Development of hydrolysates with antioxidant effects from brewers' spent grain proteins

by

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Abstract

Brewers' spent grain (BSG), the most abundant brewing by-product contains up to 24% (w/w) of protein on dry basis. Despite of a rich protein source, it is still mainly used as low value animal feed. This study was conducted to develop hydrolysates with antioxidant effects from BSG proteins by enzymatic hydrolysis, and study BSG peptide antioxidant activities in relation to molecular structures.

The BSG proteins were extracted by alkaline method with a protein content of 62.6% (w/w) and a protein recovery of 46.3%. The proteins were then hydrolyzed by selected proteases or protease combinations having different specificities. Resulting hydrolysates were analyzed for their molecular structures such as hydrolysis degree, amino acid composition, molecular weight and conformation, and their antioxidant effects were measured by reducing power, DPPH radical scavenging, ferrous chelating and superoxide radical scavenging assays. Hydrolysates prepared by alcalase and alcalase combined treatments with neutrase, flavourzyme and everlase showed the highest DPPH radical scavenging activities ranged between 72.6-74.9% at the hydrolysate concentration of 1.0 mg/mL. It could be due to specific activity of alcalase to produce amino acid sequences with greater DPPH radical scavenging potential. Similarly, high proton donation ability of the imidazole ring present in the side chain of histidine may have strong ability to scavenge the DPPH radical. The highest superoxide radical scavenging activity of 19.3% was observed in the hydrolysate resulted from alcalase and flavourzyme combined treatment and the value increased with increasing the sample degree of hydrolysis. Highly hydrolyzed proteins can be expected to have more positive charges available on the amine groups to form electrostatic interactions

with negative charges on superoxide radicals. Everlase and FoodPro PHT combined treatment was the most effective in producing ferrous ion chelating peptides. The ferrous ion chelating ability was negatively correlated to degree of hydrolysis, suggesting that longer peptides are more likely to form compact structures to trap ferrous ions. These results suggest good potential of BSG protein hydrolysates as antioxidants. The commercial potential of BSG protein hydrolysates was analyzed by comparing the highest antioxidant activities with selected commercial antioxidants. The best DPPH radical scavenging activity reported by alcalase and neutrase combined treatment (74.9%) at 1.0 mg/mL was comparable to that of BHT (42.4%) and ascorbic acid (76.3%) at 0.1 mg/mL. The highest superoxide radical scavenging activity observed at 2.0 mg/mL was greater than the activity of ascorbic acid (11.4%) at 0.01 mg/mL. None of the hydrolysates were effective in chelating ferrous ions compared to EDTA, which is a strong metal chelator. All the hydrolysates at 1.0 mg/mL showed significantly ($p < 0.05$) higher reducing power activity compared to that of ascorbic acid at 0.01 mg/mL (0.16).

There is a growing interest in using peptides as natural antioxidants due to their ability to suppress oxidative species through multiple mechanisms. The BSG protein hydrolysates can be incorporated into food formulations to retard lipid oxidation, or suppress oxidative stress and associated negative effects. Similarly, they can be potentially developed as animal feed additive to reduce oxidative stress in livestock and consequently improve animal performance, feed efficiency and productivity. Although BSG protein hydrolysates showed comparable antioxidant effects to commercial antioxidant reagents at high dosage levels, they may be added in food and feed

formulations at higher concentration with minimum impact on the product sensory quality. This study is expected to bring benefits to breweries, barley growers, food and feed industries. Also, this study gives insight to molecular structures of peptides affecting different antioxidant mechanisms.

Key words: brewers' spent grain (BSG), protein hydrolysates, protease hydrolysis, antioxidant activities

Preface

This thesis is an original work by Galhenage Thanusha Ranithri Abeynayake. I was responsible for study design, sample collection, conduct of *in vitro* experiments, data collection and analysis as well as manuscript writing. This work is part of the research project; develop value-added applications of by-products derived from food processing led by Dr. Wenzhu Yang in collaboration with Dr. Chen, supported by Agriculture and Agri-Food Canada (AAFC) Growing Forward Program (GF2#1542). Both Drs. Yang and Chen contributed to the research idea development, experimental design, data discussion and manuscript revision.

The manuscript generated based on Chapter 2 will be submitted to a peer-reviewed journal by Galhenage Thanusha Ranithri Abeynayake, Wenzhu Yang and Lingyun Chen. First part of the research was presented as a poster; optimization of brewers' spent grain protein extraction using proteases at the Cereal Innovation Symposium by Ranithri Abeynayake, Ewelina Eckert, Wenzhu Yang, Lingyun Chen. It was awarded for the 3rd best poster at the conference. Another poster; effect of protease hydrolysis on antioxidant properties of brewers' spent grain protein will be presented at the 5th International Conference on Food Chemistry and Technology by Ranithri Abeynayake, Wenzhu Yang and Lingyun Chen. The research received the Alberta Barley Award, 2017 for its potential to have a significant impact to barley producers in the near future.

Besides, during the study I also contributed to the peer-reviewed work, Shen Y., Abeynayake, R., Sun, X., Ran, T., Li, J., Chen, L. & Yang, W. (2019). Feed nutritional value of brewers' spent gain residue resulting from protease aided protein

removal. Journal of Animal Science and Biotechnology. https://doi.org/10.1186/s40104-019-0382-1. As the co-author, I was responsible for collecting BSG samples, generation as well as characterization of protein and residue.

Dedication

This thesis is dedicated to my loving family.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor, Dr. Lingyun Chen for giving me an opportunity to work in her lab. I truly appreciate her supervision, continuous support and motivation throughout the study. Also, I would like to thank Dr. Wenzhu Yang. Without his support, encouragement and guidance, I would not have completed this research successfully.

I thank all the wonderful people from 3-30 and 3-52 labs for their support. It was great sharing the laboratory with you. I would never forget the fun we have had in last two years. Many thanks to Dr. Jingqi Yang and Dr. Ewelina Eckert for their valuable suggestions and troubleshooting my experiments.

Finally, I am eternally grateful to my loving family, friends and loved ones for their unfailing support and encouragement. I would never have reached this far without all of you. Specially, I would like to thank my family for always being there for me at every step of my life and always trusting me.

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List of Abbreviations and Symbols

A+E	Alcalase and everlase combined treatment
A+F	Alcalase and flavourzyme combined treatment
A+N	Alcalase and neutrase combined treatment
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
Ala	Alanine
ANOVA	Analysis of variance
ANS	Fluorescent dye 8-anilino-1-naphthalene sulphonic acid
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Asx	Asparagine and Aspartic acid
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BSG	Brewers' spent grain
Cys	Cysteine
DH	Degree of hydrolysis
DNA	Deoxyribonucleic acid
DPPH	1,1- diphenyl-2-picryl hydrazyl
EDTA	Ethylenediaminetetraacetic acid
EY	Extraction yield
F+N	Flavourzyme and neutrase combined treatment
FAO	Food and Agriculture Organization
FTIR	Fourier transform infrared
GAE	Gallic acid equivalent
Gln	Glutamine
Glu	Glutamic acid
Glx	Glutamine and Glutamic acid
Gly	Glycine
H_0	Surface hydrophobicity

His	Histidine
Ile	Isoleucine
Leu	Leucine
LSD	Least Significant Difference
Lys	Lysine
met	Methionine
M_w	Average molecular weight
ORAC	Oxygen Radical Absorbance Capacity
P+E	FoodPro PHT and everlase combined treatment
Phe	Phenylalanine
Pro	Proline
PY	Protein yield
r	Correlation coefficient
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE-HPLC	Size Exclusion High Performance Liquid Chromatography
Ser	Serine
TCA	Trichloroacetic acid
Thr	Threonine
TNBS	Tri-nitro benzene sulfonic acid
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

Chapter 1.0

Literature review

1.1. Barley

Barley (*Hordeum vulgare L.)* was one of the first agriculturally domesticated grains as early as 10,000 years ago. In 2017, barley was the fourth largest produced cereal grain in the world after maize, rice, and wheat accounting for 141 million tonnes (FAOSTAT, 2017). Short growing season, drought tolerance, adaptability to temperate as well as tropical climates are among the factors promoting barley cultivation. Apart from its main use as animal feed, major food applications include brewing, as a component in various health foods, and bakery products. The rounded spherical shape barley grain consists of three main parts; germ (embryo), endosperm, and grain coverings.

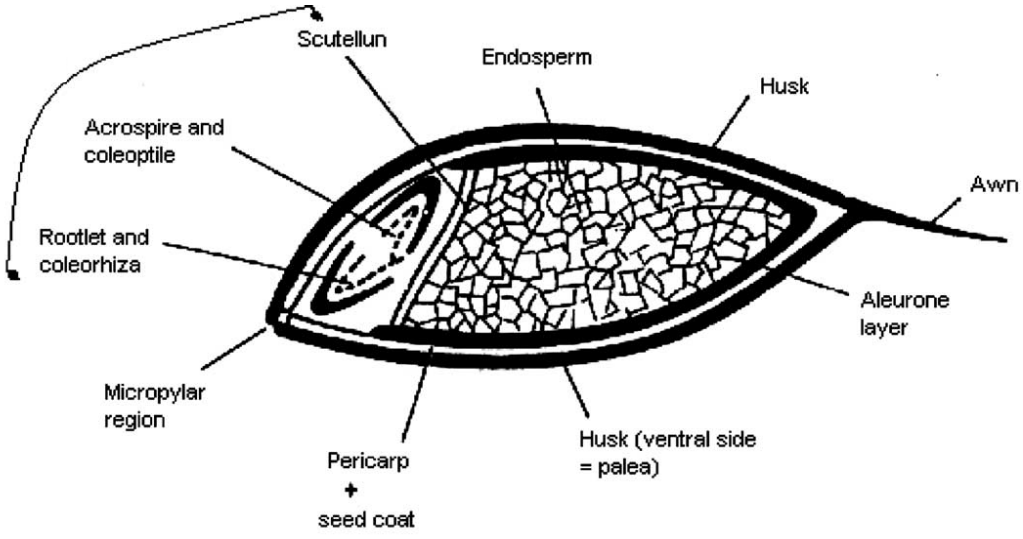


Figure1.1. Major parts of the barley kernel. Adapted from: Lewis & Young, 1995

As shown in Figure 1.1, embryo is located at the proximal end of the seed. Endosperm consisting of aleurone and starchy endosperm is the main source of nutrients for the growing embryo. The starch endosperm, the white color part in the middle of the seed is

protected by aleurone layer that contains protein and enzymes required to digest the endosperm. Embryo and endosperm are enclosed in the grain coverings; seed coat, pericarp and hull (husk). The outermost layer hull is specialized to protect the grain. The hull in hull-less barley is mostly detached during harvesting, thus have higher digestibility than hulled barley. The enzymes in aleurone, mainly amylase play an important role in digesting starchy endosperm to produce sugars during malting. The resulting soluble sugars make the major constituent of beer. In mashing, husk effectively filters soluble sugars from non-soluble fraction.

1.1.1. Barley proteins

The chemical composition of barley varies largely due to genetic and environmental factors. Data obtained from a study compared the chemical composition of four different hulled barley varieties are given in Table 1.1.

Table 1.1. Chemical composition of hulled barley grain varieties

Component (% dry matter)	Variety			
	Antek	Skarb	Nagradowicki	Granal
Starch	59.1	60.0	60.2	61.6
Fiber	18.2	20.0	19.6	21.5
Crude protein (N × 6.25)	13.6	12.5	13.0	11.7
Ash	2.1	2.2	2.3	2.6
Lipids	2.4	2.7	2.4	2.7
Sugars	3.0	2.6	2.6	2.9

Source: Biel & Jacyno, 2013

Similar to other cereals, the major constituents of barley are starch, fiber, and crude protein accounting for 59.1−61.6, 18.2−21.5, and 11.7−13.6% of dry matter, respectively.

These three components together cover more than 90% of dry matter of the grain. The remaining is made up of minor constituents: ash (2.1-2.6%), lipids (2.4-2.7%), and sugars (2.6-2.9%) (Biel & Jacyno, 2013). As the second most abundant component in barley, protein plays a significant role in determining the end use of barley.

Table 1.2. Amino acid profile of hulled barley grain varieties

Amino acid (g/16gN)	Variety			
	Antek	Skarb	Nagradowicki	Granal
Lys	3.4	3. 7	3.6	4.0
Met	1.6	1.6	1.6	1.6
Cys	1.3	1.3	1.5	1.4
Thr	2.9	3.4	3.1	3.2
Ile	3.6	3.4	3.1	3.3
Trp	1.2	1.1	1.2	1.3
Val	4.3	4.2	4.5	4.5
Leu	6.2	6.3	6.3	6.0
His	2.2	2.5	2.1	3.0
Phe	5.0	5.0	5.0	5.1
Tyr	2.5	2.6	2.5	2.5
Arg	4.0	3.8	4.4	4.0
Asp	5.9	5.6	5.9	5.6
Ser	4.0	4.0	4.2	4.0
Glu	25.8	23.8	24.8	24.5
Pro	9.3	9.7	9.3	10.0
Gly	3.8	3.6	4.0	3.5
Ala	3.9	4.0	3. 8	3.7

Source: Biel & Jacyno, 2013

Feed industry usually goes for high protein varieties aiming to obtain high feed conversion efficiency. In contrast, high protein varieties are not selected for beer making

as high protein grain usually has low starch content and also, solubilization of excessive proteins in wort causes haze formation in beer. As shown in the Table 1.2, barley proteins are characterized by relatively high proline and glutamic acid contents, but lacking in essential amino acids such as lysine and threonine (Biel & Jacyno, 2013).

Different protein fractions in the grain are essential in fulfilling nitrogen requirement of developing embryo during germination, metabolic and structural functions. Based on Osborne (1924) classification, barley proteins can be separated into albumin, globulin, hordein (prolamin), and glutelin according to their solubility in water, salt solutions, alcohol and alkaline, respectively. The relative proportions of protein fractions vary depending on barley variety, application of nitrogen fertilizer, and other cultivation practices.

Table 1.3. The relative proportions of protein fractions

Protein fraction	Weight percentage
Albumin	3-4%
Globulin	10-20%
Hordein	35–45%
Glutelin	35–45%

Source: Lâsztity, 1984

As shown in the Table 1.3, barley grain was reported to contain 3-4% albumin, 10-20% globulin, 35–45% each hordein and glutein of total proteins (Lâsztity, 1984). The protein reservoir of barley seed is the starchy endosperm filled with storage proteins hordein and

glutelin. The remaining proteins; albumin and globulin are located in aleurone and embryo functioning as enzymes and structural components of cell walls.

Hordein, the prolamin group in barley is further subdivided into five groups: A, B, C, D and γ based on their mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid composition. The sulfur rich B hordein of molecular weight 32-46 kDa is the largest group accounting for 70-80% of total hordeins. The second largest group, C hordein (49-72 kDa) which is low in sulfur covers 10-20% of hordeins. The D hordein (>100 kDa) together with sulfur rich γ hordein (< 20 kDa) is only a 5% fraction of total hordeins. The smallest polypeptide group, A hordein includes fractions less than 15 KDa (Shewry et al., 1985; Shewry & Tatham, 1990; Qi et al., 2006). The amino acid profile is an important determinant of functional, and structural properties of hordein. The intra and interchain disulphide bridges are formed between cysteine residues thus, cysteine poor C hordein naturally exists as monomers. Although, γ hordein and small part of B hordein are in the monomeric form, there are intrachain disulphide bonds formed between cysteine residues of the same chain. Rest of the B hordein and D hordein form polymer aggregates by linking the peptide chains through interchain disulphide bridges. The monomeric forms can be extracted with aqueous alcohols but, the interchain disulphide bonds retard extraction of polymeric hordeins without a reducing agent (Shewry & Tatham, 1990; Shewry, 1993; Shewry et al., 1994; Sheehan & Skerritt, 1997; Celus, 2006). Not only the functional and structural properties, but also the nutritional value is highly depending on the amino acid composition. Hordein is considered an incomplete protein source due to poor lysine content (Lange et al., 2007). Although, methionine is low in hordein, availability of cysteine makes hordein a

source of sulfur amino acids. Generally, nonpolar and polar uncharged residues in the structure limit water solubility of hordein while, is however solubility in 75% alcohol (Shewry & Miflin, 1985; Kreis & Shewry, 1989).

Glutelin, the second storage protein also accumulates only in the starchy endosperm accounting for 35–45% of total proteins (Lâsztity, 1984). Similar to hordein, it is rich in glutamine (21.0%) but, low in proline (2.1%). Also, sulfur content of glutelin is the lowest among the barley protein fractions with no cysteine and only 1.9% of methionine in the structure (Linko et al., 1989). In sequential extraction, glutelin is the final protein fraction separated. Thus, it can be contaminated with residues of other proteins while, its structure is susceptible to be altered by previous treatments. Upon alkali extraction, glutelin can be denatured to different extend depending on the strength of treatment. Inability to isolate pure glutelin due to above mentioned factors may have made glutelin the least studied barley protein.

The metabolic proteins albumin and globulin located in embryo and aleurone are dissolved in dilute salt solutions, later they can be separated with dialysis based on their differential water solubility. Albumin and globulin are the most important protein fractions in deciding beer quality attributes including flavor, haze formation and foam stability (Fox & Henry, 1995). The water-soluble albumin, also known as leucosin represents 3-4% of total barley proteins (Lâsztity, 1984). It consists of three sub units: α-amylase, protein Z, and lipid transfer proteins with molecular weights ranging between 14-58 kDa (Linko et al., 1989). The globulin (edestin) fraction, which comprises of 10-20% of total proteins (Lâsztity, 1984), is further divided in to four sub groups namely, α, β, γ, and δ (Go¨rg et al., 1992). As shown in the Table 1.4, glutamine is the most

abundant amino acid in barley proteins, followed by glycine and asparagine (Linko et al., 1989).

Table 1.4. Amino acid profile of barley protein fractions, expressed as mol %

Amino acid	Albumin[a]	Globulin[a]	Hordein[b]				Glutelin[a]
			D	C	B	γ	
Ala	9.3	7.9	3.2	1.5	2.6	2.1	10.0
Asx	10.6	10.5	1.5	1.5	0.7	2.4	11.5
Cys	6.5	5.6	1.5	-	2.9	3.5	-
Glx	19.2	14.5	28.0	3.8	32.1	30.1	21.0
Gly	10.8	12.7	15.7	0.6	2.9	3.1	12.5
Ilu	3.8	3.0	0.7	3.4	4.4	3.8	6.0
Leu	6.8	8.4	4.1	8.6	8.0	7.0	10.9
Lys	3.4	7.8	1.2	0.9	0.7	1.8	0.8
Met	2.5	1.6	0.4	-	1.1	1.8	1.9
Phe	3.6	4.4	1.3	7.7	4.7	5.6	5.3
Pro	6.1	7.3	10.5	29.1	19.4	16.8	2.1
Ser	6.7	7.9	10.5	2.5	4.7	5.6	9.7
Tyr	4.4	3.0	4.2	1.8	2.6	2.1	1.1
Val+Thr	6.4	5.5	5.3	0.9	6.9	8.0	7.2

Source: [a] Linko et al., 1989; [b]Lange et al., 2007

1.2. Brewing process

History of brewing or production of beer from starchy plant parts runs as far to the ancient Egyptian and Mesapotamian civilizations. Since, progression of small scale or cottage level beer making to the industry scale, it became an important economic contributor in leading beer producing countries. In 2018, the world beer production was estimated 1.94 billion hectoliters with the main contribution from China, the United States, and Brazil (Statista, 2018). The main ingredients used in brewing are fermentable

starch source and water. Apart from most widely preferred starch source barley, less popular sources such as wheat, rice, millet, cassava and sorghum. are also fermented in brewing. In most cases, starch hydrolysis is facilitated by the activity of brewers' yeast while, hops are added to create the specific flavor. Sometimes, sugar and secondary starches are added in aiming to reduce production cost and to improve favorable properties of beer.

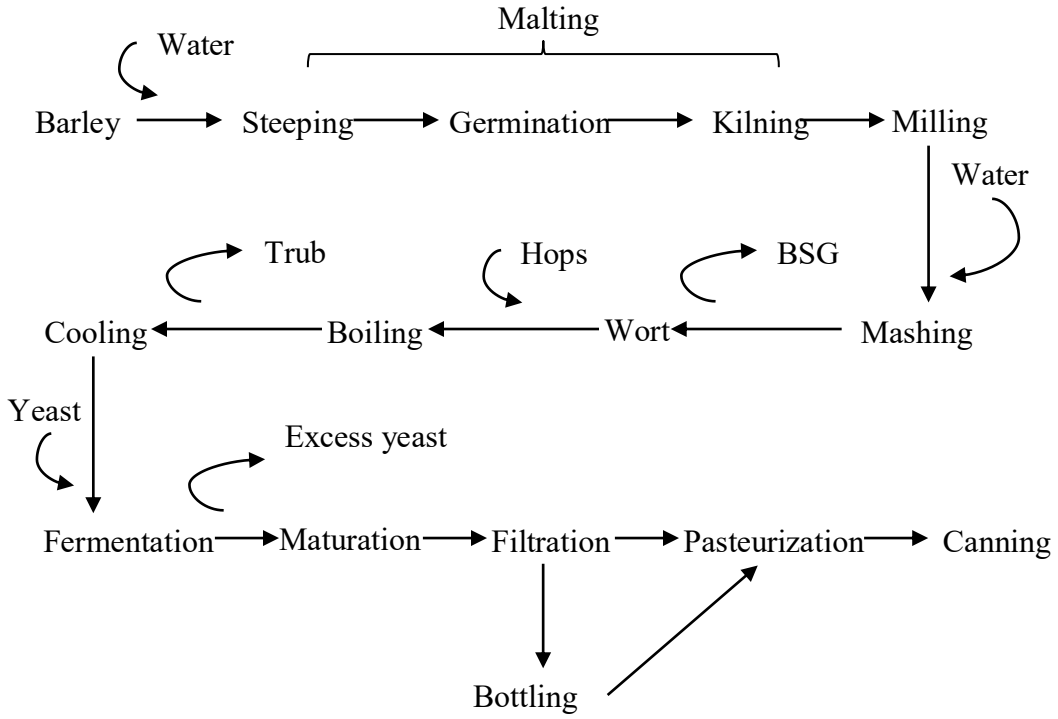


Figure 1.2. Schematic representation of commercial brewing process

As shown in Figure 1.2, brewing process consists of several steps: malting, mashing, lautering, boiling, fermenting, conditioning, filtering, and packaging. Malting is defined as the controlled germination of cereals, to ensure a given physical and biochemical change within the grain, which is then stabilized by grain drying. Barley is malted in three steps: steeping, germination and drying (kilning) to activate endogenous barley

enzymes and to hydrolyze endosperm to different degrees. During steeping, barley is mixed with water and allowed to soak between 5-18 ^{o}C for about 48 hours. Water enters to the seed increases the moisture content of grain from 12% to at least 40%, initiating the aleurone metabolism. Germination starts with spreading the moist grain on the floor or germination vessel. During, 5-7 days of germination period, synthesis and activation of enzymes in the aleurone and starchy endosperm happen. Activity of endogenous enzymes fully or partially modify the endosperm by the end of germination. Drying at 40-60 ^{o}C for several hours is done mainly to reduce moisture content so, the malted barley can be stored without microbial spoilage. Also, numerous flavor compounds, that are later dissolved in the wort are generated. Then, malted barley is milled to expose starch and sugars stored in the endosperm. In mashing, milled malt is dissolved in water followed by gradually increasing the temperature of the mixture from 37 to 78 ^{o}C to facilitate further hydrolysis of constituents. There, mainly starch is broken down to fermentable sugars while, proteins are partially degraded to peptides and amino acids. The resulting sugar rich liquid or wort is filtered in the lautering process to be fermented in next step. The residue removed after wort filtration is known as brewers spent grain, the first and most abundant by-product generated in brewing. The wort transferred to the brewing kettle is boiled with hops and sometimes with secondary ingredients. The main aim of boiling is to cease enzyme activity. Also at this stage precipitation of proteins, concentration of wort, as well as development of specific flavoring, aroma and color compounds from constituents extracted from hops take place. Once the boiling is completed, wort is settled in whirlpool to separate trub containing insoluble hop components, and precipitated proteins. After that, wort is pumped to a plate heat exchanger to rapidly cool down to a

temperature at which yeast can survive. The cooled wort is filled to a fermentation tank, followed by addition of selected varieties of yeast. Yeast begins the fermentation process to convert sugars to ethanol and carbon dioxide. The fermented wort is called beer and is transferred to maturation tank for aging after removal of spent yeast. Aging is the process in which compounds responsible for strong flavor are removed to make beer with smooth flavor. The conditioning period may be weeks or months depending on the type of the beer produced. Finally, beer is filtered, pasteurized and filled in to bottles or cans (Mussatto & Roberto, 2005; Gupta et al., 2010).

1.2.1. Modification of barley proteins in brewing

Modification of barley proteins starts at malting stage. Although, the main changes to the proteins happen during malting, the later step mashing also gives a significant contribution. In malting, endosperm of the barley kernel is degraded by series of endogenous enzymes. As a result, endosperm cell walls, starch granules, and complex protein structures are hydrolyzed releasing break down products from the bound matrix. When considering proteins, especially the storage proteins in the starchy endosperm, hordein and glutelin are partially decomposed to peptides by endoproteases, and to amino acids by carboxypeptidases (Osman et al., 2002). Although, the activity of these enzymes is not yet fully known, five seine carboxypeptidases and at least 40 different endoproteinases belong to four common proteinase classes were identified in germinating barley (Mikola, 1983; Zhang & Jones, 1995). According to Jones & Budde (2005), cysteine proteases clearly have the highest efficacy in solubilizing proteins during malting, which is almost similar to the activity of metallo proteases. This is followed by aspartic proteases and lastly, serine proteases with little or zero activity. These

endoproteases are not inactivated during malt kilning or mashing so, have potential to hydrolyze storage as well as other proteins during mashing (Jones et al., 2000; Jones & Marinac, 2002). Similar to the carboxypeptidases in the endosperm, aminopeptidases in scutellum release amino acids by protein hydrolysis. Except hydrolyzing to amino acids and peptides, barley proteins are also folded during malting. This is a function of the catalytic activity of disulfide isomerase to form disulfide bridges (Steiner, 2011). Poyri et al., (2002) observed formation of gel protein aggregates by oxidation of free thiol groups of proteins to disulfide bridges during mashing. Furthermore, another study suggested that C hordeins are entrapped in disulfide linked aggregates formed by B hordeins during mashing (Celus et al., 2006). On the other hand, the same study implied that naturally existing interchain disulphide bridges are reduced and B and D hordein aggregates are proteolytically hydrolyzed during malting. Although, the storage proteins are mainly solubilized in wort after proteolytic hydrolysis to form peptides and amino acids, the water-soluble proteins, albumin and globulin passed to the wort remain almost intact or modified to limited level due to their resistant nature to proteolysis, temperature and pH (Slack et al., 1979; Evans & Hejgaard, 1999; Osman, 2003). For an instance, barley proteins, Z and LTP1 survive in the brewing process relatively intact, retaining their apparent molecular weights of 43,000 and 9,700 Da (Evans & Hejgaard, 1999). The insoluble barley proteins remain with BSG, the residue after malting process account for approximately 15-24% (w/w) on dry basis (Mussatto & Roberto, 2005). The brewing process is also designed to removes extra proteins solubilized in the wort. Firstly, proteins coagulated during wort boiling are removed in whirlpool. Next, proteins are precipitated by decrease of pH due to ethanol formation in fermentation. Finally, excess

proteins stick to brewers' yeast can be removed (Bamforth, 1999). At the end of brewing process, it is accounted that no more than one third of the total barley grain proteins are passed into the finished beer (Steiner et al., 2011).

1.2.2. Beer Proteins

The total protein content in beer including proteins, peptides and amino acids is approximately 500 mg/L (Hejgaard & Kaersgaard, 1983). These fractions are fallen in between the molecular weight range of 5 to 100 kDa (Sorensen & Ottesen, 1978) with majority of molecular weights 10 to 40 kDa (Leiper et al., 2003). The amino acids and peptides are mainly originated from endogenous proteases hydrolysis of storage proteins, hordein and glutelin during malting and mashing. Water soluble proteins, albumin and globulin passed to beer mostly intact or somewhat modified due to their resistant nature to proteolysis (Slack et al., 1979; Evans & Hejgaard, 1999; Osman et al., 2003). Although, hordeins are known to be insolubilized in water, Taner et al., (2013) identified hordeins in some of the beer varieties tested. The impact of proteins to quality of beer such as flavor, haze formation, foam stability, texture, color, and nutritional value has been studied. Beer foam is an important quality parameter and is characterized by its stability. Two albumins; protein Z and lipid transfer protein 1 (LTP1) have ability to stabilize beer foam (Hao, 2006). Also, polypeptides rich in hydrophobic moieties are foam active thus, hordein peptides that are rich in proline and glutamine are considered important foam stabilizers (Shewry, 1993). Comparatively, albumin is more foam active than that of the hordein fractions (Kapp & Bamforth, 2002). Beer haze is first formed as chill haze when polypeptides and polyphenols are bound non-covalently at 0 °C. This is solubilized and disappeared at temperatures above 20 °C. Chill haze is sometimes

converted to permanent haze when insoluble complexes are formed by covalent linkages (Siebert, 1996). Permanent haze is considered a quality problems in beer varieties that are expected to be very clear but, is a desirable aspect in certain other beer varieties. Several studies observed high proline content in proteins present in beer haze, so proline was further studied in relation to haze formation. Previous study observed that haze stability is a function of proline distribution within the protein structure (Outtrup, 1987). Another study reported that major haze active proteins are proline rich peptides in the molecular weight range of 15–35 kDa (Nadzeyka, 1979). Apart from haze formation and foam stability, impact of proteins and their hydrolyzed products on other quality characteristics like flavor were also found important.

1.3. Brewers spent grain

Brewers' spent grain removed after mashing is the most abundant brewing by-product accounting for 85% of total by-products generated. Approximately, 20 kg of wet BSG is generated per 100 L of beer produced (Reinold, 1997). This largely available low value by-product is mainly used as animal feed, and the remaining being deposited in landfill. Mostly, it is made up of the residues of malted barley after removing water soluble components, and sometimes residues of adjuncts such as wheat, rice or maize added during mashing (Reinold, 1997). Figure 1.3 shows visual and electron microscopic appearance of BSG.

Figure 1.3. (A) Visual (B) Scanning electron microscopic (x100) (C) Scanning electron microscopic (x300) appearance of BSG (Scanning electron microscopic images adapted from Mussatto et al., 2006).

1.3.1. Chemical composition of BSG

The soluble components extracted to wort include most of the starch and a portion of proteins in barley. Thus, leaving water insoluble proteins, lipids, minerals and cell wall residues of the husk, pericarp and seed coat consisting cellulose and non-cellulose polysaccharides and lignin with BSG (Townsley, 1979). However, incomplete mashing process may remain fractions of starchy endosperm and walls of empty aleurone cells with BSG (Mussatto et at., 2006). Apart from the conditions applied in brewing, the chemical composition of BSG is largely determined by several other factors including agricultural practices, genetic and environmental factors, as well as quality of grains. BSG contains considerably high moisture content, varies between 72-76%, covering approximately three fourth of its composition (Niemi et al., 2012; Vieira et al., 2014). Table 1.5 compares the chemical composition of BSG on dry basis as reported by several authors.

Table 1.5. Chemical composition of BSG, expressed as % dry matter.

Component	BSG[a]	BSG[b]	BSG[c]	BSG[d]	BSG[e]	
					Pale	Black
Protein	15.2	24.0	26.4	22.8	23.1	26.9
Lipid	nd	10.6	7.5	11.0	13.5	10.0
Ash	4.6	2.4	4.1	4.7	3.3	2.1
Carbohydrates						
Non-starch carbohydrates	-	-	-	-	34	nd
Cellulose	16.8	25.4	9.9	-	-	-
Arabinoxylan	28.4	21.8	26.2	22.2	-	-
Beta-glucan	-	-	10.4	17.1	-	-
Starch	-	-	-	-	1.5	0.85
Lignin	27.8	11.9	-	19.3	23.4	nd
Polyphenols	-	-	-	-	1.7	2.6

Source: [a] Mussatto & Roberto, 2005; [b] Kanauchi et al., 2001; [c] Vieira et al., 2014; [d] Niemi et al., 2012; [e] Connolly et al., 2013.

According to Mussatto et at., (2006), BSG is a lignocellulosic material rich in protein and fiber. As a result of removal of starch in malting and mashing, other components get concentrated in BSG. Carbohydrates including cellulose, arabinoxylan, beta-glucan, and traces of starch comprise around half of the dry mass of BSG. Lignin which is a polyphenolic macromolecule has been found to vary between 12-27% in BSG (Kanauchi et al., 2001; Mussatto & Roberto, 2006). Mostly, the insoluble protein in barley remains with BSG, making around 15-27% of dry weight (Mussatto & Roberto, 2006; Connolly et al., 2013). The lipid and mineral content of BSG range between 7-13% and 2-5% of dry weight, respectively (Kanauchi et al., 2001; Niemi et al., 2012; Connolly et al., 2013; Vieira et al., 2014). Approximately, 25% of BSG minerals are in the form of silicates (Kunze, 1996). Husk is the source with considerable amount of silica and most of the

polyphenolics in barley grain (Macleod, 1979). Thus, it can be expected that husk to be the major BSG fraction consisting of minerals and phenolic compounds. BSG phenolics, mainly ferulic and *p*-coumaric acids belong to the group of hydrocinnamic acid (Faulds et al., 2002).

1.3.2. BSG protein

The barley protein ranges between 11.7−13.6% of dry matter is partially modified during malting and mashing (Biel & Jacyno, 2013). After separation from wort, BSG was observed to contain all four protein fractions to different degrees with hordein being the major part. Approximately, 65% of total proteins are remained in BSG without getting solubilized in wort (Celus et al., 2006). Despite the fact that all four protein fractions in barley kernel are solubilized in the wort to different degrees, BSG has high protein content compared to that of barley. The increase of protein level from around 11.7−13.6% to 15%-24% protein (w/w) on dry basis is due to removal of sugars yielded from hydrolysis of carbohydrates, mainly starch stored in the endosperm (MacGregor & Fincher, 1993; Kanauchi et al., 2001; Mussatto & Roberto, 2005; Biel & Jacyno, 2013). The BSG can be expected to contain structurally modified proteins compared to that of barley. In detail, it may include gel protein aggregates formed by oxidation of free thiol groups to disulfide bridges and C hordeins entrapped in disulfide linked aggregates formed by B hordeins (Poyri et al., 2002; Celus et al., 2006; Steiner, 2011).

Table 1.6. Amino acid composition of barley and BSG

	Barley[a]	BSG[b]
Amino acids (g /100 g)		
Asp	5.9	6.4
Glu	25.8	21.4
Ser	4.0	4.3
His	2.2	2.4
Gly	3.6	3.3
Arg	4.0	4.2
Ala	3.8	6.3
Pro	9.3	10.3
Val	4.3	5.3
Thr	2.9	3.7
Met	1.6	1.9
Cys	1.3	1.1
Phe	5.0	5.4
Ile	3.6	3.9
Leu	6.2	10.7
Lys	3.4	2.9
Tyr	2.5	nd
Trp	1.2	nd

Source: [a]Biel & Jacyno, 2013; [b]Ajanaku et al., 2011

Table 1.6 compares the amino acid profiles of barley and BSG. Accordingly, the amino acid profile of BSG is considerably different from that of barley due to solubilization of proteins, peptides and amino acids in wort. In terms of nutrition perspective, the essential amino acids represent approximately 35% of the total protein content in BSG. However, Ajanaku et al., (2011) didn't report the tryptophan content of BSG. Glutamine is the most abundant amino acid in BSG followed by leucine and proline with content of 21.4, 10.7, and 10.3 g /100 g respectively. Depending on the type and quality of barley used as well

as the conditions applied in brewing, protein composition of BSG can vary significantly thereby, modifying the amino acids profile of BSG.

1.3.3. Extraction of BSG protein

Several methods have been tested to optimize the extraction of BSG protein. However, extraction of most of the BSG protein without damaging to their native structures is still in experiment. The most common approaches are alkaline extraction or protease aided extraction. Also, pre-treatments such as hydrolysis with carbohydrases and physical treatments to break down cell walls were tested in aiming to release proteins from bound matrices and cells walls. Niemi et al., (2013) observed 76% of protein solubilization when milled BSG was pre-hydrolysed with carbohydrate degrading enzyme (*Humicola insolens* Depol 740L) followed by alcalase aided extraction, and 53% of protein solubilization when extracted without alcalase. However, the drawbacks associated with protease aided extraction are inability to precipitate all the solubilized protein as it contains small peptides and amino acids, and alteration of the native properties of protein with hydrolysis.

Sequential extraction can be applied to separate protein fractions from BSG. In detail, the sequential extraction starts with water followed by salt solution, 75% alcohol, and alkaline to remove albumin, globulin, hordein, and glutelin in respective order (Osborne, 1924). Although, the monomeric hordeins can be extracted with aqueous alcohols, the polymeric forms containing interchain disulphide bonds can't be extracted without a reducing agent. Since, BSG protein contains hordein polymers resulted from C hordeins entrapped in disulfide linked aggregates formed by B hordeins and insoluble B

and D hordein polymer aggregates present in barley, alcohol together with reducing conditions are expected to facilitate hordein extractability (Celus et al., 2006).

Since, alkali conditions are known to solubilize all four protein fractions together, they can be applied to extract BSG protein (Bishop, 1928). It has been shown that 41% (w/w) protein can be recovered on dry basis, when extracted with 0.1 M NaOH for 1h at 60 ^{o}C (Celus et al., 2007). In a research studying the effect of NaOH concentration (40-200 mM), temperature (20-60 ^{o}C) and substrate to solvent ratio (1:15-1:40) on protein extraction from BSG, the most efficient conditions were found to be 100 mM, 60 ^{o}C and 1:20 respectively (Connolly et al., 2013). Facilitation of protein release at elevated temperatures and alkalinity could be due to loosen cell wall matrix to release bound proteins. However, high alkalinity and temperatures may hydrolyze and even denature proteins, changing their native physicochemical and functional properties. Not only the proteins, but also other compounds such as polyphenols and lignin can be released at these conditions. If BSG is previously dried and not used wet, it may need even higher alkalinity and temperatures to break interactions formed during drying (Niemi et al., 2013). In addition, despite the harsh extraction conditions, the protein yields may be as low as 20–30% (Chiesa & Gnansounou, 2011). The pH requires for iso-electric precipitation is another important determinant of protein yield. Previous studies used different precipitation pH values: 3.8 (Connolly et al., 2013), 4.0 (Celus et al., 2007) and 5.3 (Wu et al., 1979).

1.3.4. Protease hydrolysis of BSG protein

Lack of solubility at neutral pH is a barrier that limits the more extensive use of BSG protein. The alkaline extracted BSG protein precipitated at pH 4.0, shows the

maximum solubility at alkaline pH. Below pH 8.0, the solubility gradually declines, giving rise to the lowest around pH 4.0. According to Bilgi & Celik (2004) it is only around 20% solubility at pH 7.0. Protease hydrolysis is often applied to increase the solubility of proteins. The increased solubility could be due to decreased molecular weight, unfolding or surface exposure of polar groups (Cellus et al., 2007). With increased solubility, it expands the potential applications of BSG protein as gelling, emulsifying, foaming, water holding, binding agents. Also, biological properties are expected to modify due to increased solubility as well as generation of different amino acid sequences.

Proteases are the enzymes that catalyze break down of proteins into peptides and amino acids by cleaving peptide bonds. Exopeptidases like aminopeptidase and carboxypeptidase hydrolyze terminal peptide bond of the protein chain while, endopeptidases like alcalase, trypsin, pepsin only cleave the internal bonds. The proteases having both endo and exopeptidase activities act on both internal and external peptide bonds. For an instance, flavourzyme is an endo and exopeptidase and it was reported to yield high degree of hydrolysis percentages compared to that of alcalase and pepsin belong to endopeptidases (Bamdad et al., 2011). Based on the catalytic residue, proteases are categorized into seven groups: serine, cysteine, threonine, aspartic, glutamic, metallo and asparagine peptide lyase proteases. Table 1.7 describes some of the widely used commercial proteases.

Table 1.7. Description of some of the widely used proteases

Enzyme	Origin	Enzyme type	Conditions	
			Temperature (^{o}C)	pH
Flavourzyme	*Aspergillus oryzae*	Endo & exopeptidase	50	5-7
Alcalase	*Bacillus licheniformis*	Endopeptidase	30-65	7-9
Neutrase	*Bacillus amyloliquefaciens*	Endopeptidase	40-50	7
Everlase	*Bacillus sp.*	Endopeptidase	30-70	8-11
FoodPro PHT	*Geobacillus sp.*	Endopeptidase	65-75	6-9
Trypsin	Pancreatic cells	Endopeptidase	37	7.8-8
Chymotrypsin	Pancreatic cells	Endopeptidase	37	7.8-8
Pepsin	Gastric mucosa	Endopeptidase	37-42	1.5-2.0
Papain	Papaya latex	Endo & exopeptidase	70-90	5-7.5
Bromelain	Pineapple stem	Endopeptidase	40-65	6-8.5
Savinase	*Bacillus sp.*	Endopeptidase	30-70	8-10
Esperase	*Bacillus sp.*	Endopeptidase	30-70	8-10
Carboxypeptidase	Pancreatic cells	Exopeptidase	37	7.8-8
Aminopeptidase	*Aeromonas proteolytica*	Exopeptidase	37	7.8-8

Source: Product sheet-flavourzyme (2001); Proteases for biocatalysis (2016); Food protease enzymes selection guide (2019)

Naturally, proteases are involved in several biological reactions to digest protein food, biodegrade plant and animal material, and continue cell signaling pathways. Also, they are playing an important role in a wide range of industry applications such as detergent, leather, silver recovery, dairy, baking, beverages, meat and pharmaceutical. Benefits of using proteases in industry scale include higher yields, mild reaction conditions, lower energy costs, shortened synthesis routes, reduction of waste products and less residual solvents due to reduced solvent usage.

1.3.5. Potential applications of brewers spent grain

Despite the fact that BSG is a largely produced, readily available by-product rich in valuable chemical components, it receives a marginal commercial value. Besides, it is considered an environmental burden due to difficulties in disposal. Considerable amount of BSG goes to animal feeding and the rest is deposited in landfill. Since last few decades, several research attempts have been made to discover the potential commercial applications of BSG. The high nutrient content particularly, protein and fiber make it a potential feed ingredient. Huige (1994) suggested BSG as an excellent feed ingredient for ruminants since it can be combined with inexpensive nitrogen sources like urea. In addition, BSG incorporated diet given to cows was reported to increase milk yield, milk total solids and milk fat (Sawadogo et al., 1989; Belibasakis & Tsirgogianni, 1996; Reinold, 1997). Not only as a ruminant feed ingredient, it has also potential to be incorporated into feed of other animals.

According to Huige (1994), BSG is a suitable candidate as a food ingredient due to several reasons including high fiber and protein contents, low fat, lower calorie content compared to most cereal flours, high water absorption capacity, presence of valuable

minerals such as Ca, P, Fe, Cu, Zn and Mg, and mildly roasted aroma. However, it was found too granular when directly incorporated into flakes, whole wheat bread, biscuits, and aperitif snacks thus, must be converted to flour first (Hassona 1993; Miranda et al., 1994a, Miranda et al., 1994b; Ozturk et al., 2002). A high protein flour prepared from BSG was successful in several bakery products (Townsley, 1979; Huige, 1994). Consumption of BSG incorporated products may be associated with health benefits: increase fecal weight, accelerate transit time, increase cholesterol and fat excretion and decrease in gallstones (Fastnaught, 2001). Therefore, BSG is not only a potential food ingredient rich in protein and fiber, but also an agent providing several health benefits.

Recently, numerous attempts have been made to extract valuable components concentrated in BSG such as ferulic and *p*-coumaric acids, xylose, arabinose, protein, lignin, and beta-glucan. Ferulic and *p*-coumaric acids, arabinoxylan, and beta-glucan have potential applications as health promoting compounds in food and nutraceutical industries. However, use of suitable extraction methods to release these compounds from bound matrices without altering the natural structures is of importance. The precursors of BSG polysaccharides can be yielded upon hydrolytic degradation. Cellulose is hydrolyzed to glucose, whereas non-cellulose polysaccharides hydrolyzed to xylose, arabinose, mannose, galactose, acetic and hydroxycinnamic acids (Palmqvist & Hahn-Hagerdal, 2000; Mussatto & Roberto, 2004). Some of these products can be used as energy sources in microbial fermentation, while some others can be fermented to generate valuable compounds like xylitol, which is an alternate to sucrose. BSG protein can potentially be incorporated into food to increase protein value, and as a source of essential amino acids and bioactive compounds. Also, techno-functional properties of

BSG protein can be used in food industry to give rise emulsifying, forming, gelling, and flavor binding properties. Poor solubility that limits the applications of BSG protein can be overcome by applying chemical, enzyme or physical treatments.

Other potential applications of BSG include energy production, paper manufacture, substrate for cultivation of microorganisms and enzyme production. In energy production, BSG can be directly combusted or aerobically fermented to generate biogas. However, direct combustion needs pre-drying to reduce moisture content. Also, another disadvantage associated with direct combustion is emission of toxic gases like sulfur dioxide and oxides of nitrogen. The efficiency of aerobic fermentation can be increased to a certain extent by breaking BSG into small particles by a suitable method. High fiber content in BSG makes it suitable to convert into paper and paper based products. BSG has been successful as the substrate for cultivation of mushroom species including *Pleurotus, Agrocybe* and *Lentinus* (Schildbach et al., 1992). Further studies are a necessity to find the suitability of BSG as the substrate to cultivate a wide variety of microorganisms. Several research attempts have been made to find the suitability of BSG as the nitrogen and energy source in production of enzymes, suggesting its suitability as a substrate for microbial enzyme production. For an instance, Bogar et al., (2002) used BSG as the substrate in production of alpha amylase by *Aspergillus oryzae.*

Although, BSG has several potential commercial applications, its applicability is limited due to high susceptibility to microbial spoilage characterized by high moisture and nutrient contents. Drying is one of the methods that can be used to preserve BSG by reducing moisture content and even killing microbes. However, use of high temperatures might destroy valuable, heat sensitive components.

1.4. Antioxidants

Antioxidants are the molecules that inhibit oxidation of other molecules through multiple pathways. They can interfere with oxidation process by scavenging free radicals, chelating catalytic metals ions or by donating protons to reduce reactive species. Over the past several decades, antioxidants such as vitamin E and C, phenols, peptides, selenium have been widely studied. Incorporation of natural as well as synthetic antioxidants to foods and nutraceuticals is practiced in aiming to retard oxidation of biological molecules and associated negative changes. Reactive species mainly included chemically active oxygen containing molecules such as superoxide radical, hydrogen peroxide, and hydroxyl radical and also, nitrogen containing molecules such as nitric oxide and peroxynitrite radicals. In biological systems, reactive species accumulated in elevated quantities may trigger oxidative stress. This is caused by generation of reactive species by endogenous and exogenous sources in elevated levels, or a significant decrease in the effectiveness of antioxidant defense mechanisms. The ultimate result of oxidative stress could be the destruction of biological molecules such as DNA, proteins and lipids causing diseases like atherosclerosis, neurodegenerative diseases and cancers. Not only the biological molecules in living systems, but also unsaturated lipids in food and feed are also subjected to oxidation. Autoxidation of polyunsaturated lipids involves free radical chain reactions affecting nutritional, textural and sensory properties of food.

The sources of reactive species in aerobic systems are cellular respiration and environmental factors, such as air pollutants or cigarette smoke. Mitochondria generates ATP through series of oxidative phosphorylation reactions. During this process, one or two electron reductions may occur instead of four electron reductions, leading to

formation of $O_2^{\bullet-}$ or H_2O_2, and later, H_2O_2 can be converted to $OH^{\bullet}$ through Fenton reaction when ferrous ion is available. The $OH^{\bullet}$ triggers chain reactions to produce various other free radicals causing irreversible damages to biological molecules. Aerobic organisms have integrated systems, that consist of enzyme and non-enzyme antioxidants effective in suppressing destructive effects of reactive species. However, pathological conditions may cease the antioxidant systems leading to accumulate reactive species. The major enzymatic antioxidants are superoxide dismutase, catalase, and glutathione peroxidase. Non-enzymatic antioxidants mainly include vitamins C and E, β-carotene, uric acid and glutathione (Birben, 2012).

The nutritional quality, wholesomeness, safety, color, flavor, and texture of lipid containing foods and feed are largely affected by autoxidation. The autoxidation of polyunsaturated lipids is usually initiated by light, heat, ionizing radiation, metal ions, and lipoxygenase enzyme.

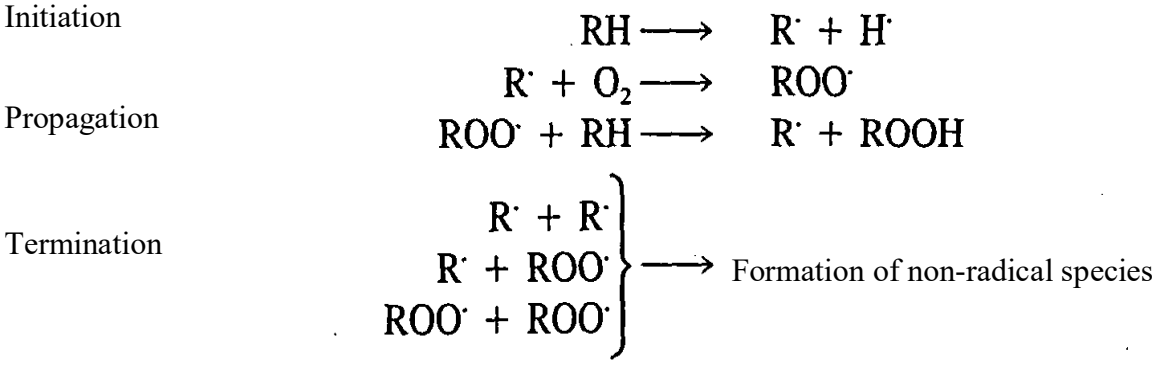


Figure 1.4. The route of autoxidation reaction

Adapted from: Shahidi et al., 1992

As shown in the Figure 1.4, autoxidation reaction happens in three distinct steps: initiation (production of lipid free radicals), propagation and termination (production of

non-radical products). The most commonly applied antioxidants in retarding lipid oxidation include butylated hydroxytoluene, hydroxyanisole and propyl gallate (Shahidi et al., 1992).

1.4.1. Antioxidant activity of proteins, peptides and amino acids

There is a growing interest in identifying antioxidant properties of plant and animal proteins, as they are generally considered safe, available, known for functional and nutritional properties. Protease hydrolysis may produce different amino acid sequences having ability to suppress reactive species. The antioxidant activity of protein hydrolysates has been reported from different sources including soy protein (Moure et al., 2006), fish protein (Mendis et al., 2005; Sun et al., 2013), zein protein (Kong & Xiong, 2006; Li et al., 2008), rice endosperm protein (Zhang et al., 2009), flaxseed protein (Marambe et al., 2008), whey protein (Pena-Ramos et al., 2004), egg yolk (Park et al., 2001), chickpea protein (Li et al., 2008; Ghribi et al., 2015), barley hordein (Bamdad et al., 2011) and wheat gluten (Kong et al., 2008). Protein hydrolysates act as antioxidants through multiple pathways including participation in redox reactions, scavenging free radicals, and chelating pro-oxidant metal ions. These activities are closely related to their structural and chemical properties: amino acid composition, peptide sequences, surface hydrophobicity, size of the peptides and interactions with other components in the matrix, which are mainly determined by the protein source, pre-treatments and hydrolysis conditions.

One of the key factors in determining the antioxidant activity of a protein hydrolysate is amino acid composition. The hydrolysates containing higher percentage of hydrophobic amino acids: phe, val, leu, Ile and met may have significantly higher

antioxidant activity. For an example, Pownall et al., (2010) related strong superoxide radical scavenging activity of pea peptides to proton donation ability of hydrophobic amino acids. However, very high hydrophobic amino acid content might be associated with reduced antioxidant properties due to poor solubility of proteins. Beside the hydrophobic nature, the same study observed higher amount of aromatic amino acids in protein fractions with high radical scavenging and metal chelating properties. Donation of protons to electron deficient species, while maintaining stability via resonance structures gives aromatic amino acids an ability to scavenge free radicals. Histidine was observed to involve in several antioxidant mechanisms. For an example, the chickpea peptides with reducing power, free radical scavenging and cellular antioxidant activities contained histidine in the sequences (Torres-Fuentes et al.,2015). Similarly, histidine supplemented diets prevented copper induced oxidative stress in grass carp (Jiang et al., 2016). The comparatively high proton donor activity of histidine is believed to be due to presence of imidazole ring in the side chain (Mendis et al., 2005). Upon proteolytic degradation, surface hydrophobicity is generally increased due to surface exposure of hydrophobic amino acids buried in the core of proteins. The surface exposed hydrophobic amino acids are expected to elevate antioxidant activity through donation of protons to neutralize reactive species. Amino acid sequence, an indication of interactions between the amino acids in a peptide sequence is also an important factor in determining the antioxidant capacity. Table 1.8 shows some of the amino acid sequences identified to have potent antioxidant activities through multiple mechanisms.

Table 1.8. Amino acid sequences identified to have potent antioxidant activities

Protein source	Assay	Amino acid sequence	Reference
Barley hordein	DPPH, superoxide radical scavenging	QPYPQ	Bamdad, & Chen, 2013
Chicken egg white	ORAC	AEERYP, DEDTQAMP	Nimalarathne et al., 2015
Tilapia gelatin	Hydroxyl radical scavenging	LSGYGP	Sun et al., 2013
Rice residue protein	DPPH, ABTS radical scavenging, FRAP	RPNYTDA, TSQLLSDQ, TRTGDPFF, NFHPQ	Yan et al., 2015
Oyster	DPPH radical scavenging	LANAK,	Umayaparvathi et al., 2014
Chickpea protein	DPPH radical scavenging	PSLVGRPPVGKLTL, VGDI, DHG	Ghribi et al., 2015

Adapted from: Zou et al., 2016

The secondary structures stabilized by intra and intermolecular hydrogen bonds are denatured by proteases to varying degrees depending on the strength of hydrolysis. There are only a limited number of studies analyzed the role of secondary structures on antioxidant activity thus, still the data is scarce on the effect of secondary structures on antioxidant mechanisms. Molecular weight distribution or size of the fractions in hydrolysate is another structural feature that changed upon protease hydrolysis. Several studies have attempted to show and explain the relationship between molecular weight and the antioxidant activity. Xia et al., (2012) studied the antioxidant activities of barley gluteline hydrolysate, separated into different molecular weight fractions: >10 kDa, 1–10 kDa and <1 kDa by ultra-filtration. Accordingly, the large peptides (>10 kDa) showed higher DPPH scavenging activity and reducing power, whereas peptides below 1kDa demonstrated greater hydroxyl radical scavenging and Fe^{2+} chelating activity. Notably, these fractions exhibited higher antioxidant activities than the hydrolysate before separation. In another study, casein derived peptides below 1000 Da exhibited the best antioxidant activities in 2, 2′-azinobis (3-ethylbenzothiszoline-6-sulphonic acid) diammonium salt (ABTS+), hydroxyl radical scavenging and ORAC (Chen & Li, 2012) assays. Therefore, it can be assumed that effect of molecular weight on antioxidant capacity varies depending on the antioxidant mechanism. Although, hydrolysis is generally considered to increase antioxidant activity, Tironi et al., (2010) observed partial loss of linoleic acid inhibition capacity of naturally occurring amaranth peptides after protease hydrolysis. This signifies the importance of controlled hydrolysis in producing antioxidant peptides.

1.4.2. Antioxidant activity of phenols

Phenolic compounds are large and heterogeneous group of secondary plant metabolites. Phenols possess one or more hydroxyl groups bonded to an aromatic ring. The structures of natural phenols vary from simple molecules such as phenolic acids to highly polymerized compounds such as condensed tannins (Table 1.9; Harborne, 1980).

Table 1.9. The major classes of phenolic compounds

Carbon	Skeleton	Class	Examples
6	C6	Simple phenols	Catechol
		Benzoquinones	
7	C6-C1	Phenolic acids	Gallic acid
8	C6-C2	Acetophenones	3-Acetyl-6-methoxybenzaldehyde
		Tyrosine derivatives	
		Phenylacetic acids	
9	C6-C3	Hydroxycinnamic acids	Ferulic
		Phenylpropenes	
		Coumarins	
		Isocoumarins	
		Chromones	
10	C6-C4	Naphthoquinones	Juglone
13	C6-C1-C6	Xanthones	Mangiferin
14	C6-C2-C6	Anthraquinones	Emodin
		Stilbenes	
15	C6-C3-C6	Flavonoids	Quercetin
		Isoflavonoids	
18	$(C6-C3)_2$	Lignans	Pinoresinol
		Neolignans	
30	$(C6-C3-C6)_2$	Biflavonoids	Amentoflavone
n	$(C6-C3)_n$	Lignins	Tannic acid
	$(C6)_n$	Catechol melanins	
	$(C6-C3-C6)_n$	Condensed tannins	

Source: Harborne, 1980

The polyphenol rich foods include sorghum, millet, barley, peas, apple, berries, grapes, onion, tea, wine etc. Antioxidant properties of phenolic compounds are achieved mainly via two main mechanisms firstly, suppression of reactive oxygen and nitrogen species synthesis by inhibiting specific enzymes or chelating trace metals involved in free radical generation and secondly, donating protons to scavenge radical species such as reactive oxygen and nitrogen species (Figure 1.5). The phenoxy radical formed as an intermediate of the reaction is relatively stable due to resonance and therefore, a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates act as terminators of propagation route by reacting with other free radicals (Dai & Mumper, 2010).

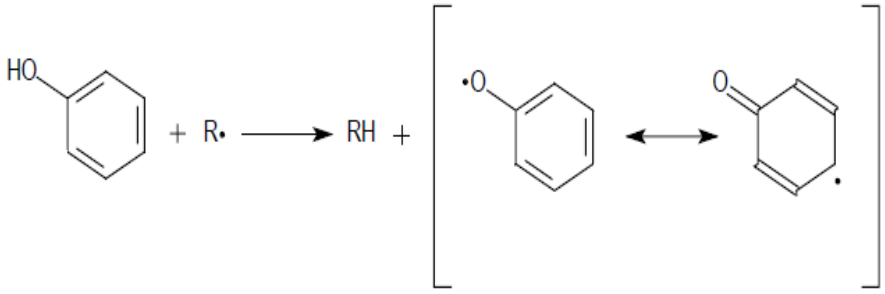


Figure 1.5. Formation and stabilization of phenoxy radical intermediate

Adapted from: Dai, & Mumper, 2010

1.4.3. Assessment of antioxidant activity

Measurement of antioxidant activity is not a straightforward process due to heterogeneous nature of antioxidant compounds. A variety of *in vitro* techniques such as, uv-visible spectroscopy, gas chromatography, mass spectroscopy and high-performance liquid chromatograph are commonly used in assessing antioxidant activity and quantifying metabolites having antioxidant properties. Despite wide usage of these *in*

vitro methods, their ability to predict *in vivo* activity has not been demonstrated. Most of the chemical antioxidant assays do not reflect cellular physiological conditions and do not consider the bioavailability, uptake, and metabolism of the antioxidants. Biological systems are much more complex than the chemical mixtures and antioxidant compounds may operate via multiple mechanisms. At the same time, there might be interactions and synergies between antioxidants from foods and antioxidant enzymes in the body influence the overall antioxidant status. On the other hand, sometimes antioxidants may act as prooxidants at higher consumption levels. The animal models and human studies are the best methods in determining the actual efficacy of antioxidants in the body, but they are expensive and time consuming and not suitable for initial antioxidant screening.

1.4.3.1. Spectroscopic assays in measuring antioxidant activity

Antioxidants may suppress reactive species through various pathways. Thus, assessment of different antioxidant mechanisms such as metal ion chelation, suppression of reactive oxygen species, scavenging free radicals, and participating in redox reactions are expected to give better understanding of the activity. Some of the most widely accepted spectroscopic assays in measuring antioxidant activity are reducing power, DPPH radical scavenging activity, metal chelating activity and superoxide radical scavenging capacity. Generally, antioxidant activity of a sample is expressed in relative to that of standards measured under the same conditions.

1.4.3.1.1. Reducing power assay

Total antioxidant capacity can be measured using reducing power assay. This assay quantifies the ability of antioxidants to reduce ferric irons to ferrous ions. As shown

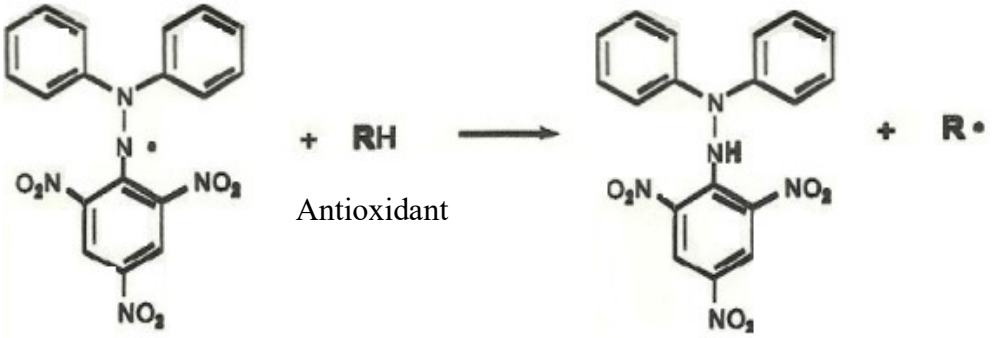
below, the antioxidant compound (ArOH) participates in a redox reaction with potassium ferricyanide (Fe^{3+}) to form the reduced form potassium ferrocyanide (Fe^{2+}) and the oxidized form of the antioxidant ($ArOH^{+}$).

$$K_3[Fe(CN)_6] + (ArOH) \longrightarrow K_4[Fe(CN)_6] + (ArOH+)$$

As a result, yellow color of potassium ferricyanide is converted to various shades of green color of potassium ferrocyanide. The samples with greater proton donation ability form high concentration of potassium ferrocyanide giving high absorbance at 700 nm. Therefore, absorbance is directly proportional to reducing power or antioxidant activity of the sample at 700 nm. However, if the reaction mixture contains other compounds having reduction potential except antioxidants such as high oxidation status metal ions, those compounds also may take part in the reaction giving rise to an over estimation to total antioxidant activity.

1.4.3.1.2. Free radical scavenging activity by DPPH assay

This assay measures the ability of a compound to act as an antioxidant in a non-aqueous medium by measuring reduction of synthetic radical DPPH. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. It can be scavenged by an antioxidant through donation of a proton forming reduced DPPH (Figure 1.6). The color changes from purple to yellow after reduction is quantified by measuring decrease of absorbance at wavelength 517 nm. The degree of discoloration indicates the free radical scavenging potentials of the sample (Gupta et al., 2013**)**. The antioxidant compound after donation of a proton remains with an un paired electron but, fairly stable nature prevents it from acting as an oxidizing agent.



DPPH radical

Reduced DPPH

Figure 1.6. Reduction of DPPH radical by an antioxidant

Adapted from: Gupta et al., 2013

1.4.3.1.3. Ferrous ion chelating activity assay

Ferrous ion chelating ability of the sample can be measured with the method proposed by Kong & Xiong (2006). Ferrous ions together with ferrozine form a pink color chromophore, ferrous-ferrozine complex that can be spectrophotometrically measured at 562 nm (Figure 1.7).

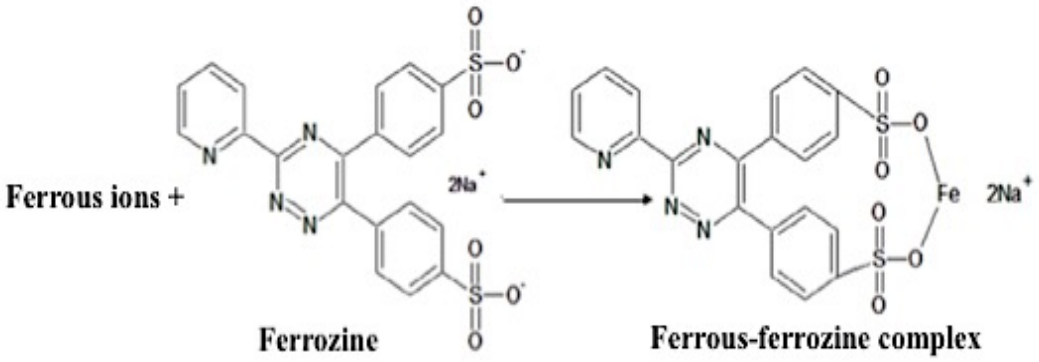


Figure 1.7. Formation of ferrous-ferrozine complex

Adapted from: Santosa et al., 2017

However, in presence of the antioxidant compounds, formation of the metallic complex is disrupted due to binding of Fe^{2+} with antioxidants. This leads to a decrease in the absorbance at 562 nm. Therefore, color intensity is an estimation of the binding ability of the extract. Higher the absorbance at 562 nm, weaker the ferrous iron binding ability or the antioxidant activity of the sample and vice versa.

1.4.3.1.4. Superoxide radical scavenging activity assay

Among other methods used to analyze superoxide radical scavenging activity such as cytochrome c reduction, nitrotetrazolium blue chloride and high-performance liquid chromatography, spectrophotometric monitoring of pyrogallol (1,2,3-trihydroxybenzene) autoxidation inhibition is widely used as it doesn't require expensive instruments, chemicals or biological agents. Pyrogallol autoxidizes to produce superoxide radicals through a complicated pathway (Figure 1.8).

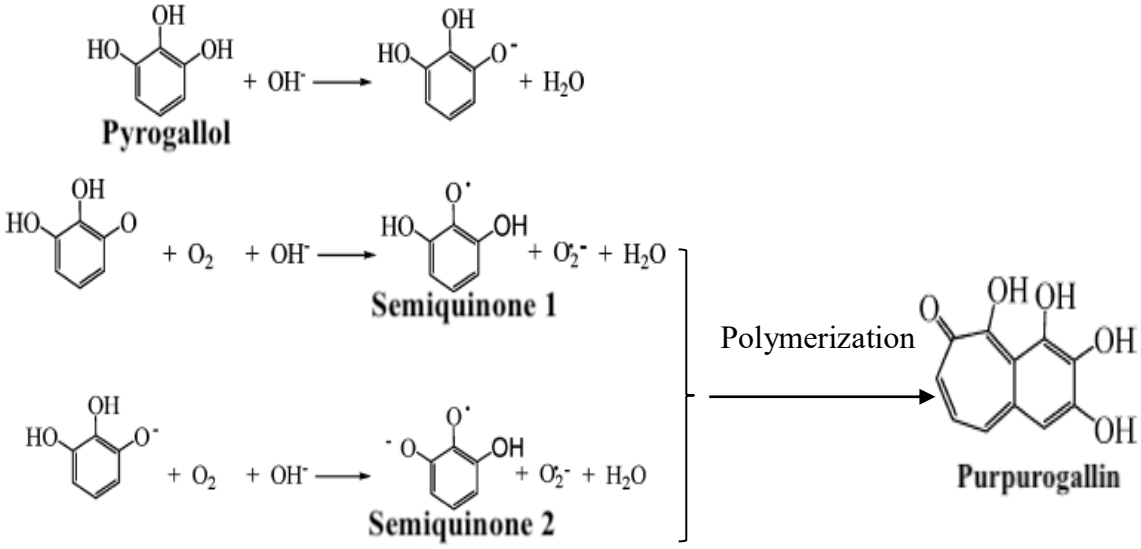


Figure 1.8. Pyrogallol autoxidation reaction to form superoxide radical

Adapted from: Li, 2012

Briefly, in mild alkaline medium pyrogallol produces semiquinones (1 and 2) and superoxide radicals as intermediates by reacting with oxygen. Semiquinones are polymerized to form purpurogallin, which can be detected at 320 nm by measuring the emission, thus it is an indication of both purpurogallin and superoxide radical generation. Reduction of emission after 5 min signifies the superoxide radical scavenging capacity of test compound (Li, 2012). Monitoring of pH around 8.3 is important to facilitate mild alkaline conditions to support the autoxidation reaction.

1.4.3.1.5. Total phenol content by Folin-Ciocalteu assay

Folin-Ciocalteu reagent contains phosphomolybdic and phosphotungstic acid complexes. The assay relies on transfer of electrons from phenolic compounds to phosphomolybdic and phosphotungstic acid complexes in alkaline medium to form blue color complexes that can be measured spectrophotometrically at 760 nm (Singleton & Rossi, 1965; Singleton et al., 1999). Gallic acid is widely used as the comparison standard. The other oxidizing substrate present in a given extract sample can interfere the total phenolic measurement in an inhibitory or additive manner (Singleton et al., 1999; Singleton & Rossi, 1965). The inhibitory effect could be due to the oxidizing substrates competing with Folin-Ciocalteu reagent after the sample is made alkaline. For this reason, the Folin-Ciocalteu reagent is added ahead of alkali (Singleton et al., 1999). Additive effect occurs from compounds such as aromatic amines, high sugar levels, sulfites, sulfur dioxide or ascorbic acid in the samples. However, despite these disadvantages, the Folin-Ciocalteu assay is simple and reproducible and has been widely used for quantification of phenolic compounds in plant materials.

1.5. Summary of the key justifications for the research

Based on above the literature review, BSG could be a promising natural source of antioxidant peptides. As reported by Ajanaku et al., (2011), BSG is a source of histidine (2.4%), which is known for antioxidant properties. Histidine involves in several antioxidant mechanisms including free radical scavenging and cellular antioxidant activities (Torres-Fuentes et al., 2015). Although, methionine is not detected, it is notable that BSG contains high amount of other hydrophobic amino acids: phe, val, leu, and Ile. They are known antioxidants, probably because of the ability to stabilize free radicals by donating protons (Pownall et al., 2010; Chi et al., 2015; Nourmohammadi et al., 2017). Also, aromatic amino acids: tyrosine and phenylalanine may give BSG peptides the radical scavenging and metal chelating properties due to their ability to donate protons to electron deficient species while maintaining stability via resonance structures (Pownall et al., 2010). Protease hydrolysis is an effective means of generating bioactive peptides from BSG proteins. Barley hordein hydrolysate contains the peptide QPYPQ with DPPH and superoxide scavenging activities (Bamdad & Chen, 2013). It is reasonable to expect antioxidant effects of protein hydrolysates from BSG. Moreover, peptides have several mechanisms to inhibit oxidation of molecules, including chelating metal ions suppressing reactive oxygen species, scavenging free radicals, and participating in redox reactions. Therefore, BSG protein hydrolysates have potential to be used as antioxidant ingredients in food, feed and nutraceuticals.

1.6. Hypothesis and objectives

Based on the knowledge generated in the previous studies, this thesis research tested the following hypotheses: Enzymatic hydrolysis of BSG proteins can release

peptides with antioxidant effects. The overall objective of this research was to develop peptides with antioxidant activities from BSG proteins by enzymatic hydrolysis.

The specific objectives were:

1. To develop a processing to extract proteins from BSG
2. To prepare protein hydrolysates from BSG by enzymatic hydrolysis and characterize their structures
3. To study the antioxidant activities of BSG protein hydrolysates in relation to their structures.

This project is expected to bring significant economic, health and environmental benefits. The targeted beneficiaries include barley growers, nutraceutical sector, feed producers, food industries mainly including brewing. If BSG protein hydrolysates contain significant antioxidant properties, they have a potential to be used as antioxidant components in food and nutraceuticals. This will not only increase the economic value of BSG, but also promote public health. Similarly, the BSG protein hydrolysates with antioxidant activity can be potentially developed as animal feed additive to reduce or suppress oxidative stress in livestock and consequently improve animal performance, feed efficiency and productivity. Although, it can be widely seen that small scale livestock farmers use BSG as a feed ingredient, the commercial feed preparations hardly contain BSG as an ingredient. Confirmation of antioxidant properties of BSG protein would be an encouragement to use this readily available, low cost by-product in commercial feed formulas. Other than reduction of feed production cost, it will further encourage livestock farmers to get BSG directly from brewery gates for feed. On the other hand, analysis of different antioxidant pathways in relation to the structural and

chemical properties of BSG protein and hydrolysates would allow rational design of peptide structure from BSG with improved antioxidant effects for food and feed applications.

Chapter 2.0

Development of hydrolysates with antioxidant effects from brewers' spent grain proteins

2.1. Introduction

The cereal grain barley (*Hordeum vulgare L.*) was one of the first agriculturally domesticated grains as early as 10,000 years ago. According to FAO statistics, barley was the fourth largest produced cereal grain in the world in 2017, accounting for 141 million tonnes (FAOSTAT, 2017). Apart from its main use as animal feed, major food applications include brewing. Barley, the primary grain source used in brewing, contains 10-12% protein (w/w) on dry basis (MacGregor & Fincher, 1993). Hordeins (A, B, C, D) also known as barley prolamins are the main barley protein, and the rest consists of glutelins, globulins and albumins. In brewing, malted barley is produced by controlled germination of barley for a short period of time. This is followed by mashing to remove soluble constituents, mainly the sugars resulted from starch hydrolysis. Although, barley proteins are partially hydrolyzed to amino acids and peptides by endogenous proteases during malting, part of the proteins remain insoluble. On the other hand, due to removal of carbohydrates by endosperm solubilization, the protein content is increased in mashing. Brewers' spent grain (BSG) removed after mashing is the most abundant brewing by-product accounting for 85% of total by-products generated (Reinold, 1997). It is reported to contain 15%-24% of protein (w/w) on dry basis (Kanauchi et al., 2001; Mussatto Roberto, 2005). Except D hordeins, BSG constitutes all the other protein fractions in barley, but in different quantities. The most abundant protein fraction, hordeins (A, B, and C) comprises more than 50% of the proteins. This is followed by

glutelins and less abundant proteins including albumins and globulins. Furthermore, it was suggested that C hordeins are entrapped in disulfide-linked aggregates formed by B hordeins during mashing (Celus et al., 2006). Although, numerous attempts have been made to explore the commercial applications of BSG, still the main use of this protein rich by-product is low value animal feed, with the rest deposited in landfill. Approximately, 20 kg of BSG is generated per 100 L of beer produced (Reinold, 1997). Numerous benefits could be gained by identifying higher value-added applications of BSG.

With increasing research evidences to associate oxidative stress to occurrence and progression of chronic diseases such as neurodegenerative diseases, type 2 diabetes and coronary heart diseases, the consumers' interest in antioxidant containing foods as well as antioxidant supplements are increasing. Oxidative stress, is created by continuous exposure to reactive species or decreased functioning of antioxidant defense mechanisms like catalase, glutathione peroxidase and vitamin E. Higher degree of oxidative stress can destruct macromolecules in biological systems such as proteins and DNA. In living organisms, reactive oxygen species are produced in cellular metabolism. Superoxide radical and hydrogen peroxide are formed in electron transport chain and Fenton reaction further produces hydroxyl radical from hydrogen peroxide when ferrous ion is available. Exogenous reactive species include oxygen and nitrogen species produced by environmental pollutants. On the other hand, autoxidation of polyunsaturated lipids in food systems involves a free radical chain reaction leading to undesirable sensory properties and deterioration of nutrition quality of foods. Natural as well as synthetic antioxidants are incorporated to lipid containing foods and nutraceuticals to avoid

undesirable effects of reactive species. Over the past several decades, natural antioxidants such as polyphenols, vitamin C, carotenoids, proteins and peptides have attracted considerable consumers' attention as they are generally considered safe, less expensive compared to synthetic antioxidants and availability.

The antioxidant activity of protein hydrolysates has been reported from different sources such as soy protein (Moure et al., 2006), fish protein (Mendis et al., 2005; Sun et al., 2013), rice endosperm protein (Zhang et al., 2009), whey protein (Pena-Ramos et al., 2004), egg yolk (Park et al., 2001), chickpea protein (Li et al., 2008; Ghribi et al., 2015), and barley hordein (Bamdad et al., 2011). Peptides can exert antioxidant effects through multiple mechanisms including chelating metal ions such as ferrous, suppressing reactive oxygen species, scavenging free radicals, and participating in redox reactions. These activities are closely related to structural and chemical properties of the peptides. The BSG protein may be a potent source of antioxidants due to its high hydrophobic amino acid content: phe, val, leu, Ile and met. In addition, QPYPQ in barley protein showed good DPPH and superoxide scavenging activities (Bamdad & Chen, 2013). Thus, it is expected that BSG might be a good protein source to prepare hydrolysates with antioxidant effects.

The objective of this study was to analyze the effect of protease hydrolysis in modifying structural properties and improving antioxidant activity of brewers' spent grain protein. Proteases with different optimum conditions and specificities were used to prepare hydrolysates. Resulting hydrolysates were analyzed for amino acid composition, secondary structures, molecular weight distribution, surface hydrophobicity, and solubility. The peptide antioxidant activities were measured by reducing power, DPPH

radical scavenging activity, ferrous chelating activity and super oxide radical scavenging assays. The effect of BSG peptide structural and conformational properties in relation to their antioxidant effects was discussed. Although, several studies reported the antioxidant activity of plant proteins and their hydrolysates, data is hardly available about the impact of polyphenols. Due to protein-polyphenol interactions, plant protein extracts may contain polyphenols that are known antioxidants even at minor concentrations. Therefore, this study also evaluated the effect of total polyphenol content on antioxidant activities. Furthermore, the commercial antioxidants; BHT, L-ascorbic acid and EDTA were used as positive controls to understand the potential of using BSG protein hydrolysates as antioxidant ingredients in food applications.

2.2. Materials and Methods

2.2.1. Materials

Three BSG samples from different batches were obtained from a local brewing company (Edmonton, Alberta, Canada) and stored at -20 ^{o}C until used. FoodPro PHT was obtained from DuPont Industrial Biosciences (Denmark). Flavourzyme (500 U/g), alcalase (≥ 2.4 U/g), everlase (≥ 16 U/g), neutrase (≥ 0.8 U/g), sodium dodecyl sulphate (SDS), tri-nitro benzene sulfonic acid (TNBS), L- leucine, fluorescent dye 8-anilino-1-naphthalene sulphonic acid (ANS), Foiln- Ciocalteau reagent, gallic acid, 1,1- diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxytoluene (BHT), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulphonic acid)-1,2,4- triazine (ferrozine), ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), and standard molecular markers for HPLC analysis (protein standard mix, 15 - 600 kDa; thyroglobulin, 670 kDa; beta- amylase, 200 kDa;

BSA, 66 kDa; alpha- amylase, 51 kDa; ovalbumin, 44.3 kDa; cytochrome C, 12.3 kDa; and aprotinin, 6.5 kDa) were obtained from Sigma Aldrich (St. Louis, MO, USA). L-ascorbic acid, potassium ferricyanide, and pyrogallol were obtained from Fischer Scientific (Edmonton, AB, Canada). All the other chemicals were of analytical grade.

2.2.2. Extraction of BSG protein

Brewers spent grain protein was prepared using alkali extraction followed by acid precipitation. In brief, BSG was milled passing through 0.5 mm sieve. Then the ground BSG powder was dispersed in 0.1 M NaOH to make 20% (w/v) solution. Protein was extracted at 50 ^{o}C for 2 h with continuous stirring. After 2 h extraction the supernatant was collected by centrifuging at 8,000×g for 15 min at 20 ^{o}C (Beckman Coulter Avanti J-E Centrifuge System, CA, USA). Protein was precipitated by centrifugation at 8,000×g for 15 min at 20 ^{o}C after adjusting to pH 4.0. The precipitated protein was freeze dried followed by storage at 4 ^{o}C until further used. Protein content was determined by nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) using nitrogen conversion factor 6.25. The extraction yield (EY%), and protein yield (PY%) were calculated using the following equations;

EY% = (Dry weight of freeze dried protein (g) / Dry weight of sample used (g)) × 100%

PY% = (EY% × Protein % of the freeze-dried extract) / Protein % of the sample

2.2.3. Preparation of BSG protein hydrolysates

An aqueous suspension of 2% (w/v) BSG protein was hydrolyzed with different proteases or protease combinations for 4 h. For treatments with combined enzymes, the pH and temperature were set up be between the ranges of individual enzymes. For

example, 50 ^{o}C and pH 7.0 were used in alcalase and neutrase combined treatment, as the conditions were common to both enzymes. (Table 1). The substrate was mixed with 4% (w/w) enzyme (based on protein dry weight). For treatment with two combined proteases, 2% (w/w) of each enzyme was added to the BSG suspension. The pH and temperature were monitored throughout the hydrolysis. At the end of hydrolysis, enzymes were inactivated by heating at 80 ^{o}C for 20 min. Centrifugation at 8,000×g for 15 min at 20 ^{o}C was done to separate solubilized protein from insoluble substances. The collected supernatant was adjusted to pH 7.0 and freeze dried.

2.2.4. Degree of hydrolysis % (DH%)

The DH% was determined according to TNBS method (Adler-Nissen, 1979) with modifications. Samples (50 µL) were mixed with 950 µL of 1 % (w/v) sodium dodecyl sulfate. It was heated at 75 ^{o}C for 20 min to inactivate enzymes. After inactivation of enzymes, 0.25 mL of sample was mixed with 2.0 mL sodium phosphate buffer (0.2 M, pH 8.2), and 2.0 ml of 0.1% (w/v) TNBS reagent, followed by incubation at 50 ^{o}C for 60 min in a water bath. At the end of incubation, 4.0 mL of 0.1 M HCl was added to terminate the reaction. The solutions were cooled to room temperature for 30 min, and the absorbance was measured at 340 nm. Standard curve was constructed using L-leucine ranging from 0 - 4.0 mM. The DH% values were calculated using the following equation:

$$DH\ \% = (h/h_{total}) \times 100$$

where h is the number of peptide bonds broken during hydrolysis (mM/g of protein) and h_{total} is the total amount of peptide bonds in BSG protein (7.75 mM/g of protein) (Celus, et al., 2006).

2.2.5. Fourier transform infrared (FTIR) spectroscopy

Samples were dissolved in D_2O to prepared 5% (w/v) solutions and kept shaking for 24 h to ensure complete H/D exchange. Prepared samples were placed between CaF_2 windows separated by a 25 mm polyethylene terephthalate film spacer. Infrared spectra were recorded at room temperature at a resolution of 4.0 cm^{-1} and a total of 128 scans for each sample (Nicolet 6700 spectrometer, Thermo Scientific, Madison, WI, USA). The spectrometer was continuously supplied with nitrogen. To study the amide I region (1600–1700 cm^{-1}) of the protein, Fourier self-deconvolutions were performed using the Omnic 8.1.210 software. Band narrowing was achieved with a full width at half maximum of 20 – 25 cm^{-1} and with a resolution enhancement factor of 2.0 – 2.5 cm^{-1}.

2.2.6. Solubility

Solubility was determined by weighing method. Aqueous samples of 1% (w/v) were stirred for 1 h after adjusting to pH 7.0. Then, the resulting solutions were centrifuged for 30 min at 4,000×g and 30 ^{o}C. Precipitates obtained after removing supernatants were freeze dried until reaching a constant weight. Solubility % was calculated using the following equation:

Solubility % = [1- (weight of precipitate/ weight of initial sample)] × 100

2.2.7. Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

Samples were analyzed for average molecular weight (M_w) by SE-HPLC using an Agilent 1100 series HPLC system coupled with a BiosuiteTM 125/5 mm HR-SEC column (7.8 300 mm, Waters Corp., Mass., USA). The mobile phase, phosphate buffer (0.1 M) at pH 7.0 containing 0.1 M NaCl was flowed at a rate of 0.5 mL/min at 25 ^{o}C.

Twenty microliters of samples were injected into the HPLC system and absorbance was monitored at UV wavelength of 220 nm. Standard molecular markers were also analyzed using the same procedure. Log M_w of the markers were plotted against and their respective elution times.

2.2.8. Surface hydrophobicity (H_0)

Surface hydrophobicity was determined using the method described by Kato & Nakai (1980) with modifications. All the samples and reagents were dissolved in 0.l M phosphate buffer at pH 7.4. First, samples were dissolved to prepare 0.1% (w/v) stock solutions followed by further dilutions to made concentration series ranging from 0.0025% to 0.04% (w/v). Twenty microliters of 8.0 mM apolar flurescent dye (ANS) was mixed with 4.0 mL of sample. Fluorescence intensity was measured after 15 min at excitation and emission wavelengths at 390 and 470 nm, respectively (Jasco FP-6300 spectrofluorometer Tokyo, Japan). Blank contained phosphate buffer instead of sample. The relative fluorescence intensity was plotted against concentration. Slope of the plot which is surface hydrophobicity was calculated by linear regression analysis.

2.2.9. Amino acid composition

For amino acid analysis, samples were dissolved in deionized water to a concentration of 10 mg/mL. Thirty microliters of each sample were dried and hydrolyzed under vacuum in 6 M HCl for 24 h at 110 ^{o}C. The hydrolyzed samples were again dried and then dissolved in 20 mM HCl. Amino acid analysis was done using Waters AccQ-Tag method. High-performance liquid chromatography system (Agilent series 1200, Palo Alto, CA, USA) equipped with AccQ-Tag 3.9 x 150 mm C18 column was used in

separating the derivatives. Samples were pumped at a flow rate of 1.5 mL/min using gradient solvent system (AccQ-Tag eluent, acetonitrile, and water), and finally detected at 254 nm wavelength. Data processing was controlled by ChemStation software.

2.2.10. Total phenol content

The total phenolic content was determined by Folin- Ciocalteu method according to the procedure reported by Singleton & Rossi (1965). Briefly, 100 µL of 1.0 mg/mL sample was mixed with 1.0 mL of 10% (v/v) Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 3 min, followed by addition of 800 µL of 7.5% (w/v) sodium carbonate. After 30 min incubation at room temperature, absorbance was measured at 765 nm against the blank. Blank contained all the reagents except the sample. Absorbance of gallic acid ranging from 0.04 to 0.2 mg/mL was used in developing the standard plot. Total phenol content was expressed as mg GAE (gallic acid equivalents)/mg of sample on dry weight basis.

2.2.11. DPPH radical scavenging activity

The radical scavenging activity of samples were evaluated by DPPH assay according to the method described by Blois (1958). One milliliter of samples (1.0 mg/mL) were mixed with 1.0 mL of 0.1 mM DPPH in anhydrous ethanol. The mixture was shaken vigorously and incubated at room temperature for 30 min under light protection. Ascorbic acid and BHT at concentrations of 0.1 and 0.01 mg/mL were used as positive controls. The reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm. The ability of the samples to scavenge DPPH free radicals was calculated according to the following equation;

% inhibition of DPPH radicals = $(A_0\text{-}A)/A_0$ x 100 %

Where A_0 and A represent the absorbance of the control and hydrolysate sample (control contained everything except hydrolysate), respectively.

2.2.12. Superoxide radical scavenging activity

Superoxide radical scavenging activity was measured by monitoring the inhibition of pyrogallol autoxidation and polymerization (Marklund, 1974). Briefly, 80 µL of 2.0 mg/mL sample was mixed with 80 µL of 0.05 M Tris–HCl buffer (pH 8.3) containing 1.0 mM EDTA, followed by addition of 40 µL of 1.5 mM pyrogallol in 1.0 mM HCl. Absorbance was measured at 320 nm for 5 min at room temperature against the blank. Tris–HCl buffer was used instead of samples in the blank. Ascorbic acid at 0.01 and 0.1 mg/mL was used as the positive control. Superoxide radical scavenging activity was calculated using following equation:

Superoxide radical scavenging activity = $[(\Delta A_0/\text{min}) - (\Delta A_s/\text{min})]/ (\Delta A_0/\text{min}) *100$

Where A_0 and A represent absorbance of the blank and the sample, respectively.

2.2.13. Ferrous ion chelating activity

The method described by Kong & Xiong (2006) was used in measuring ferrous ion chelating activity of the samples. Briefly, 1.0 mL of 20 µM $FeCl_2$ was mixed with 0.5 mL of 1.0 mg/mL sample, and then 1.0 mL of 0.5 mM ferrozine was added to initiate the reaction. Mixture was incubated at room temperature for 15 min prior to measuring absorbance at 562 nm. The EDTA at concentrations of 0.1 and 0.01 mg/mL were used as positive controls. Ferrous ion chelating ability was calculated by the following equation;

% Ferrous ion chelating ability = $(B_0\text{-}B)/B_0$ x 100 %

Where B and B_0 represent the absorbance of the sample and the control (distilled water instead of hydrolysates), respectively.

2.2.14. Reducing power

Reducing power was measured according to the method of by Oyaizu (1986). In short, 1.0 mL of 1.0 mg/L sample was mixed with 1.0 mL of 1% (w/v) potassium ferricyanide and 1.0 mL of 0.2 M phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. Reaction was stopped by adding 1.0 mL of 10% (w/v) TCA. After centrifugation at 1,000×g, 10 min at 20^0C, 1.0 mL of supernatant was collected. It was mixed with 1.0 mL of distilled water and 0.2 mL of 0.1% (w/v) $FeCl_3$. After 10 min reaction, absorbance of the resulting solution was measured at 700 nm. BHT and ascorbic acid at concentrations of 0.1 and 0.01 mg/mL were used as positive controls. The blank contained everything except the sample.

2.2.15. Statistical analysis

Results were given as mean ± standard deviation values from three independent experiments. SAS student version (SAS Institute, Inc., Cary, NC) was used in statistical analysis. The comparisons were made with one-way analysis of variance (ANOVA) followed by least significant difference (LSD) procedure. Differences were considered significant at $p < 0.05$. The correlations were evaluated by calculating the correlation coefficient (r) values using the excel CORREL function.

2.3. Results and discussion

2.3.1. Extraction and hydrolysis of BSG protein

The BSG had higher protein content compared to that of barley grain. The increase of protein content from 10-12% to 24% (w/w) on dry basis is due to removal of sugars yielded from hydrolysis of carbohydrates, mainly starch stored in the endosperm (MacGregor & Fincher, 1993; Kanauchi et al., 2001; Mussatto & Roberto, 2005). The protein extract of BSG contained 62.6% (w/w) of protein on dry basis. The extraction yield (EY%) and protein recovery (PY%) were 17.8% and 46.3% respectively. Usually, in mashing water soluble albumins and globulins are removed from barley to a greater extent. Albumins were identified to add unique flavor to beer together with other flavor compounds and also acting as a foaming agent (Hao, 2006). Also, hordein and glutelin hydrolyzed by endogenous barley proteases are solubilized in the wort during mashing (Osman et al., 2002). Although, hordeins are not known to be solubilized in water without getting hydrolyzed, Taner et al., (2013) identified hordeins in some of the beer varieties tested. Sodium hydroxide was selected as the extraction medium, aiming to solubilize all protein fractions in BSG including albumin, globulin, hordein, and gluteline (Osborne, 1924; Bishop, 1928). In a research, studying the effect of NaOH concentration (40-200 mM), temperature (20-60 ^{o}C) and substrate to solvent ratio (1:15 to 1:40) on protein extraction from BSG, the most efficient conditions were found to be 0.1 M, 60 ^{o}C and 1:20 respectively (Connolly et al., 2013). Thus, 0.1 M NaOH, and 1:20 substrate to solvent ratio were selected as the extraction conditions. The extraction was done at 50 ^{o}C temperature, instead of 60 ^{o}C to avoid any denaturation of proteins. However, 53.7% protein remained in insoluble component, likely due to the cellulose-protein interactions

in BSG restricting protein extraction. Also, previous work observed failure to extract B and C hordeins under non-reducing conditions, suggesting C hordeins are entrapped in disulfide linked aggregates formed by B hordeins during mashing (Celus et al., 2006). In the present study, reducing conditions such as dithiothreitol were not applied to increase protein extractability considering the safety of the final products.

The antioxidant activities of proteins are known to have significant variations upon proteolytic hydrolysis depending on the enzyme specificity and hydrolysis conditions applied. Therefore, BSG protein was hydrolyzed with selected proteases or protease combinations having different specificities under the optimum hydrolysis conditions for the selected enzymes (Table 2.1). Combined enzyme treatments were developed by combining two proteases with similar optimum pH and temperature ranges. The extent of hydrolysis was calculated by DH%, which expresses the number of peptide bonds cleaved as a percentage of total number of peptide bonds in the substrate. As shown in the table, DH% ranging from 5.9% to 24.1% was observed at the end of 4 h hydrolysis. Flavorzyme, the fungal protease showed to be the most effective with a DH% value of 24.07%. This is explained by endo and exopeptidase activity of flavorzyme, while all the other proteases used were endo peptidases. Other than hydrolyzing the internal peptides bonds, flavorzyme releases amino acids by hydrolyzing external peptide bonds. The proteolytic activity of flavourzyme was followed by A+F combined treatment reporting 23.2% of DH%, a value more towards flavorzyme treatment rather than alcalase treatment. The DH% values of other combined protease treatments were fallen in between the values of individual enzymes.

Table 2.1. Protease treatment conditions used to prepare BGS protein hydrolysates, and the relevant DH% values

Enzyme/Enzyme combination	Origin	Specificity	Conditions		DH%
			pH	Temp (^{o}C)	
Flavourzyme	*Aspergillus oryzae*	Acidic endo and exopeptidase	6	50	24.1[a]
Alcalase	*Bacillus licheniformis*	Alkaline endopeptidase specific	8	50	17.7[b]
Everlase	*Bacillus sp.*	Alkaline endopeptidase	9.5	50	8.1[f]
Neutrase	*Bacillus amyloliquefaciens*	Neutral endopeptidase	7	50	6.7[g]
FoodPro PHT	*Geobacillus sp.*	Thermostable endopeptidase	8	65	5.9[g]
Alcalase and neutrase (A+N)			7	50	11.4[e]
Alcalase and flavourzyme (A+F)			7	50	23.2[a]
Alcalase and everlase (A+E)			8	50	13.0[d]
Flavourzyme and neutrase (F+N)			7	50	15.3[c]
FoodPro PHT and everlase (P+E)			8	65	6.5[g]

Means with different superscript letters differ significantly at ($p < 0.05$)

In general, hydrolysates with higher DH% can be expected to have lower molecular weight peptides and vice versa. Not only the peptides with different molecular weights, but also the specificity op proteases might have resulted hydrolysates containing different amino acid sequences.

2.3.2. FTIR spectrum measurements

The amide I region (1700-1600 cm^{-1}) of FTIR spectrum measures C=O stretch vibrations, which is an indicator of protein secondary structures. Although, it is not fully understood, the knowledge about the likeliness of proteases to denature different secondary structures to varying degrees could be useful in designing properties of the hydrolysates depending on the end use. BSG protein showed several major peaks in amide I region, which were assigned to secondary structures (Kong & Yu, 2007): β-turn (1691, 1669, 1659 cm^{-1}), β-sheet (1679, 1630, 1620 cm^{-1}), and 3_{10} helix (1641 cm^{-1}). The most distinct peak at 1641 cm^{-1} is accompanied by a shoulder at 1648 cm^{-1} confirming the presence of random coils. The band at 1608 cm^{-1} is due to vibration of amino acid residues (Yu, 2006). Effectiveness of proteases in altering secondary structures can be interpreted by comparing the changes in spectrums. Accordingly, it can be seen that BSG hydrolysates were denatured by all the treatments, but to different degrees. In the case of everlase, neutrase, FoodPro PHT and P+E treatments, most of the characteristic absorptions disappeared, even though these hydrolysate samples showed relatively lower DH% (Figure 2.1). In the hydrolysates obtained from flavorzyme or flavorzyme combined (A+F, F+N) treatments, vibration of amino acid residues increased significantly (Figure 2.2). This confirms higher amount of amino acid residues exposed outside probably due to exopeptidase activity of flavourzyme. Similarly, upon

flavorzyme included treatments, increase of β-sheets at the wavelength 1620 cm^{-1} was identified. Alcalase treatment was only able to reduce the intensity of the peak identified at 1620 cm^{-1}, despite its greater ability to hydrolyze peptide bonds. The alcalase combined treatments: A+N and A+E were able to denature β-sheets identified at 1630 and 1620 cm^{-1}, while A+F treatment increased the intensity of the peaks at 1608 and 1620 cm^{-1} (Figure 2.3).

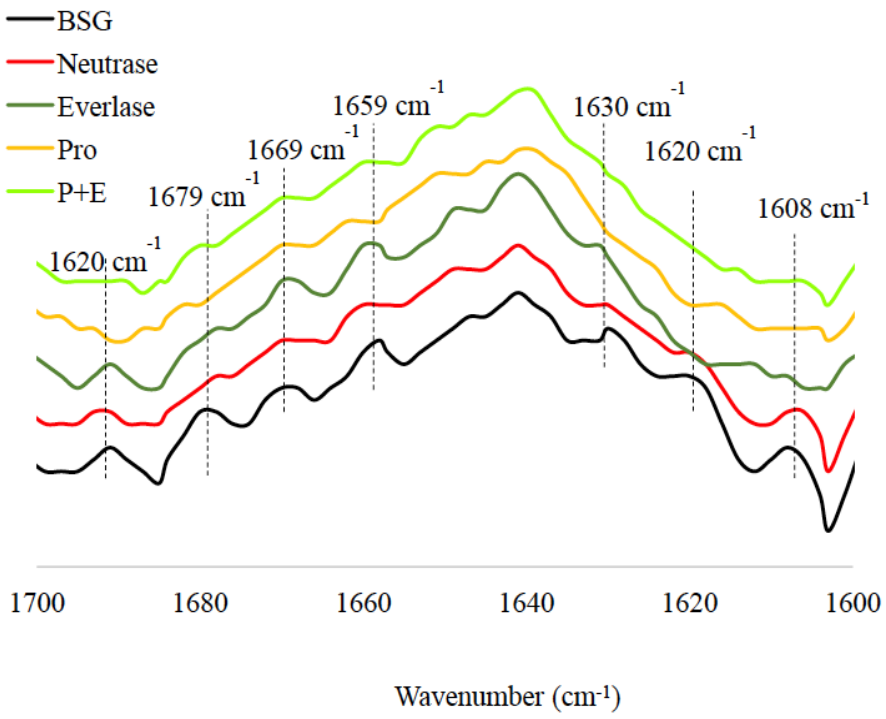


Figure 2.1. Disappearance of most of the characteristic absorbance of BSG protein upon everlase, neutrase, FoodPro PHT and FoodPro PHT+everlase treatments

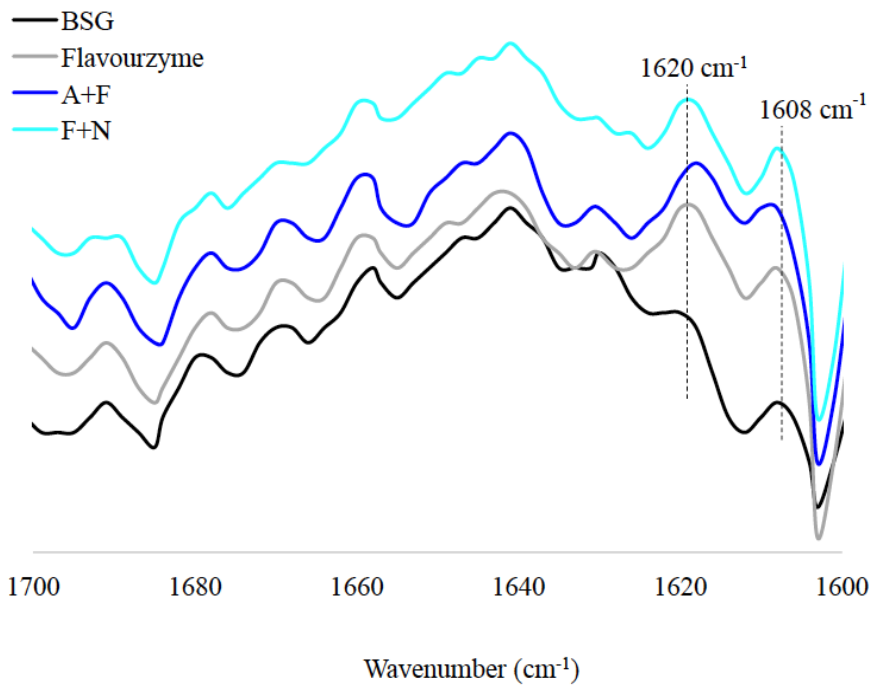


Figure 2.2. Increase of amino acid vibrations by flavorzyme containing treatments

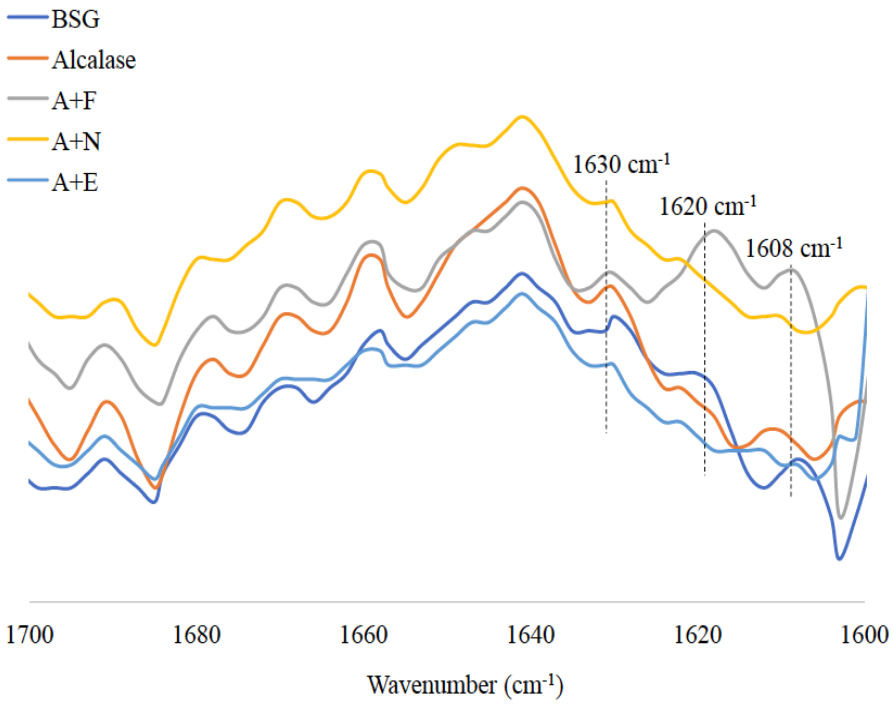


Figure 2.3. Alcalase treatments in altering secondary structures of BSG protein

2.3.3. Solubility

In general, poor water solubility at neutral pH is one of the factors that limit applications of most plant proteins. Water solubility of barley proteins extracted with NaOH was found to be around 20% at pH 7.0 (Bilgi & Celik, 2004). This low solubility could be resulted from high hydrophobic amino acid content of barley hordein and glutelin such as phe, val, leu, Ile and met. Elevated solubility of BSG protein (36.2%) compared to that of barley protein (20%) (Bilgi & Celik, 2004) can be attributed to hydrolysis by endogenous proteases during brewing. Especially, hordein and glutelin are partially decomposed to peptides by endoproteases, and to amino acids by carboxypeptidases and aminopeptidases (Osman et al., 2002).

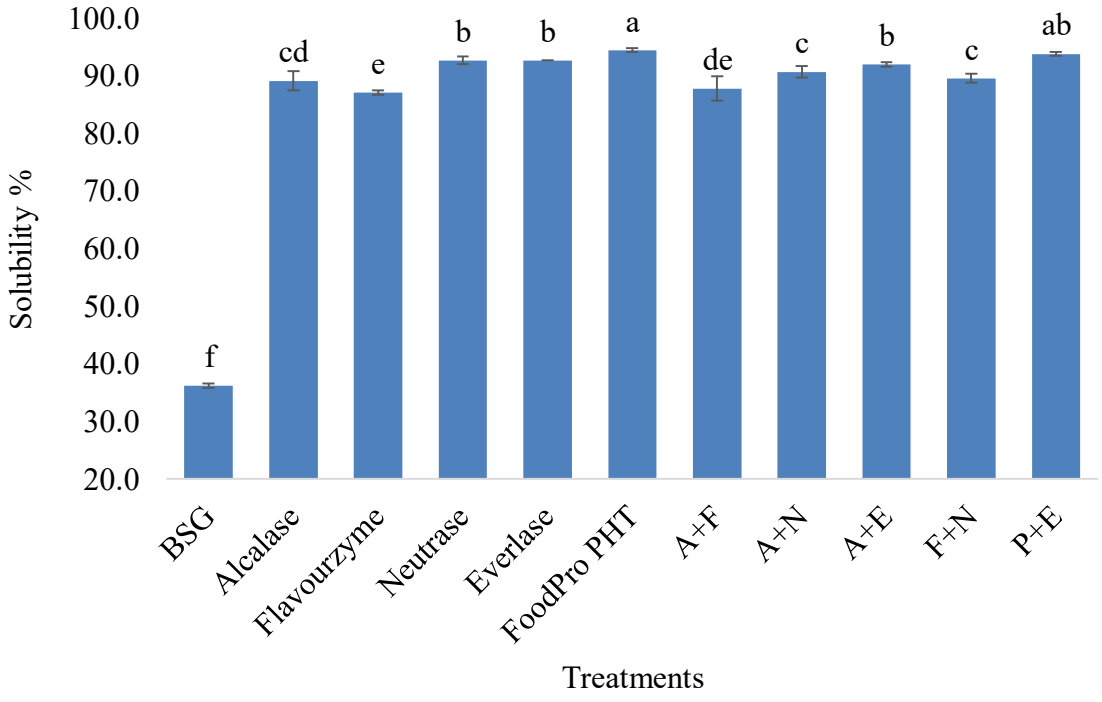


Figure 2.4. Solubility % of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

Although, the activity of these enzymes is not yet fully known, five seine carboxypeptidases and at least 40 different endoproteinases belong to four common proteinase classes were identified in germinating barley (Mikola 1983; Zhang & Jones, 1995). Enzyme hydrolysis has been widely applied to increase the solubility and functional properties of proteins.

Figure 2.4 compares solubility of BSG protein and hydrolysates in water at neutral pH. Protease hydrolysis led to significant ($p < 0.05$) increase of BSG protein solubility up to 94.4%. However, there was no correlation existed between BSG protein solubility and DH%, despite the fact that protein solubility usually increases with increasing DH%. Rather, a relationship was observed between solubility and denaturation of secondary structures. Samples with relatively lower solubility were observed to have higher β-sheets and β-turns in the secondary structures, and vice-versa. For example, β-turns, and β-sheets observed in secondary structure of BSG protein were not seen in FoodPro PHT hydrolysate, the sample with the highest solubility. Breakdown of intermolecular hydrogen bonds in denaturation of β-turns and β-sheets must have exposed the amino acids on to the surface, facilitating formation of interactions with water molecules to increase the solubility

2.3.4. Molecular weight distribution

The SE-HPLC chromatograms of BSG protein and hydrolysates are shown in Figure 2.5. Limited solubility of BSG protein at pH 7.0 made it difficult to obtain molecular weight distribution of all constituent protein fractions. The chromatogram of BSG protein consisted of three major peaks; one broad peak at molecular weight 23.9 kDa, and two narrow peaks of smaller molecular weights (0.44 and 0.10 kDa). Also,

minor peaks can be seen between 23.9 and 0.44 kDa. These protein fractions must be resulted from hydrolysis of barley proteins by endogenous proteases during brewing. Interestingly, there are two isolated peaks at higher molecular weights (1792 and 1061 kDa), confirming presence of protein aggregates. Although, the mechanism is not known, unfolded proteins and peptides can self-assemble to form soluble or insoluble protein aggregates. The aggregates observed in BSG protein could be formed during brewing as a result of protein hydrolysis under conditions favorable to aggregate formation. Apart from shifting the broad peak to a lower molecular weight region, protease hydrolysis also changed the amplitude of the peaks to different degrees. All the proteases and protease combinations tested were capable to hydrolyze BSG protein of 23.9 kDa, giving rise to peptides around 8.0 kDa as the major protein fraction in hydrolysates. Also, reduction of the intensity of peaks at 0.44 and 0.10 kDa to different degrees evident the effectiveness of proteases to hydrolyze small peptides. The amino acids and peptides resulted from hydrolysis might have combined with existing aggregates to form larger and more complex structures. Marked variations can be seen in the intensity of the peaks for protein aggregates depending on the pH used in hydrolysis. It seems that hydrolysates prepared under acidic or neutral pH (flavourzyme, neutrase, and F+N) contained relatively higher amount of protein aggregates when compared to that treated under alkaline conditions (Figure 2.6). Hollar et al., (1995) also observed increased soluble protein aggregate formation between pH 6.6 - 6.8 in whey proteins. The SE-HPLC chromatograms obtained are in align with the DH% as measured by TNBS method. For an instance, flavourzyme hydrolysate reported the most deviating chromatogram from that of BSG protein, confirming its effective proteolytic activity as suggested by DH% results.

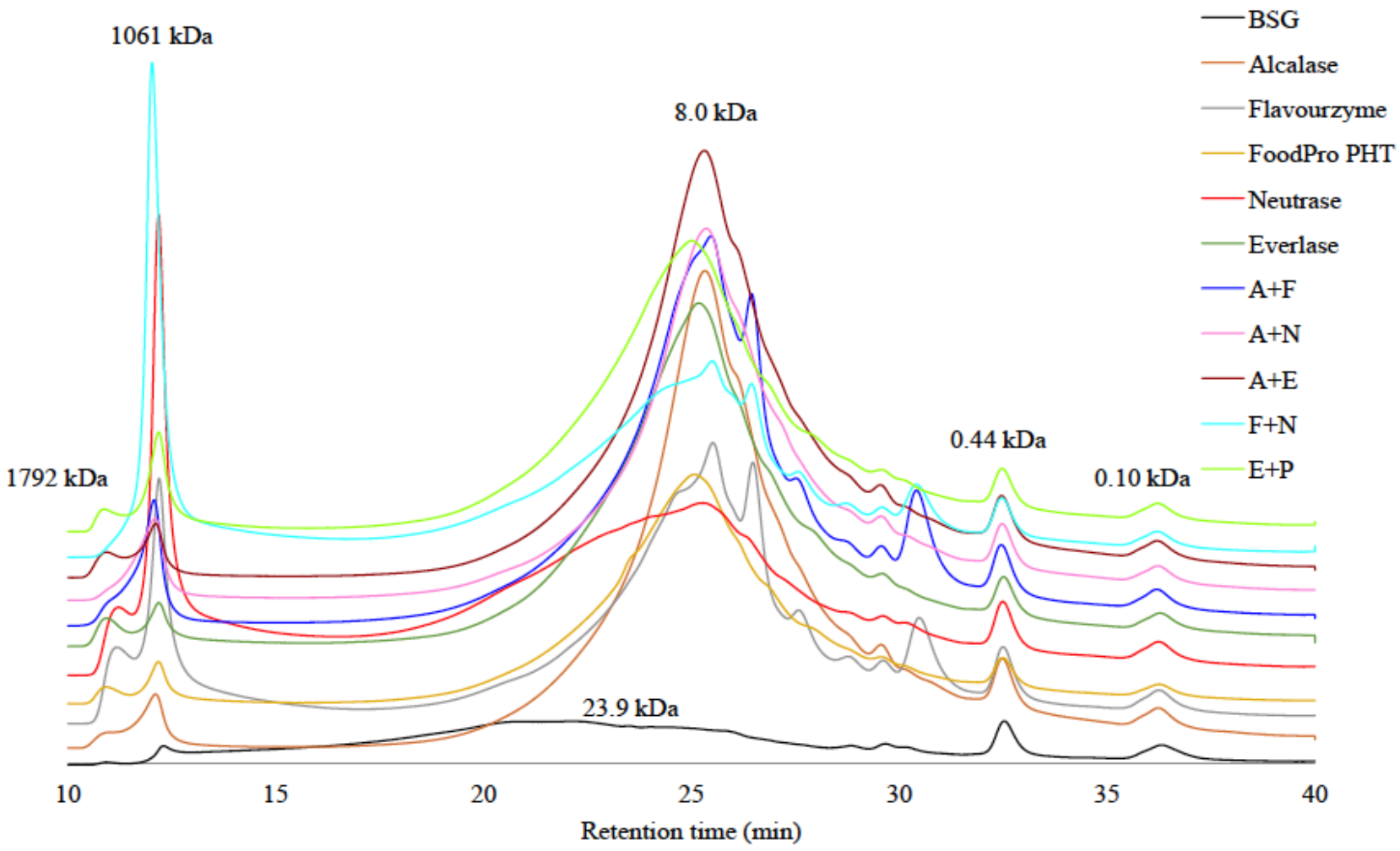


Figure 2.5. Molecular weight distribution of BSG protein and hydrolysates

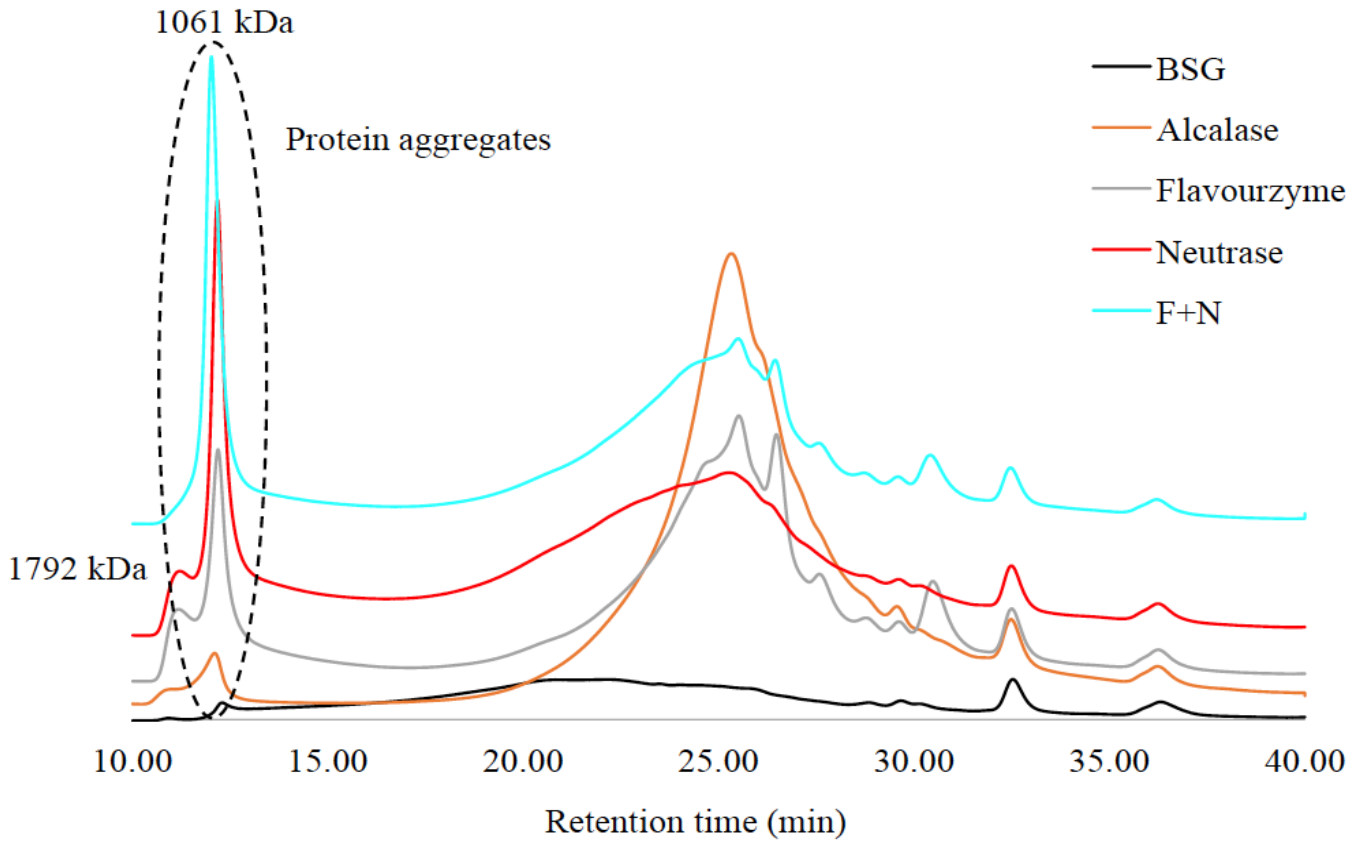


Figure 2.6. Comparison of aggregates formed in hydrolysates prepared under acidic or neutral pH (flavourzyme, neutrase, and F+N) with BSG protein and hydrolysate prepared at alkaline pH (alcalase)

2.3.5. Surface hydrophobicity (H_0)

Surface hydrophobicity is a measure of hydrophobic groups on protein surface in contact with the aqueous phase. It has a significant impact on protein functional properties such as gelation, foaming, water absorption and solubility. It was found that surface hydrophobicity is not a function of a single factor, but several factors including amino acid composition, size and shape of protein, hydrolysis conditions, cross-linking, and secondary structures (Zhongjiang et al., 2014). In many globular proteins, the hydrophobic groups in the core are surrounded by hydrophilic groups. Hydrolysis of such proteins exposes the hydrophobic moieties on the surface. Thus, it can be expected to increase the surface hydrophobicity upon hydrolysis.

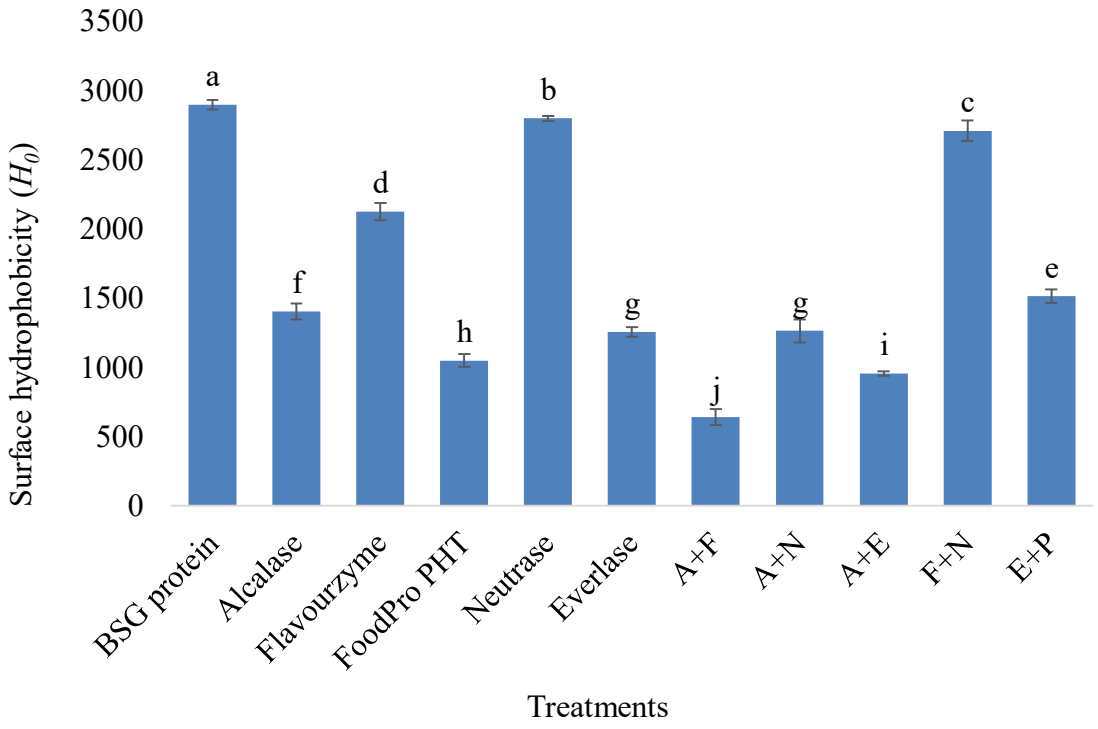


Figure 2.7. Surface hydrophobicity of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

However, as shown in Figure 2.7, it was observed that protease hydrolysis decreased the surface hydrophobicity of BSG protein to different degrees. Several other authors also reported gain as well as loss of surface hydrophobicity as a result of protease hydrolysis (Celus, 2007; Liu et al., 2010; Bamdad et al., 2011). Not like many other globular proteins, barley hordeins and glutenlins are hydrophobic proteins. Their hydrophobic groups might be exposed outside in natural status. The hydrolysis unfolded the BSG proteins to different extend, thus expose the hidden hydrophilic groups, leading to reduced H_0 value (Bamdad et al., 2011). There could be a relationship between surface hydrophobicity and aggregate formation, as the hydrolysates reported the high content of aggregates (neutrase, F+N, and flavourzyme) also reported the highest values for surface hydrophobicity. Configuration of peptides and amino acids in aggregates could be in a way that hydrophobic moieties arrange more on the surface.

2.3.6. Amino acid composition

Amino acid profiles of proteins and hydrolysates were identified to play a significant role in several antioxidant mechanisms. The amino acid composition expressed as mM/100g of protein is shown in Table 2.2. Except cysteine, BSG contains all the essential amino acids in the composition. Thus, cysteine is the limiting amino acid in BSG and its hydrolysates. Apart from that, flavourzyme hydrolysate also lacks in methionine, making it a sulfur deficit source. The hydrophobic amino acids were observed to be potent antioxidants, probably because of their ability to stabilize free radicals by donating protons (Pownall et al., 2010; Chi et al., 2015; Nourmohammadi et al., 2017). Also, due to non-polar nature, hydrophobic amino acids can be expected to have a great affinity towards fatty acid radicals formed in lipid oxidation.

Table 2.2. Amino acid composition of BSG protein and hydrolysates given as mM/100 g

Residue	BSG	Flaourzyme	Everlase	Neutrase	Alcalase	FoodPro PHT	A+N	A+F	F+N	P+E	E+A
asx	15.2	29.9	21.2	18.3	39.4	26.8	26.9	28.6	39.7	41.2	43.8
ser	11.2	23.1	16.3	14.2	30.5	20.8	20.6	22.9	31.4	32.0	34.8
glx	41.8	86.4	61.4	51.9	116.8	78.4	78.3	84.1	117.4	120.4	134.6
gly	12.0	27.9	18.0	17.7	35.6	23.1	24.2	23.6	35.8	36.3	40.9
his	2.4	7.5	4.2	6.2	9.2	5.4	6.9	6.4	11.4	9.1	11.1
arg	6.3	13.5	5.7	6.1	11.8	6.6	6.9	8.3	12.4	11.9	13.5
thr	8.8	16.8	12.3	11.1	21.4	13.3	14.3	15.8	22.3	22.6	25.3
ala	14.4	28.2	19.3	17.2	37.0	23.5	24.2	26.5	37.3	38.5	42.2
pro	25.3	52.9	36.3	31.9	70.9	46.6	47.2	50.5	71.0	75.0	82.5
cys	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
tyr	5.9	12.5	8.9	7.8	16.3	10.7	10.6	12.5	18.2	17.0	18.9
val	12.5	26.8	17.9	15.4	34.6	23.5	22.6	25.8	36.1	36.2	39.2
met	3.1	0.0	4.9	4.1	9.3	5.8	6.1	7.2	9.6	9.9	10.7
lys	6.4	12.8	8.4	7.5	15.8	10.6	10.8	11.7	15.9	16.0	18.2
ile	8.3	19.3	12.7	10.9	23.3	15.9	14.4	18.5	25.0	24.3	27.4
leu	17.0	35.2	24.9	21.7	45.2	30.7	29.1	32.5	46.6	47.7	54.9
phe	9.5	20.4	14.3	12.3	27.6	17.3	18.2	19.6	28.1	28.0	32.3

Asx represents Asn and Asp; Glx represents Gln and Glu

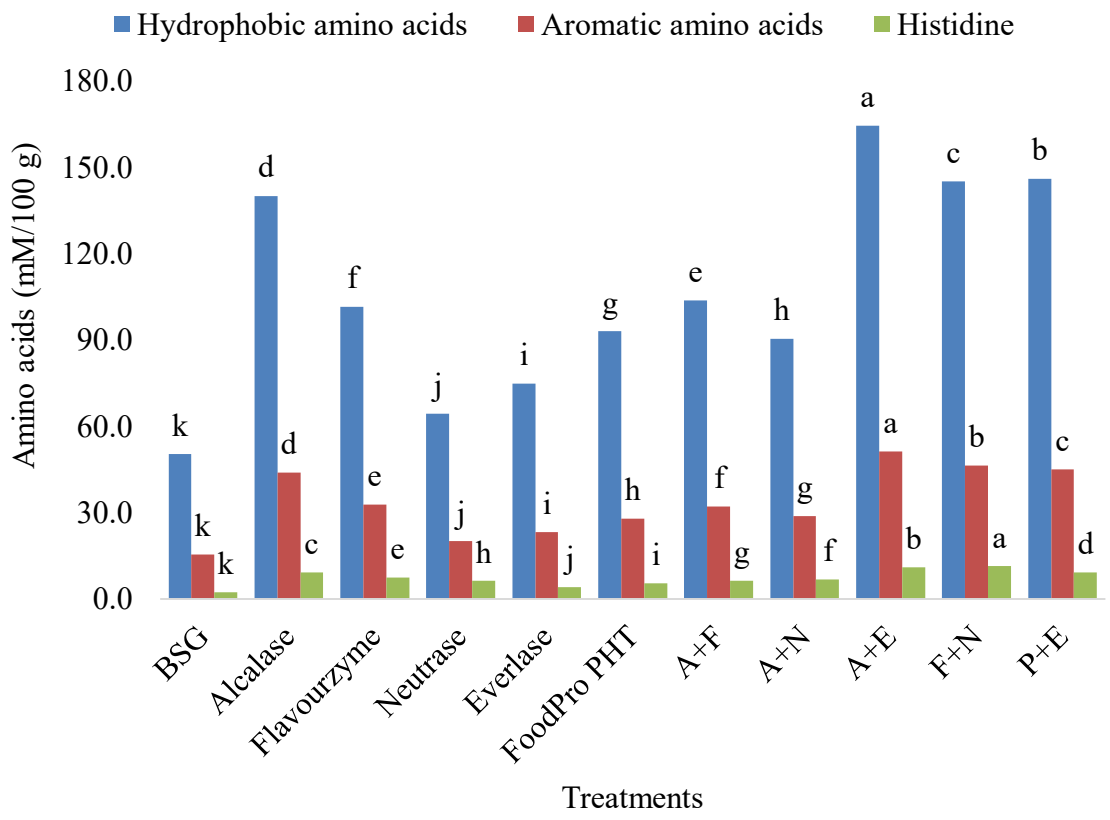


Figure 2.8. Total hydrophobic, aromatic, and histidine amino acid contents. Means with different letters in each color category differ significantly at ($p < 0.05$)

The hydrophobic amino acid content increased from 50.4 to 164.5 mM/100g upon protease hydrolysis. The A+E treatment reported the highest hydrophobic amino acid content among the samples tested. Beside the hydrophobic nature, Pownall et al., 2010 observed higher amount of aromatic amino acids: tyrosine and phenylalanine in protein fractions with high radical scavenging and metal chelating properties. Donation of protons to electron deficient species while maintaining stability via resonance structures gives aromatic amino acids an ability to scavenge free radicals. In similar to hydrophobic amino acids, A+E treatment also reported the highest aromatic amino acid content, which is 51.5 mM/100g. Histidine was observed to involve in several antioxidant mechanisms.

For instance, the chickpea peptides with reducing power, free radical scavenging and cellular antioxidant activities contained histidine in the sequences (Torres-Fuentes et al., 2015). Similarly, histidine supplemented diets prevented copper induced oxidative stress in grass carp (Jiang et al., 2015). The comparatively high proton donor activity of histidine is believed to be due to presence of imidazole ring in the side chain (Mendis et al., 2005). All the treatments showed significantly ($p < 0.05$) higher histidine content compared to that of BSG protein (2.4 mM/100g). The sample hydrolyzed with F+N combined enzyme treatment is the histidine richest (11.4 mM/100g), followed by A+E treated sample (11.1 mM/100g) (Figure 2.8).

2.3.7. Total phenol content

Phenolic compounds are heterogeneous group of secondary plant metabolites, known to have antioxidant properties even at small concentrations. Although, structure of natural phenols varies from simple molecules such as phenolic acids to highly polymerized compounds such as condensed tannins, the common structural feature is one or more hydroxyl groups bonded to an aromatic ring. Phenols behave as antioxidants mainly via donation of protons. The resulting phenoxy radicals are stabilized by resonance thus, new chain reactions are not easily initiated. BSG is expected to contain phenolic acids, mainly ferulic acid and *p*-coumaric acid coming from barley grain husk. However, BSG cannot be expected to contain all the phenols in barley due to their water-soluble nature and susceptibility to temperatures used in brewing. Also, brewing might destroy phenols due to oxidation by polyphenol oxidases. According to Figure 2.9, BSG protein extract contained phenol content of 0.028 mg GAE/mg of sample on dry basis, as measured by Folin- Ciocalteu method. Vieira et al., (2017) also observed presence of

phenols in BSG protein extract (0.021 mg GAE/mg of sample on dry basis). Strong interactions between protein and phenols must have facilitated release of phenols with protein from BSG during the extraction. Also, alkaline extraction might have broken the interactions between phenols and other matrix components, releasing phenols to the extract. BSG protein hydrolysates showed significantly ($p < 0.05$) higher phenol content when compared to that of BSG protein with values ranging from 0.051 to 0.055 mg GAE/mg of sample on dry basis. This significant increase is probably due to concentration of phenols upon freeze drying of the hydrolysates.

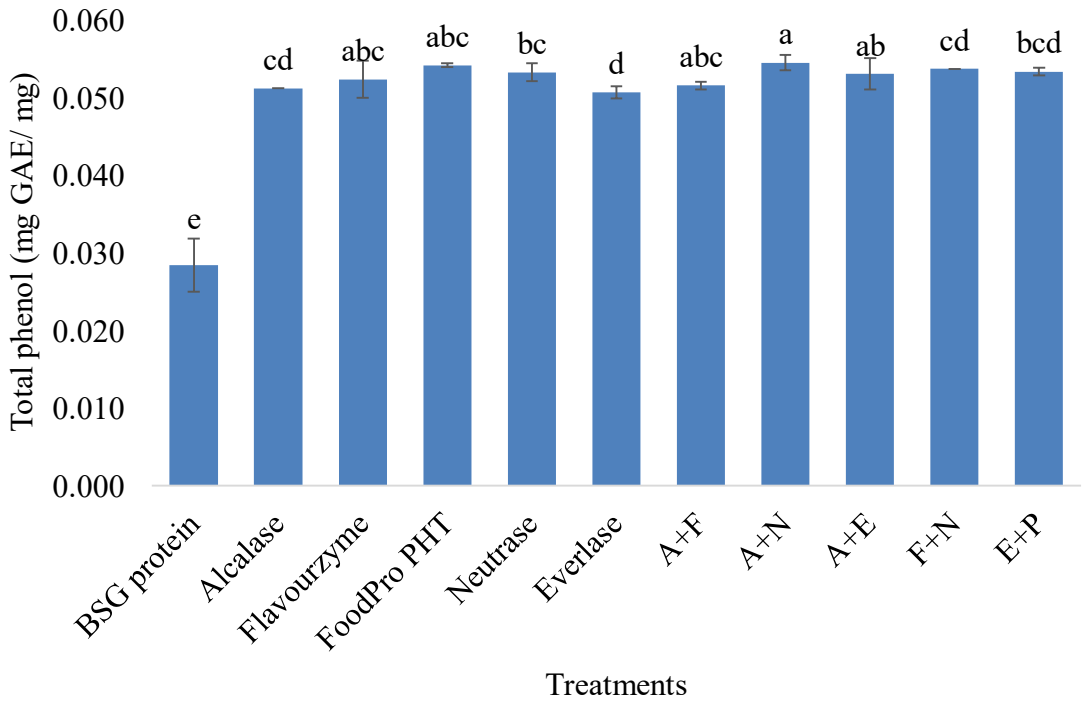


Figure 2.9. Total phenol content of BSG and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

2.3.8. Antioxidant activities

The antioxidant activities of BSG protein and hydrolysates were measured by DPPH radical scavenging, superoxide radical scavenging, reducing power, and ferrous ion chelating activity assays to compare the effectiveness of hydrolysis with different proteases in increasing antioxidant capacity. Different assays were performed since antioxidants were found to take different pathways in reacting with or neutralizing reactive species. Commercial antioxidants were tested as positive controls to see the potentiality of BSG protein and hydrolysates to be used in commercial applications. Therefore, EDTA which is a strong metal chelator was used as a positive control in ferrous ion chelating ability assay. Ascorbic acid, a natural antioxidant known to play a pivotal role to suppress reactive species developed in biological systems including superoxide radical, was compared with the superoxide radical scavenging activity of test samples. BHT and ascorbic acid were used representing a synthetic and natural antioxidant respectively, as the positive controls in DPPH radical scavenging and reducing power assays.

2.3.8.1. DPPH radical scavenging activity

The synthetic radical, DPPH is commonly used to analyze the ability of a compound to act as radical scavengers by donating protons in ethanol medium. Thus, it could be a better measure in assessing antioxidant activity in polar as well as non-polar environments representing biological systems and lipid containing foods, respectively. The odd electron in DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. At the same wavelength, reduced DPPH formed by accepting a proton from antioxidant gives yellow color thus, the degree of discoloration indicates free radical

scavenging potentials of samples. The DPPH radical scavenging activity calculated as percentage inhibition is given in Figure 2.10. The lowest scavenging activity of was observed for BSG protein (36.2%). Protease hydrolysis was observed to increase DPPH radical scavenging activity of BSG protein significantly ($p < 0.05$) due to the release of bioactive peptides responsible for antioxidant effect by protease treatment. Interestingly, the hydrolysates reported the highest DPPH scavenging activity were produced by alcalase or alcalase combined enzyme treatments (A+N, A+F, A+E, and alcalase). It could be due to specific activity of alcalase to give rise amino acid sequence with greater DPPH scavenging potential.

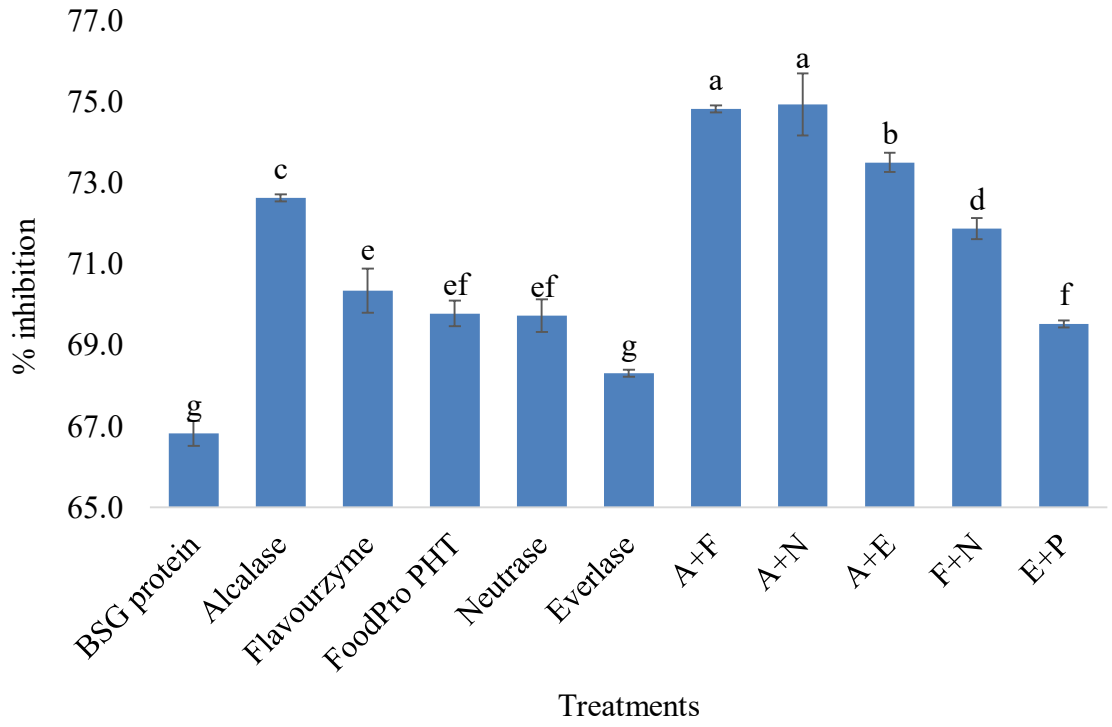


Figure 2.10. DPPH radical scavenging activity of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

Previous studies also reported relatively higher DPPH radical scavenging activity of alcalase hydrolysates (Chi et al., 2015; Nourmohammadi et al., 2017). When the DPPH scavenging activity is considered without alcalase containing treatments, it was moderately correlated with total hydrophobic amino acids; phe, val, leu, Ile, met (r = 0.73), aromatic amino acid content (r = 0.75). The amino acids included in these group must be excellent proton donators to scavenge DPPH radicals. The higher proton donation capacity of hydrophobic amino acids could be a result of them being surface oriented in ethanol medium. Aromatic amino acids can donate protons to electron deficient species like DPPH, maintaining stability via resonance structures. Interestingly, there was a strong correlation (r = 0.9) with available histidine. As suggested by Mendis et al., (2005), the greater DPPH radical scavenging activity of histidine could be due to high proton donation ability of imidazole ring present in the side chain.

2.3.8.2. Superoxide radical scavenging activity

The superoxide anion radical produced in aerobic respiration is a signaling molecule and also essential in regulating apoptosis, and aging. However, over production or malfunctioning of antioxidant defense mechanisms may cause oxidative stress induced by superoxide radicals. Thus, several attempts have been made to find ways to control the harnessing effects of superoxide radicals, including identification of antioxidants having superoxide radical scavenging activity. Among other *in vivo* and *in vitro* methods, spectrophotometric monitoring of pyrogallol autoxidation inhibition is widely used in analyzing superoxide radical scavenging activity. The activity measured in aqueous medium is a better estimate of superoxide radical scavenging activity of a compound in biological systems. Pyrogallol autoxidizes in mild alkaline mediums to produce

semiquinones and superoxide radicals as intermediates. Semiquinones are polymerized to form purpurogallin, which can be detected at 320 nm, thus the absorbance is an indication of both purpurogallin and superoxide radical generation. Reduction of absorbance signifies the superoxide radical scavenging capacity of test compound (Li, 2012). As shown in Figure 2.11, protease hydrolysis increased superoxide radical scavenging activity of BSG protein from 7.8% to 19.3%. The highest activity was reported by hydrolysates with A+F treatment.

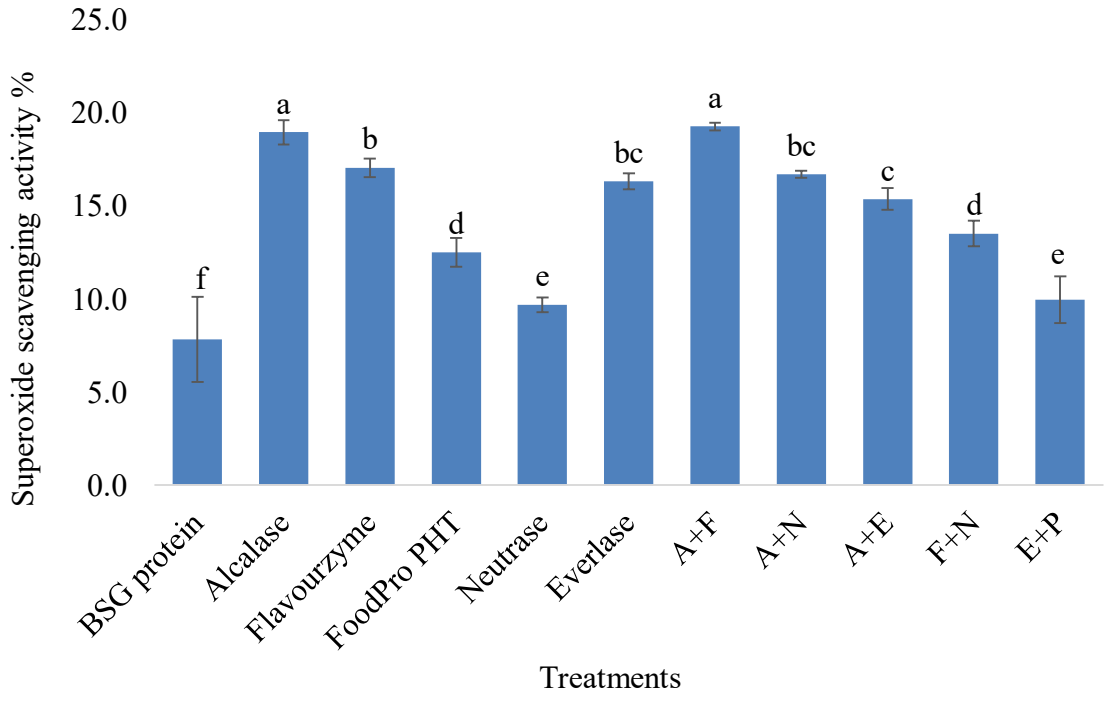


Figure 2.11. Superoxide radical scavenging activity of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

Previous studies also observed increment of superoxide radical scavenging activity upon protease hydrolysis. When considering the mechanism how hydrolytic break down of peptide bonds increased the scavenging activity, some authors linked it with amino acid

composition. For example, Pownall et al., (2010) related higher concentration of proline, and hydrophobic amino acids in pea peptide fractions to highest superoxide radical scavenging activity. Another study conducted in our lab proposed that higher superoxide radical scavenging activity of hordein hydrolysates could be due to high His, Pro, Tyr, and Try content in hordein (Bamdad et al., 2011). However, the present study found no relationship between amino acid composition and superoxide radical scavenging activity of BSG protein. Instead, there was a strong correlation (r = 0.82) between DH% and superoxide radical scavenging activity. The correlation was further increased (r = 0.9) when it is considered without hydrolysates containing markedly higher amount of protein aggregates (flavourzyme, F+N, and neutrase treated samples). The protein hydrolysates of skipjack tuna prepared with five proteases also showed a correlation between DH%, and superoxide radical scavenging capacity (Chi et al., 2015). It can be suggested that with the increasing DH%, there were more positive charges available on the amine groups of peptides, and amino acids to form electrostatic interactions with negative charges on superoxide radicals. The lower activity of flavourzyme, F+N, and neutrase could be due to formation of protein aggregates, making positive charges less available to form interactions with radicals.

2.3.8.3. Ferrous ion chelating activity

Iron is essential in regulating normal body functions however, in excess it shows pro-oxidant activity to catalyze various oxidation reactions in the body, and also takes part in Fenton reaction. Hydrogen peroxide produced as a by-product in aerobic respiration can be converted to hydroxyl radicals via Fenton reaction when ferrous ion is available. The hydroxyl radicals trigger chain reactions to produce various other free

radicals causing irreversible damages to biological molecules. Removal of excess ferrous ions in living systems by metal chelators have potential to cease progression of Fenton reaction thus, avoid accumulation of reactive species. The ferrous chelating activity measured in aqueous medium indicates the efficacy of test compounds to act as antioxidants by chelating ferrous ions in biological systems. In this study, ferrous chelating activity was measured by ability of samples to inhibit formation of pink chromophore, ferrous-ferrozine complex.

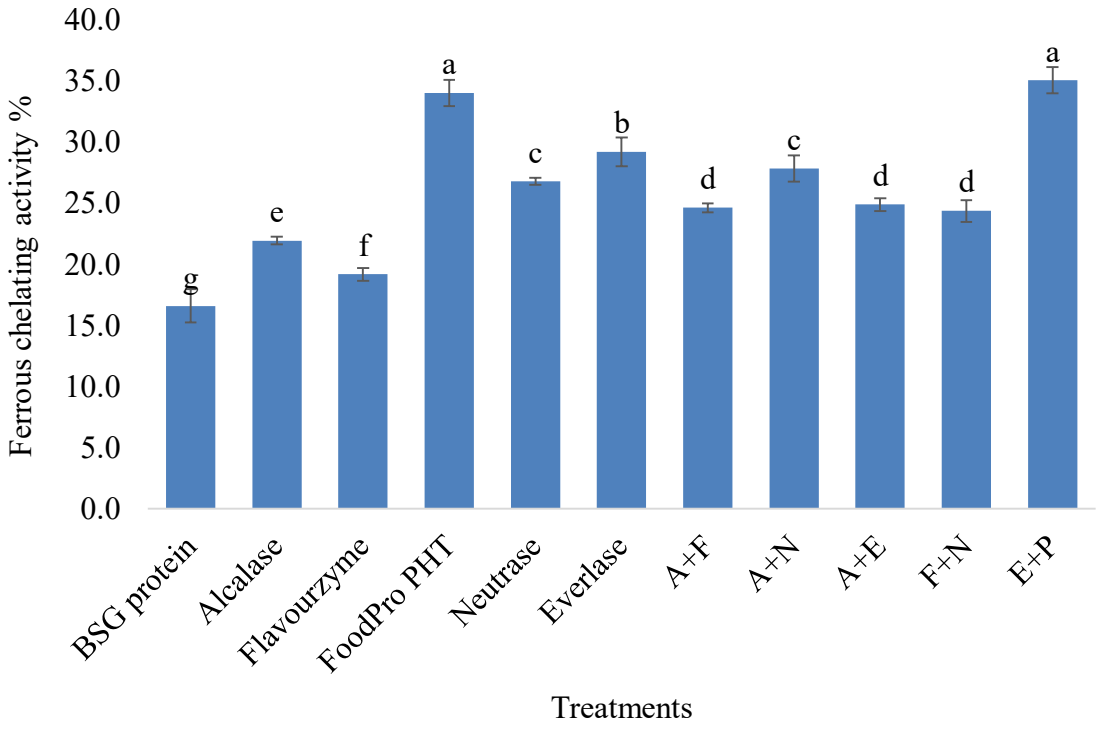


Figure 2.12. Ferrous ion chelating activity of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$)

As shown in Figure 2.12, protease hydrolysis was effective in increasing ferrous ion chelating activity of BSG protein (16.6%). Upon protease hydrolysis, the ferrous ion chelating activity was approximately increased by two folds. The highest activity, 35.1%

was reported by sample treated by E+P. Although, several authors (Pownall et al., 2010; Nourmohammadi et al., 2017) reported that high hydrophobic and aromatic amino acids in hydrolysates showed greater ferrous ion chelating activity, there was no such relationship observed in this study. Instead, a strong and negative correlation (r = - 0.84) existed between the DH% and ferrous ion chelating activity of the hydrolysates. Thus, it can be expected that hydrolysates with long peptide chains to have greater ferrous ion chelating activity. According to Zhang et al., (2009) metal chelating activity of proteins and peptides could be via structures that have ability to trap metal ions. Long peptide chains might have comparatively higher ability to form such structures than that of the shorter peptides and amino acids. Although, BSG protein must have contained the longest peptide chains compared to hydrolysates, its poor ferrous ion chelating activity can be explained by low solubility.

2.3.8.4. Reducing power

Among several pathways, donation of protons to neutralize reactive species is one of the most potent antioxidant mechanisms. Reducing power assay quantifies the ability of a compound to reduce ferric ion to ferrous ion by donating protons in aqueous medium, thus represents the total antioxidant capacity in biological systems. Yellow color of the test solution changes to various shades of green depending on the reducing power of test compound. The samples with greater proton donation ability give high absorbance at 700 nm. Therefore, absorbance is proportional to reducing power or antioxidant activity of sample. As given in Figure 2.13, hydrolysates showed significantly ($p < 0.05$) higher reducing power compared to that of BSG protein (0.3). Poor solubility at pH 7.0 could be the fact that limited the reducing power of BSG protein. The reducing power of

the hydrolysates ranged between 0.34 to 0.44. FoodPro PHT was the most effective in producing hydrolysate with proton donation ability, followed by neutrase and P+E treatments.

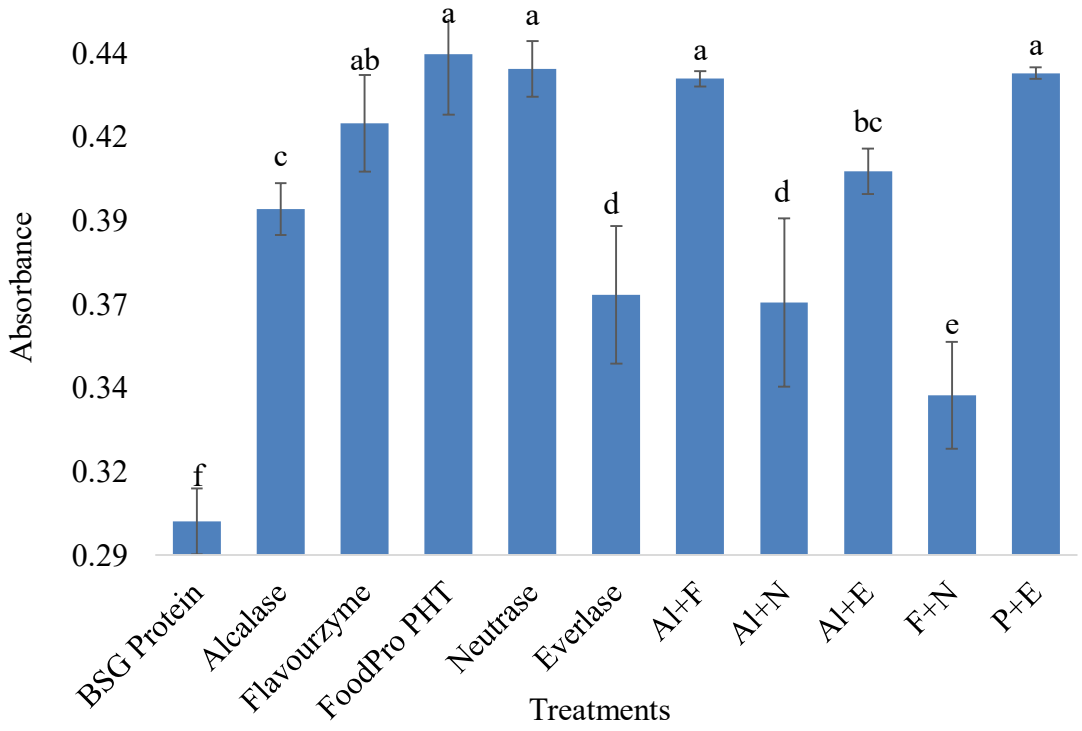


Figure 2.13. Reducing power of BSG protein and hydrolysates. Means with different letters differ significantly at ($p < 0.05$).

Previously, several attempts have been made to identify the factors that change the reducing power upon protease hydrolysis. According to Bamdad et al., (2011), increase of reducing power can be attributed to exposure of electron dense amino acid side chains moieties as a result of hydrolysis. Free amino acids in the hydrolysates also can be expected elevate reducing power as they are a source of protons and electrons to maintain high redox potential (Zhu et al., 2008). Another study suggested that the strong reducing

power observed in loach protein hydrolysate could be due to the amino acids: tyrosine, methionine, histidine, lysine, and tryptophan (You et al., 2009). However, in this study there was no strong correlation observed between reducing power and the amino acid composition or structural properties of the samples. Thus, it is not clear what is the key factor determined the reducing power of BSG and its hydrolysates. It can be suggested that it was multiple factors that determined the reducing power, but not a single fact. The strong proton donation ability of phenols might have contributed to the reducing power of the hydrolysates to any degree together with the other influencing factors.

2.3.8.5. Effect of phenol content on antioxidant activities

Phenols are the most studied among the natural antioxidants. Higher proton donation capacity, even under low concentrations makes them strong antioxidants. As shown previously, BSG contained phenol content of 0.028 mg GAE/mg of sample on dry basis, as measured by Folin- Ciocalteu assay. Phenol content was significantly ($p < 0.05$) increased by protease treatments (0.051 – 0.055 mg GAE/mg of sample on dry basis). Although, the antioxidant activities of BSG and its hydrolysates were measured by different assays, it is not known that whether the activity is contributed by phenols to any degree. Therefore, the correlations between antioxidant activities and total phenol content of BSG protein and hydrolysates were calculated in order to see the impact of phenols on antioxidant activities. The correlation (r) values were 0.55, 0.47. 0.59, and 0.67 for DPPH radical scavenging activity, superoxide radical scavenging activity, ferrous chelating ability, and reducing power, respectively. Thus, it can be suggested that the total phenols were not a major determinant in antioxidant activities of BSG protein, and hydrolysates.

3.9. Comparison with commercial antioxidants

Incorporation of natural as well as synthetic antioxidants to lipid containing foods and nutraceuticals is widely practiced to retard oxidation of biological molecules and the associated negative changes. However, the safety concerns, availability and low cost take natural antioxidants such as proteins and peptides to the platform. In order to assess the applicability in industry scale, the BSG protein hydrolysates reported the highest activity in each antioxidant assay were compared with selected positive controls, which are known commercial antioxidants. The highest DPPH radical scavenging activity reported by A+N treatment (74.9%) at 1.0 mg/mL was comparable to that of BHT (42.4%) and ascorbic acid (76.2 %) at 0.1 mg/mL. The A+F hydrolysate at 2.0 mg/mL showed the best capacity to scavenge superoxide radicals (19.3%). It was comparable to ascorbic acid (11.4%) at 0.01 mg/mL, a widely used natural antioxidant in food systems, and also a key contributor to antioxidant defense mechanisms in biological systems. EDTA, a strong metal chelator was used as the positive control in ferrous ion chelating ability assay. None of the hydrolysates were effective in chelating ferrous ions compared to EDTA. In this study, all the samples at 1.0 mg/mL showed significantly ($p < 0.05$) higher reducing power compared to that of ascorbic acid at 0.01 mg/mL (0.16). Even, the reducing power of FoodPro PHT hydrolysate, 0.44 was closer to the reducing power of 0.1 mg/mL BHT. Although, the antioxidant activities of hydrolysates were comparable to lower concentrations of positive controls, they have a potential to be used in food, feed, and nutraceutical applications as they can be incorporated at high concentrations. On the other hand, the same protein hydrolysates can be used to give functional properties to the food

systems such as gelling, foaming, emulsifying etc. and also as essential amino acid supplements.

2.4. Conclusion

Protease hydrolysis is an effective way of improving antioxidant activity of BSG protein. The protease treatments, A+N, A+F, E+P and FoodPro PHT have the highest DPPH radical scavenging, superoxide radical scavenging, ferrous ion chelating, and reducing power activates, respectively. In detail, alcalase or alcalase combined treatments are more effective in producing peptides with DPPH radical scavenging activity. It could be due to specific activity of alcalase to produce amino acid sequences with greater DPPH radical scavenging potential. Also, histidine content has a strong effect in increasing DPPH radical scavenging activity. It may be attributed to high proton donation ability of imidazole ring present in the side chain. The superoxide radical scavenging activity increases with DH% as more positive charges may allow peptides to form electrostatic interactions with negative charges on superoxide radicals. The hydrolysates with less DH% showed stronger ferrous chelating activity. The longer peptide chains in the composition may be able to form compact structures to trap ferrous ions inside. The reducing power is not related with amino acid composition or structural properties of the hydrolysates. It may be a function of multiple factors which needs further investigation. Although, the antioxidant activities of hydrolysates are comparable to lower concentrations of positive controls, they have a good potential to be used in food, feed, and nutraceutical applications as they can be incorporated at high concentrations without impacting food and feed sensory qualities.

Chapter 3.0

General Discussion and Conclusion

3.1. Summary of findings and conclusion

Despite the high protein content up to 24% (w/w) on dry basis, the only commercial application of BSG so far is low value animal feeding. It is of current importance to explore new applications to add economic value to this by-product. Based on the previous knowledge, BSG can be a good protein source to generate bioactive peptides. Therefore, the overall objective of this work was to develop hydrolysates with antioxidant effects from BSG proteins by enzymatic hydrolysis, and study BSG peptide antioxidant activities in relation to its molecular structures. In the first step, BSG proteins were extracted by alkaline method. The protein extract contained 62.6% (w/w) of protein on dry basis, and the extraction yield and protein recovery were 17.8% and 46.3%, respectively. Since, the antioxidant activities of protein hydrolysates significantly vary depending on the enzyme and hydrolysis conditions applied, BSG proteins were then hydrolyzed by different proteases and protease combined treatments: flavourzyme, alcalase, everlase, neutrase, FoodPro PHT, A+N, A+F, A+E, F+N and P+E. The generated samples showed a wide range of hydrolysis degree (DH%), ranging from 5.9% to 24.1%. Flavorzyme was the most effective enzyme to hydrolyze BSG proteins with a DH% value of 24.1% after 4h of reaction. This is explained by endo and exopeptidase activity of flavorzyme. The DH% values of the combined protease treatments were fallen in between the values of individual treatments. As shown in SE-HPLC chromatogram, BSG protein mainly consisted of molecular weight fractions, 23.9 kDa, 0.44, 0.10 kDa and higher molecular weights protein aggregates of 1792 and 1061 kDa. All treatments

were capable of hydrolyzing BSG protein of 23.9 kDa to 8.0 kDa. Proteases also showed ability to hydrolyze smaller molecular weight fractions (0.44 and 0.10 kDa). Protein denaturation was also observed during BSG proteolytic degradation as reflected by the disappearance of major secondary structures of BSG proteins in FTIR spectra. In the hydrolysates obtained from flavorzyme or flavorzyme combined treatments (A+F, F+N), vibration of amino acid residues increased significantly. This confirms higher amount of amino acid residues exposed outside probably due to exopeptidase activity of flavourzyme. Poor water solubility at neutral pH is one of the factors that limit applications of most plant proteins. Protease hydrolysis significant ($p < 0.05$) increased the solubility of BSG protein from 36.2% up to 94.4% due to reduced molecular weight. Moreover, breakdown of intermolecular hydrogen bonds in denaturation of β-turns and β-sheets could have exposed the amino acids buried in the core of proteins on to the surface, facilitating formation of interactions with water molecules to increase the solubility.

Protease hydrolysis is an effective way of improving antioxidant properties of BSG protein. The high DPPH radical scavenging activities were reported by alcalase (72.6%), A+N (74.9%), A+F (74.8%), and A+E (73.5%). It could be due to ability of alcalase to produce amino acid sequences with greater DPPH radical scavenging potential. The strong correlation (r = 0.9) of histidine content to DPPH radical scavenging activity was observed due to high proton donation ability of imidazole ring present in the histidine side chain. Superoxide radical scavenging activity of BSG protein (7.8%) increased to up to 19.3% due to protease treatments. With the increasing DH%, there may be more positive charges available on the amine groups to form electrostatic interactions

with negative charges on superoxide radicals. The increase of ferrous ion chelating activity was approximately by two folds upon the hydrolysis. The highest activity, 35.1% was reported by sample treated with E+P. The strong and negative correlation (r = - 0.84) between DH% and ferrous ion chelating activity could be due to ability of long peptide chains to form compact structures that can trap ferrous ions. The reducing power of the hydrolysates (0.34 to 0.44) were significantly ($p < 0.05$) higher compared to that of BSG protein (0.3).

The BSG protein and hydrolysates were found to contain phenols, which are potent antioxidants. The protease hydrolysis increased total phenol content as measured by Folin- Ciocalteu method from 0.028 to 0.055 mg GAE/mg of sample on dry basis. The correlations (r) between antioxidant activities and total phenol content of BSG protein and hydrolysates were calculated to be 0.55, 0.47. 0.59, and 0.67 for DPPH radical scavenging activity, superoxide radical scavenging activity, ferrous chelating ability, and reducing power, respectively. Thus, it can be suggested that the total phenols were not a major determinant of antioxidant activities tested.

In order to assess the commercial potential of BSG protein hydrolysates as antioxidants, the highest activities reported for each antioxidant assay were compared with selected positive controls, which are known commercial antioxidants. The highest DPPH radical scavenging activity reported by A+N treatment was 74.9 at 1.0 mg/mL, which is comparable to BHT (42.4 at 0.1 mg/mL) and ascorbic acid (76.3 at 0.1 mg/mL). The A+F hydrolysate at 2.0 mg/mL showed the best capacity to scavenge superoxide radicals (19.3%). It was comparable to ascorbic acid (11.4%) at 0.01 mg/mL. None of the hydrolysates were effective in chelating ferrous ions compared to EDTA, which is a

strong metal chelator. All the samples at 1.0 mg/mL showed significantly ($p < 0.05$) higher reducing power compared to that of ascorbic acid at 0.01 mg/mL (0.16). These results suggest good potential of BSG hydrolysates as antioxidant ingredients.

3.2. Significance of the research

This research has extended the commercial applications of BSG. Approximately, 20 kg of wet BSG is generated per 100 L of beer produced (Reinold, 1997). Since, the only commercial application of BSG is animal feeding, considerable amount has to be deposited in the landfills, which is an extra cost to the breweries. This study was able to recover (PY%) 46.3% of proteins from BSG using a non-toxic, simple and less expensive alkaline treatment. The extracted protein has an economic potential to be used as functional ingredient in food preparations and also as a nitrogen supplement in food and feed formulas. As the second step of the study, different proteases and protease combined treatments were tested to prepare BSG protein hydrolysates with antioxidant properties: DPPH radical scavenging, reducing power, superoxide radical scavenging and ferrous chelating activities. Although, the activities were comparable to lower concentrations of commercially available antioxidants, they are likely to be successful in industry applications, as they can be incorporated at higher concentrations. Also, due to the fact that proteins and their hydrolysates have multiple antioxidant mechanisms, there is a growing interest in using them in foods and nutraceuticals applications. A major factor limiting the commercialization is the cost of starting peptides, mostly prepared by expensive and inefficient peptide synthetic chemistry. Therefore, being a naturally derived, abundant and less expensive source, BSG protein hydrolysates are more economically feasible antioxidant supplements. Similarly, the consumer preference for

natural food additives over synthetic ones will further strengthen the applicability of BSG protein hydrolysates as antioxidants. In this context, development of antioxidant protein hydrolysates from BSG can be expected to add economic returns to breweries and also to barley growers, while promoting health in the long run. Likewise, BSG protein hydrolysates with antioxidant property may be potentially used to reduce or suppress oxidative stress and associated negative effects including poor growth performance, reduced feed efficiency and productivity in livestock. Although, it can be widely seen that small scale livestock farmers locally use BSG as a feed ingredient, the commercial feed preparations hardly contain BSG as an ingredient because of its high moisture and high cost related to the transportation and storage. Confirmation of antioxidant properties would encourage to use BSG in commercial feed formulas. In addition, determination of the factors affecting different antioxidant mechanisms of BSG protein hydrolysates, could be useful in future studies to understand the antioxidant pathways in greater depth. In summary, this study would not only bring significant benefits to breweries, barley growers, food, feed and nutraceutical industries, but also provide insight to molecular structures of peptides affecting different antioxidant mechanisms.

3.3. Recommendations for future work

This research demonstrated the antioxidant activities of BSG protein and hydrolysates using *in vitro* methods. However, the ability of these methods to predict *in vivo* activity has not been demonstrated. They do not reflect cellular physiological conditions and do not consider the bioavailability, uptake, and metabolism of the antioxidants. Therefore, *in vivo* studies using animal models is a necessity to understand the actual efficacy of BSG protein hydrolysates as antioxidants in biological systems.

There were several chemical and structural properties of the hydrolysates identified as the factors affecting the antioxidant mechanisms tested. The hydrolysates showed the highest activities can be further studied with technologies like MS/MS to identify the specific amino acid sequences responsible for the activities. In this study, the proteins extracted from BSG and its hydrolysates were only tested for antioxidant properties. In this context, further studies can be recommended to identify other biological activities including antimicrobial, anti-hypertensive, anti-diabetic and anti-carcinogenic activities. Proteins derived from different sources play a significant role in food industry as functional ingredients. The BSG protein and hydrolysates can also be analyzed for applicability as functional ingredients in food systems including emulsifying, forming, gelling, and flavor binding agents. The successfulness of any food product in the market depends mainly on consumer acceptance, which is collectively determined by factors like cost, quality, sensory attributes and nutrient composition of the product. Different foods incorporated with BSG protein hydrolysates must be further tested for such attributes to claim their suitability as an ingredient in food applications. Although, the protein extracted from BSG can potentially be incorporated into food, feed or nutraceuticals as antioxidants, the residue after protein extraction is left without any commercial application. It will become an economic burden in terms of waste disposal. Therefore, feasibility of using BSG residue in economic applications must be assessed to complete the value addition to BSG. The fiber rich residue could be used in energy production, paper manufacture, development of cellulose nano-crystals, substrate for cultivation of microorganisms and enzyme production.

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