosides content. The carbohydrase complex may have induced the de-esterification of malonylglucosides into β-glucosides that normally occurs in moist heating above 100°C, in which the malonylglucosides lose their stability (Chien et al., 2005).

The concentration of aglycone (Table 10) increased ($p<0.05$) in the control sample throughout the treatment, probably due to the action of the endogenous soybean enzyme β-glucosidase; because the experiments were carried out under its optimum temperature (50°C) and pH (5.5) (Baú & Ida, 2015; Sutil et al., 2008). For most samples treated enzymatically with Viscozyme L, the increase of enzyme concentration resulted in significantly ($p<0.05$) greater aglycone content than in the control sample, suggesting that the carbohydrase complex

also favored the interconversion of conjugated isoflavones into aglycone. Vong et al. (2017) and Vong and Liu (2019) reported that the concentration of aglycone in okara increased due to hydrolysis of glycosidic bonds by the endo-β-gluconases present in Viscozyme L. Then, analyzing the isoflavone profiles, this reaction may have been responsible for the hydrolysis of malonylglucosides to aglycone (Table 10), in which a strong negative Pearson's correlation was obtained (r = -0.77; data not shown). Moreover, this result could also be attributed to the release of isoflavones from the plant cell interior after its hydrolysis through Viscozyme L action. As consequence, there was a greater exposition of the malonyglucosides to the endogenous β-glucosidase, converting them to aglycones. The conversion of isoflavone glycosides to aglycone by β-glucosidase has also been reported by Wei et al. (2018) during enzymatic-assisted extraction from soy flakes, corroborating the results found in this work. Because the aglycones have stronger hydrophobicity than their glycosidic conjugates, hydrophobic interactions between polypeptide chains and aglycone may occur during the extraction process, so they were more easily leached with the extracted proteins (Lu, Chen, Wang, Yang, & Qi, 2016; Wei et al., 2018). Soybase containing high aglycone content is interesting from a nutritional point of view, because, due to their low molecular weight, they are more prone to be absorbed by the intestine than their glucosylated conjugates (Barnes et al., 2011). The genistein and daidzein aglycones are the most important isoflavones in soybean and its derivatives, which play a significant role in the reduction and control of different types of cancer, as they act in reducing the oxidative stress of the cells' DNA (Li, Luo, & Qiao, 2012). Daidzein has higher bioavailability, whereas genistein is mainly attributed to an anticancer effect, since this isoflavone acts to control the body's hormones, blocking the availability of vital nutrients and restricting the oxygen supply to the tumor (Ahmad et al., 2014; Li et al., 2012).

Thus, owing to its known nutritional properties, several researchers have been looking for alternative processes to favor the conversion of conjugated isoflavones to aglycones in soy-based foods. For instance, Baú and Ida (2015) reported that the thermal treatment of soybean paste at 50°C for 3h in soymilk processing yielded aglycone content 16 times higher than that of control samples. Ultrasound pretreatment in hydrated soybeans or soybean paste induced the formation of aglycones, increasing from 36% (control sample) to 57% of aglycone in relation to total isoflavone in soymilk (Morales-de la Peña et al., 2018).

The current work raised important results to enhance soy-based food processing. The carbohydrase-assisted extraction has shown promise for future studies.

3.4. Conclusion

The application of enzymatic pretreatment using a carbohydrase complex in soybean paste disrupted the cell wall, and increased the extraction of soluble solids in soybase. In addition, a greater amount of soybase was obtained, consequently reducing the generation of okara by-product, making this method an environmentally friendly food processing method. Increases in the enzyme concentration resulted in higher percentages of isoflavone aglycones in soybase. Therefore, pretreatment with Viscozyme L in the processing of soybase was effective in improving the recovery of proteins and isoflavones when compared to samples obtained through standard processing. This process can be applied to obtain soybean products, such as soymilk and tofu, which can be richer in these bioactive compounds.

Declaration of interest

The authors report no conflicts of interest.

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CAPÍTULO 4. APPLICATION OF HYDROLYSIS BY PROTEASE DURING THE SOYBASE PREPARATION AND ITS EFFECTS ON THE PROTEIN RECOVERY, ISOFLAVONE RECOVERY AND PROFILE

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APPLICATION OF HYDROLYSIS BY PROTEASE DURING THE SOYBASE PREPARATION AND ITS EFFECTS ON THE PROTEIN RECOVERY, ISOFLAVONE RECOVERY AND PROFILE

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ABSTRACT

Soymilk and tofu are plant-based products that have gained prominence due to being the main substitutes for cow's products. In the filtration stage of their production, soybase and insoluble residue okara are generated. Okara retains compounds of nutritional interest, such as proteins and isoflavones. The aim of this work was the application of the commercial protease Protamex enzyme during the soybase production, and the evaluation of proteolysis on the protein and isoflavone recoveries, as well the degradation and interconversion among isoflavone forms. Three concentrations of enzyme (1, 2, and 4%) were applied, as well as a treatment without enzyme (control process), and evaluated in relation to standard process. The only treatment that showed a significant increase in protein recovery, in relation to standard sample, was 2% enzyme and 15 min of hydrolysis. Isoflavones were degraded due to the pH and temperature parameters used, but protein hydrolysis contributed to a higher degradation. A significant recovery of non-degraded isoflavone was achieved, but no influence of proteolysis was observed. The control and enzymatic treatments influenced the interconversion among isoflavones forms, with a decrease in malonylglucosides, predominance of β-glucosides forms and an increase in aglycone forms, in relation to standard process.

Keywords: Protamex, proteolysis, soymilk, okara, biovalorization.

4.1. Introduction

The substitution of animal proteins for vegetables goes far beyond eating habits, directly impacting the reduction of pollution and the need for resources (Hartmann & Siegrist, 2017). In developing countries, vegetable proteins have become a major source of dietary protein (Du et al., 2012). Among them, soybean stands out due to the availability, quantity produced, and versatility of products.

Soymilk and tofu are soybean products that gained prominence for being substitutes for milk derivatives. The processing of these products includes the followed steps: soaking of soybeans, homogenization in water, and filtration to obtain the soybase (corresponding to the soluble fraction), which can be pasteurized to obtain soymilk or undergo a coagulation process for the tofu production (Jackson et al., 2002). However, in the filtration step, there is also the generation of a large amount of solid waste (called as okara) that contains around 15-33% of protein, resulting in economic and nutritional losses, since it is a material mainly destined to animal feed (B. Li et al., 2012). On the other words, this indicates that a considerable amount of soy compounds are not lixiviated to the soybase, being retained in the solid waste.

To increase the use of okara, some methods, such as chemical, enzymatic or microbiological, have been studied for the extraction of its proteins (Kasai et al., 2004; Orts et al., 2019; Vong et al., 2017). Thus, it would also be interesting to evaluate the application of these methods during the processing of soy products in order to avoid the loss of compounds of interest. However, chemical methods could be drastic and difficult to control during processing, just like microbiological ones. The application of proteases, in turn, could break down soybean protein, increasing its solubility for soybean extract, under mild conditions, without decrease of the nutritional value of the protein source (Tavano, 2013).

In this work, we hypothesized that the enzymatic hydrolysis by protease during the soybase processing can enhance the protein extraction. Protamex, an endopeptidase derived from *Bacillus sp,* can break the soy protein during soybean processing into peptides, increasing exposure of the hydrophobic groups. As consequence, the aqueous protein solubility is increased, reducing loss for the insoluble residue (Molina Ortiz, Puppo, & Wagner, 2004). The raise of protein solubility of different plant matrices due to the action of

the commercial protamex enzyme was previously reported for peanut flour, corn glutelin, and isolated soy protein (Yoo & Chang, 2016; G. Zhao et al., 2013; Zheng et al., 2015).

Additionally, enzymatic hydrolysis of protein can improve isoflavones extraction and the conditions used can change their profile. Isoflavones in soy products are present in 12 chemical structures, grouped into four main categories, which are malonylglucosides, acetylglucosides, β-glucosides, and aglycone (H. J. Wang & Murphy, 1994). However, okara retains about 12-40% of the total isoflavone initially present in raw soybeans, and, as part of these isoflavones are complexed with proteins, they could also be leached into the aqueous part of the soybase with the proteolysis (Jankowiak, Kantzas, Boom, & Van Der Goot, 2014; Orts et al., 2019; H.-J. Wang & Murphy, 1996). The isoflavones forms can undergo changes due to processing parameters; therefore, the enzymatic treatment by protease could influence the transformation of glycated isoflavones into aglycone, increasing the amount of these forms in the soybase.

Then, the aim of this work was to evaluate the application of the commercial protease Protamex during the soybean process to improve the protein and isoflavone extractions to the soybase product. The specific objective was to evaluate the effect of protease concentration (1, 2, and 4%) and hydrolysis time (15, 30, 60, and 120 min) on protein and isoflavone recoveries, as well the interconversion and degradation of isoflavones.

4.2. Material and Methods

4.2.1. Materials

Soybean grains of cultivar BRS 257, lipoxygenase enzyme free, crop 2015-2016,were provided by SL Cereais e Alimentos (Mauá da Serra, Brazil).The endopeptidase enzyme Protamex (Novozymes Inc., Bagsvaerd, Denmark) derived from *Bacillus sp* was used. The standard isoflavone acetyldaidzin (6”-O-acetyldaidzin), acetylgenistin (6”-O-acetylgenistin), acetylglycitin (6”-O-acetylglycitin), malonyldaidzin (6”-O-malonyldaidzin), malonylgenistin (6”-O-malonylgenistin), and malonylglycitin (6”-Omalonylglycitin) were purchased from Wako Pure (Osaka, Japan). Daidzin, genistin, glycitin, daidzein, genistein, and glycitein were supplied from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

4.2.2. Preparation of soybean paste

The preparation of the soybean paste was carried out by selecting the soybean grains, soaking for 14 h in distilled water at 4 °C (1:3, w/v), then being drained and homogenized with distilled water (1: 8, w/v) in a domestic blender, as described by Baú & Ida (2015).

4.2.3. Enzymatic pretreatment of soybean paste

Protein enzymatic hydrolysis was performed in duplicate on a Dubnoff incubator-shaker with temperature control (TE-053, Tecnal, Piracicaba, Brazil). In 250 ml erlenmeyer flasks, 50 g of soybean paste were placed and kept under agitation at 35 rpm. When the temperature of 50 °C was reached, pH solution was adjusted to 8.0 with 1 M sodium bicarbonate solution and the enzyme was added at concentrations of 1, 2, and 4% (g/100 g protein in soybean paste). After different hydrolysis time (15, 30, 60, and 120 min), the soybean paste was heated in a water bath (Cole-Parmer, Brazil) at 80 °C (internal temperature) for 10 min, to inactivate the protease, and then cooled in an ice bath. Then, the soybean paste was again placed in the incubator-shaker at 50 °C for 30 min for protein solubilization. The protein enzymatic hydrolysis parameters were defined based on the preliminary tests and the optimal temperature and pH for the enzyme's performance defined by the manufacturer.

The differences between the hydrolyzed and control experiments, as well the standard process are defined in the flowchart of the experiments (Figure 3). The control sample corresponded to that obtained under enzymatic hydrolysis conditions without protease addition (submitted only to thermal treatments). The standard sample corresponded to that obtained by the soymilk/tofu processing industries, without protease addition and thermal treatment. To obtain the soybase and okara, the soybean paste was filtered through a 100% cotton cloth.

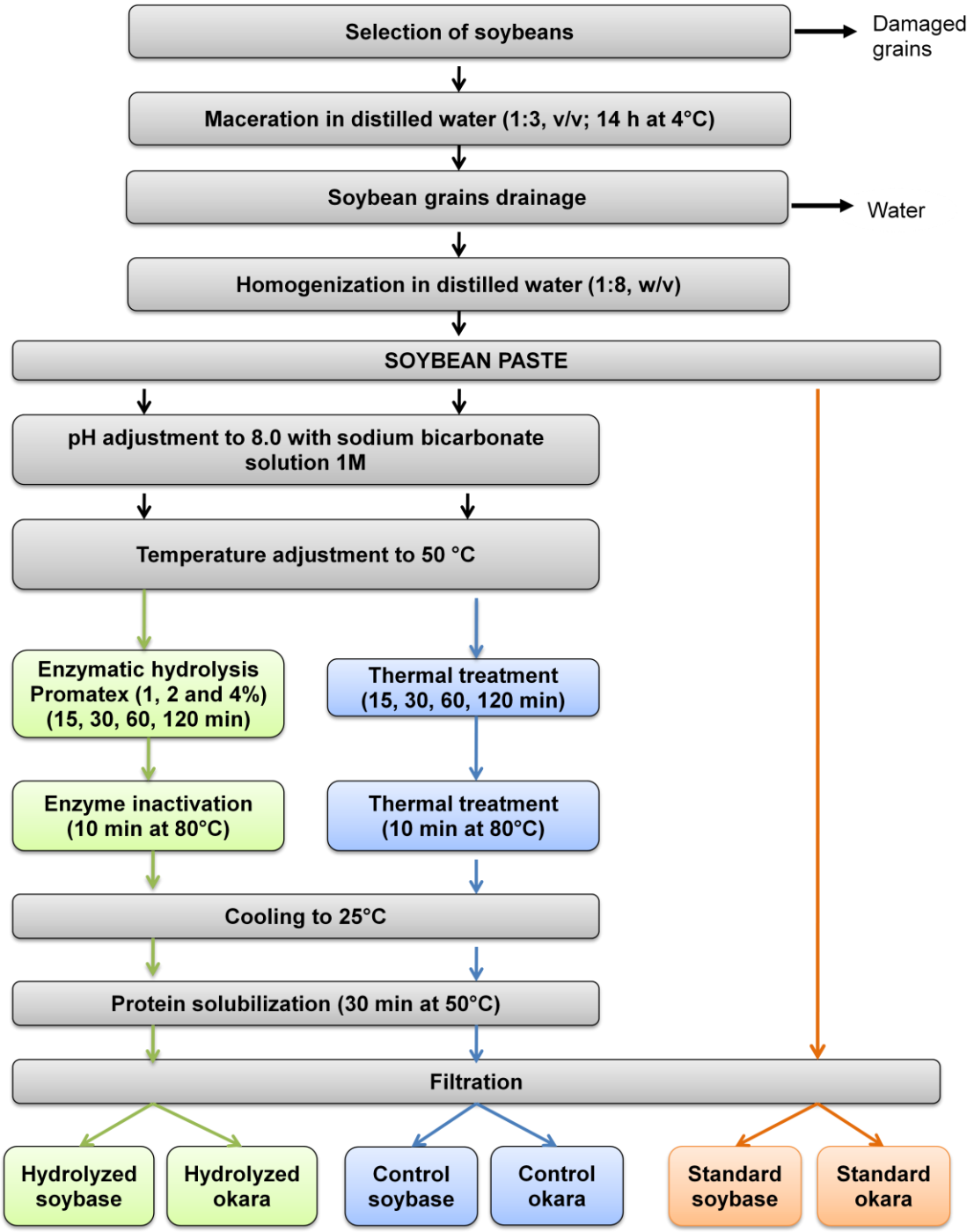

Figure 3.Schematic flowchart of the enzymatic hydrolysis of protein, control experiments and standard processing.

The soybean paste, standard, control and hydrolyzed soybase samples were analyzed for protein content. Protein recovery (PR, %) was calculated to assess the efficiency of protein extraction (Eq. 1).

$$PR = \frac{M_{protein\ ,soybase}}{M_{protein\ ,paste}} \times 100\% = \frac{PC_{soybase} \times M_{soybase}}{PC_{paste} \times M_{paste}} \times 100\% \quad (1)$$

Where: $M_{protein,\ soybase}$ and $M_{protein,\ paste}$ are the masses of protein in soybase and soy paste, respectively (g); $PC_{soybase}$ and PC_{paste} are the protein contents of soybase and paste, respectively (g/100 g); and $M_{soybase}$ and M_{paste} are the masses of soybase and paste, respectively (g).

All soybase and okara samples were lyophilized (Liotop LP820, Liobras, São Carlos, Brazil), and analyzed for isoflavone identification and quantification, to verify isoflavone degradation during enzymatic pretreatment by mass balance (Eq. 2).

$$TI_{soybase\ +okara} = \frac{M_{isoflavone\ ,soybase} + M_{isoflavone\ ,okara}}{M_{soybase} + M_{okara}} = \frac{TI_{soybase} \times M_{soybase} + TI_{okara} \times M_{okara}}{M_{soyba\ se} + M_{okara}} \quad (2)$$

Where: $TI_{soybase+okara}$ is the sum of total isoflavone concentration in soybase and okara (µmol/g soybase + okara); $M_{isoflavone,soybase}$ and $M_{isoflavone,okara}$ are the masses of isoflavones in soybase and okara, respectively; $TI_{soybase}$and TI_{okara} are the total isoflavone concentrations in soybase and okara (µmol/g), respectively; and $M_{soybase}$ and M_{okara} are the masses of soybase and okara, respectively.

From Equation 2, total isoflavone recovery in soybase (TIR, %) was also calculated as an index of the isoflavone extraction efficiency (Eq. 3).

$$TIR = \frac{M_{isoflavone\ ,soybase}}{M_{isoflavone\ ,soybase\ +okara}} = \frac{TI_{soybase} \times M_{soybase}}{TI_{soybase\ +okara} \times (M_{soybase} + M_{okara})} \times 100\% \quad (3)$$

Where: $M_{isoflavone,\ soybase+okara}$ is the sum of the masses of the isoflavone in soybase and okara.

4.2. Analytical methods

4.2.1. Protein content

The nitrogen contents of the soybean paste and soybase samples were determined in duplicate by the combustion method, using the NDA 701 equipment (Velp Scientifica, Italy) and the data acquisition was performed using the DUMASoftTM software (version 2.2.9). A nitrogen conversion factor of 5.46 (Mosse, 1990) was used to obtain the protein content. The results were expressed as g protein/100ml of soybase.

4.2.2. Molecular weight distribution

Molecular weight profiles of soy proteins (Laemmli,1970), and peptides (Gomes & Kurozawa, 2020) resulting from standard sample and enzymatic hydrolysis by 2 and 4% of protease was determined by SDS-PAGE under reducing conditions using β-mercapto-ethanol. Samples were diluted at a 1:1 ratio with buffer solution (0.5 M Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 0.5% coomassie blue, v/w/w) and heated at 37 °C for 15 min. A 4% stacking gel was used and the separating gels were 12.5% and 10%, for soy protein standard and protein hydrolysates, respectively. For hydrolysates, the separation was carried out using a tris-tricine running buffer (Bio-Rad). Gel fixation was carried out using an aqueous methanol/acetic acid/water (5/1/4) solution with 0.1% coomassie brilliant blue G250. The approximate molecular weights (MW) of the soy protein and protein hydrolysates were estimated using standard markers with MW ranges of 3.45–26.6 kDa (Bio-Rad, Hercules, CA, USA) and 21.5–116.25 kDa (Sigma-Aldrich, St Louis, MO, USA), respectively.

4.2.3. Identification and quantification of isoflavones by UPLC

The identification and quantification of isoflavones were performed according to the methods proposed by Handa et al. (2014), with modifications as described by Falcão et al. (2018). The freeze-dried soybase and okara were deffated with hexane (1:10, w/v) for 1h under constant agitation (300 rpm) in a water bath shaker (Tecnal, Brazil), at 25 °C. The isoflavone extraction was performed in duplicate using a solvent composed of ultrapure water, ethanol, and acetone (1: 1: 1, v/v/v). The samples were filtered using 0.22 µm Millex filters and the compounds were detected at 260 nm by a diode array detector using Acquity UPLC® (Waters, Milford, MA, USA). The quantification of isoflavones was performed using calibration curves and the results are expressed in µmol isoflavones/100 g.

4.2.4. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were used to estimate the statistical parameters. Variables with a level of significance above 95% ($p<0.05$) was considered significant.

4.3. Results and Discussion

4.3.1. Protein content and protein recovery

The results of protein content (g/100ml) in hydrolyzed soybase, control, and standard samples are shown in Table 11. In general, a richer soybase in protein was achieved using 2% of protease, in which 15 min of hydrolysis reaction was enough to result in greater values ($p<0.05$) when compared to the standard sample. By 1% of protease, protein content in soybase was significantly ($p<0.05$) higher than the standard sample after 30 min of hydrolysis. However, the enzymatic hydrolysis by 4% protease did not increase significantly the protein content in relation to the standard sample ($p>0.05$). For the control treatment, without enzyme use, the protein content was significantly ($p<0.05$) higher than the standard sample in samples after 30 min of treatment.

Table 11. Protein content (g/100 ml) of soybase samples obtained by standard procedure, enzymatic hydrolysis by different protease concentrations (1, 2, and 4%), and control experiment.

Treatment	Time (min)	Control	1% Protease	2% Protease	4% Protease
			Protein Content		
Hydrolysis	15	2.65 ± 0.00^{BCb}	2.66 ± 0.00^{Cb}	3.14 ± 0.06^{Aa}	2.80 ± 0.16^{Aab}
	30	2.74 ± 0.01^{BCb}	2.88 ± 0.04^{Bab}	3.19 ± 0.09^{Aa}	2.84 ± 0.14^{Aab}
	60	2.86 ± 0.10^{ABb}	2.92 ± 0.03^{Bb}	3.17 ± 0.00^{Aa}	2.74 ± 0.09^{Ab}
	120	3.01 ± 0.12^{Aa}	3.19 ± 0.10^{Aa}	3.15 ± 0.00^{Aa}	3.04 ± 0.17^{Aa}
Standard		2.61 ± 0.01^{C}	2.61 ± 0.01^{C}	2.61 ± 0.01^{B}	2.61 ± 0.01^{A}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscripts in capital letter are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscripts in lowercase letter are significantly different at $p < 0.05$ using Tukey's test in the same line. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.Standard represents the sample obtained only by filtration of soybean paste.

To verify the efficiency of extraction, protein recovery was calculated (Table 12). The hydrolysis with 2% protease for 15 min was the only treatment sufficient to increase significantly ($p<0.05$) the recovery of proteins in soybase, in relation to standard sample, thus increasing the extraction efficiency.

Disregarding the action of enzyme, it was expected that the hydrolysis conditions used in the current work (alkaline pH and 50 °C) would increase the solubility of soy proteins and, as consequence, protein recovery to soybase control sample. This is because, when the pH of the solution is increased, there is a higher repulsion of charges within the protein due to the increase of charged groups, destabilizing its conformation and inducing the unfolding (Chi, Krishnan, Randolph, & Carpenter, 2003). In addition, soy protein is mainly composed of glycinin and β-conglycinin protein fractions, representing 40% and 30% of the total protein, respectively. The simultaneous presence of them contributes to the high solubility of soy protein in alkaline pHs, as demonstrated by Kuipers et al. (2006). However, temperature and pH conditions did not significantly influence the increase in protein extraction from soybean paste to soybase. This may be related to the fact that the proteins retained in the insoluble fraction contain peptides with a higher content of hydrophobic amino acids. Such

proteins are complexed with the fibrous component, thus hindering interactions with water, decreasing solubility (O'Toole, 1999a; Orts et al., 2019).

Table 12. Protein recovery (%) of soybase samples obtained by standard procedure, enzymatic hydrolysis by different protease concentrations (1, 2, and 4%), and control experiment.

Treatment	Time (min)	Control	1% Protease	2% Protease	4% Protease
		Protein Recovery (%)			
Hydrolysis	15	51.8 ± 3.0^{Ab}	52.2 ± 0.5^{Ab}	69.6 ± 2.5^{Aa}	55.9 ± 1.5^{Ab}
	30	55.3 ± 1.8^{Aa}	55.7 ± 3.5^{Aa}	60.9 ± 0.9^{ABa}	58.4 ± 2.7^{Aa}
	60	55.6 ± 1.4^{Aab}	54.6 ± 1.0^{Ab}	60.0 ± 1.5^{ABa}	53.7 ± 1.3^{Ab}
	120	55.9 ± 1.6^{Aa}	54.0 ± 0.1^{Aa}	60.2 ± 4.9^{ABa}	60.1 ± 4.0^{Aa}
Standard		54.8 ± 1.4^{A}	54.8 ± 1.4^{A}	54.8 ± 1.4^{B}	54.8 ± 1.4^{A}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscripts in capital letter are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscripts in lowercase letter are significantly different at $p < 0.05$ using Tukey's test in the same line. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.Standard represents the sample obtained only by filtration of soybean paste.

Regarding to hydrolyzed samples, with the exception of 2% protease treatment and 15 min, the enzymatic hydrolysis by protease did not influence significantly the protein extraction from soybean paste ($p>0.05$). Different results was achieved in corn protein, using the same enzyme, where the increase in hydrolysis time resulted in an increase in protein recovery (Zheng et al., 2015). The 1% protease was not sufficient to hydrolyze soluble peptides into soybase. The inefficiency of soy protein extraction from hydrolyzed samples by 4% enzyme may be related to the tendency of aggregation of peptides released during hydrolysis by protease, as reported by Fischer et al. (2002), making them insoluble in the soybase. The incidence of peptide aggregates in extruded soy flour hydrolyzed by Protamex was reported by Surówka et al. (2004), corroborating this hypothesis for low protein extraction for 4% enzyme hydrolyzed soybase samples. In addition, okara is known to be rich in cellulose, which represents approximately 50% of the dry weight of soybean (S. Li et al.,

2013). Fischer et al. (2002) indicated a correlation between insoluble cellulose and the formation of non-extractable hydrolyzed proteins in soybean meal. These authors also demonstrated that the aggregation of peptides is favored by the neutral ph, and under conditions of ph > 9, the solubility of the peptides increases due to electrostatic repulsion. So this also may be had contributed to prevent further protein extraction from the insoluble matrix of the soybean paste in this study, since the pH used was about 8.

The SDS-PAGE analysis of soy intact protein and its hydrolysates by 2 and 4% enzyme is shown in Figure 4 and confirm these considerations. The protein subunit bands present in standard sample (soy protein) were the subunits of β-conglycinins (~45-72 kDa), acidic and basic subunits of glycinin (~20-35 kDa). These results are in accordance with that reported by Lamsal et. al (2007) and Meinlschmidt et al (2016), in which the main subunits molecular weight profiles of most of the soy proteins range between 47-72 kDa and 22-33 kDa.

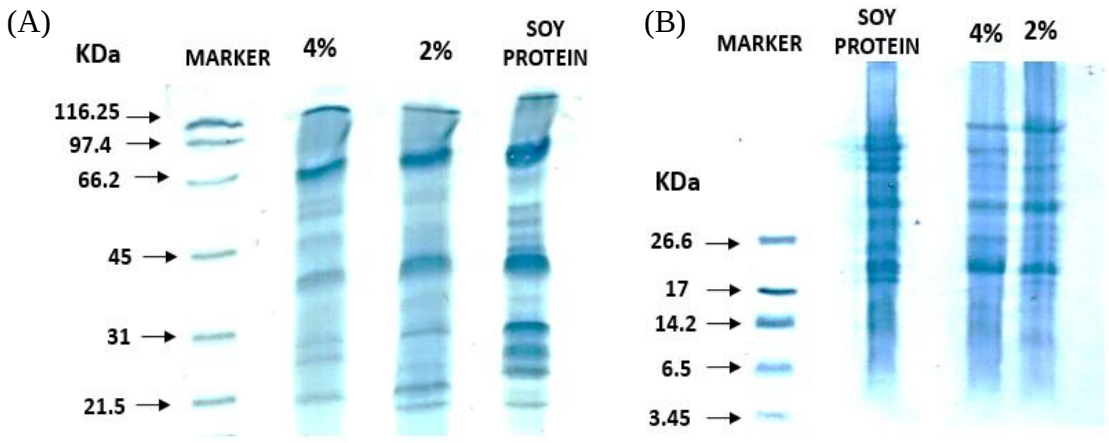

Figure 4.Distribution of high molecular weight (A) and low molecular weight (B) of the standard (soy protein), 4% and 2% hydrolysated soybase samples.

Upon hydrolysis, several bands disappeared accompanied by appearance of new subunits. Since there is a correlation between protein hydrolysis and decrease in the intensity of bands, a significant reduction in molecular weight bands of subunits was observed with the increase of enzyme concentration (from 2 to 4%) of the samples. The significant reduction of soy protein subunit bands suggests that these bands are susceptible to Protamex hydrolysis.

This endopeptidase enzyme could influence the quaternary and tertiary conformations of proteins by cleaving peptide bonds within individual or aggregated proteins to produce smaller peptides and/or smaller protein sub-units. Moreover, the hydrolysates showed new bands at approximately 21 kDa weak bands are also observed.

However, the enzymatic action using 2 and 4% enzyme was not so efficient, because molecular weight profile of the hydrolyzed samples did not show other bands or lighter bands when compared to the standard sample, for the lower molecular weight peptides. As a result, there may not have been a large exposure of hydrophobic groups or an increase in ionizable groups, reducing the possibility of more interactions with water, which may have contributed for low protein extraction in the current work (Tavano, 2013).

4.3.2. Degradation, extraction, recovery and interconversion of isoflavones in soybase

Isoflavones are phenolic compounds that can undergo degradation and/or changes into their different forms due to processing conditions used during treatments. Table 13 shows the mass balance of total isoflavone in soybase and okara in order to verify its degradation during hydrolysis process.

Table 13. Mass balance of total isoflavone concentration in soybase and okara (μmol total isoflavone/100g of soybase + okara).

Treatment	Time (min)	Control	1%	2%	4%
Hydrolysis	15	17.4 ± 4.6^{Ba}	17.0 ± 0.8^{Ba}	11.7 ± 1.8^{Ba}	12.3 ± 0.5^{Ba}
	30	15.6 ± 0.2^{Ba}	15.8 ± 1.4^{BCa}	10.8 ± 0.8^{Bb}	11.6 ± 0.1^{Bb}
	60	13.7 ± 0.3^{Ba}	11.4 ± 0.6^{Cab}	10.3 ± 0.9^{Bab}	10.2 ± 1.3^{BCb}
	120	11.0 ± 0.6^{Bab}	13.1 ± 1.8^{BCa}	8.3 ± 0.4^{Bb}	8.6 ± 0.7^{Cb}
Standard		32.1 ± 0.0^{A}	32.1 ± 0.00^{A}	32.1 ± 0.0^{A}	32.1 ± 0.0^{A}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscripts in capital letter are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscripts in lowercase letter are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

When compared to the standard procedure, the total isoflavone was degraded during control process due to thermal treatment and pH used. Mathias et al (2006) also demonstrated that high temperature (80 and 100°C) and pH (7 and 10) influenced the loss of total isoflavone content, whether it may be degraded or interconverted into other small compounds. Moreover, in the present study, it is found that the 2 and 4% protease concentrations with 120 min hydrolysis time, the higher was the total isoflavone degradation. Due to their polyphenolic nature, isoflavones are believed to associate with the inner portion of the native form of globular soy proteins, and the protein may have a protective effect against isoflavone degradation during processing (Achouri, Boye, & Belanger, 2005; Nufer, Ismail, & Hayes, 2009). Therefore, the enzymatic hydrolysis of protein probably resulted in the disruption of the protein-isoflavone interactions, making them more susceptible to degradation. The significant loss in the total isoflavone content due to the application of an enzyme-assisted extraction with trypsin was previously reported by Nufer et al. (2009), confirming that the processing temperature, pH and interactions with proteins can affect the isoflavone degradation. Wu & Muir (2010) also reported that the pH condition during soy flour hydrolysis by proteases played an important role in the stability of conjugated isoflavones. These studies corroborated our results, where, in general, the isoflavone stability was mainly affected by process parameters when compared with the influence of enzyme.

In all samples, the content of isoflavones was lower compared to the standard sample (Table 14). With the exception of the 120-minute hydrolysis sample by 2% enzyme, there is no influence of time in the isoflavone content ($p>0.05$). The same behavior is observed in relation to the percentage of enzyme, as the samples do not differ significantly in relation to the control sample, without enzyme use ($p>0.05$).

The non-degraded isoflavones recovery in the control soybase obtained was higher than in the standard procedure, due to the thermal and alkaline treatment used (Table 14). On contrary, the enzymatic proteolysis did not influence the recovery of the non-degraded isoflavones, because, in general, there was no significant difference between control samples and enzymatic groups. Although it contributed to the occurrence of degradation, the alkaline pH and thermal treatments contributed to a better extraction of the percentage of isoflavones in the soybean samples, in relation to the standard processing. As previously mentioned, heating and alkaline pH can induce the unfolding of the protein, thus exposing the hydrophobic groups and the isoflavones associated with them, resulting in an increase in the

extraction (Nufer et al., 2009). Orts et al. (2019) demonstrated that the alkaline pH values have a greater effect on the solubilization of total isoflavones. However, with the use of the subtilisin enzyme, they reported a 76% increase in isoflavone extraction, unlike this study in which the Protamex enzyme did not contribute to an increase in this extraction.

Table 14. Total isoflavone content (µmol/100g of soybase) and isoflavone recovery (%) in soybase samples obtained by standard procedure, enzymatic hydrolysis by different protease concentrations (1, 2, and 4%), and control experiment.

Treatment	Time (min)	Control	1% Protease	2% Protease	4% Protease
		Total Isoflavone Content (µmol/100g of soybase)			
Hydrolysis	15	17.3 ± 4.1^{ABa}	15.6 ± 1.3^{Ba}	11.2 ± 1.3^{Ba}	11.0 ± 0.7^{Ba}
	30	14.5 ± 0.6^{Ba}	14.7 ± 1.9^{Ba}	10.8 ± 0.2^{Ba}	11.0 ± 0.3^{Ba}
	60	13.3 ± 0.9^{Ba}	11.7 ± 0.4^{Bab}	10.0 ± 1.0^{BCab}	9.5 ± 1.1^{Bb}
	120	11.0 ± 0.8^{Bab}	12.0 ± 1.4^{Ba}	7.4 ± 0.8^{Cb}	8.6 ± 0.4^{Bab}
Standard		22.9 ± 0.0^{A}	22.9 ± 0.0^{A}	22.9 ± 0.0^{A}	22.9 ± 0.0^{A}
		Isoflavone Recovery (%)			
Hydrolysis	15	60.3 ± 0.6^{Aab}	57.3 ± 2.6^{ABb}	67.6 ± 3.7^{Aa}	56.7 ± 0.1^{Bb}
	30	59.8 ± 3.8^{Aa}	56.8 ± 2.2^{ABCa}	62.5 ± 3.9^{Aa}	61.3 ± 1.9^{ABa}
	60	59.7 ± 1.1^{Aa}	60.4 ± 2.6^{Aa}	58.0 ± 2.0^{ABa}	58.0 ± 0.3^{Ba}
	120	60.7 ± 2.8^{Aa}	49.1 ± 2.6^{BCb}	58.3 ± 2.0^{ABab}	67.2 ± 3.6^{Aa}
Standard		47.5 ± 1.5^{B}	47.5 ± 1.5^{C}	47.5 ± 1.5^{B}	47.5 ± 1.5^{C}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscripts in capital letter are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscripts in lowercase letter are significantly different at $p < 0.05$ using Tukey's test in the same line. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme. Standard represents the sample obtained only by filtration of soybean paste.

Owing to the relationship between the nutritional bioavailability of isoflavones and their different forms, it is important to evaluate if and how isoflavones interconversion occurred during enzymatic treatment. Each form was evaluated by its percentage in relation to the total isoflavones (Table 4). For all samples, there were significant ($p<0.05$) changes in the isoflavone profile in relation to the standard processing. The percentage of malonylglucosides was reduced after enzymatic hydrolysis, which may mean their interconversion to other

isoflavone forms. A strong Pearson correlation of -0.9813 for all treatments was found between the percentages of malonylglucosides and β-glucosides, indicating that there was a de-esterification of malonyglucoside to β-glucoside. Little amount of acetylglucoside form were only found in the standard (2.90%) and control sample with 15 min of treatment (3.91%), being completely converted or degraded, after 30 minutes of heat treatment, and also with any concentration of enzyme studied. Acetylglucoside forms are transient, which can easily be converted into other forms depending on the processing parameters, and are generally found in small proportions in relation to the total isoflavone content (Kao et al., 2004; Mathias et al., 2006; Nufer et al., 2009). Wu & Muir (2010) also reported that protease hydrolysis under high pH conditions, resulted in the complete loss of acetylglucoside conjugates in both the hydrolyzed soybean flour and the residue.

Table 14. Percentages of different isoflavone forms in relation to the total isoflavones presents in soybase samples obtained by standard procedure, enzymatic hydrolysis by different protease concentrations (1, 2, and 4%), and control experiment**.**

Treatment	Time (min)	Enzyme concentration			
		Control	1% Protease	2% Protease	4% Protease
		Malonylglucosides			
Hydrolysis	15	24.0 ± 0.0^{Ba}	18.3 ± 0.1^{Cb}	23.1 ± 1.1^{Ca}	17.9 ± 0.1^{Cb}
	30	23.8 ± 0.1^{Bb}	19.2 ± 0.0^{Bc}	25.8 ± 0.0^{Ba}	17.8 ± 0.1^{Cd}
	60	22.4 ± 0.3^{Cb}	17.0 ± 0.1^{Dd}	23.0 ± 0.0^{Ca}	18.6 ± 0.1^{Bc}
	120	22.7 ± 0.0^{Ca}	16.8 ± 0.1^{Dd}	21.3 ± 0.3^{Cb}	18.0 ± 0.1^{Cc}
Standard		38.7 ± 0.0^{A}	38.7 ± 0.0^{A}	38.7 ± 0.0^{A}	38.7 ± 0.0^{A}
		β-glucosides			
Hydrolysis	15	63.2 ± 0.1^{Bd}	69.0 ± 0.1^{Aa}	65.8 ± 0.7^{Bc}	67.3 ± 0.1^{Db}
	30	66.9 ± 0.1^{Ab}	68.2 ± 0.2^{Aa}	64.4 ± 0.1^{Cc}	68.3 ± 0.1^{Ca}
	60	66.9 ± 0.1^{Ac}	69.1 ± 0.2^{Ab}	66.1 ± 0.0^{Bd}	70.0 ± 0.2^{Aa}
	120	67.2 ± 0.1^{Ac}	68.6 ± 0.3^{Ab}	67.7 ± 0.1^{Ac}	69.3 ± 0.1^{Ba}
Standard		49.7 ± 0.2^{C}	49.7 ± 0.2^{B}	49.7 ± 0.2^{D}	49.7 ± 0.2^{E}
		Aglycones			
Hydrolysis	15	8.9 ± 0.1^{Cd}	12.7 ± 0.2^{Bb}	11.1 ± 0.5^{Ac}	14.7 ± 0.1^{Aa}
	30	9.3 ± 0.2^{BCc}	12.5 ± 0.3^{Bb}	9.8 ± 0.1^{Bc}	13.9 ± 0.0^{Ba}
	60	10.7 ± 0.3^{Ab}	14.0 ± 0.1^{Aa}	10.8 ± 0.0^{Ab}	11.4 ± 0.3^{Db}

	120	10.1 ± 0.0^{ABd}	14.6 ± 0.2^{Aa}	11.0 ± 0.2^{Ac}	12.6 ± 0.0^{Cb}
Standard		8.7 ± 0.1^{C}	8.7 ± 0.1^{C}	8.7 ± 0.1^{C}	8.7 ± 0.1^{E}

Values are expressed as mean ± standard deviation (n=2). Means followed by different superscripts in capital letter are significantly different at $p < 0.05$ using Tukey's test in the same column. Means followed by different superscripts in lowercase letter are significantly different at $p < 0.05$ using Tukey's test in the same line. Standard represents the sample obtained only by filtration of soybean paste. Control represents the sample submitted under enzymatic pretreatment conditions without addition of enzyme.

The percentage of aglycones in relation to the total isoflavones was higher in all samples when compared to the standard sample, indicating that the treatments (control and protein hydrolysis) favored the formation of aglycone. Considering the decrease in the percentage of malonylglucosides, the formation of aglycone during the experiments may have occurred by two ways: (i) direct hydrolysis of malonylglucosides to aglycone by action of the endogenous β-glucosidase or (ii) de-esterification of malonylglucosides to β-glucosides followed by partial hydrolysis to aglycone by β-glucosidase. The optimum temperature and pH of the β-glucosidase are 50 °C and 5.0-7.0, respectively (Baú & Ida, 2015; Sutil et al., 2008). Despite the pH used in our experiments (8.0) was far away from optimum pH of this enzyme, the hydrolysis of conjugated isoflavones to aglycone can be occurred at a slow rate. This can be seen by the low increase in the percentage of aglycone formed. The protein hydrolysis seems to have contributed to increase this rate, mainly in the proportions of 1 and 4% enzyme. The use of enzymatic hydrolysis by Protamex in the conditions evaluated in this work indirectly influenced the interconversion of isoflavones, resulting, in general, in the reduction of malonylglucosides with an increase in β-glycosides and aglycone forms.

4.4. Conclusion

The enzymatic treatment by Protamex endopeptidase during soybean processing to obtain the soybase was evaluated in order to recover proteins and isoflavones that would be retained in the okara insoluble residue. In general, treatments by 1 and 2% of enzyme increased the protein content in the soybase in relation to the sample obtained by standard experiment. In addition, treatment by 2% enzyme and 15 min of hydrolysis showed a significant increase of approximately 15% in the protein recovery, compared to the standard procedure.

In relation to isoflavones, there was degradation due to the pH and temperature conditions used in the treatments, which was also influenced by the amount of enzyme used. The isoflavone recovery was influenced only by pH and temperature parameters. Overall, all treatments resulted in the significant conversion among isoflavone forms, in relation to the standard sample. The protein hydrolysis affected the isoflavone profile, increasing the β-glucosides content in relation to the total amount of isoflavones in the soybase. In conclusion, we evaluated that the enzymatic treatment using 2% of Protamex protease for 15 min resulted in a soybase with higher protein extraction, a higher profile of β-glucosides and aglycone compared to the standard procedure.

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CAPÍTULO 5. DISCUSSÃO GERAL

O extrato hidrossolúvel de soja é a bebida vegetal mais consumida em todo o mundo e a principal substituta de leite de vaca para as pessoas intolerantes ou alérgicas. Durante sua produção é gerada uma grande quantidade de resíduo okara que retém compostos de interesse como proteínas e isoflavonas, as quais não são lixiviadas ao produto principal (O'TOOLE, 1999). Ainda que alguns processos químicos e enzimáticos sejam empregados nesse resíduo para a recuperação de proteínas e isoflavonas, torna-se desejável o emprego de tecnologias durante o processamento do extrato hidrossolúvel de soja, que sejam capazes de aumentar a lixiviação dos compostos de interesse nutricional ao produto principal.

A aplicação do complexo enzimático de carboidrases Viscozyme L® na pasta de soja mostrou-se significativamente ($p<0,05$) eficiente na recuperação de proteínas para todas as concentrações estudadas, sendo que, em geral, quanto maior a concentração de enzima, maior a recuperação de proteínas. Isso se deve à maior degradação dos carboidratos da parede celular, a qual pode ser constatada através do aumento do teor de sólidos solúveis (°Brix). Quanto maior a degradação da parede celular, maior a liberação dos compostos intracelulares, aumentando a extração à matriz líquida. Já a aplicação da protease Protamex® foi capaz de aumentar significativamente ($p<0,05$) a recuperação de proteína na base de soja somente com 2% de enzima e 15 minutos de tratamento. A concentração de 1% de protease demonstrou-se ser insuficiente na solubilização de peptídeos na base de soja, e, inesperadamente, a concentração de 4% não aumentou a recuperação de proteínas. Isso foi atribuído à agregação dos peptídeos de menor peso molecular, com consequente insolubilização na matriz líquida (FISCHER et al., 2002). Além disso, o alto teor de fibras insolúveis presentes no okara pode ter contribuído para a baixa extração, como reportado também por esses autores.

Quanto às isoflavonas, foram avaliados os fenômenos de degradação, extração e interconversão entre as suas formas, bem como a recuperação na base de soja. As condições de pH e temperatura empregados para a atuação do complexo de carboidrases Viscozyme L não influenciaram na degradação do seu conteúdo, diferentemente da protease Protamex. No emprego da Viscozyme L foi utilizado pH de 5,5, enquanto que para Protamex foi usado pH alcalino de 8,0. Os tratamentos térmicos de ambos os experimentos foram realizados com as mesmas temperaturas. Além disso, foi constatado que a hidrólise das proteínas por Protamex também influenciou no aumento da degradação de isoflavonas. Isso pode ter ocorrido devido

à quebra das interações entre proteínas e isoflavonas, tornando-as mais susceptíveis à degradação. Outros autores também reportaram a degradação de isoflavonas devido ao emprego do pH alcalino, confirmando os resultados deste estudo (MATHIAS et al., 2006; ORTS et al., 2019).

Os fenômenos de extração e interconversão para os tratamentos com o complexo de enzimas carboidrases e para a protease também foram investigados. Na extração de isoflavonas, Viscozyme L foi capaz de aumentar a recuperação devido aos parâmetros de processamento empregados, mas a enzima também apresentou contribuição, especialmente nas concentrações de 4 e 6%. Então, quanto mais intensa a hidrólise dos carboidratos da parede celular, maior a liberação de isoflavonas intracelulares, aumentando a capacidade de extração desses compostos para a base de soja. Para as amostras hidrolisadas com a protease Protamex, o aumento da extração de isoflavonas não degradadas não apresentou correlação com o uso da enzima, mas sim somente com o pH alcalino e as temperaturas dos tratamentos térmicos. Ou seja, não houve diferença significativa ($p>0,05$) entre o grupo controle e amostras pré-tratadas enzimaticamente. Isso pode ser atribuído ao desdobramento da proteína da soja no pH alcalino, ocorrendo consequentemente liberação de isoflavonas associadas, aumentando a extração para a base de soja.

Todas as formas de isoflavonas foram encontradas nas amostras de base de soja, nos tratamentos realizados com Viscozyme L. Já para os tratamentos com Protamex, as formas de acetilglicosídeos foram totalmente degradadas ou interconvertidas, devido ao pH alcalino empregado. Quanto às formas de malonilglicosídeos, constata-se que, para ambas as enzimas, houve diminuição do conteúdo devido aos tratamentos enzimáticos. Essas formas são termicamente instáveis e podem ter sido interconvertidas nas formas de β-glicosídeos através de reações de desesterificação, ou em agliconas, através da hidrólise da molécula de açúcar (ORTS et al., 2019).

Entre as formas de isoflavonas, as agliconas têm maior interesse, devido aos seus potenciais benefícios à saúde. Nos experimentos realizados com carboidrases, foi constatado um aumento da porcentagem dessas formas de isoflavonas em relação às demais para todas as condições avaliadas, principalmente com 6% de enzima, a partir de 60 min de hidrólise. Isso foi provavelmente às condições favoráveis (pH 5,5 e temperatura de 50°C) para atuação da enzima endógena β-glucosidase, bem como maior tempo para essa interconversão (BAÚ; IDA, 2015). Nos experimentos realizados com a protease Protamex, também houve aumento

significativo ($p<0,05$) nas agliconas, em relação à amostra padrão. O aumento não foi relacionado à concentração da protease, constatado que não houve uma correlação exata com a quantidade de enzima. Nesse caso, a reação enzimática da β-glucosidase pode ter ocorrido a uma taxa de reação mais lenta, não correlacionada com a proteólise.

CAPÍTULO 6. CONCLUSÃO GERAL

Embora alguns processos químicos e enzimáticos sejam empregados no resíduo okara para a recuperação de proteínas e isoflavonas, torna-se desejável o emprego de tecnologias durante o processamento do extrato hidrossolúvel de soja, que sejam capazes de aumentar a lixiviação dos compostos de interesse nutricional ao produto principal. Essa tese objetivou a aplicação de hidrólise enzimática.

A aplicação de enzimas carboidrases na pasta de soja demonstrou ser capaz de desintegrar a parede celular, liberando mais compostos intracelulares, aumentando assim a recuperação de proteínas e isoflavonas na base de soja. Principalmente, constatou que, com a solubilização das fibras do resíduo, o complexo enzimático de carboidrases foi capaz de aumentar o rendimento do processo. Esses resultados mostram a possibilidade da utilização desse complexo enzimático de carboidrases durante o processamento para a obtenção de base de soja com maior rendimento, menor geração de resíduo okara, maior recuperação de proteínas e isoflavonas no produto principal, bem como maior porcentagem de isoflavonas agliconas.

A aplicação de enzima protease foi capaz de aumentar a solubilidade dos peptídeos somente na concentração de 2% e 15 minutos de tratamento. Além disso, os parâmetros de processamento utilizados para a ação enzimática resultaram em degradação das formas de isoflavonas. Ainda assim, houve uma recuperação significativa de isoflavonas não degradadas, superior ao processo padrão. Então, para uma maior recuperação das proteínas do okara, a aplicação desse tratamento poderia ser avaliada.

Sendo assim, a aplicação de hidrólise enzimática por carboidrases foi evidenciada como uma tecnologia ecologicamente correta que pode ser aplicada industrialmente para melhorar a extração de compostos bioativos do resíduo okara, aumentando o seu conteúdo na base de soja, resultando em um extrato de soja mais rico em proteínas e isoflavonas agliconas.

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ANEXO A. COMPROVANTES DA SUBMISSÃO DO ARTIGO DO CAPÍTULO 2 E PERMISSÃO PARA O USO DO ARTIGO CORRESPONDENTE AO CAPÍTULO 3

1. Submissão do artigo referente ao Capítulo 2

2. Permissão para uso do artigo referente ao Capítulo 3