Ultrasound-assisted Enzymatic Extraction of Protein Hydrolysates from Brewer's Spent Grain

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SCIENTIFIC ABSTRACT

Brewer's spent grain (BSG) is the most abundant by-product of the brewing industry and its main application is limited to low-value cattle feed. Since BSG contains 20 to 25% of proteins, it has the potential to provide a new protein source to the food industry. In this research, an ultrasound-assisted enzymatic extraction was designed to extract protein hydrolysates from BSG. Original BSG and ultrasound pretreated BSG were hydrolyzed under different enzyme (Alcalase) loadings and incubation times. Centrifugation was applied to separate solubilized proteins from insoluble BSG residue. When the enzyme loading increased from 1 to 40 μL /g BSG, the solubilized proteins increased from 34% to 64.8%. The application of ultrasound further increased the solubilized proteins from 64.8% to 69.8%. Solubilized proteins from ultrasound pretreated BSG was significantly higher ($p < 0.05$) than that from the original BSG. Particle size distribution analysis showed that the application of ultrasound pretreatment reduced the BSG particle size from 331.2 to 215.7 μm. Scanning electron microscopy images revealed that the BSG particle surface was partially ruptured by the ultrasound pretreatment. These two phenomena might have contributed to the increased protein separation efficiency with ultrasound pretreatment. The solubility (pH 1.0 to 11.0) of protein hydrolysate increased by the application of ultrasound and the ultrasound did not lead to the change of the amino acid composition of the separated protein hydrolysates. Based on sodium

dodecyl sulfate-polyacrylamide gel electrophoresis profile, the protein was degraded to peptides which had molecular weights lower than 15 kDa. The color of the separated protein hydrolysates by enzymatic hydrolysis was brighter and lighter than the original BSG. The application of ultrasound did not affect the color of the separated protein hydrolysates. Overall, the ultrasound pretreatment prior to enzymatic hydrolysis enhanced the extraction of proteins from BSG in terms of higher protein separation efficiency, lower enzyme loadings, and reduced incubation time. This study developed a novel and green method to effectively extract value-added protein hydrolysates from the low-value food processing byproducts.

Ultrasound-assisted Enzymatic Extraction of Protein Hydrolysates from Brewer's Spent Grain

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PUBLIC ABSTRACT

Brewer's spent grain (BSG) is the most abundant waste generated by beer industries after beer production and it is mainly used to feed cattle. Since BSG contains 20 to 25% proteins, it has the potential to provide a new protein source to food industries. The aim of this research is to study if the ultrasound technology can assist the enzymatic extraction of proteins from BSG. If it can, the cost of the protein extraction from BSG can be reduced. In this research, the original BSG and the BSG pretreated with ultrasound were incubated under different enzyme loadings and incubation times. The protein-rich liquid was separated from fiber-rich solids using a centrifuge. When the enzyme loading increased from 1 to 40 μL /g BSG, 34% to 64.8% of proteins were separated from the original BSG. The application of ultrasound further increased the solubilized proteins from 64.8% to 69.8%. For the BSG pretreated with ultrasound, there were significantly more proteins separated from BSG compared to the original BSG. Particle size of the original BSG and the ultrasound pretreated BSG was measured, and the results showed that the application of ultrasound pretreatment decreased the BSG particle size from 331.2 to 215.7 μm. Scanning electron microscopy images were taken to investigate the effect of ultrasound on the surface of BSG particles. Based on the photos, we found that the BSG particle surface was partially broken by the ultrasound pretreatment. The surface was rough and contained large amounts of holes instead of being flat and smooth observed without ultrasound. Therefore, there were more locations for the enzyme to attack.

These two phenomena might have contributed to extracting more proteins from BSG. Protein solubility (pH 1.0 to 11.0) increased by the application of ultrasound. The nutritional value of the protein extracted was not altered by the ultrasound. The extracted protein hydrolysates had a small molecular weight and the application of ultrasound did not affect the color of the extracted protein hydrolysates. The ultrasound pretreatment prior to enzymatic hydrolysis increased the extraction of proteins from BSG, decreased the enzyme consumption and incubation time. This study developed a novel and green method to effectively extract value-added protein hydrolysates from the low-value food processing byproducts.

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Chapter 1 Introduction

Brewer's spent grain (BSG) is the most abundant by-product generated by the brewing industry, accounting for up to 85% of the total byproducts generated during beer brewing (Reis & Abu-Ghannam, 2014). Approximately 38.6 million tons of BSG were produced worldwide every year, with 2.7 million tons produced in the US (Mussatto, 2014). Although BSG is produced in large amounts every year, it is currently used as low-value cattle feed or disposed in landfills, leading to substantial resource losses. Besides, BSG spoils quickly due to its high content of water and other nutrients (nitrogen and phosphorus), which would result in highly polluted wastewater and other environmental issues. Finally, since the phosphorus content in BSG surpasses the requirement of cattle, feeding BSG to dairy or beef cattle increases phosphorus content of the manure, causing environmental concerns of eutrophication (Knowlton, Radcliffe, Novak, & Emmerson, 2004). Therefore, from economic and environmental perspectives, there is a growing demand to develop novel uses for BSG.

The most valuable component in BSG is protein. Studies have been conducted to separate proteins from BSG using physical, chemical, and enzymatic methods (Kanauchi & Agata, 1997; Kishi, Kimura, Minami, & Kobayashi, 1992; Treimo, Aspmo, Eijsink, & Horn, 2008). Physical separation process combining milling and sieving is simple and cheap, but it is not efficient since the simple milling cannot separate proteins and fibers completely (Kanauchi & Agata, 1997). Chemical separation using alkali usually needs prolonged treatment with concentrated alkaline solutions at elevated temperatures, which would cause denatured and low-quality proteins (Kishi, Kimura, Minami, & Kobayashi, 1992). In recent years, enzymatic extraction has emerged as a mild and more environmentally attractive method to separate proteins from

BSG. In this process, BSG proteins are hydrolyzed by protease to the aqueous soluble peptides, followed by centrifugation or membrane filtration to separate soluble protein hydrolysates from insoluble solids (e.g, fibers) (Treimo, Aspmo, Eijsink, & Horn, 2008). However, the relatively high cost and the required high concentration of food-grade enzymes hinder the commercialization of this process. A way to overcome the limitations of enzymatic separation is to use a chemical or physical pretreatment step to facilitate enzymatic hydrolysis, thus reduce enzyme usage to hydrolyze proteins.

Currently, pretreatments of BSG using chemicals (e.g., acid, alkali) or physicochemical methods (e.g., steam explosion) have been reported by other studies; however, these pretreatments usually involve high-temperature and high-pressure conditions, which may adversely change protein quality or structure (Kemppainen, Rommi, Holopainen, & Kruus, 2016b; Mussatto, Dragone, Fernandes, Milagres, & Roberto, 2008; Xiros, Topakas, Katapodis, & Christakopoulos, 2008b). Compared to harsh thermal and chemical pretreatments, non-thermal pretreatment is essential to improve enzymatic hydrolysis of BSG which preserves the quality of proteins. Ultrasound is considered as an emerging non-thermal and environmentally friendly technology because it is energy saving and free of using chemicals. In recent years, ultrasound-assisted extraction (UAE) has been studied for natural product separation, such as antioxidant from pomegranate peel (Pan, Qu, Ma, Atungulu, & McHugh, 2011), carotenoids from tomato waste (Luengo, Condón-Abanto, Condón, Álvarez, & Raso, 2014), natural dyes from plant materials (Sivakumar, Vijaeeswarri, & Anna, 2011). During pretreatment, ultrasound is propagated via a series of compression and rarefaction waves, which form cavitation bubbles from gas nuclei existing within the fluid. When these cavitation bubbles

grow and violently collapse, it leads to energy accumulations in the hot spot, generating extreme pressures and producing considerable shear energy waves and turbulence. These phenomena help break down large particles and change the surface characteristics of target materials, making materials more accessible to enzymes (Soria & Villamiel, 2010). Several studies reported that ultrasound pretreatment improved protein separation from agricultural products such as rice bran (Phongthai, Lim, & Rawdkuen, 2016), defatted soy flakes (Karki, Lamsal, Jung, van Leeuwen, Pometto, Grewell, et al., 2010) and olive kernel (Roselló-Soto, Barba, Parniakov, Galanakis, Lebovka, Grimi, et al., 2015). To date, to our best knowledge, no study has been reported using ultrasonication to improve the enzymatic hydrolysis of proteins from BSG.

Hence, the objective of the work is to develop an ultrasound-assisted enzymatic method to separate proteins from currently under-utilized BSG. The effects of ultrasound on protein separation efficiency were investigated under different enzyme loadings and incubation times. Solubility, amino acid profile, molecular size, and color of the resulting proteins were also investigated to reveal the process impact on the physicochemical properties of separated proteins. The extracted proteins will be named after protein hydrolysates (PH) with a protein purity of 30 ~ 80%.

Chapter 2 Literature Review

2.1 Beer and BSG

Beer is one of the most popular alcoholic beverages in the world and it can be made from barley, wheat, and sorghum, of which barley is the most commonly used grain for beer making. The overall process of beer production is shown in Figure 1. Raw barley is not appropriate for brewing beer due to several reasons: a) it is too hard to mill; b) it does not contain most of the enzymes for producing fermentable components in wort; c) it contains complicated viscous components that may lead to clarity problems in beer (Bamforth, 2006). Therefore, the raw barley needs to undergo malting prior to brewing and the malted barley is called malt. After milling and mashing, malt will be separated to two parts: liquid and solid. The liquid part is called wort which is fermented to produce beer; and the solid part is known as BSG (Kim, Hwang, Song, Lee, Choi, Lim, et al., 2013). BSG is the major by-product generated by breweries, accounting for up to 85% of the total byproducts generated during beer brewing (Reis & Abu-Ghannam, 2014). BSG is composed mainly of husks, bran, and residues of endosperm of barley. Approximately 38.6 million tons of BSG were produced worldwide every year, with 2.7 million tons produced in the U.S (Mussatto, 2014). Although BSG is produced in a large

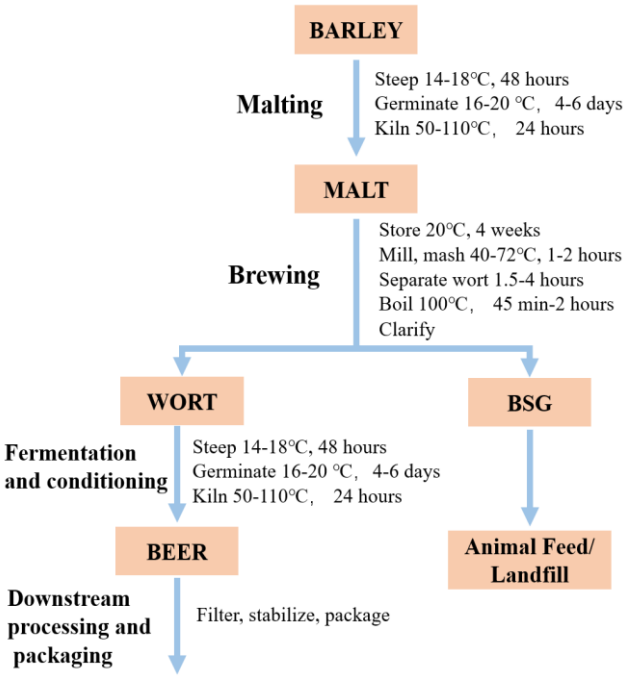

Fig. 1 Overview of malting and brewing.

amount every year, it receives little attention and is mainly used as animal feed (Mussatto & Roberto, 2006) .

2.2 Chemical composition of BSG

BSG has higher protein and fiber contents than the native barleys because starch is removed during the mashing process (S. Mussatto, Dragone, & Roberto, 2006). BSG usually contains 60-70% fiber, 20-25% protein, 5-13% fat and 3-5% ash (Table 1). The chemical composition of BSG differs based on barley varieties, harvest time, malting and mashing conditions and the qualities and types of adjuncts added during brewing process (Xiros, Topakas, Katapodis, & Christakopoulos, 2008a). Chemical composition of BSG reported in the literature was summarized in Table 1.

Table 1. Chemical composition of BSG as reported in the literature.

Components (% dry weight)	Dietary Fiber			Protein	Ash	Lipid
	Cellulose	Hemicellulose	Lignin			
Mussato et al. (2005)	16.8	28.4	27.8	15.3	4.6	-
Reis et al. (2014)	60.5			20.8	3.2	4.5
White et al. (2008)	67.9			22.4	-	-
Xiros et al. (2008)	11.5	40.0	11.9	14.0	3.2	13.0
Forssell et al. (2008)	45.0			21.5	-	9.0
Meneses et al. (2013)	21.7	19.3	19.4	24.7	4.2	-
Ktenioudaki et al. (2013)	60.5			20.8	3.2	4.5

Proteins in barley are mainly located inside the aleuronic cells and also in the starchy endosperm (Forssell, Kontkanen, Schols, Hinz, Eijsink, Treimo, et al., 2008). During malting, barley proteins are partially degraded to small peptides and amino acids (Celus, Brijs, &

Delcour, 2006). According to Celus et al. (2006), approximately 65% of malt proteins were retained in the BSG after processing and the other 35% were hydrolyzed and present in the wort. The protein concentration in BSG is about 20 to 25% depending on different sources. Proteins in BSG can be classified into four categories based on their extractabilities: hordeins (barley prolamins), glutenins, globulins, and albumins. Hordeins, soluble in alcohol, is the main storage proteins and account for 35-55% of proteins in BSG (Celus, Brijs, & Delcour, 2006; Niemi, Martins, Buchert, & Faulds, 2013). Glutenins are the second abundant proteins in BSG, they are soluble in dilute acid or base and insoluble in water or alcohol (Niemi, Martins, Buchert, & Faulds, 2013). Globulins are dilute salt or water soluble. The protein fraction in lowest concentration of BSG is water-soluble albumin which only accounts for 2% of proteins in BSG (Vieira, Rocha, Coelho, Pinho, Saraiva, Ferreira, et al., 2014).

The fiber in BSG is mainly composed of cellulose, hemicellulose and lignin. **Cellulose** is one of the most important structural components of the plant cell walls and it is fibrous, tough and water-insoluble (O'Sullivan, 1997). It is a liner polymer made up of 7000-15000 glucose units linked by β-1,4-glycosidic bonds (Gibson, 2012). The β configuration between two glucoses not only allows the production of linear, unbranched chains but also increases the opportunity for hydrogen bonding to increase the interaction between adjacent glucoses, which leads to the formation of micro-fibrils (Bamforth, 2006). Cellulose is composed of ordered crystalline and disordered amorphous regions (Sun, Xu, Sun, Xiao, & Sun, 2005). It is resistant to hydrolysis by most solvents such as strong alkali. Exoglucanase is used for converting crystalline cellulose to short chain polymer and endoglucanase is used for amorphous cellulose (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). In the cell walls, cellulose presents

in the form of micro-fibrils enclosed by the other two components: hemicellulose and lignin. Cellulose associates with hemicellulose physically and with lignin physically and chemically (Mussatto, Fernandes, Milagres, & Roberto, 2008).

Hemicellulose is a heterogeneous polymer composed of 500-3000 pentose units including xylose, mannose, galactose, rhamnose and arabinose (Gibson, 2012). In contrast to cellulose, hemicellulose has a highly branched random structure and is mainly amorphous (Morán, Alvarez, Cyras, & Vázquez, 2008). It is highly susceptible to dilute acid or base and high temperatures (White, Yohannan, & Walker, 2008). According to Beldman et al. (1989), dilute sulfuric acid hydrolysis at temperature between 90-130 ºC led to nearly complete (89%) hydrolysis of hemicellulose in BSG (Beldman, Hennekam, & Voragen, 1987) which indicated that dilute acid hydrolysis was one of the most efficient methods to hydrolyze hemicellulose to liberate pentose (Mussatto & Roberto, 2006). Hemicellulose binds with pectin to cellulose to form cross-linked fibers network of the cell walls. Arabinoxylan is the main hemicellulose in BSG and it has long linear chains of xylose of which a small amount substituted by arabinose residues (Steinmacher, Honna, Gasparetto, Anibal, & Grossmann, 2012). The main pentose in acid hydrolysates of BSG is arabinose and xylose due to the liberation of arabinoxylans (Reis & Abu-Ghannam, 2014).

Lignin is another important structural component in cell walls and its function is to provide the material cell walls with cohesion and rigidity and to prevent the microbial attack by forming a physicochemical barrier (Mussatto, Fernandes, & Roberto, 2007). It is an aromatic heteropolymer consisting of aromatic units such as guaiacyl, syringyl and phenylpropane (Morán, Alvarez, Cyras, & Vázquez, 2008). Lignin and hemicellulose create a

dense matrix in which cellulose microfibrils are embedded. It supports the cell wall tissues by filling the spaces between cellulose, hemicellulose, and pectin components. The difference between BSG and most of other crop residues is that BSG has higher lignin content (Mussatto & Roberto, 2006).

Lipids are also an important component in BSG and account for 4.5% to 13% of the dry mass of BSG. According to Niemi et al. (2012), triglycerides were the most abundant lipids in BSG (55%) followed by free fatty acids (30%), phospholipids (9.1%) and diglycerides (5.7%). BSG contains more free fatty acids than unmalted barley due to the endogenous lipase activity during malting and mashing. Endogenous lipase activity helps release free fatty acids from triglycerides and phospholipids. BSG is rich in fatty acids- linoleic, palmitic and oleic acids, while other fatty acids also present but only in a small amount such as stearic and linolenic (Niemi, Tamminen, Smeds, Viljanen, Ohra-aho, Holopainen-Mantila, et al., 2012).

Some **minerals** are detected in BSG such as calcium, sodium, potassium, magnesium, aluminum, iron, barium, strontium, manganese, copper, zinc, phosphorus, sulfur, chromium and silicon (Mussatto & Roberto, 2006). Silicon content in BSG is similar to that in cereal straws ashes and BSG has a higher phosphorus and calcium content than cereal straws. The extractives separated from BSG are composed of waxes, fats, gums, starches, resins, tannins, essential oils and some other cytoplasmatic constituents (Mussatto & Roberto, 2006). BSG contains vitamins biotin, choline, folic acid, niacin, pantothenic acid, riboflavin, thiamin and vitamin B_6.

2.3 Current applications of BSG

Because of its high fiber and protein concentrations, BSG now is used as low-value cattle feed or disposed in a landfill, leading to substantial resource losses. In addition, BSG spoils

quickly due to its high content of water and other nutrients (N and P), which would result in highly polluted wastewater or other environmental issues. Finally, since the phosphorus content in BSG exceeds the requirement of cattle, feeding spent grain to dairy or beef cattle increases phosphorus content of the manure, causing environmental concerns of eutrophication which means the lakes change into rich nutrition and high productivity condition. Heavy use of high P byproducts like BSG (aka 'brewer's spent grain') in the dairy and beef industry is being questioned with the widespread adoption of environmental regulations to limit manure P application (Knowlton, Radcliffe, Novak, & Emmerson, 2004). These problems will only be intensified due to the continuous growth of beer production, especially the rapid growth of the small and independent craft beer producers in the U.S. (Association, 2013). Thus, from economic and environmental perspectives, there is an increasing demand and pressure to develop novel uses for BSG.

2.3.1 Direct incorporations of BSG in food manufacturing

Due to its high concentrations in protein and dietary fiber, studies have been conducted to use BSG as a protein and fiber ingredient for human food especially in conventional baked products including bread, cookies, and snacks (Özvural, Vural, Gökbulut, & Özboy - Özbaş, 2009). The addition of BSG powder to baked foods can enrich not only the protein and dietary fiber contents but also the phenolic content in final products (Kissell, Prentice, & Lindsay, 1979; Reis & Abu-Ghannam, 2014). Moreover, the addition of BSG can increase water absorption and mixing time of the dough (Dreese & Hoseney, 1982). Nevertheless, there are limitations of direct incorporation of BSG in food products. For example, adding BSG powder has some negative effects on the overall qualities of foods such as appearance, texture and taste

(Ktenioudaki, Chaurin, Reis, & Gallagher, 2012). The color of breadsticks containing BSG was darker due to the color of BSG, the texture was less crispy, and the loaf volume decreased (Ktenioudaki, Chaurin, Reis, & Gallagher, 2012). Due to these limitations, only a small portion (10%) of the flour can be replaced by BSG powder to avoid obvious off-flavor, off-color and worse texture of the final bakery products.

2.3.2 Feedstock for chemical production

Monosaccharides and xylitol

BSG is rich in cellulose and hemicellulose; thus it has been used as feedstock to produce hexose (glucose) and pentose (xylose and arabinose) by hydrolyzing cellulose and hemicellulose, respectively. (Mussatto & Roberto, 2006). Due to the high concentration of fermentable sugars especially xylose, BSG hydrolysate is a good feedstock to produce xylitol, a rare sugar alcohol in nature (Mussatto & Roberto, 2008). Studies have shown that yeasts *Debaryomyces hansen ii* and *Candida guilliermondii FTI 20 037* were used to convert xylose from BSG to xylitol effectively (Carvalheiro, Duarte, Medeiros, & Gírio, 2007; Mussatto & Roberto, 2005). Xylitol is valuable because it can be used as a sweetener for people with diabetes. Furthermore, it can help treat illnesses such as disorders in lipid metabolism and parenteral and renal lesions and help prevent lung infection, otitis and osteoporosis (Mussatto & Roberto, 2005).

Phenolic acids

Phenolic acids have many benefits to human health (Mandalari, Faulds, Sancho, Saija, Bisignano, LoCurto, et al., 2005). Medically, they can help prevent certain coronary and chronic diseases, lower cholesterol in serum and liver, and increases sperm viability due to their antioxidant and antiradical properties (Meneses, Martins, Teixeira, & Mussatto, 2013).

BSG is an excellent source of phenolic acids, such as ferulic acids and p-coumaric acids. These phenolic compounds are esterified with the arabinose molecules in BSG (Bartolomé, Santos, Jiménez, Del Nozal, & Gómez-Cordovés, 2002). Bound phenolic compounds are approximately five times higher in BSG than in barley due to the concentration effect of malting and mashing processes (Szwajgier, Waśko, Targoński, Niedźwiadek, & Bancarzewska, 2010). Mono- and dimeric ferulic acids can be released from BSG by fungal feruloyl esterases or ferulic acid esterases from *Lactobacillus acidophilus K2* (Faulds, Sancho, & Bartolomé, 2002; Szwajgier, Waśko, Targoński, Niedźwiadek, & Bancarzewska, 2010). Mussatto et al. (2007) reported that 6.6% of ferulic and p-coumaric acids were extracted by alkaline hydrolysis of BSG (Mussatto, Dragone, & Roberto, 2007). Meneses et al. (2013) evaluated the extraction of antioxidant phenolic compounds from BSG by different solvents including pure methanol, ethanol, acetone, hexane, ethyl acetate, water and methanol, ethanol, acetone solution of different concentrations (Meneses, Martins, Teixeira, & Mussatto, 2013). The authors found that a mixture of acetone and water (60% v/v) was most efficient in recovering phenols from BSG. In another study, it was reported that microwave-assisted extraction was five times more efficient than conventional solid-liquid extraction for polyphenol extraction from BSG under the optimal condition (Moreira, Morais, Barros, Delerue-Matos, & Guido, 2012). The optimal condition was 15 min extraction time, 100 ºC extraction temperature, 20 mL solvent (methanol) and maximum stirring speed.

Lactic acid

Lactic acid is not only an important acidulant, flavorant and preservative in foods but also an excellent hydroxycarboxylic acid used in pharmaceutical, textile, leather, chemical,

cosmetic, and polymer industries (Mussatto, Fernandes, Dragone, Mancilha, & Roberto, 2007; Mussatto & Roberto, 2006). Lactic acid is a relatively low-cost product, but pure sugars such as glucose, sucrose and starch, used as carbon sources to produce lactic acid are not cheap (Mussatto, Fernandes, Dragone, Mancilha, & Roberto, 2007; Mussatto & Roberto, 2006). Therefore, it is more economical to find a cheaper carbohydrate source to produce lactic acid. Given the richness of lignocellulose content, BSG is an excellent feedstock to produce lactic acid. Mussatto et al. (2007) used BSG as a raw material to produce lactic acid (Mussatto, Fernandes, Dragone, Mancilha, & Roberto, 2007). Acid pretreatment was first applied to reduce the recalcitrant structure of BSG, followed by saccharification with cellulase to convert cellulose to glucose. After centrifugation, the liquid fraction was used as fermentation medium to produce lactic acid. A lactic acid yield of 0.73 g/g (73% efficiency) was obtained with 1 g cells l^{-1} inoculum level without nutrients supplementation. This result indicated that BSG was a good material to produce lactic acid. Furthermore, if cheap nutritional sources can be supplemented along with the pH being controlled during fermentation, a higher lactic acid yield can possibly be obtained possibly (Mussatto & Roberto, 2006).

Bioethanol

Ethanol is considered to be a substitute or additive to gasoline for transportation (Mussatto, Fernandes, Dragone, Mancilha, & Roberto, 2007). Currently, the production of ethanol mainly uses sugarcane and cereals like wheat and corn by fermentation. As these raw materials are also used as human foods (Rana, Janveja, & Soni, 2013), it leads to a conflict between food and energy production (Rana, Janveja, & Soni, 2013). Industrial residues, such as lignocellulosic material from BSG which does not compete with human foods, can be used as

an alternative feedstock for bioethanol production to avoid these conflicts. Xiros and Christakopoulos (2009) used BSG as a substrate to produce bioethanol (Xiros & Christakopoulos, 2009). Alkali pretreatment was applied to convert cellulose and hemicellulose to a sugar mixture (glucose and xylose), which was fermented to produce ethanol by *Fusarium oxyxporum* under submerged conditions. An ethanol yield of 109 g ethanol per kg of dry BSG was obtained, corresponding to 60% of the theoretical yield based on the total glucose and xylose content of BSG. In another study, White et al. (2008) applied dilute acid pretreatment with HNO_3 and enzymatic digestion with cellulase and hemicellulase to hydrolyze BSG to reducing sugars (White, Yohannan, & Walker, 2008). Fermentation of the hydrolysates was conducted for 48 h by *Pichia stipitis* and *Kluyveromyces marxianus* which produced 8.3 and 5.9 g L^{-1} ethanol corresponding to ethanol conversion yields of 0.32 and 0.23g ethanol (g substrate) $^{-1}$ respectively.

2.4 Other applications and potential applications

BSG is a good substitute for substrates used to develop the fermentation system such as α-amylase induction (Hashemi, Razavi, Shojaosadati, & Mousavi, 2011). By chemical activation of BSG lignin, BSG could be converted to an excellent absorbent which can absorb phenolic compounds and metallic ions (mainly Ni, Fe, Cr, and Si) (Mussatto, Fernandes, Rocha, Órfão, Teixeira, & Roberto, 2010). BSG can also be used to produce bleached pulp for papermaking (Mussatto, Rocha, & Roberto, 2008). According to José, Prinsen, & Gutiérrez (2013), BSG has high potential to be a valuable source of phytochemicals used in pharmaceutical, cosmetic, food or other industries due to its high content of lipids (José, Prinsen, & Gutiérrez, 2013).

2.5 Protein separation from BSG

Protein is one of the most valuable components in BSG. Studies have been conducted to separate proteins from BSG using physical, chemical, and enzymatic methods (Table 2). Early in 1977, Kanauchi et al. applied a physical method combining milling and sieving to separate proteins from BSG (Kanauchi & Agata, 1997). This physical separation process is simple and cheap, but it is not efficient since the simple milling cannot separate proteins and fibers completely. The proteins separated are usually not qualified as food ingredients due to other impurities, such as fiber and ashes.

Table 2. Current existing methods to separate proteins from BSG.

Methods	Description	Advantages	Limitations	Reference
Physical Separation	Milling and sieving to separate proteins from fibers	• Simple • Cheap	• Low protein Separation efficiency	Kanauchi & Agata, 1997
Chemical Separation	Using alkaline solution to dissolve proteins	• Alkaline is relatively cheap • Easy to be scaled up	• Low-quality proteins • Large amounts of inorganic salts generated as waste	Kishi, Kimura, Minami, & Kobayashi, 1992
Enzymatic Separation	Using protease to hydrolyze proteins to soluble peptides	• Mild treatment, resulting in high-quality proteins • Environmentally friendly	• High concentrations of enzyme required • High cost of enzymes	Treimo, Aspmo, Eijsink, & Horn, 2008

Wet separation methods using different chemicals have also been developed (Kishi, Kimura, Minami, & Kobayashi, 1992). Alkaline extraction using sodium hydroxide is a traditional method to separate proteins from plant materials, especially from soybean meals (Ortiz & Wagner, 2002). In 2007, Celus et al. used alkaline extraction to prepare BSG protein concentrate (BPC) from BSG. Approximately 41% of the proteins in BSG were present in BPC by alkaline extraction of BSG (17% w/v) with 0.1 M NaOH at 60 ºC followed by acid precipitation (Celus, Brijs, & Delcour, 2007). This method has been tested but with limited

success. Since proteins and fibers are tightly interconnected in BSG, protein separation using alkaline solution usually needs prolonged treatment with concentrated alkaline solutions at elevated temperatures, which usually cause denatured and low-quality proteins and formation of high quantities of inorganic salts as waste (Kishi, Kimura, Minami, & Kobayashi, 1992).

Recent years, enzymatic extraction has emerged as a milder and more environmentally attractive method to separate proteins from BSG. In this process, BSG proteins are hydrolyzed by protease to aqueous soluble peptide, followed by centrifugation or membrane filtration. It is conducted under moderate temperature and pH which can protect valuable components from being damaged. Celus et al. used three commercially available proteases (Alcalase, Favourzyme, and pepsin) to obtain protein concentrates from BSG and evaluated the techno functional properties of the resulting protein concentrates (Celus, Brijs, & Delcour, 2007). Treimo et al. (2008) used and compared different commercial enzymes (Alcalase 2.4 L, Neutrase 0.8L, and Protamex, etc.) to hydrolyze and then separate proteins from BSG (Treimo, Aspmo, Eijsink, & Horn, 2008). The results showed that Alcalase 2.4 L was the most effective protease for solubilization of BSG proteins. Alcalase solubilized 77% of proteins in BSG under its optimal condition of pH 8.0, reaction temperature of 60 ^{o}C, 3.33% w/v dry BSG concentration, and reaction time of 4 hours. Since the enzymatic hydrolysis is mild and does not damage amino acids, the separated protein hydrolysates are of high quality.

In BSG, most proteins are found inside the aleuronic cells and the starch endosperm fragments. The cell wall barrier formed by lignin, cellulose and hemicellulose restricts the contact between protein substrates and proteases. Breaking down the cell wall first can

facilitate enzymatic hydrolysis. Treimo et al. (2009) used a combination of carbohydrases and peptidases to extract proteins from BSG, with a hypothesis that carbohydrases can disrupt the cell wall barrier to increase protein solubility (Treimo, Westereng, Horn, Forssell, Robertson, Faulds, et al., 2009). In this study, Alcalase alone solubilized 77% of proteins in BSG (reaction condition: pH 8.0, 4 h reaction time, 60 ºC reaction temperature, 3.33% w/v dry BSG concentration, and 20 μL enzyme/g DM of BSG). When BSG was hydrolyzed first by Depol 740 following by Alcalase, the solubility of protein was increased to 87% (reaction time was 2 ×4 hours with two enzyme preparations). However, the relatively high cost and the required high concentration of enzymes (20 - 40 μL/g BSG) hinder the commercialization of this process.

2.6 Ultrasound-assisted protein separation from agricultural products

A way to overcome the limitations of enzymatic separation is to use a chemical or physical pretreatment step to facilitate enzymatic hydrolysis instead of enzymatic pretreatment, thus reducing enzyme dosages. Pretreatments that decrease particle size and reduce recalcitrance of biomass structure improve enzymatic digestibility by making the material more accessible to enzymes (Mosier, Wyman, Dale, Elander, Lee, Holtzapple, et al., 2005). This approach (using pretreatments) has been widely used to improve carbohydrate digestions of different biomaterials (Hendriks & Zeeman, 2009; Mosier, et al., 2005). Currently, most pretreatments of BSG use chemicals such as acid, alkali, or physicochemical methods, such as steam explosion and hot water pretreatment, as reviewed by Mussatto et al. (Mussatto & Roberto, 2006). These pretreatments usually involve high temperature and high-pressure conditions, which may adversely change protein quality or structure. For example, steam explosion was studied as a means to improve the enzymatic digestibility of BSG (Kemppainen, Rommi, Holopainen, & Kruus, 2016a). The BSG was held at 180 ºC, 10 bar for 5 min before instant

pressure release for biomass explosion. This high-temperature and high-pressure process caused partial protein degradation and the insoluble protein appeared to become more strongly associated with fiber. Compared to harsh thermal and chemical pretreatments, non-thermal pretreatment is essential to improve enzymatic hydrolysis of BSG while preserves the high quality of proteins.

Ultrasound pretreatment is considered an emerging non-thermal and environmentally friendly technology because it is relatively cheap, energy saving, and free of using chemicals (Chemat & Khan, 2011). Ultrasound is sound waves having a frequency that exceeds the hearing limit of the human ear (~20 kHz). Based on the frequency range, ultrasound can be divided into two categories: low-intensity ultrasound (LIU) and high-intensity ultrasound (HIU). LIU has frequencies higher than 100 kHz but intensities lower than 10 W/cm^2, which can be used for non-invasive food analysis, such as composition analysis of fish and meats (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Ghaedian, Coupland, Decker, & McClements, 1998; Griffin, Savell, Recio, Garrett, & Cross, 1999; Tait, 2016). HIU, on the other hand, has low frequencies between 20 and 500 kHz but intensities higher than 10 W/cm^2, which are disruptive and induce effects on the physical, mechanical, and biochemical properties of foods. It was also called power ultrasound (Betts, Williams, & Oakley, 1999). During HIU pretreatment, ultrasound is propagated via a series of compression and rarefaction waves, which form cavitation bubbles from gas nuclei existing within the fluid. When these cavitation bubbles grow and violently collapse, it leads to energy accumulations in hot spot, generating extreme pressures and producing very high shear energy waves and turbulence (Soria & Villamiel, 2010). This large shear energy and turbulence can break down large

particles and change the surface characteristics of target materials, making samples more accessible to enzymes.

In recent years, ultrasound-assisted extraction has been studied for natural product separation, such as antioxidant from pomegranate peel, carotenoids from tomato waste, natural dyes from flowers (Luengo, Condón-Abanto, Condón, Álvarez, & Raso, 2014; Pan, Qu, Ma, Atungulu, & McHugh, 2011; Sivakumar, Vijaeeswarri, & Anna, 2011). The results of these studies show that ultrasound-assisted extraction has significant advantages over conventional maceration and Soxhlet extraction methods in terms of increased extraction efficiency, reduced extraction time, and less solvent consumption. These advantages are mainly attributed to the combined mechanisms of ultrasound treatment: fragmentation, erosion, local shear stress and destruction-detexturation (Chemat & Khan, 2011).

Several studies reported that ultrasound pretreatment improved protein separation from agricultural products. Karki et al. (2010) reported using HIU pretreatment to enhance protein separation from soybean meal (Karki, et al., 2010). The authors found that HIU treatment at a high amplitude gave the increase in protein yield by 46% compared with non-treated samples, which is mainly attributed to the fragmentation effect of ultrasound treatment. Karki et al. (2009) also studied the effect of ultrasound pretreatment on the soy protein isolate (SPI) extraction yield from defatted soy flakes and the functionality of the produced SPI was determined (Karki, Lamsal, Grewell, Pometto, Leeuwen, Khanal, et al., 2009). The solubility of the resulting SPI was increased by ultrasound pretreatment while the emulsification and foaming capacities were decreased. Zhu et al. (2009) studied the application of ultrasound-assisted procedure for the separation of proteins from defatted wheat germ by reverse micelles

(Zhu, Sun, & Zhou, 2009). Under the optimum ultrasound treatment conditions, the separation efficiency of proteins can reach 57%, much higher than the traditional alkaline extraction method. In another study, pilot-scale ultrasound trials were conducted to separate proteins from soybean meal (Moulton & Wang, 1982). The continuous HIU application separated 54% and 23% more protein for aqueous and alkali extraction, respectively. All these studied pointed out that ultrasound pretreatment has a positive effect on protein separation. However, to our best knowledge, there are few published studies on the ultrasound-assisted separation of protein from BSG (TaNg, TiaN, He, Li, Hu, & Li, 2010), and no studies on using the combination of ultrasound pretreatment and enzymatic hydrolysis to improve protein separation from BSG. Moreover, the effects of ultrasonic treatment on the properties of BSG proteins are unknown. Therefore, the physicochemical properties of resulting proteins need to be evaluated. If the proteins in BSG can be extracted effectively without damaged properties by ultrasound-assisted enzymatic extraction, the cost will be decreased, and it will upgrade the value of low-value BSG and promote the sustainable development.

Chapter 3 Long-term goal, Hypothesis and Objectives

The **long-term goal** of this project is to separate high-value proteins from currently under-utilized BSG through the fractionation process. The **hypothesis** of this study is that protein separation from BSG can be promoted by applying ultrasound pretreatment along with enzymatic hydrolysis. Ultrasound will not only promote the protein separation from BSG but also contribute to improved physicochemical properties of extracted proteins as a food ingredient. The **objectives** of this study are as follows:

Objective 1: To establish an ultrasound-assisted enzymatic extraction method to extract protein from BSG.

Objective 2: To determine the physicochemical of the resulting protein hydrolysates (PH), including solubility, amino acid composition, molecular weight and color.

Chapter 4 Materials & Methods

4.1 Materials & Reagents

BSG was obtained from Devils Backbone Brewing Company and stored at -20 ºC until use. The frozen BSG sample was dried at 60 ºC in a convection oven until it reached a constant weight. The dried sample was then milled using a hammer mill equipped with a No. 20 sieve (0.841mm). The milled BSG was stored inside a sealed plastic bag at 4 ºC until use.

Alcalase 2.4 L, a protease derived from Bacillus licheniformis, was kindly provided by Novozymes Inc. (Franklinton, NC). The optimum pH for this enzyme is 8.0, and the optimum temperature is 60 ºC enzymatic activity of Alcalase 2.4 L is 2.40 AU/g according to the product specification sheet (Alcalase® Food Grade Product Sheet, Novozymes). Other chemicals, such as sodium hydroxide and hydrogen chloride, were purchased from Fisher Scientific Company (Pittsburgh, PA).

4.2 BSG composition analysis

The moisture content was determined by oven drying method at 135 ºC for 2 hours according to the AOAC 930.15 (AOAC, 2005a). The ash content was determined by weight difference before and after incineration in a muffle furnace at 550 ºC for 12 hours according to the AOAC 942.05 (AOAC, 2005b). The crude protein content was determined by the total nitrogen content by the Kjeldahl method, followed by multiplying a factor 6.25 (AOAC, 2005d). The crude fat content was determined by AOAC 2003.05 (AOAC, 2005c). Briefly, the BSG sample was extracted by petroleum ether for 4 hours using the Soxtec extractor (2055 Soxtec, Foss, Sweden). And the crude fat was determined by weight difference before and after

extraction. The neutral detergent fiber content was measured by ANKOM Filter Bag System (ANKOM 2000 Automated Fiber Analyzer, Macedon NY).

4.3 Ultrasound pretreatment

Two grams of dried BSG sample was dispersed in deionized water (5% w/v) in a 50-mL bottle. The suspension was sonicated by an ultrasound processor (Model 505, Fisher Scientific Inc., Waltham, MA) equipped with a 3.0-mm-diameter ultrasound probe which was placed 1 cm below the liquid surface. Power output (amplitude 40%) and treatment time (10 min) was applied with a pulse duration of 5s on and 3s off. The bottle was placed in an ice water bath to maintain the temperature. The untreated BSG was also dispersed in deionized water (5% w/v) and set aside for 10 min.

During the ultrasonic treatment, part of the ultrasonic energy is lost in the form of heat when the ultrasound penetrated the samples (Niemi, Martins, Buchert, & Faulds, 2013). During treatment, the temperature was measured by a thermometer to determine the temperature changes. Based on the temperature changes, the acoustic power applied to the BSG suspension was estimated using the following equation:

$$\mathrm{P} = \mathrm{m}C_P\left(\frac{dT}{dt}\right)_{t=0} \tag{1}$$

where m is the mass of the sonicated liquid (g); C_p is the specific heat of the BSG solution at a constant pressure, which is dependent upon the composition and the volume of the solution ($\mathrm{J} \cdot K^{-1} \cdot g^{-1}$); and $\frac{dT}{dt}$ is the slope at the origin of the curve of temperature against time (Jiang, Wang, Li, Wang, Liang, Wang, et al., 2014). The ultrasonic intensity (UI, expressed in $\mathrm{W} \cdot \mathrm{cm}^{-2}$) was determined using the following equation:

$$\mathrm{UI} = \frac{4P}{\pi D^2} \tag{2}$$

where P is the ultrasonic power (W) and D is the probe diameter (cm).

4.4 Enzymatic hydrolysis

Two control experiments (control 1 and control 2) were conducted independently. For the two control experiments, there were no ultrasound or enzyme treatments to extract proteins. For the control 1, the pH was not adjusted. For the control 2, the pH was adjusted to 8.0 which was the optimal pH of Alcalase. The incubation time was set at 4 hours, before protein separation.

For the enzymatic hydrolysis, the original BSG and ultrasound-pretreated BSG were subjected to enzymatic hydrolysis at different incubation times and enzyme loadings. The pH of the pretreated BSG sample was adjusted to 8.0 by using 5 M NaOH or 2.5 M HCl solutions, followed by adding the calculated amount of Alcalase 2.4 L. A pH of 8.0 was selected because Alcalase has the best performance based on the enzyme product sheet. The sample was incubated in a water bath (60 °C) with shaking at 110 rpm (939XL, Amerex Instrument, Inc., Concord, CA). After the designed incubation time, the reaction was then stopped by centrifugation at 13751 × g for 10 min in a pre-cooled (4 °C) centrifuge (5804R, Eppendorf Inc., Hauppauge, NY). The supernatant, rich in hydrolyzed proteins, was carefully collected with pipettes. The solid residue was washed twice with 25 mL of deionized water and the resulting supernatant was collected and combined with the previously collected supernatant. Both the supernatant and residue were collected in the pre-weighed boats and dried in a convection oven (at 60 °C) until they reached constant weights. After drying, the weights of supernatant and residue was measured respectively to calculate the percentage of solubilized solids. The crude protein content in supernatant and residue was analyzed by the Kjeldahl

method and converted to protein content by multiplying a factor of 6.25 (Treimo, et al., 2009). To investigate the effect of ultrasound pretreatment on the following enzymatic protein separation, both ultrasound pretreated and original BSG were tested under different enzyme concentrations and incubation times. Two series of experiments were designed here:

1). With fixed enzyme loading at 10.0 μL Alcalase/g dry mass BSG, incubation time was 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 24.0 hours.

2). With fixed incubation time at 4 hours, enzyme loading was 1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 μL Alcalase/g dry mass BSG; The incubation times and enzyme loadings were selected based on the previous literature (Treimo, Aspmo, Eijsink, & Horn, 2008).

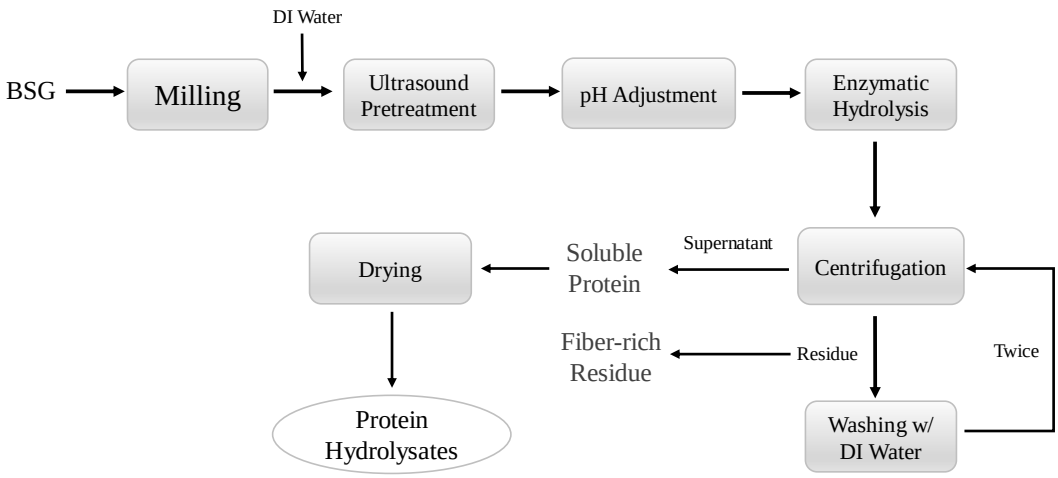

Fig. 2 The scheme of ultrasound-assisted enzymatic extraction of proteins from BSG.

4.5 Separation efficiency determination

Separation efficiency was evaluated by both the percentage of solubilized solids and solubilized proteins. The percentage of solubilized solids was calculated using the following equation:

$$\text{Solubilized Solids (\%)} = \frac{W_{supernatant}}{W_{solids} + W_{supernatant}} \times 100 \quad (3)$$

where W_{solids} is the mass of dried solids and $W_{supernatant}$ is the mass of dried supernatant. The percentage of solubilized proteins was calculated by the following equation:

$$\text{Solubilized Proteins (\%)} = \frac{W_{P(supernatant)}}{W_{P(solids)}+W_{P(supernatant)}} \times 100 \quad (4)$$

where $W_{P(solids)}$ is the mass of the protein in dried solids and $W_{P(supernatant)}$ is the mass of the proteins in the dried supernatant.

4.6 Particle size and surface morphology analysis

To investigate the effect of ultrasound pretreatment on the particle size of BSG, the particle size distribution of the original BSG sample and ultrasound pretreated BSG was determined by a Laser Diffraction Particle Size Analyzer (LA-950, HORIBA, Ltd., Texas). Two grams of dried BSG sample was dispersed in deionized water (5% w/v) and was sonicated by an ultrasound processor to get the ultrasound pretreated BSG. For comparison, untreated BSG samples was also dispersed in deionized water. The prepared sample suspension was then diluted by the auto-dilution system of the particle size analyzer for another time to reach the appropriate concentration for laser scanning.

The surface morphology and microstructure of the ultrasound pretreated and original BSG were investigated using the scanning electron microscope (SEM). Ultrasound pretreated BSG was freeze-dried, fixed on the silicon wafer first, and sputtered with iridium to get a thickness of 15 nm. For a comparison purpose, the original BSG was also fixed on silicon wafer and sputtered. The prepared samples were analyzed by SEM (LEO 1550 Field-emission Scanning Electron Microscopy, Zeiss, Germany) for the surface morphology and microstructure changes under magnifications of 150X and 500X.

4.7 Physicochemical properties determination

4.7.1 Protein solubility as a function of pH

Protein solubility was determined by the method described by Celus et al. with some modifications (Celus, Brijs, & Delcour, 2007). Briefly, 100 mg lyophilized protein hydrolysates were dispersed in 10.0 mL deionized water followed by the adjustment of pH to 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 using either 0.1 M HCl or 0.1 M NaOH solution. The suspensions were shaken for 30 min at room temperature followed by centrifugation at 13751 × g for 10 min. Protein concentration was quantified in the supernatant with the biuret method as described by Morr et al. with some modifications (Morr, et al., 1985). One milliliter of protein solution was mixed with 4 ml of Biuret reagent in a test tube. The test tube was vortexed and left at room temperature for 20 min to develop color. The absorbance was measured by a spectrophotometer (Genesys 10-S, Thermo Electron Corporation, Madison, WI) at 540 nm. The protein content was calculated based on the standard curve developed with Bovine serum albumin (BSA). Protein solubility was calculated as the percentage of the soluble protein content in supernatant over the total protein in the protein hydrolysates using the following equation:

$$\text{Protein Solubility (\%)} = \frac{PC_s}{PC_c} \times 100 \qquad (5)$$

where PC_s is the protein content solubilized in the supernatant and PC_c is the protein content of the protein hydrolysates.

4.7.2 Amino acid composition analysis

Amino acid analyses were performed on both the original BSG and protein hydrolysates according to the standard AOAC official method 982.30 E (a, b) (AOAC, 2006). In brief,

sample was hydrolyzed by 10 mL 6M HCl and filtered. The filtered hydrolysate was dried at 65 ºC under vacuum and dissolved in buffer until analyzed in amino acid analyzer. This hydrolysate was used to determine all amino acids except methionine, cystine and/or cysteine, and tryptophan by chromatograph (HPLC). To determine the methionine and cystine/cysteine, sample was soaked in 2 mL cold performic acid and then mixed with 3 mL cold HBr and 0.04 mL 1-octanol (antifoam) in an ice-water bath following by drying at 40 ºC under vacuum. Afterwards, the sample was hydrolyzed in 10 mL 6 M HCl and undergo the above-mentioned acid hydrolysis. And the hydrolysates were send to HPLC for quantification.

4.7.3 Molecular weight determination

BSG protein-enriched isolates (BSG-PI) was prepared by alkaline extraction as described by Tang et al. with some modifications (Tang, Yin, He, Hu, Li, Li, et al., 2009). Milled BSG was dispersed in deionized water (5% w/v) and the pH was adjusted to 12.0 by using 5.0 M NaOH. After a 60-min incubation, the suspension was centrifuged at 13751 × g at 4 ºC for 10 min. The supernatant was collected and then acidified to pH 4.0 by 2.0 M citric acid to precipitate proteins. The acquired protein precipitates were lyophilized to get BSG-PI.

Lyophilized protein hydrolysates and BSG-PI suspensions were respectively prepared by mixing them with deionized water at a solids concentration of 4% (w/v). Then, 90 μL of the sample solution was mixed with 10 μL B-mercaptoethanol and 60 μL Laemmli solution, and boiled for 3 min. After cooling, the prepared samples and the protein standards (molecular weight ranged from 10 kDa to 250 kDa) were loaded onto Mini-Protein® TGX™ Precast Gel with 4% stacking gel and 4-20% separating gel (Bio-Rad Laboratories, Inc., California, USA). The running currency was 25 mA, constant current and the running time was approximately 57

min to 60 min. Afterward, the gel was stained by using 0.02% Brilliant Blue R-250, 50% methanol, 7.5% acetic acid and destained by destain solution (5% methanol, 7.5% acetic aicd) until no apparent blue color on the gel. A standard curve was conducted based on the protein standards. Then, the migration of the band from the gel baseline was measured to calculate the RF values, which were used to estimate the molecular weight of unknown proteins.

4.7.4 Color measurement

The color of the BSG-PI and protein hydrolysates was determined by a Minolta Chrome Color Meter (Model CR-300, Minolta Camera Co., Ltd., Osaka Japan) with the port size of 8 mm, equipped with a pulsed xenon lamp and the diffuse illumination/0° viewing geometry system. The color measurement values were calculated based on the CIE standard illuminant C (CIE L*a*b* values will be measured to compare the lightness, redness, and yellowness between samples). A white plate was used for calibration. The total color difference between the resulting products and the original BSG was calculated by the following formula (Celus, Brijs, & Delcour, 2007):

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5} \quad (6)$$

4.8 Statistical analysis

All hydrolysis reactions and ultrasound-assisted hydrolysis reactions, as well as analytical methods, were performed in duplicates unless otherwise mentioned. The results presented were the mean values of duplicates (triplicates for particle size analysis and ultrasound intensity determination), and the standard deviations were shown as error bars in figures. Two-way ANOVA statistical analysis was conducted using JMP (13.0.0, SAS Institute Inc, NC) to test

whether the treatments had a significant effect on the protein separation efficiency. One-way ANOVA analysis for the color measurement results was also conducted using JMP. Statistical significance was accepted at a level of $p < 0.05$.

Chapter 5 Results & Discussion

5.1 Chemical composition of BSG

After oven drying at 60 ºC and being milled, the BSG powder had a moisture content of 2.17 ± 0.19%. The BSG powder contained 23.4 ± 0.2% protein, 51.0% ± 0.7 neutral detergent fiber (NDF), 9.4 ± 0.1% fat, and 4.1 ± 0.1% ash, on a dry matter basis (Table 3). The BSG chemical composition was compared with the results reported by other studies (Table 3). The levels of protein, fat and ash were similar to the ones reported by other studies, but the level of NDF in our BSG was higher compared with other studies. The difference could be due to different barley varieties, harvest time, malting and mashing conditions during beer production, and the adjuncts added during brewing process (Hardwick, 1994; Santos, Jiménez, Bartolomé, Gómez-Cordovés, & del Nozal, 2003).

Table 3. Chemical composition of BSG.

Component (% dry wt)	BSG[1]	BSG[2]	BSG[3]	BSG[4]
Protein	23.4 ± 0.2	23.4	22.6	22.8
NDF	51.0 ± 0.7	45.9	43.5	42.2
Fat	9.4 ± 0.1	nd	11.4	11.0
Ash	4.1 ± 0.1	nd	4.1	4.7

[1] BSG used in this experiment. Data represent the average value of 2 replications (n = 2).

[2] BSG from Treimo et al. (2009).

[3] BSG from Kemppainen et al. (2016).

[4] BSG from Niemi et al. (2013). nd, not determined.

5.2 Ultrasound intensity determination

The temperature increase of the BSG suspension was monitored by a thermometer during the ultrasound pretreatment. The temperature increased linearly with increased sonication time (Figure 3). After 10 minutes of the ultrasound pretreatment, the temperature increased by 32.7

± 0.3 ºC. Based on the temperature change, the acoustic power applied to the BSG suspension was estimated using the equation (1) and (2). The calculated ultrasound intensity was 128.6 $W \cdot cm^{-2}$ which was in the range of high intensity sonication (10 – 1000 $W \cdot cm^{-2}$) (Betts, Williams, & Oakley, 1999). Without the ice-water bath, the temperature of the BSG suspension increased to around 56 ºC which was still lower than the following enzymatic incubation temperature of 60 ºC. Therefore, the ultrasound pretreatment did not lead to a high temperature which may cause the potential heat damage of the extracted proteins.

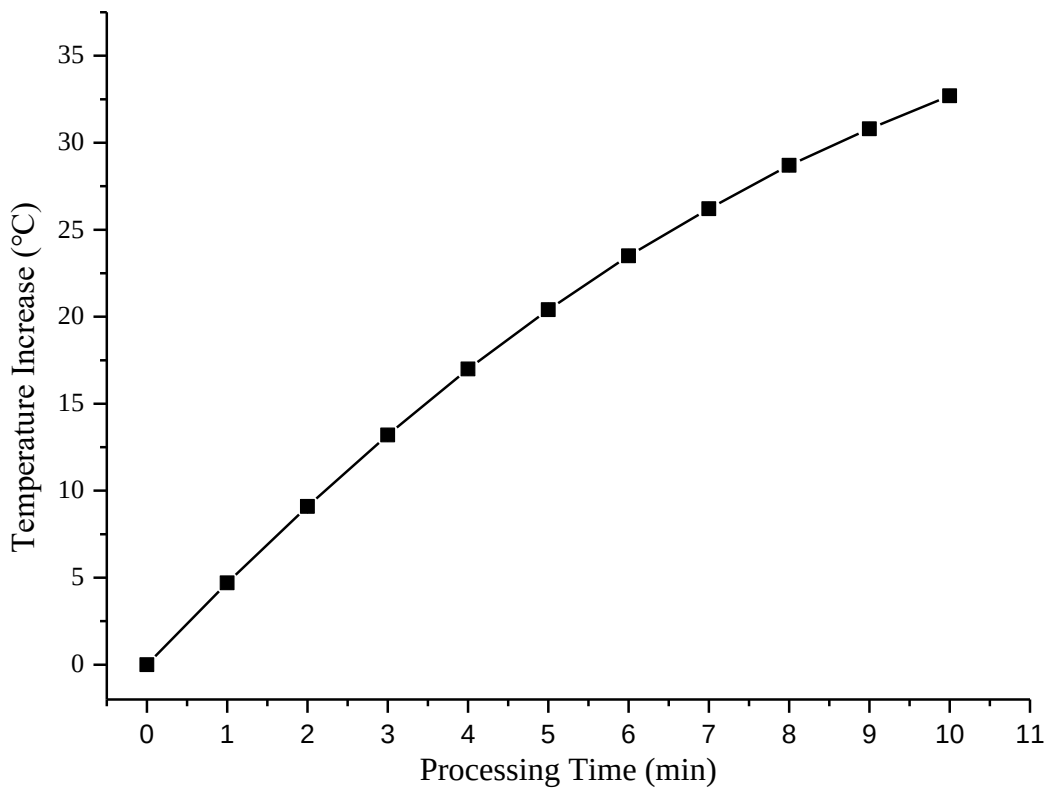

Fig. 3 Temperature increase of the BSG sample suspension with increased ultrasound processing time. Data represent the average ± standard deviation (SD) for 3 replications (n = 3).

5.3 Protein separation efficiency

Protein separation efficiency was determined by solubilized solids (%) and solubilized proteins (%) under different enzyme loadings and incubation times, with and without ultrasound pretreatment. Two control experiments were conducted independently. The results were summarized in Table 4 only 8.8 % of BSG solids and 3.0% of BSG proteins were solubilized for the control experiment. By adjusting the pH to 8.0, the solubilized solids (%) was increased to 9.3% and the solubilized proteins (%) was increased to 3.3%. The low solubilized proteins in the control experiments indicated that simply physical methods (i.e., milling and centrifugation) were not able to effectively separate proteins from BSG.

Table 4. Protein separation efficiency of control groups. Data represent the average ± standard deviation (SD) for 2 replications (n = 2).

Group	Solubilized Proteins (%)	Solubilized Solids (%)
Control 1 (no pH adjustment)	3.0 ± 1.1	8.8 ± 0.4
Control 2 (pH adjusted to 8.0)	3.3 ± 0.9	9.3 ± 0.4

The effect of enzymatic incubation time on the protein solubilization of original and ultrasound pretreated BSG samples were investigated (Figure 4). The enzyme loading was 10 μL /g BSG for all treatments. The results showed that solubilized proteins from the original BSG increased consistently as the incubation time increased from 0.25 to 8 hours. When the incubation time further increased to 24 h, no increase of solubilized proteins was observed. For the ultrasound pretreated BSG, the solubilized proteins reached the plateau at the incubation time of 4 hours; and no further increase was observed when the incubation time increased to 8 and 24 hours. This result indicates that ultrasound pretreatment can decrease the enzymatic

incubation time, which can potentially reduce the size of the reactor and the energy consumption for the incubation process. Based on the statistical analysis shown in Table 5, the application of ultrasound and the change of the incubation times had the significant effect on the protein separation efficiency ($p < 0.0001$). As the incubation time increased from 0.25 to 24 h, the solubilized solids (%) increased from 15.1% to 24.5% (Figure 6). The application of the ultrasound increased the solubilized solids to 26.8% at 24 h. The application of ultrasound had a significant effect on increasing the solubilized solids (%) (Table 6).

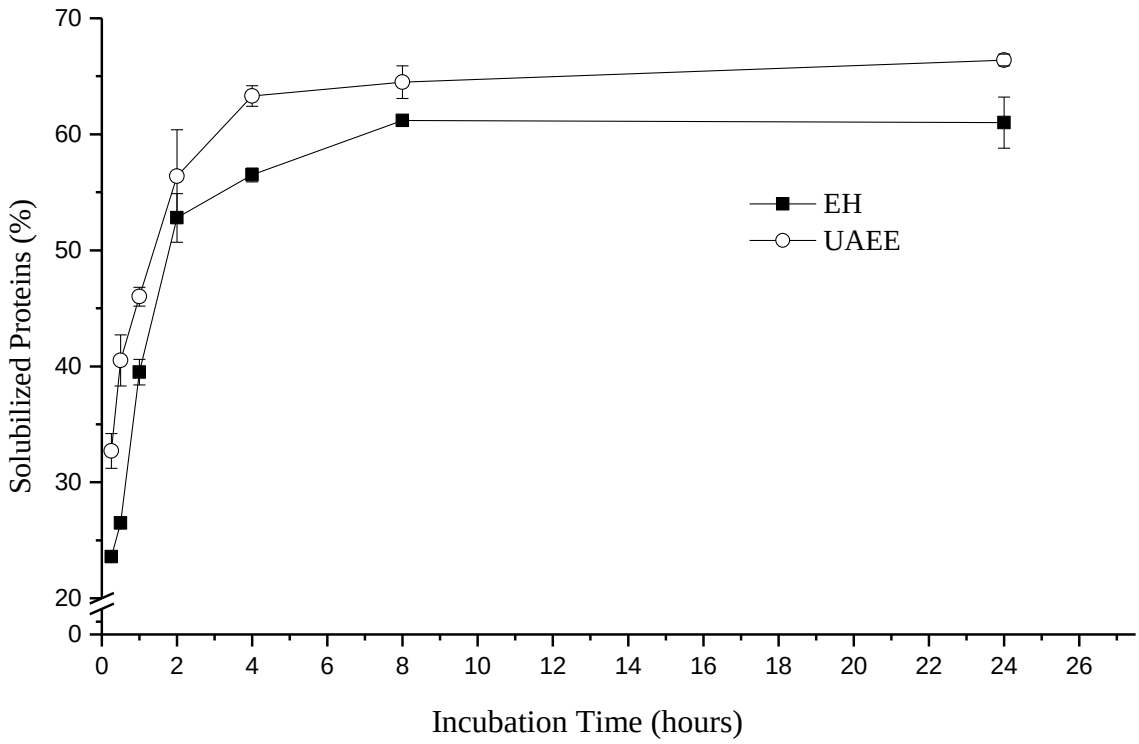

Fig. 4 Solubilized Proteins (%) after enzymatic hydrolysis (—■—, EH) and ultrasound-assisted enzymatic extraction (—○—, UAEE) under different incubation times (from 0.25 to 24 hours). Data represent the average ± SD for 2 replications ($n = 2$).

Table 5. *p* values of two-way ANOVA analysis for Solubilized Proteins (%) under different incubation times.

	p value
Ultrasound-assisted or not	< 0.0001
Time	< 0.0001
Interaction	0.0123

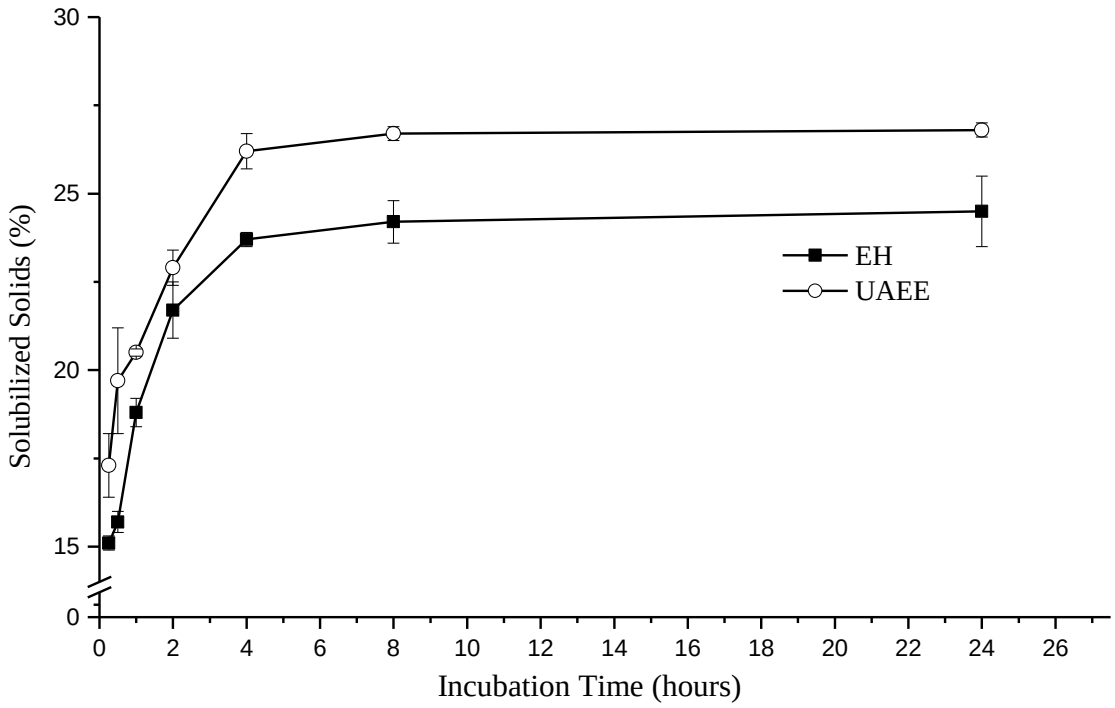

Fig. 5 Solubilized Solids (%) after enzymatic hydrolysis (—■—, EH) and ultrasound-assisted enzymatic extraction (—○—, UAEE) under different incubation times (from 0.25 to 24 hours). Data represent the average ± SD for 2 replications ($n = 2$).

Table 6. *p* values of two-way ANOVA analysis for Solubilized Solids (%) under different incubation times.

	p value
Ultrasound-assisted or not	< 0.0001
Time	< 0.0001
Interaction	0.1875

Original BSG and ultrasound-treated BSG samples were also incubated with enzymes at different loadings to solubilize proteins that can be separated by centrifugation. Figure 6 shows the percentages of the solubilized proteins and solubilized solids after running the enzymatic hydrolysis and centrifugal separation. For the original BSG, when the enzyme loading increased from 1 to 40 μL /g BSG, the solubilized proteins increased from 34% to 64.8% (Figure 6). When ultrasound pretreatment was applied, the solubilized protein percentage increased significantly compared with unpretreated BSG (Table 7, $p < 0.0001$). At the enzyme loading of 10 μL /g BSG, the application of ultrasound pretreatment increased the solubilized proteins from 55% to 67%, a 12% increase. Besides solubilized proteins, we have also measured the total solubilized solids from BSG, which is another important indicator showing the process selectivity of protein separation. The results showed that the solubilized solids (%) increased from 17.8% to 26.7% when the Alcalase loading increased from 1 to 40 μL /g BSG. This value further increased to 27.8 % by the application of ultrasound treatment. Overall, the results herein indicate that ultrasound pretreatment has the potential to significantly increase the solubilized protein content, but only leads to a slight increase of solubilized solids.

It is also important to note that the ultrasound pretreatment could reduce the enzyme dosage based on the results (Figure 6). For example, at protein separation effciency of 64%, only 15 μL of enzyme was needed for the ultrasound pretreated BSG; whereas 40 μL enzymes was required for the BSG sample without ultrasound pretreatment. This result support our hypothesis that the application of the ultrasound pretreatment could reduce the enzyme dosages to achieve the same level of protein recoverty. Additionally, the proteins extracted by

enzymatic hydrolysis (EH) and ultrasound-assisted ezymatic extraction (UAEE) had a protein concentration between 30~ 80%.

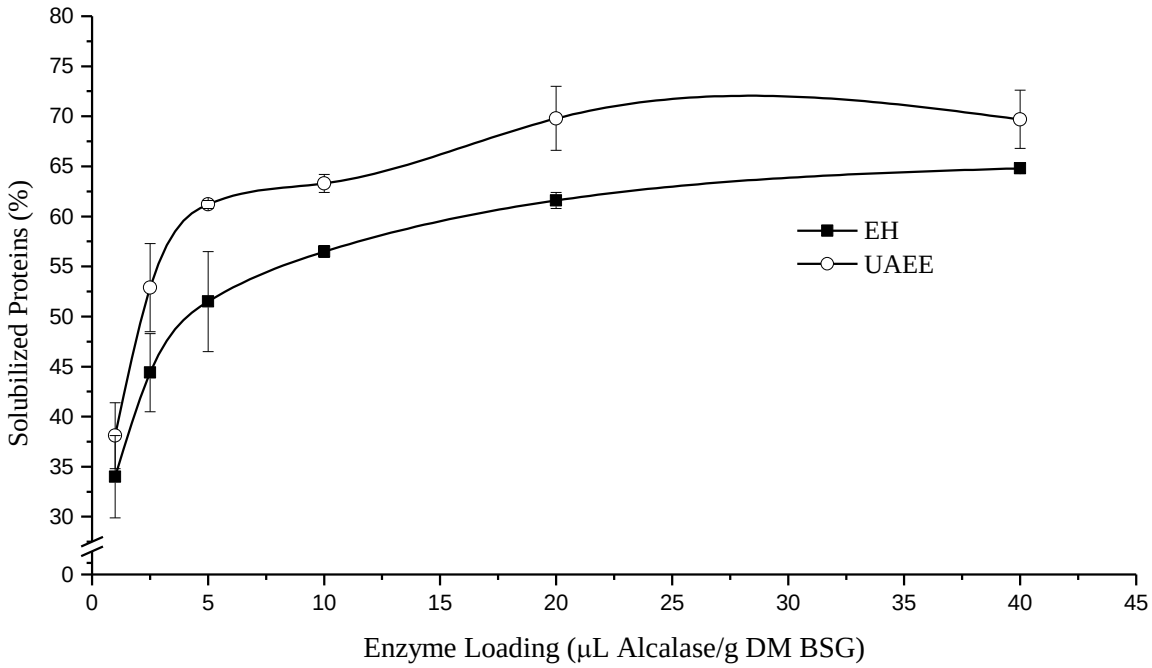

Fig. 6 Solubilized Proteins (%) after enzymatic hydrolysis (—■—, EH) and ultrasound-assisted enzymatic extraction (—○—, UAEE) under different enzyme loadings (from 1.0 to 40 μL Alcalase/g dry mass BSG). Data represent the average ± SD for 2 replications (n = 2).

Table 7. p values of two-way ANOVA analysis for Solubilized Proteins (%) under different enzyme loadings.

	p value
Ultrasound-assisted or not	< 0.0001
Enzyme loading	< 0.0001
Interaction	0.7482

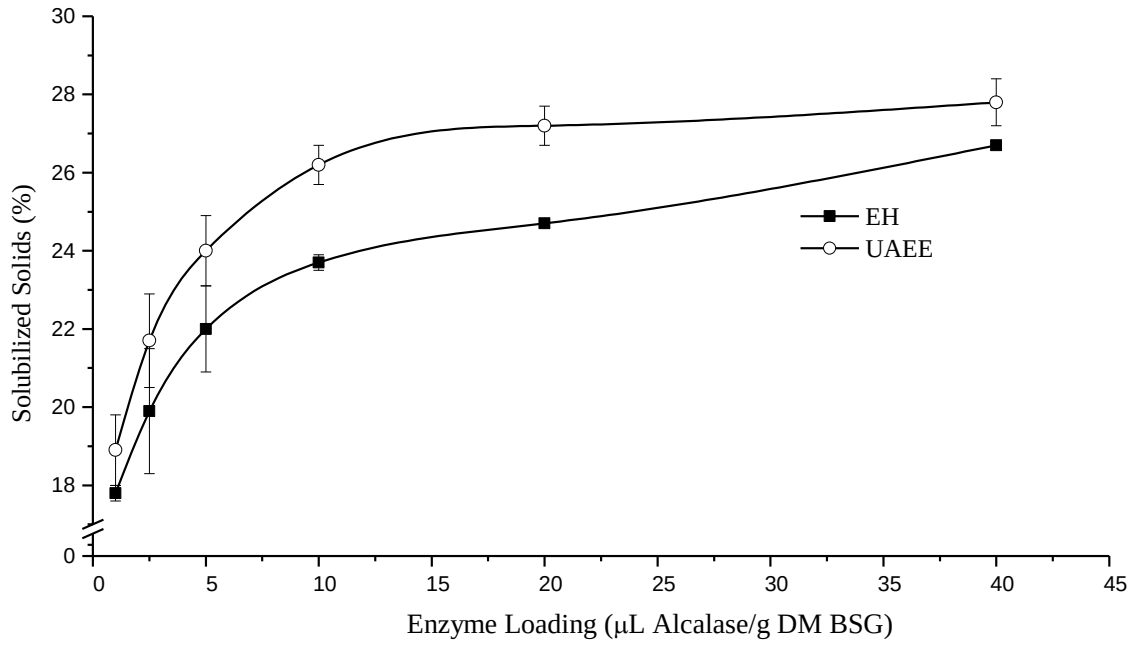

Fig. 7 Solubilized Solids (%) after enzymatic hydrolysis (—■—, EH) and ultrasound-assisted enzymatic extraction (—○—, UAEE) under different enzyme loadings (from 1.0 to 40 μL Alcalase/g dry mass BSG). Data represent the average ± SD for 2 replications (n = 2).

Table 8. p values of two-way ANOVA analysis for Solubilized Solids (%) under different enzyme loadings.

	p value
Ultrasound-assisted or not	< 0.0001
Time	< 0.0001
Interaction	0.6561

5.4 Particle size distribution and surface morphology of BSG

The particle size distribution and surface morphology of original BSG and ultrasound pretreated BSG were characterized to reveal the impact of ultrasound pretreatment on BSG. The information could assist in explaining why ultrasound pretreatment can improve protein solubilization and reduce enzyme incubation time. The particle size distribution was conducted using the Diffraction Particle Size Analyzer. After ultrasound pretreatment, the sample

geometric mean size decreased from 331.2 ± 6.3 μm to 215.7 ± 28.5 μm. The sample particle-size distribution before and after ultrasound pretreatment are shown in Fig. 8. We observed that the particle size distribution of untreated BSG was a unimodal distribution. The peak appeared at the particle size of 201 ~ 400 μm and 80% of the particles stayed in the particle size range of 0~ 800 μm. After ultrasound pretreatment, 90% of the particles stayed in the particle size range of 0~800 μm. The peak moved to smaller particle size and 37% of particles had a particle size from 0 to 200 μm compared to only 21% for the original BSG particles. The results supported our statement that ultrasound pretreatment could break down the BSG particles to smaller sizes, thus increasing the surface area for the enzyme to attack. In this way, the ultrasound helped increase the protein separation efficiency and had the potential to decrease the usage of expensive enzymes.

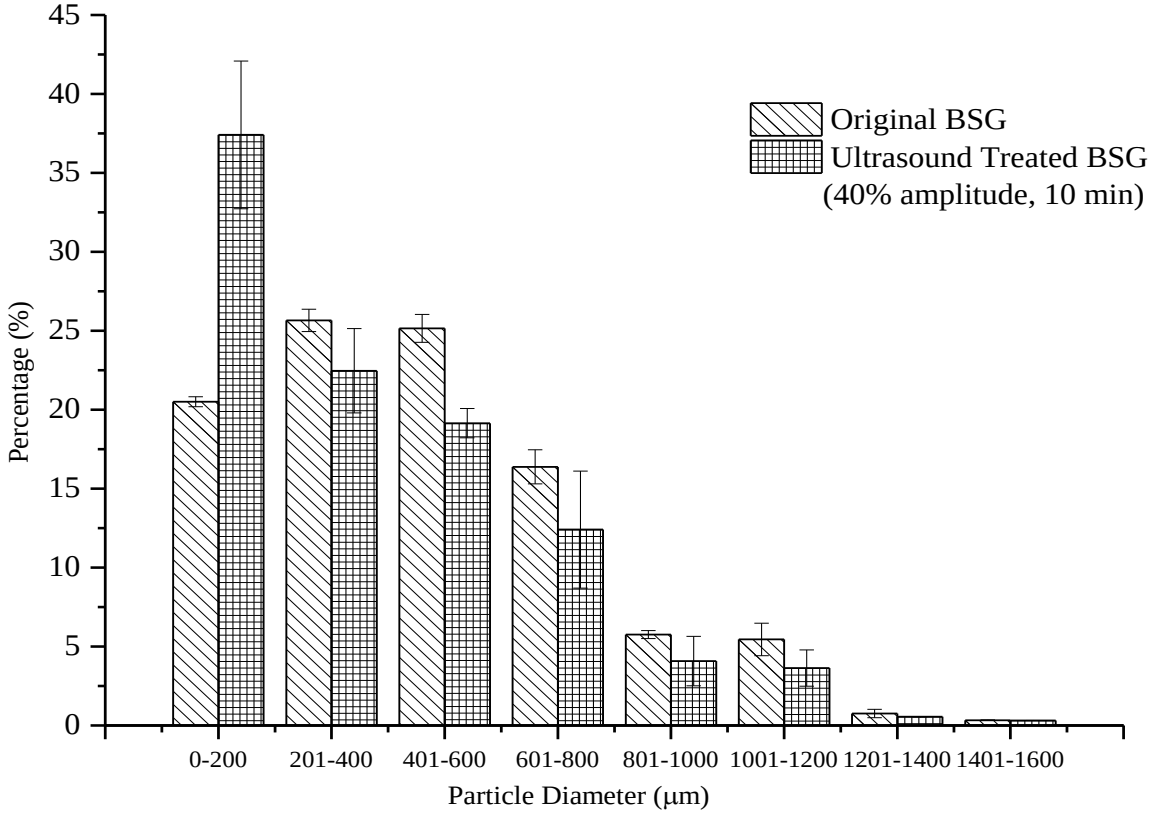

Fig. 8 Particle size distribution of original and ultrasound-treated BSG. Data represent the average ± SD for 3 replications (n = 3).

The surface morphology of the original and ultrasound pretreated BSG was investigated by SEM and the images are shown in Figure 9. SEM photo taken under magnification factor of 150X for the original BSG sample (a) showed large and intact BSG particles and the surface of the particle had a smooth appearance which was observed more clearly under higher magnification factor of 500X (b). After ultrasound pretreatment, more fragmented particles were observed under 150X magnitude (c). This was confirmed by the decrease of the particle size caused by ultrasound pretreatment. Additionally, the flat surface was partially ruptured by ultrasound as shown in Figure 9 (d). The surface of the BSG particles became rough and polyporous which provided enzymes more sites to attack. These visual observations proved that the application of ultrasound changed the surface properties of BSG sample materials and

made them more accessible to enzymes. Therefore, the ultrasound could accelerate the enzyme hydrolysis and reduce incubation time.

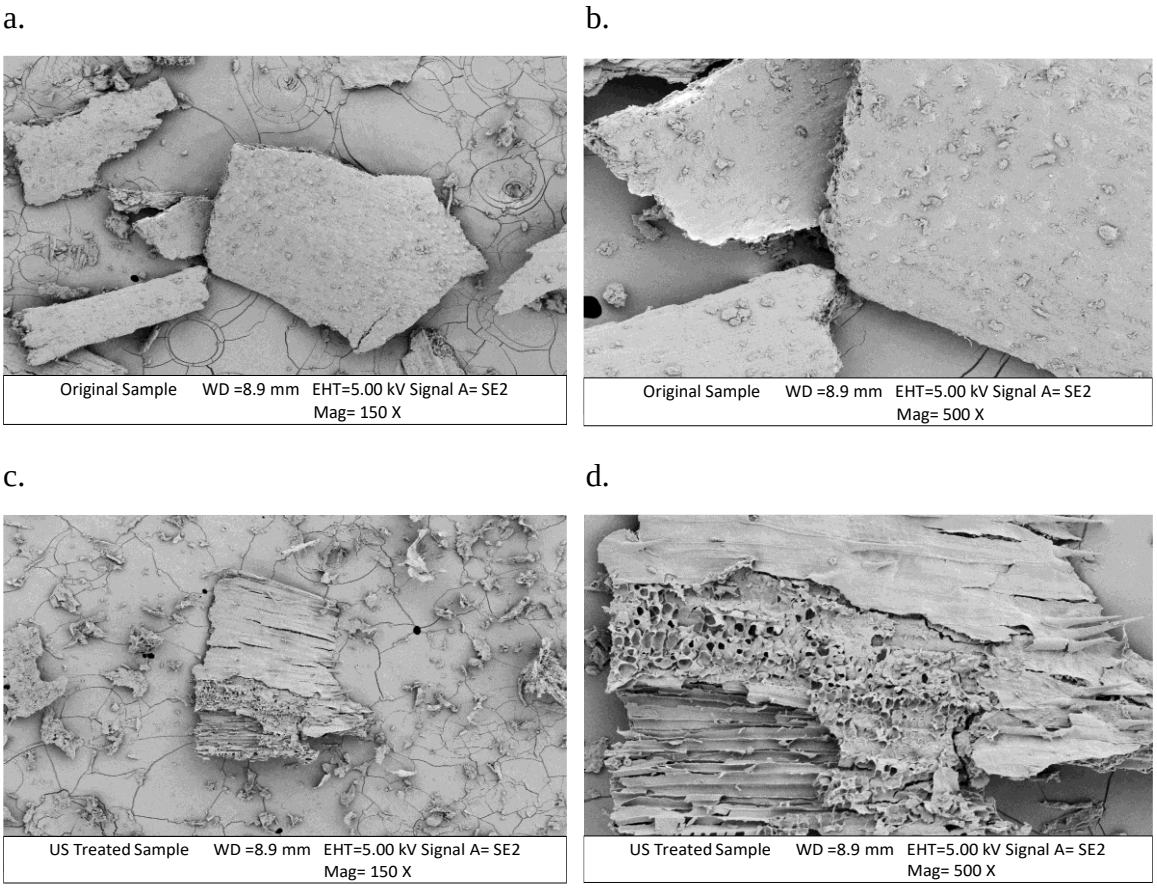

Fig. 9 SEM microphotographs of (a) (b) the surface of original BSG samples, (c) (d) the surface of ultrasound treated BSG samples.

5.5 Solubility of protein hydrolysates as a function of pH

Protein solubility was regarded as the most practical index of protein physicochemical properties. Figure 10 shows protein solubility as a function of pH for protein hydrolysates (PH) extracted EH and protein hydrolysates extracted by UAEE from BSG. The PH extracted by both EH and UAEE had a high solubility at both acid and alkaline conditions. As the figure shown, the protein solubility for PH from EH was 75.8% at pH 1.0; and the solubility increased to 85.6% as the pH increased to 3.0. No significant increase in PH solubility was observed

when we further increased pH until 11.0; the solubility value stayed at 86%. The high solubility of PH was probably attributed to the decreased molecular weight of proteins during the enzymatic hydrolysis using Alcalase (Celus, Brijs, & Delcour, 2007). In addition, enzymatic hydrolysis could also lead to the unfolding of proteins to expose both nonpolar and polar amino acid groups, which were buried inside protein molecules. The exposed polar amino acids may interact with water molecules through hydrogen bonds and electrostatic interactions, thereby increasing protein solubility. Similar results were also reported by Wu et al (Wu, Hettiarachchy, & Qi, 1998), who used Papain, a protease with both exo- and endo-peptidase activity, to increase the solubility of soy protein from 56% to 94% at pH 7.0.

Application of ultrasound pretreatment prior to enzymatic extraction could further increase the protein solubility of extracted PH significantly (Table 9, $p < 0.05$). As shown in Figure 10, the PH extracted by UAEE had significantly higher solubility than that extracted by EH. At the extreme low acid condition (pH 1.0), the protein sobubility of PH from UAEE was as high as 86.0%. This value increased to above 90% at all other pH conditions (3.0 to 11.0), except for pH 5.0 at which the solubility was 88.7%. This high protein solubility at acid pH makes it a good protein supplement for vitamin C-enriched or other types of beverages. This result agrees well with the study of Jambrak et al, who reported that ultrasound treatement could increase the protein solubility of whey protein concentrates and protein isolates at pH 7.0 (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008). The ultrasound increases the solubility probably by assisting the unfolding of the proteins. Jiang et al. also reported that ultrasound combined with pH-shifting treatment could improve the solubility of pea protein isolates (S. Jiang, Ding, Andrade, Rababah, Almajwal, Abulmeaty, et al., 2017).

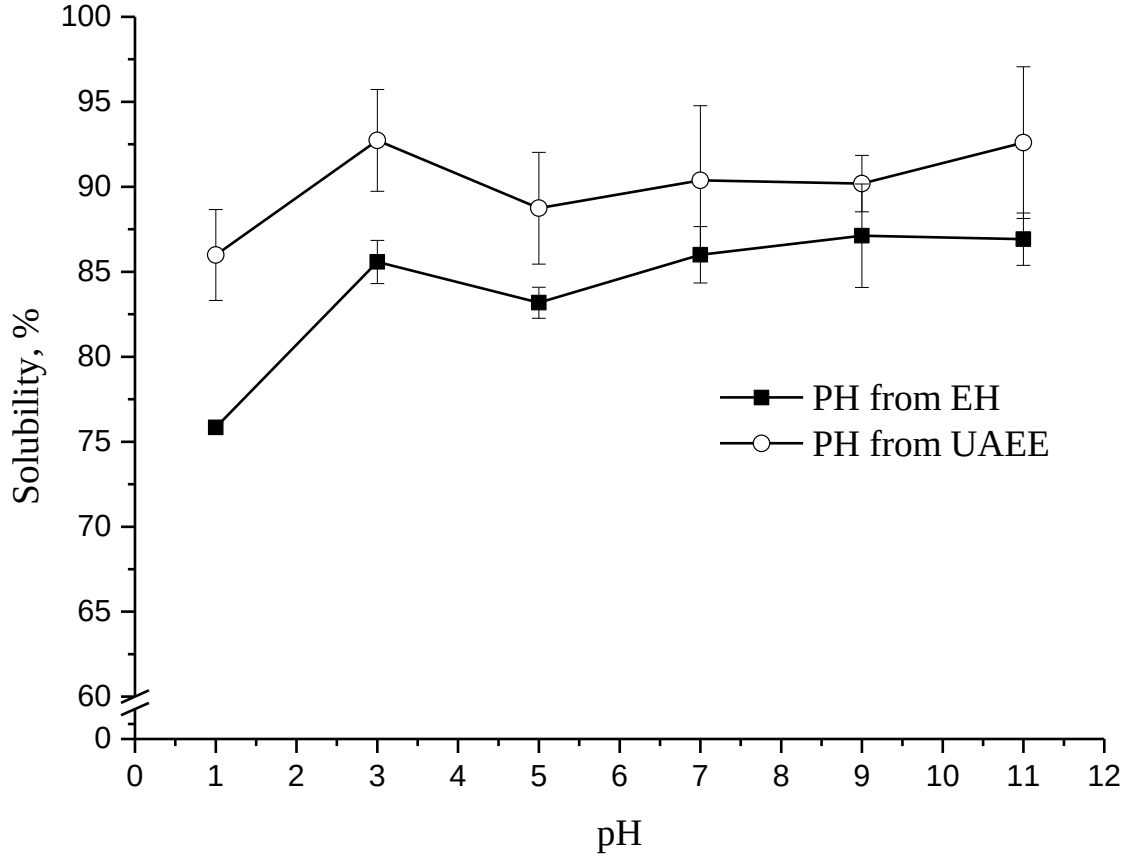

Fig. 10 Protein solubility as a function of pH for protein hydrolysates (PH) extracted by enzymatic hydrolysis (—■—, PH from EH) and PH extracted by ultrasound-assisted enzymatic extraction (—○—, PH from UAEE) from BSG. Data represent the average ± SD for 2 replications (n = 2).

Table 9. p values of two-way ANOVA analysis for protein solubility as a function of pH.

	p value
Ultrasound-assisted or not	0.0001
pH	0.0049
Interaction	0.5459

5.6 Amino acid profile

Figure 11 shows the amino acid composition (g/100g AA) of PH extracted by EH and UAEE, as well as the amino acid composition of protein in original BSG. As shown in Figure 11, glutimine and proline were the main amino acids for both PH and the original BSG. This

results matched with the amino acid composition reported by Treimo et al. in 2008 (Treimo, Aspmo, Eijsink, & Horn, 2008). Glutimine and proline are the main components of hordeins which represent the main storage protein in barley. After extraction by EH or UAEE, the amount of glutimine and proline were higher in PH than those in original BSG which indicated that the Alcalase may work better on solubilizing glutamine and proline as explained by Treimo et al. The percentage of methionine was around 2% which was not affected by hydrolysis and ultrasound treatment while the percentage of lysine decreased. The increase in the percentages of glutimine and proline in PH may cause the decrease of the relative percentages of other amino acids. When comparing the amino acid composition of PH extracted by EH with PH extracted by UAEE, there was no difference. This results indicated that the application of ultrasound did not change the amino acid profile of the PH products.

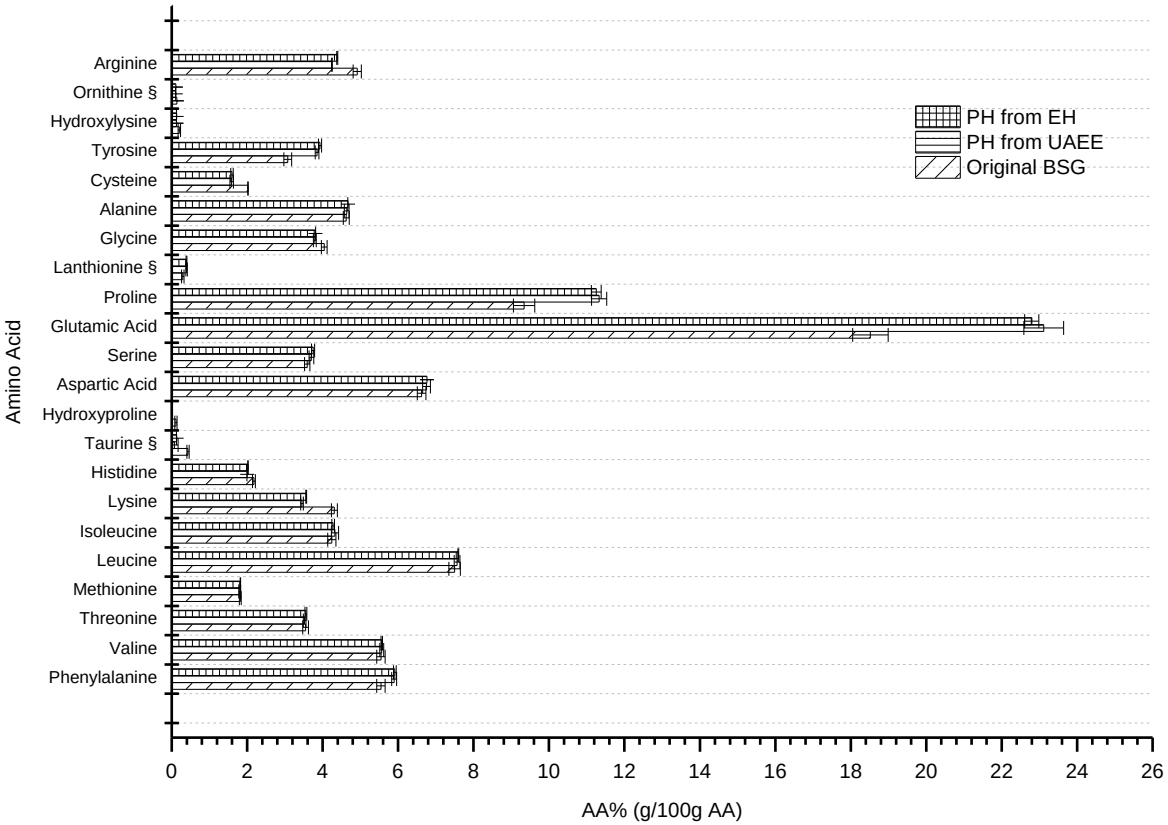

Fig. 11 Amino acid composition analysis. The figure was the amino acid composition of PH from EH (protein hydrolysates extracted by enzymatic hydrolysis), PH from UAEE (protein hydrolysates extracted by ultrasound-assisted enzymatic extraction) and original BSG. Data represent the average ± SD for 2 replications (n = 2).

5.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Figure 12 is the SDS-PAGE profile of protein-enriched isolates (BSG-PI), PH extracted by EH, PH extracted by UAEE, and the enzyme Alcalase. SDS-PAGE was used to estimate the molecular weight (MW) of the protein products. Since the enzyme was also a protein and had a potential effect on the results, we also added Alcalase as a control group. The MW of the three major bands of BSG-PI were >250 kDa, 50 kDa and 37 kDa respectively (Figure 12). The band with a MW larger than 250 kDa was also found in the enzymatically solubilized proteins extracted by Acid Protease A, Promod 144 and Alcalase as reported by Niemi et al (Niemi, Martins, Buchert, & Faulds, 2013). The other two bands of BSG-PI with a MW of 50

kDa and 37 kDa were found in BSG samples used by Kemppainen (Kemppainen, Rommi, Holopainen, & Kruus, 2016b). There were three major bands for PH from EH and UAEE. However, the band with a MW close to 25 kDa and the band with a MW smaller than 10 kDa might come from the Alcalase when compared to the bands of just Alcalase. Therefore, compared to the BSG-PI, the PH extraced by EH and UAEE had a much smaller MW which were smaller than 15 kDa and around 10 kDa. The same observation was reported by Niemi et al (Niemi, Martins, Buchert, & Faulds, 2013), the MW of the enzymatically solubilized proteins extracted by Alcalase also had a MW lower than 15 kDa. This decrease in the MW was caused by the action of the Alcalase which breaks the peptide bonds. When comparing the PH from EH and UAEE, there was no difference between them, which indicated that ultrasound pretreatment didn't change the MW of the PH products. The SDS-PAGE results indicated that the proteins degraded into smaller size proteins and peptides after the enzymatic hydrolysis by Alcalase. This degradation doesn't affect the nutritive value of the small proteins or peptides. However, the functional properties such as foaming properties or emusifying properties might be affected.

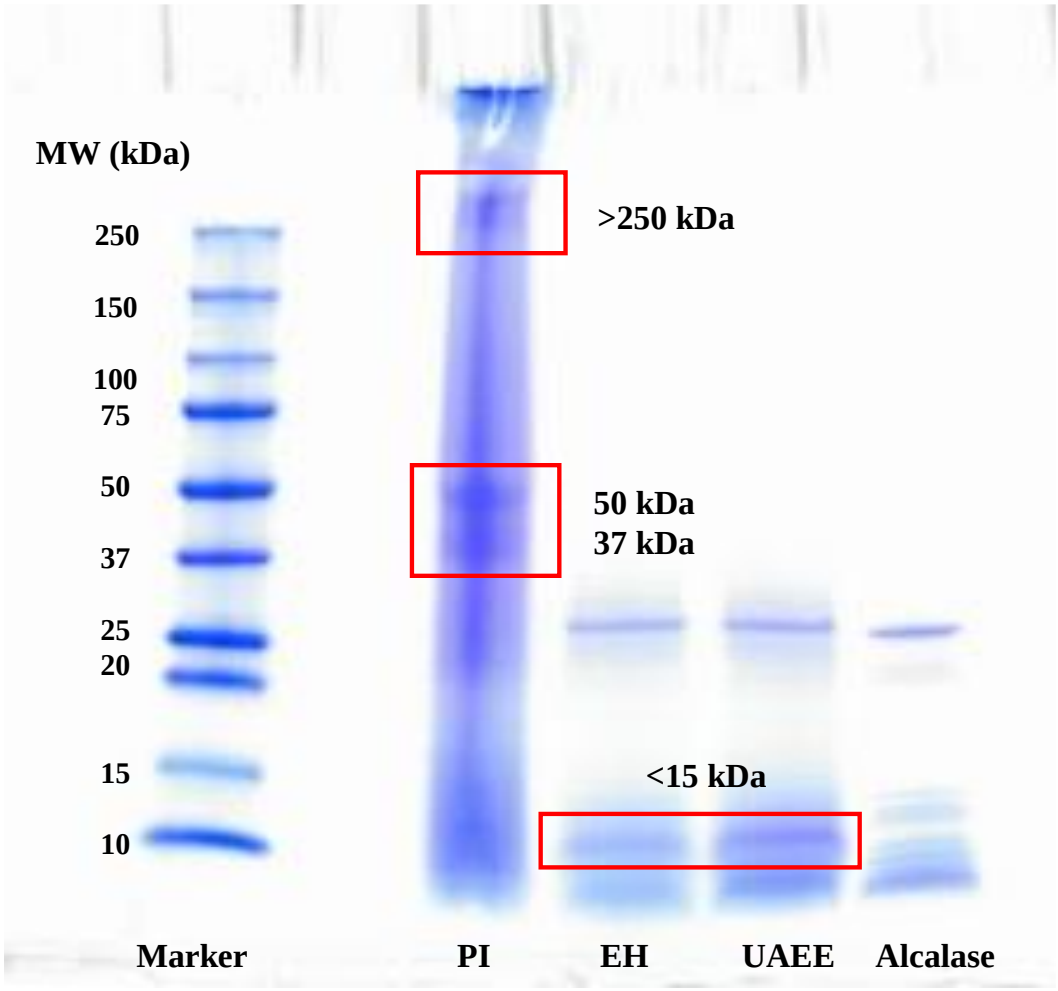

Fig. 12 SDS-PAGE electrophoretic profiles of molecular weight marker (marker), protein-enriched isolates (PI), protein hydrolysates of EH (EH), protein hydrolysates of UAEE (UAEE) and Alcalase.

5.8 Color

The colors of the original BSG, extracted protein hydrolysates, and fiber-rich residues were determined using CIE L*a*b* scale. Color difference between samples was measured by the total color difference **(**ΔE^*). The color of fiber-rich residue from EH and UAEE didn't have a big difference (2.60 and 1.66 respectively) from original BSG samples while the extracted PH did, based on the ΔE^* values (Table 10). The individual color parameters L^*(brightness) and b^* (yellowness) of PH samples were higher than the original BSG while a^* (redness) was lower than original BSG which means the color of protein hydrolysates was brighter and lighter.

There was no difference between the color of the protein hydrolysates between EH and UAEE, indicating that the application of ultrasound didn't change the sample color. Based on the color measurement results, the protein hydrolysates can be a good protein supplement for foods since it had a lighter color which will not affect the color of the foods or beverages. The photos of the real protein and fiber products were listed in Appendix C, Figure 15.

Table 10. Color of BSG, protein hydrolysates extracted by EH and UAEE, and the fiber-rich residue of EH and UAEE. Data represent the average ± SD for 2 replications (*n* = 2). [abc] For each value, the data marked by different letters in the same column indicates a significant difference ($p < 0.05$).

	L^*	a^*	b^*	ΔE^*
Original BSG	62.66 ± 0.07 [b]	4.63 ± 0.23 [a]	15.95 ± 0.34 [ab]	0.00
PH from EH	76.72 ± 0.83 [a]	3.48 ± 0.26 [a]	16.17 ± 0.49 [a]	14.11
PH from UAEE	76.25 ± 4.38 [a]	3.52 ± 0.65 [a]	16.13 ± 0.46 [a]	13.64
Fiber from EH	60.77 ± 1.66 [b]	4.01 ± 0.14 [a]	14.28 ± 0.48 [c]	2.60
Fiber from UAEE	62.70 ± 0.65 [b]	4.00 ± 0.06 [a]	14.42 ± 0.01 [bc]	1.66

Chapter 6 Conclusions

This study devleoped an ultrasound-assisted enzymatic extraction method to effectively separate proteins from BSG. The main factors (i.e. particle size distribution and surface morphology) contributing to the improved protein separation by ultrasound treatment were investigated. Lastely not unimportant, the solublity, amino acid compositon, molecular weight and color of the separated PH were determiend.

The first specific aim was to evaluate the protein separation efficiency of original BSG and ultrasound-treated BSG under different incubation times and enzyme loadings. The solubilized proteins (%) of ultrasound-treated BSG reached plateau at the incubation time of 4 hours compared to 8 hours of the original BSG. The solubilized proteins (%) was significantly increased by the application of ultrasound ($p < 0.05$). The result indicated that ultrasound can decrease the enzymatic incubation time and had the potential of decreasing the energy consumption of the incubation process. Additionally, the application of ultrasound significantly increased the protein separation efficiency at all different enzyme loadings. At the enzyme loading of 10 μL /g BSG, the solubilized proteins (%) was increased by 12%. Overall, ultrasound treatment prior to enzymatic hydrolysis enhanced the separation of proteins from BSG in terms of higher protein separation efficiency, lower enzyme loadings, and reduced incubation time. Particle size distribution was determined to investigate the effect of ultrasound pretreatment on the BSG particle size. The particle size decreased by 34.9% (from 331.2 to 215.7 μm). According to the particle size distribution, the percentage of smaller particles increased after ultrasound treatment. SEM photos showed a rough and polyporous particle surface caused by ultrasound pretreatment. Both particle size distribution results and SEM

photos provide proof that decreased particle size and polyporous surface of BSG after ultrasound treatment could be the reason for the improved proteins separation efficiency.

The second specific aim was to study the physicochemical properties of the resulting PH, including solubility, amino acid composition, molecular weight and color. Enzymatically extracted PH had a high solubility (above 75%) at different pH conditions, and the application of ultrasound further increased the solubility (above 85%) of the extracted PH. Compared to the amino acid composition of original BSG, the amount of proline and glutamic acid in both EH extracted PH and UAEE extracted PH increased due to the function of Alcalase. However, the amino acid composition of EH extracted PH and UAEE extracted PH had no difference, which indicates that ultrasound did not change the amino acipid profile of the PH products. The SDS-PAGE profiles revealed that the molecular weights of the extracted PH by both EH and UAEE were mainly smaller than 15 kDa, due to the enzymatic cleavage of peptide bonds of large proteins. The PH extracted by EH and UAEE had a similar color which was bright and light.

The hypotheses of this research were that the application of ultrasound pretreatment can promote the enzymatic separation of proteins from BSG, and that the ultrasound can contribute to the improved physiochemical properties of extracted PH. It can be stated that the protein separation efficiency is increased by the application of ultrasound by shortening the incubation time and decreasing the enzyme consumption. Although only the solubility was measured to determine the physiochemical properties of extracted PH, the application of ultrasound did increase the solubility of the resulting PH. Therefore, we can consider that both hypotheses were proved.

This study developed a novel and green method to effectively separate value-added proteins from the low-value food processing byproducts. The findings in this study prove that ultrasound can be used as a pretreatment to assist the enzymatic extraction of proteins from BSG. It has the potential to decrease the enzyme usage and enzymatic incubation time and it might have the potential to reduce the energy consumption and the overall cost of the extraction process.

Chapter 7 Future prospects

More functional properties such as surface hydrophobicity, emulsifying properties (emulsifying activity index and emulsion stability) and foaming properties need to be determined. By measuring these functional properties, it will be easier to find an appropriate way to utilize the separated PH. Based on the utilization of the other plant-based proteins, the potential applications of PH from BSG are: 1) protein supplements for infant foods or pet foods; 2) meat replacement in meat products; 3) emulsifier for non-dairy drinks or meat products; 4) protein source or water-retaining agent for vegetarian products; 5) protein beverages. Because the PH extracted in this study has a high solubility even under acid pH, it will be a good ingredient to make vitamin C-enriched protein beverage.

After the proteins are separated, fiber is the main component of the insolubilized residue. Therefore, a better way to utilize the fiber-rich residue could be investigated in the future. For example, the BSG fiber can be used to produce glucose, xylose, mannose, galactose and arabinose or it can be used as the dietary fiber supplement for human foods or the other non-food utilization. It would also be interesting to use the BSG fibers to produce food packaging materials.

Once the BSG was fully utilized, techno-economic analysis can be conducted to evaluate the economic feasibility and the cost assessment of the ultrasound-assisted enzymatic extraction. Techno-economic analysis is also one of the most important steps to realize the industrialization of the developed extraction process.

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Appendices

Appendix A: Temperature (T) and temperature change (ΔT) of BSG samples after ultrasound pretreatment.

Table 11. Temperature (T) and temperature change (Δ*T*) of BSG samples after ultrasound pretreatment.

Treatment	1		2		3	
Time (min)	T (ºC)	ΔT (ºC/K)	T (ºC)	ΔT (ºC/K)	T (ºC)	ΔT (ºC/K)
0	24.4	0.0	23.2	0.0	23.3	0.0
1	29.1	4.7	27.9	4.7	28.1	4.8
2	33.7	9.3	32.1	8.9	32.4	9.1
3	37.8	13.4	36.1	12.9	36.5	13.2
4	41.6	17.2	40.0	16.8	40.4	17.1
5	44.9	20.5	43.4	20.2	43.8	20.5
6	48.0	23.6	46.5	23.3	46.9	23.6
7	50.6	26.2	49.2	26.0	49.7	26.4
8	53.0	28.6	51.7	28.5	52.3	29.0
9	55.1	30.7	53.8	30.6	54.3	31.0
10	57.1	32.7	55.7	32.5	56.3	33.0

Appendix B: Supplementary data

Enzymatic hydrolysis with high enzyme loading

For ultrasound-pretreated BSG, solubilized proteins did not increase further when enzyme loading doubled from 20 μL/g BSG to 40 μL/g BSG (Figure 6). There might be a limitation for the protein extraction from the BSG we used. Therefore, high enzyme loading was used to test the maximum amount of proteins which can be extracted from this original BSG. In this experiment, BSG suspension was subjected to enzymatic hydrolysis at the enzyme loading of 80 μL/g BSG and the incubation time of 4 hours. Centrifugation was then applied to separate protein-rich supernatant and fiber-rich residue. As a result, 69.3 ± 1.4% of proteins was solubilized (Table 12). This result indicated that protein separation efficiency of 70% was the limit of this extraction method. And, this solubilized protein percentage has already been reached by 20 μL/g BSG enzyme loading with ultrasound pretreatment. This result further proved that the application of ultrasound pretreatment can decrease the usage of enzyme.

Table 12. Solubilized proteins (%) and solubilized solids (%) of the extraction at the enzyme loading of 80 μL/g BSG for original BSG and the extraction at the enzyme loading of 10 μL/g BSG for MTS-pretreated BSG.

Group	Solubilized Proteins (%)	Solubilized Solids (%)
Enzyme loading of 80 μL/g BSG	69.3 ± 1.4	29.7 ± 0.1
MTS	67.0	28.4

Manothermosonication-assisted enzymatic separation of proteins from BSG

After ultrasound pretreatment, the sample geometric mean size decreased from 331.2 ± 6.3 μm to 215.7 ± 28.5 μm. At this point, another research question arises: when more powerful ultrasound is applied, and the particle size becomes much smaller, the protein separation efficiency can be increased further or not. Therefore, manothermalsonication (MTS), the currently most powerful ultrasound treatment with the highest cavitation intensity was chosen to pretreat the BSG to see if the protein separation efficiency was further improved.

MTS is a treatment which combines ultrasound treatment under pressure and a mild thermal treatment (Bermudez-Aguirre, 2017). MTS is conducted when a low hydrostatic pressure is applied to a sono-reactor. When pressure is applied, ultrasound can transmit more power into the samples. Currently, it has been used to inactive the microbial in foods. An experimental setup of MTS is shown in Figure 13. In this experiment, two grams BSG sample was dispersed in deionized water in a bottle. The suspension was sonicated by an ultrasound processor (VC-750, Sonics & Materials, Inc., Newtown, CT, USA) equipped with a 13.0-mm-diameter ultrasound probe. Power output (amplitude 67%) and treatment time (10 min) was applied with a pulse duration of 5s on and 3s off. The pressure was set at 4-time standard

atmosphere (4 atm). The temperature of the bottle was controlled under 39 ºC. After MTS treatment, the sample was incubated at 60 ºC with enzyme loading of 10 µL/g BSG.

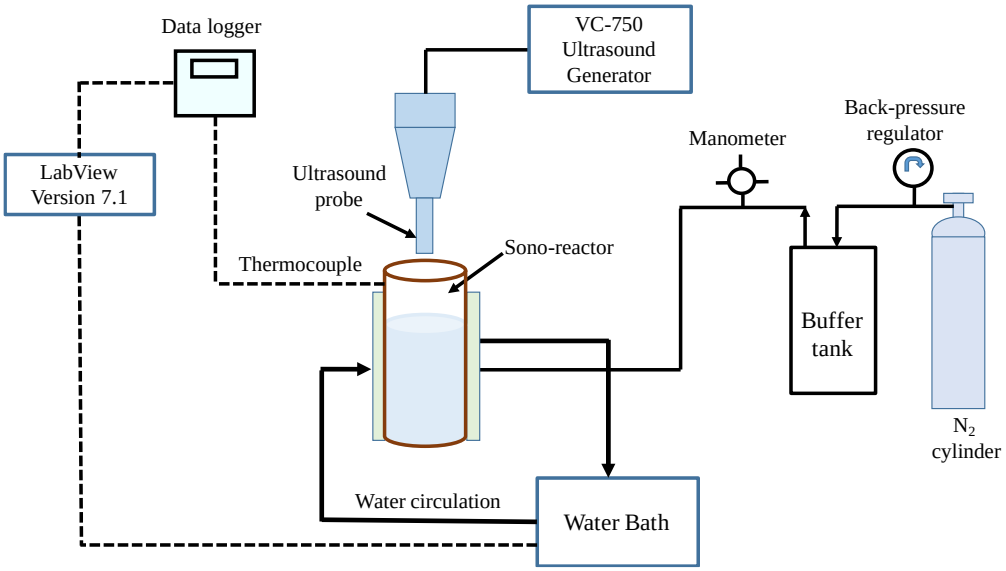


Fig. 13 Bench-top high-intensity manothermalsonication (MTS) system setup

Particle size was determined before and after the MTS treatment. The sample particle size decreased further to 49.7 ± 22.9 µm. The sample particle-size distribution of original BSG, ultrasound-treated BSG and MTS-treated BSG are shown in Figure 14. We observed that the percentage of smaller particles (0-200 µm) increased further to 74.6 ± 12.9% and there was no particle having a particle size higher than 800 µm. Although the particle size was further decreased, the solubilized proteins (%) was 67.0% which was still lower than 70.0% and the solubilized solids (%) was 28.4%. This result might give another proof that the maximum protein separation efficiency was 70.0% for this extraction method and the BSG used in this research.

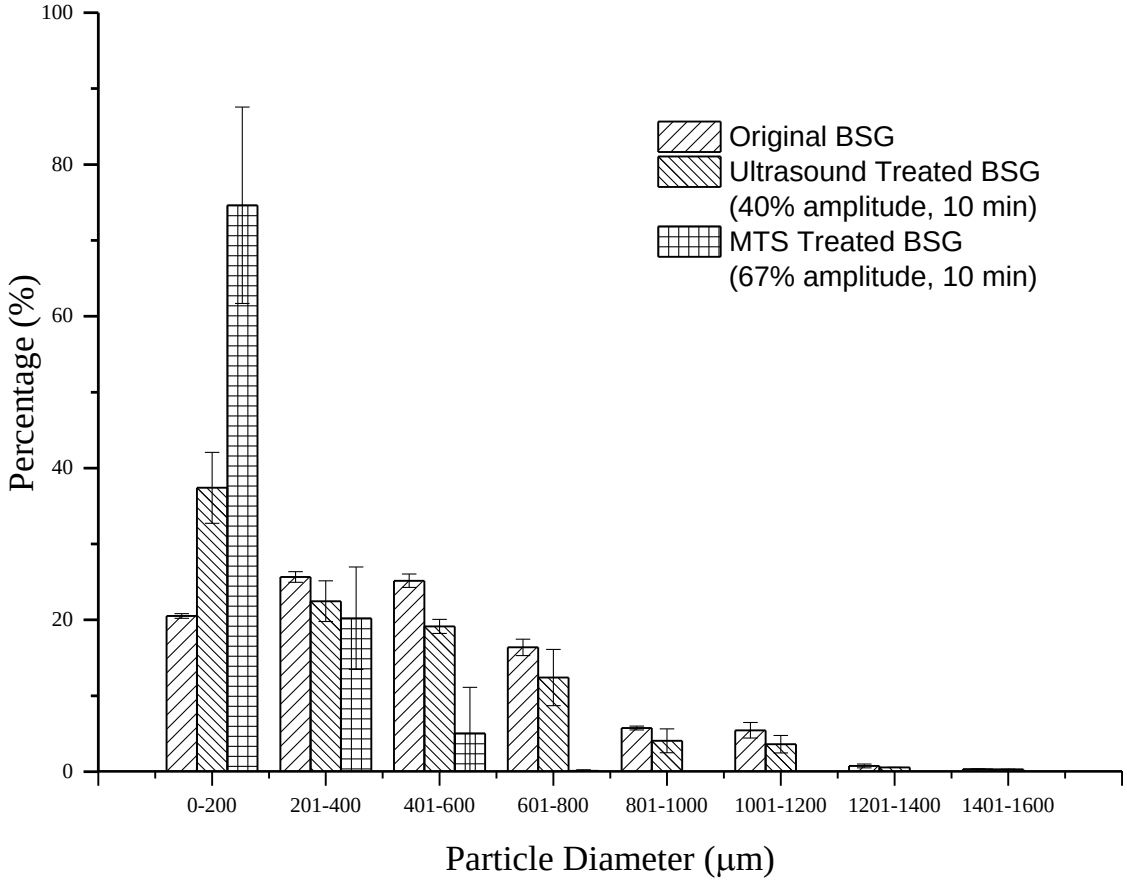


Fig. 14 Particle size distribution of original, ultrasound-treated BSG and MTS-treated BSG. Data represents the average ± SD for 3 replications (n = 3).

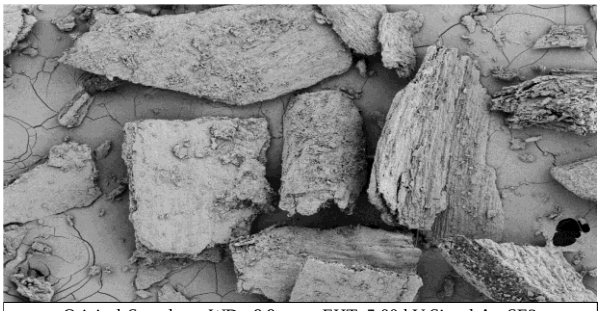
Appendix C: Photos of real protein products and fiber products

a. b.

Fig. 15 Photos of protein hydrolysates extracted (a) and the fiber rich residue (b).

Appendix D: Additional SEM microphotographs of the surface of original BSG samples and the surface of ultrasound treated BSG samples

a.

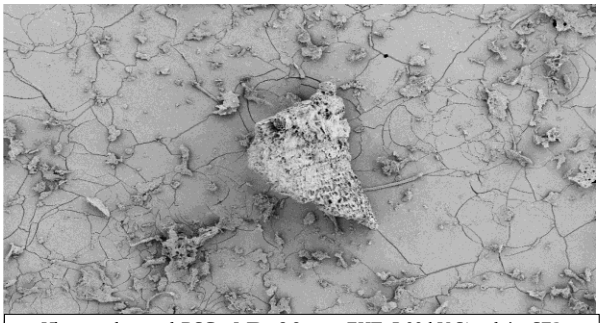


Original Sample WD =8.9 mm EHT=5.00 kV Signal A= SE2
Mag= 150 X

b.

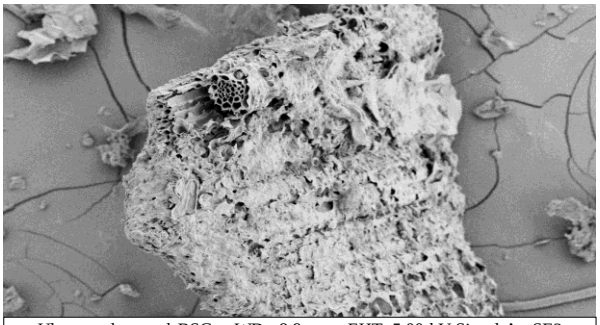
Original Sample WD =8.9 mm EHT=5.00 kV Signal A= SE2
Mag= 500 X

c.



Ultrasound-treated BSG WD =8.9 mm EHT=5.00 kV Signal A= SE2
Mag= 150 X

d.



Ultrasound-treated BSG WD =8.9 mm EHT=5.00 kV Signal A= SE2
Mag= 500 X

Fig. 16 Additional SEM microphotographs of the surface of original BSG samples (a) (b) and the surface of ultrasound treated BSG samples (c) (d).