CHARACTERIZATION AND BIOACTIVE PROPERTIES OF YOUNG AND MATURE SOYBEAN AND THEIR PROTEIN HYDROLYSATES

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CHARACTERIZATION AND BIOACTIVE PROPERTIES OF YOUNG AND MATURE SOYBEAN AND THEIR PROTEIN HYDROLYSATES

Amna Khalifa Mohammed Alnuaimi

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Food Science

Under the Supervision of Dr. Sajid Maqsood

April 2021
Declaration of Original Work

I, Amna Khalifa Mohammed Alnuaimi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Characterization and Bioactive Properties of Young and Mature Soybean and their Protein Hydrolysates”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Sajid Maqsood, in the College of Food and Agriculture at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Copy ___ of ___
Abstract

Soybeans are known for its high protein content that could be substituted to animal proteins. The potential bioactive properties of mature soybean (MS) proteins and their hydrolysates have been widely explored. The aim of this study was to investigate and compare various in-vitro bioactive properties (antioxidant, anti-diabetic, anti-obesity, and anti-inflammatory) of young soybean (YS) and MS flour and their protein hydrolysates when subjected to in-vitro simulated gastrointestinal digestion (SGID) and enzymatic hydrolysis, respectively. In the first phase, SGID of YS and MS flour was carried out to mimic the human digestion, while in the second phase, enzymatic hydrolysis of the soybean protein isolate was carried out by alcalase, bromelain, and flavourzyme for 2, 4, and 6 h in order to produce different protein hydrolysates. The results showed that upon in-vitro SGID, the total phenolic content (TPC) significantly increased, and was higher in YS compared to MS (P<0.05). YS and MS flours varied in their inhibitory activity against α-amylase (AA), dipeptidyl peptidase IV (DPP-IV), pancreatic lipase (LIP), and cholesterol esterase (CE) enzymes when subjected to simulated gastric digestion (P<0.05) followed by a decrease in the inhibitory activity upon simulated intestinal digestion stage. Furthermore, MS flour exhibited higher antioxidant and anti-inflammatory (AI) activities than YS when subjected to SGID (P<0.05). The results of the enzymatic hydrolysis in the second phase revealed that the YS and MS protein hydrolysates displayed enhanced inhibitory activity against AA, DPP-IV, LIP, and CE enzymes when hydrolyzed by different enzymes for different time periods (P<0.05). Antioxidant and AI activities were also found to be higher in hydrolysates compared to intact proteins for both YS and MS proteins (P<0.05). In conclusion, the bioactive properties of YS and MS flour upon SGID and proteins upon enzymatic hydrolysis were enhanced compared to unhydrolyzed samples. Further research is needed to identify the sequence of bioactive peptides responsible for different bioactive properties of YS and MS proteins.

Keywords: Soybean, young soybean, proteins, hydrolysates, SGID, enzymatic hydrolysis, bioactive properties.
الوصف والخصائص النشطة بيولوجياً لفول الصويا الصغير والناضج ولهيدروليزات البروتينات الخاصة بهم

الملخص

تُعرف فول الصويا بمحتواها العالي من البروتينات التي يمكن أن تحل محل البروتينات الحيوانية. تم استكشاف الخصائص النشطة بيولوجياً المحتملة لبروتينات فول الصويا ولهيدروليزاتها بعد تحللها المائي على نطاق واسع. كان الهدف من هذه الدراسة هو البحث والمقارنة بين مختلف الخصائص النشطة بيولوجياً في المختبر (مضادات الأكسمدة، ومضادة لمرض السكري، ومضادة للسمنة، ومضادة للالتهابات) لدقيق فول الصويا الصغير والناضج ولهيدروليزات البروتينات الخاصة بهم عند تعرضهم لمحاكاة معدية معوية الهضم في المختبر والتحلل المائي الأنزيمي. في المرحلة الأولى، تم إجراء محاكاة معدية معوية الهضم لدقيق فول الصويا الصغير والناضح لمحاكاة الهضم البشري، بينما في المرحلة الثانية، تم إجراء التحلل المائي الإيزيمي لبروتيني فولي الصويا الصغير والناضح المعزولين بواسطة استخدام ثلاث إنزيمات محالة للبروتينات - أنزال، بروميدين، فلافيورزمي لمدة 2 و 4 و 6 ساعات من أجل إنتاج تحلل بروتيني مختلف. أظهرت النتائج أنه عند إجراء محاكاة معدية معوية الهضم في المختبر زاد المحتوى الفينولي الكلي بشكل ملحوظ، وكان أعلى في فول الصويا الصغير مقارنة بفول الصويا الناضج. أظهر دقيق فولي الصويا الصغير والناضح نشاطًا مثبطًا مثبئًا مثبطًا مثبئًا مثبئًا ضد إنزيمات ألفا أميليز، ودييبيتيديل بيتيدازالرابع، والليلاباز، استعراض الكولينترول عند تعرضهم لمحاكاة الهضم المعزول. علاوة على ذلك، أظهر دقيق فول الصويا الناضج مضادات الأكسمدة والمضادة للالتهابات أعلى من فول الصويا الصغير عند التعرض إلى محاكاة معدية معوية الهضم. أظهرت نتائج التحلل المائي الأنزيمي لبروتينات فول الصويا الصغير والناضح نشاطًا مثبطًا مثبئًا ضد إنزيمات ألفا أميليز، ودييبيتيديل بيتيدازالرابع، والليلاباز، وانعكاسات استعراض الكولينترول عند تحللهم بواسطة إنزيمات مختلفة لفترات زمنية مختلفة. تم العثور على خصائص مضادات الأكسمدة وأنشطة المضادة للالتهابات أعلى في هيدروليزات البروتينات مقارنة بالبروتينات السليمة لكل من بروتينات فول الصويا الصغير والناضج. في الختام، تم تحسين الخصائص النشطة بيولوجياً لدقيق فول الصويا الصغير والناضج عند إجراء محاكاة معدية معوية الهضم والبروتينات عند التحلل المائي الأنزيمي مقارنة بالعينات.

Title and Abstract (in Arabic)
غير المتطلة. هناك حاجة إلى مزيد من البحث لتحديد تسلسل البيوتيدات النشطة بيولوجيًا المسؤول عن الخصائص النشطة بيولوجيًا المختلفة لبروتينات فول الصويا الصغير والناضج.

مفاهيم البحث الرئيسية: فول الصويا، فول الصويا الصغير، بروتينات، هيدروليزات، محاكاة معدية معوية الهضم، التحلل الإنزيمي، خصائص نشطة بيولوجيًا.
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Dedication

To my beloved parents and family
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>α-amylase</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AI</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol esterase</td>
</tr>
<tr>
<td>Corolase PP</td>
<td>Food-grade porcine pancreatic proteolytic preparation</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DH</td>
<td>Degree of hydrolysis</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl</td>
</tr>
<tr>
<td>DSEC</td>
<td>Diclofenac sodium equivalent capacity</td>
</tr>
<tr>
<td>E:S</td>
<td>Enzyme: substrate</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric-reducing antioxidant power</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic acid equivalents</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric inhibitory polypeptides</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HMGCoAR</td>
<td>3-hydroxy-3-methylglutaryl CoA reductase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MS</td>
<td>Mature soybean</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OPA</td>
<td>O-phthalaldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>pNPM</td>
<td>p-nitrophenyl-α-D-maltohexaoside</td>
</tr>
<tr>
<td>R6</td>
<td>Full seed stage</td>
</tr>
<tr>
<td>R8</td>
<td>Full maturity stage</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SGD</td>
<td>Simulated gastric digestion</td>
</tr>
<tr>
<td>SGID</td>
<td>Simulated gastrointestinal digestion</td>
</tr>
<tr>
<td>SGJ</td>
<td>Simulated gastric juice</td>
</tr>
<tr>
<td>SIJ</td>
<td>Simulated intestinal juice</td>
</tr>
<tr>
<td>SPH70</td>
<td>Soybean protein hydrolysate produced at 70°C</td>
</tr>
<tr>
<td>SPH90</td>
<td>Soybean protein hydrolysate produced at 90°C</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic content</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tripyridyl-s-triazine</td>
</tr>
<tr>
<td>Trolox</td>
<td>6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid</td>
</tr>
<tr>
<td>YS</td>
<td>Young soybean</td>
</tr>
<tr>
<td>11S</td>
<td>Glycinin</td>
</tr>
<tr>
<td>7S</td>
<td>β-conglycinin</td>
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</tbody>
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Chapter 1: Introduction

1.1 Overview

Soybean (Glycine max) L. is an important crop worldwide, which is an excellent source of protein for human consumption (Berk, 1992). More than 50% of the soybean world’s production is accounted to the United States of America (USA) (Palomo et al., 2011). Soybean is a leguminous plant native to Eastern Asia, north and central China. Later the cultivated varieties were introduced into Korea and Japan approximately before 2000 years ago. Until this day, soybean is consumed as an essential component of different region’s traditional diets (Berk, 1992). The demand for soybean is increasing worldwide, because for various reasons such as it is one of the oils crops, it is also used for feed for livestock and aquaculture, and as an important source of protein and biodiesel feedstock (Masuda & Goldsmith, 2009).

There are a variety of products being produced from soybean including fermented products such as soy sauce, tempeh, miso, and douche (Chatterjee et al., 2018), soy flour, tofu, soy milk, oil, edamame (green soybean) (Agyei, 2015), Soybean is composed of ~35–40% protein, ~20% lipids, ~9% dietary fiber, and ~8.5% moisture based on the dry weight of mature raw seeds (Chatterjee et al., 2018). In addition, soybean contains different components that are known to have a biological activity such as proteins or peptides, isoflavones, saponins, and protease inhibitors (Isanga & Zhang, 2008). Moreover, soybean varieties may differ in the color of their mature pods, and the brown and tan colors are the most common (Fehr & Caviness, 1977). The soybean is considered mature when it reaches R8 (Full maturity stage), unlike the young soybean which is harvested before full maturity when the green seeds are fully developed in R6 (physiological stage) (Mozzoni & Chen, 2019).
In Asian cultures, young soybean consumption has remained a favorite for many years, especially in Japan for 400 years (Wszelaki et al., 2005). Moreover, the popularity of young soybean has been seen a recent surge in its consumption especially in the USA due to its health-related functional properties (Mozzoni & Chen, 2019), and also is considered a functional food crop because it is rich in phytochemicals and is high in nutrients, that are beneficial to human health (Mentreddy et al., 2002). The young soybean seeds contain (22–32%) protein, (16–22%) oil, traces of oligosaccharides (dry weight basis) (Mozzoni & Chen, 2019). In addition, it contains phospholipids, iron, calcium dietary fibers (Hu et al., 2006), isoflavones and tocopherols (vitamin E) in dried seeds, and high fractions of monosaturated fatty acids in fresh green seeds (Mentreddy et al., 2002).

Soy proteins are high biological value proteins due to the presence of all essential amino acids and can be potentially used as an alternative to animal proteins (Singh et al., 2014). The two major storage soy proteins are glycine (11S) and β-conglycinin (7S) that accounts 80-90% of the whole protein, whereas the minor storage proteins include the lectin, Kunitz, and Bowman-Brik protease inhibitors. Furthermore, different ratios of glycine and β-conglycinin in soy protein may have different nutritional and physiological effects (Chatterjee et al., 2018).

Bioactive peptides have been associated with several positive health benefits including antihypertensive, anti-diabetic, anti-obesity, relaxing and, satiety-inducing effects (Hayes & Tiwari, 2015). Bioactive peptides are defined as specific protein fragments of 2-20 amino acids, linked covalently by peptide linkage and have a positive impact on body functions or conditions and may influence health (Sánchez & Vázquez, 2017). These peptides have to be bioavailable and able to exert the health effect at their target sites (Hayes & Tiwari, 2015).
Consumption of the commercial synthetic drugs such as the anti-inflammatory (AI), antihypertensive, antidiabetic, and antioxidants drugs may result in side effects with the prolonged usage. Therefore, the interest of utilizing natural compounds in therapeutic applications is increasing with an aim to have a safer alternative (Chakrabarti et al., 2014). Bioactive peptides can be used as a functional food ingredient and could be used to prevent diseases rather than treatment (Hayes & Tiwari, 2015).

Soybean peptides are well known for therapeutic role by acting as antihypertensive, anticancer, anti-obesity and, hypocholesteromic agents. Thus, these bioactive peptides are being investigated to create functional foods ingredients and drugs due to their bio-functional activity (Singh et al., 2014). The soy-derived peptides remained inactive inside their parent proteins sequence (Chatterjee et al., 2018), and become biologically active upon their release by proteolytic enzymes present in different biological processes such as gastrointestinal digestion and microbial proteolysis (Agyei, 2015).

1.2 Statement of the problem

Consumption of soybean either as an intact protein or as a bioactive peptide has been related to various beneficial health effects especially in reducing chronic diseases including insulin-resistance and type II diabetes, obesity, cardiovascular disease, and some types of cancers (Chatterjee et al., 2018). Currently, the soy peptides are being a subject of research of functional food ingredients and new drugs to regulate the absorption of nutrients and gut health due to their bioactive properties (Singh et al., 2014). On the other hand, there is a lack in research in the investigation of the hydrolysates possessing bioactive properties of young soybean either produced via proteolysis or gastrointestinal digestion. The hydrolysates produced from green
Soybean upon proteolysis were investigated for their antihypertensive activity, which could become an ingredient for the development of functional foods (Hanafi et al., 2018). Till date studies related with protein hydrolysates from young soybean proteins and their bioactive properties are scarce. Thus, this research was designed to explore comparative investigation on hydrolysates from proteins derived from young (YS) and mature soybean (MS) and to investigate their bioactive properties. The objectives of the study were to explore and compare the bioactive properties of YS and MS and their protein hydrolysates upon in-vitro gastrointestinal digestion and enzymatic hydrolysis.

1.3 Relevant literature

Soybean (*Glycine max* (L.) Merrill) belongs to Leguminosae family and is an annual growing plant with a height ranging from 0.50 to 1.25 m. Morphologically, the seeds of the soybeans are varying in shape (oval, spherical) and color and mostly present in yellowish color (Liu, 2004). In the first half of the 20th century, China was the prime producer and exporter of soybean (Qiu & Chang, 2010). In the 1950s, soybean production was increased rapidly in different regions of the USA. Now, the USA is the largest producer of soybean worldwide, followed by Brazil, and Argentina (Qiu & Chang, 2010). Soybean crop is rich in protein content, phytochemicals, essential fatty acids, vitamins, minerals, and antioxidants (Palomo et al., 2011).

The production of young soybean has flourished and expanded sharply in recent decades as a result of increased interest and awareness to its nutritional properties, and high consumption to maintain a healthier lifestyle. In the U.S, particularly in the 1980s, the importation of frozen young soybean increased from 300 – 500 tons per year, followed by an increment to 10,000 tons in 2000, and to 25,000 tons between 2000 to 2008. The United Soybean Board forecasts are expecting the
young soybean to outgrow other soy products by 2020, to have a healthier and inexpensive source of protein in the diet (Zhang et al., 2017).

Young soybean has a shorter growing season and is usually harvested at immature stage (full seed stage) (R6) than mature soybean which is harvested at reproductive stage 8 (R8) (the full maturity stage) (Zhang et al., 2017) when the color of the bean pods is green just before turning to yellow (Zeipiņa et al., 2017). Young soybean has a large seed, tender, mild taste, and easily digestible. Moreover, it has a high protein content, lower oil content, and a low percentage of gas-producing starches (Zhang & Kyei-Boahen, 2007). Young soybean can be consumed by boiling the pods, adding beans to stews, with stir-fried vegetables (Zhang & Kyei-Boahen, 2007), and it can be used in making different products such as green noodles, green milk, and green tofu (Zeipiņa et al., 2017).

Young soybean is considered as a functional food and is known to have high nutritional and medicinal values similar to mature soybean (Zeipiņa et al., 2017). It contains high protein content having contains all the essential amino acids. It is more digestible due to the lower trypsin inhibitor levels, compared to the mature soybean (Zhang et al., 2017). Young soybean also contains phosphorus, calcium, iron, vitamin E (Zeipiņa et al., 2017), rich in isoflavones, ascorbic acid, and certain oligosaccharides, and low in niacin (Mentreddy et al., 2002).

1.3.1 Compositions of young and mature soybean

The young and mature soybean compositions can vary depending on the variety, the location, and the climate of planting (Chatterjee et al., 2018). Table 1 shows the proximate composition of mature and young soybean from different studies. Redondo-Cuenca et al. (2007) has studied the proximate composition and the dietary
fiber of young and mature soybean that follows a conventional cropping practice. The moisture content in mature soybean that was reported by Redondo-Cuenca et al. (2007) was (9.82 g), which is slightly higher to moisture content values reported as 8.59 g and 8.07 g by Güzeler and Yıldırım, (2016) and Etiosa et al. (2017), respectively. However, the moisture content of young soybean (10.81 g) (Redondo-Cuenca et al., 2007) is very low than raw young soybean (67.5 g) (Ravishankar et al., 2016). Redondo-Cuenca et al. (2007) reported the protein content in mature soybean to be (40.4 g). Mateos-Aparicio et al. 2008; Güzeler and Yıldırım, 2016 and Etiosa et al. 2017 have reported relatively similar protein content of mature soybean: (36 g, 36.5 g, and 37.69 g), respectively. Redondo-Cuenca et al. (2007), reported the protein content in young soybean to be (37.1 g), while Ravishankar et al. (2016) have reported a lower protein content of 12.9 g for young soybean. Fat content in mature soybean is significantly higher than that of young soybean. Etiosa et al. (2017) has reported the fat content value in mature soybean to be (28.2 g). While relatively lower values of the fat content were reported by Redondo-Cuenca et al. (2007) 18.56 g, Mateos-Aparicio et al. (2008) 19 g, and by Güzeler and Yıldırım, (2016) 19.9 g. Young soybean has a lower fat content of 0.93 g reported by Redondo-Cuenca et al. (2007) and 6.8 g reported by (Ravishankar et al. (2016). The ash content in mature soybeans is higher as compared to the young soybeans. The reported values of ash in mature soybeans were 4.81 g, 4.9 g, and 4.29 g by (Redondo-Cuenca et al., 2007; Güzeler & Yıldırım, 2016 and Etiosa et al., 2017), respectively. Young soybean has a lower ash content (3.39 g) (Redondo-Cuenca et al., 2007) and (1.7 g) (Ravishankar et al., 2016). The dietary fiber values of mature soybean were in the range of 16.5 g – 17 g (Mateos-Aparicio et al., 2008; Redondo-Cuenca et al., 2007). While, the fiber content reported by Güzeler and Yıldırım, (2016) was 9.3 g and the crude fiber was 5.44 g (Etiosa et
Fiber content is lower in young soybean compared to mature soybean. Redondo-Cuenca et al. (2007) have reported a dietary fiber of 9.19 g, and Ravishankar et al. (2016), reported a crude fiber of 4.2 g in young soybean. Carbohydrates content also varies between the young and mature soybean crops with highest contents reported for mature soybean was 30.2 g (Güzel & Yıldırım, 2016), followed by 30 g (Mateos-Aparicio et al., 2008), 16.31 g (Etiosa et al., 2017) and 9.94 g (Redondo-Cuenca et al., 2007). Whereas the young soybean has a high carbohydrate content of 38.6 g as reported by Redondo-Cuenca et al. (2007) and a very low content of 11 g was reported by Ravishankar et al. (2016).

The two major proteins in soybean which account for 65 – 80 % and the precursor of the most isolated peptides are: glycinin and β- conglycinin (Singh et al., 2014). Glycinin is composed of five subunits: G1, G2, G3, G4, and G5, whereas β-conglycinin has three subunits (α, α’, and β) (Singh et al., 2014). Glycinin is not a conjugated protein and on the other hand, conglycinin is a glycoprotein that contains approximately 4% carbohydrates (predominantly mannose moieties). β- conglycinin is relatively resistant to hydrolysis even after removing its glycosylation sites (Isanga & Zhang, 2008). Partial hydrolysis of glycinin and β- conglycinin can result in the production of hydrolysates and peptides. The amino acid composition and sequence of these hydrolysates and peptides can be involved in biologically active functions such as anti-microbial, anti-diabetic, anti-oxidants, and anti-hypertensive activities (Agyei, 2015). Furthermore, the minor proteins of the soybean are the lectin, Kunitz and Bowman – Brik protease inhibitors (Chatterjee et al., 2018).
Table 1: Proximate composition of mature and young soybean seeds (g/100 g).

<table>
<thead>
<tr>
<th>Soybean</th>
<th>Moisture</th>
<th>Protein</th>
<th>Fat</th>
<th>Fiber</th>
<th>Carbohydrates</th>
<th>Ash</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature soybean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.82</td>
<td>40.4</td>
<td>18.56</td>
<td>16.5</td>
<td>9.94</td>
<td>4.81</td>
<td>(Redondo-Cuenca et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>8.59</td>
<td>36.5</td>
<td>19.9</td>
<td>9.3</td>
<td>30.2</td>
<td>4.9</td>
<td>(Güzeler &amp; Yıldırım, 2016)</td>
</tr>
<tr>
<td></td>
<td>8.07</td>
<td>37.69</td>
<td>28.2</td>
<td>5.44</td>
<td>16.31</td>
<td>4.29</td>
<td>(Etiosa et al., 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>19</td>
<td>17</td>
<td>30</td>
<td></td>
<td>(Mateos-Aparicio et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>8.43</td>
<td>37.29</td>
<td>17.86</td>
<td></td>
<td></td>
<td>4.99</td>
<td>(Ren et al., 2012)</td>
</tr>
<tr>
<td>Young soybean</td>
<td>10.81</td>
<td>37.1</td>
<td>0.93</td>
<td>9.19</td>
<td>38.6</td>
<td>3.39</td>
<td>(Redondo-Cuenca et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>67.5</td>
<td>12.9</td>
<td>6.8</td>
<td>4.2</td>
<td>11</td>
<td>1.7</td>
<td>(Ravishankar et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>6.12</td>
<td>42.22</td>
<td>14.96</td>
<td></td>
<td></td>
<td>4.89</td>
<td>(Ren et al., 2012)</td>
</tr>
</tbody>
</table>

1.3.2 Soybean protein hydrolysates production and their bioactive properties

Bioactive peptides are specific protein fragments that can exert a positive effect on the human body and may influence health. These bioactive peptides are organic substances composed of amino acids that are joined covalently by peptide bonds (Sánchez & Vázquez, 2017) and can vary in terms of the number, type, and sequence of amino acids.

1.3.2.1 Production of bioactive peptides

Bioactive peptides can vary in their length of amino acids they possess and usually range from 2 to 20 amino acids. Different methods are used for the release of the peptides from the sequence of amino acids in the parent proteins including,
gastrointestinal digestion, enzymatic hydrolysis, food processing, and bacterial fermentation (Chatterjee et al., 2018). The enzymes that are used for *in-vitro* enzymatic hydrolysis or bacterial fermentation can influence the peptide production and its composition and the bioactive properties of the generated peptide is determined by the sequence of the amino acid and its composition when it releases from its precursor protein where it is encrypted (Chatterjee et al., 2018; Sánchez & Vázquez, 2017). Bioactive peptides from protein sources can be obtained using any of the mentioned methods below.

1.3.2.1.1 *In-vitro* simulated gastrointestinal digestion (SGID)

The bioactive peptides can be generated upon digestion of the soybean proteins by application of gastric acid and digestive enzymes present in the stomach, small intestines, and pancreas like pepsin, chymotrypsin, trypsin, and pancreatin during the simulated conditions. The absorption of these peptides occurs through the walls of the small intestine and then to the bloodstream (Chatterjee et al., 2018). In such a model, simulated digestive juices from gastric and intestinal phase of digestion are formulated and used for digestion of protein samples. The generated hydrolysates are analyzed for identification of peptides by different advanced chromatographic techniques.

1.3.2.1.2 *In-vitro* enzymatic hydrolysis

Hydrolysis by enzymes is a common method to produce bioactive peptides by using single or multiple specific or nonspecific proteases (Rizzello et al., 2016). Enzymes require a short time to generate a similar degree of hydrolysis, and the enzymatic reaction can be controlled to produce a peptide with reproducible molecular weight profiles and compositions (Rizzello et al., 2016). Enzymatic hydrolysis is particularly used in the food and pharmaceutical industries (Singh et al., 2014). The
enzymes may be used in a combination to generate effective and stable bioactive peptide mixtures demonstrating a wide range of bioactive properties (Singh et al., 2014). Range of food-grade enzymes are currently being employed for the generation of bioactive peptides of specific amino acid sequence. Different factors like pH, temperature, enzyme to substrate ratio is being taken into consideration to optimize the production of bioactive peptides using food-grade enzymes (Baba et al., 2021).

1.3.2.1.3 Microbiological fermentation

Another method for the production of bioactive peptides and food-grade hydrolyzed proteins is by implementing microbial fermentation. Fermentation can be done through either microbial activity or microbial enzymatic activity (Chatterjee et al., 2018). Lactic acid bacteria is a large group of bacteria that is found in the human digestive system and is widely spread in nature, is frequently used in producing bioactive peptides (Singh et al., 2014). It is important to use additional enzymes (e.g. trypsin and plasma proteases) when applying fermentation, since fermentation may not fully hydrolyze specific proteins for example soy proteins due to complex tertiary structure and the presence of post-translational modification (Chatterjee et al., 2018). Natto, kinema, and chungkookjang are soybean products produced by fermenting soybean with Bacillus only, while fermentation using fungi can produce another soybean product such as sufu, tempeh, douche (Sanjukta & Rai, 2016). It is important to choose suitable cultures, due to their effect on the type, amount, and activity of the peptides produced (Sánchez & Vázquez, 2017). The microbial fermentation by the action of proteolytic enzymes of mold and bacteria can ultimately improve the flavor and enhance the absorptivity of the fermented products (Sun, 2011).
1.3.2.2 Bioactive properties of soybean protein hydrolysates

Researchers are focusing more on the soybean biologically active “bioactive” peptides upon releasing from simulated gastrointestinal digestion (SGID), food processing, and enzymatic hydrolysis. Because these peptides have been proven to possess different bio-functional properties such as anti-hypertensive, anti-oxidative, anti-obesity, anti-diabetic, anti-inflammatory (AI), and anti-cancer properties (Chatterjee et al., 2018; Singh et al., 2014). Studies have demonstrated that partial hydrolysis of the predominant proteins which account for over 85% of total soy proteins: glycinin and β-conglycinin, can produce hydrolysates and peptides which provide the aforementioned bio-functional properties (Agyei, 2015). The range of bioactive properties demonstrated by soybean protein hydrolysates are summarized in the below mentioned sections.

1.3.2.2.1 Antihypertensive properties

Hypertension is considered to be a major risk factor for several diseases including brain stroke, renal and heart diseases. The effect of hypertension was estimated that one in six people or nearly one billion worldwide will be affected by it, and the number may increase by 2025 by 1.5 million (Chockalingam, 2007). The most common food-derived peptides are the antihypertensive peptides, and their activity is exhibited by inhibiting the angiotensin-converting enzyme (ACE) (De Mejia & Ben, 2006). Several antihypertensive peptides have been derived from soybean and soybean products. Guan et al. (2018), have studied the enzymatic hydrolysis of soy protein isolates by corolase PP (food-grade porcine pancreatic proteolytic preparation) under high hydrostatic pressure conditions and estimated that the peptides generated under high hydrostatic pressure (80 – 300 MPa) had a remarkable increase in ACE inhibition
activity compared to the peptides generated at the atmospheric pressure (0.1 MPa). “Okara” is a Japanese name for the soy pulp which is a by-product of soybean curd “tofu” production, has shown an inhibition of ACE activity when examined in-vitro as a result of presence of antihypertensive peptides (Nishibori et al., 2017). A comparative study on ACE inhibition activity was conducted between two fermented tofus, the Japanese tofu and the Chinese sufu, and it was found that the Chinese sufu has higher inhibition of ACE activity than the Japanese tofu (Sanjukta & Rai, 2016). Another study was conducted to generate ACE inhibitory peptides from soy proteins by Lactobacilli fermentation. It was observed that Lactobacillus casei spp. Pseudoplanatarum have exhibited higher proteolytic activity on the soy proteins and generated two peptides associated with ACE inhibition with IC\textsubscript{50} values of approximately 17 and 30 µg/ml, respectively (Agyei, 2015).

Coscueta et al. (2016) have reported that hydrolysates which have been generated from the enzymatic hydrolysis of soybean meal protein isolate with corolase PP at two different time and temperature conditions (70°C, 1 h; 90°C, 30 min) showed an ACE inhibitory activity. These hydrolysates have been characterized and detected and the main ACE inhibiting peptides identified were IRHFNEGDLVPPGVPY, IRHFNEGDLVPPGVPYW, IYNFREGDLIAVPTG, VSIIDTNSLENQLDQMPRR, and YRAELSEQDIFVIPAG (Coscueta et al., 2016).

Hanafi et al. (2018), reported the ACE inhibitory activity of defatted green (young) soybean hydrolysates produced by different enzymes (alcalase, papain, flavourzyme, and bromelain). The highest ACE inhibitory activity was recorded for the hydrolysates generated by alcalase (IC\textsubscript{50}: 0.14 mg/ml at 6 h hydrolysis time), followed by the papain generated hydrolysates (IC\textsubscript{50}: 0.2 mg/ml at 5 h hydrolysis time), bromelain generated hydrolysates (IC\textsubscript{50}: 0.36 mg/ml at 6 h hydrolysis time), and
flavourzyme generated hydrolysates showed the least inhibitory activity (IC$_{50}$: 1.14 mg/ml at 6 h hydrolysis time). The peptides generated by alcalase that have exhibited the highest ACE inhibition were identified as EAQRLLF, PSLRSYLAELAE, PDRSIHGPQLAE, FITAFR, and RGQVLS with ACE-IC$_{50}$ values of 878 μM, 532 μM, 1552 μM, 1342 μM and 993 μM, respectively.

1.3.2.2.2 Antioxidative properties

An excessive amount of the reactive oxygen species are considered hazardous and can lead to oxidative damage to the cell membranes and ultimately causes several different chronic diseases including diabetes, cancer, and cardiovascular diseases (Zhang et al., 2018b). The antioxidant compounds in the food industries and pharmaceuticals were greatly used to inhibit the effects of the reactive oxygen species and recently, a large interest in the use of protein hydrolysates and isolated peptides as a natural source of antioxidants is being explored (Zhang et al., 2018a). The bioactive peptides having antioxidant activities show a radical scavenging activity, chelating of metal ions, and inhibition of lipid peroxidation, and there are many peptides that are generally accepted antioxidants such as tyrosine, methionine, histidine, lysine, and tryptophan (Singh et al., 2014).

A study was conducted to investigate the impact of the simulated gastrointestinal digestion, and the transepithelial transport on the antioxidant properties of soybean proteins hydrolyzed with alcalase (Zhang et al., 2018a). A wide variation was noticed in their antioxidant activities assessed using different assays such as 2,2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, ferric reducing antioxidant power, and metal ion-chelating activities and their IC$_{50}$ values were found
to be 4.22, 2.93, and 0.67 mg/ml, respectively. In addition, these hydrolysates have also inhibited the production of the intracellular reactive oxygen species in Caco-2 cells. After the simulated gastrointestinal digestion, the hydrolysates shown enhanced antioxidant activities except for ABTS (Zhang et al., 2018a). Furthermore, Yang et al. (2000) have reported the antioxidative properties of the fermented soybean broth and found that the fermented soybean broth exhibit a scavenging activity on DPPH, on hydroxyl radicals, and on the peroxide anion radicals. Fermented soybean broth was able to chelate both ferrous and cupric ions. Jara et al. (2018) studied the release of antioxidant peptides from the soybean protein isolate by using a proteolytic extract prepared from a *Maclura pomifera* (plant-protease) latex. An IC_{50} of 31.6 ± 0.2 μg/ml has been presented by the 90 min soybean hydrolysate. Hydrolysates demonstrated ABTS radical scavenging activity and oxygen–radical–absorbance capacity, to be 157.6 and 176.9 μmoles Trolox equivalent per g of the peptide, respectively. The 90 min antioxidant hydrolysates have been analyzed and identified fourteen theoretical peptides containing > 60% antioxidant amino acids.

1.3.2.2.3 Antidiabetic properties

Diabetes mellitus is a known common metabolic disorder and characterized by the dysregulation of blood glucose level. The major two forms are type 1 that is an insulin-dependent which makes up a 10%, of all diabetes cases, and type 2 the non-insulin-dependent which makes up to 90%, of all diabetes cases (Wang et al., 2019). By 2035, the number of diabetic patients is projected to increase from 350 million today to 592 million (Lee et al., 2016). The triggering factors like obesity and hyperlipidemia are frequently linked with type 1 and type 2 diabetes (Chatterjee et al., 2018).
Wang et al. (2019) have studied the different bioactivities of soybean protein hydrolysates including the antidiabetic properties. The hydrolysates were prepared with three enzymes: alkaline proteinase, papain, and trypsin. The highest $\alpha$-glucosidase inhibition was exhibited by soybean protein hydrolysates generated with alkaline proteinase. The three novel $\alpha$-glucosidase inhibitory peptides that have been identified were: LLPLPVLK, SWLRL, and WLRL with IC$_{50}$ of 237.43 ± 0.52, 182.05 ± 0.74, and 162.29 ± 0.74 $\mu$mol/l, respectively (Wang et al., 2019). In another study, germinated soybean protein isolate was subjected to the gastrointestinal digestion to produce the bioactive peptides, which were demonstrated to act as an inhibitor of dipeptidyl peptidase IV (DPP-IV), $\alpha$-amylase (AA), and intestinal $\alpha$-glucosidases and modulated the postprandial glycaemic response (González-Montoya et al., 2018a). Peptide fractions of (5 – 10 kDa) and (> 10 kDa) were potentially inhibiting the DPP-IV. On the other hand, the peptide fractions (5 – 10 kDa) and (<5kDa) were very effective in inhibiting the AA and $\alpha$-glucosidases (González-Montoya et al., 2018a).

Several studies were conducted out on soy protein hydrolysates in the in-vivo model system to investigate their anti-diabetic properties. Jamilian & Asemi, (2015), studied the effect of soy intake on women with gestational diabetes mellitus and prepared a diet with soy protein which contains (35% animal protein, 35% soy protein, and 30% other plant proteins). The diet was consumed by a group of women aged between 18 - 40 years and at week 20 – 28 of gestation for 6 weeks (n = 34). The results have shown remarkable improvement on plasma glucose, serum insulin levels, and homeostasis model of assessment-insulin resistance as well as a decrease of the hospitalization and hyperbilirubinemia incidences of the newborns in the group consuming soy protein hydrolysate, when compared to the control group of women who consumed a diet of (70% animal and 30% plant proteins) (Jamilian & Asemi,
It has been reported that aglycin is a natural bioactive peptide with 37 residues that is capable of resisting the proteolysis reactions during transit in the gastrointestinal system of mammals and can be found in plant albumins of pea seed including soybeans (Agyei, 2015). For example, Kehinde and Sharma, (2018) have reported the antidiabetic effects of aglycin in diabetic mice. Firstly, mice were fed with a fat-rich diet, followed by streptozotocin injection to induce diabetes. Aglycin was orally administered after dissolving it in saline solution (5 mg/ml) at a daily dosage (50 mg/kg) for four weeks. The results showed significant control of hyperglycemia, an increase in glucose uptake, regulated glucose homeostasis, and restored insulin signal transduction.

1.3.2.2.4 Miscellaneous bioactive properties of soybean peptides

Many studies have investigated different bioactive properties of the soybean peptides such as the anticancer (González-Montoya et al., 2018b), hypocholesteremic (Ferreira et al., 2010), anti-inflammatory (Vernaza et al., 2012), inhibition of lipid accumulation (Martinez-Villaluenga et al., 2009). Table 2 shows different examples of bioactive properties of derived peptides and hydrolysates of soybean protein. Rayaprolu et al. (2013) have tested the bioactivity of high oleic acid soybean peptides against colon, liver, and lung cancer. High oleic acid soybean was hydrolyzed by alcalase to produce hydrolysates that were fractioned into different molecular sizes. Results showed that peptides inhibitory effect increases at higher concentrations and the peptides inhibited the cell growth of colon cancer by 73%, liver cancer by 70%, and lung cancer by 68% cells. On the other hand, González-Montoya et al. (2018b) studied the AI and anti-cancer activities of geminated soybean protein after subjecting them to simulated gastrointestinal digestion using the two enzymes: pepsin and
pancreatin, followed by fractioning the peptides. The highest effect against AI and anti-cancer activities was obtained from the peptide fraction with a molecular weight between 5 – 10 kDa.

Soy protein hydrolysates rich in β-conglycinin were also investigated for their effect on lipid accumulation mainly in 3T3-L1 adipocytes. Soy protein hydrolysates were produced from different genotypes by alcalase or gastrointestinal digestion on lipid accumulation in 3T3-L1 adipocytes. With (100 µM) soy hydrolysate generated by alcalase, lipid accumulation has reduced 33 – 37% inhibition by inhibiting the gene expressions of the lipoprotein lipase and fatty acid synthase. While with 100 µM of soy hydrolysate generated by SGID, it exhibited a lower lipid accumulation 8 – 14% inhibition through down regulating the gene expression of lipoprotein lipase (Martinez-Villaluenga et al., 2009).

It has been observed that the main soy proteins β-conglycinin (7S) and glycinin (11S) were able to exert hypocholesterolemic effects when administrated at a dose of 2.75%, daily by rats that have been fed with high cholesterol diet, and the results were comparable with fenofibrate a commercial drug (Ferreira et al., 2010). Lammi et al. (2015), reported three hypocholesteremic peptides from soy glycinin (IAVPGEVA, IAVPTGVA, and LPYP) that were used as a treatment on HepG2 cells, were able to modulate the cholesterol metabolism and interfere with the catalytic activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR), which is a primary enzyme in cholesterol biosynthesis (Lammi et al., 2015)
Table 2: Examples of soybean protein derived bioactive peptides and their different bioactive properties.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme</th>
<th>Bioactive properties</th>
<th>Hydrolysis conditions</th>
<th>Peptide sequence identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted soy flakes</td>
<td>Corolase PP</td>
<td>Antihypertensive</td>
<td>(5 h) hydrolysis time</td>
<td>LPDNAK, VQACSQEEVKN, ENFVYVAK, VLTHTPPPLK and GVAIMDFILR.</td>
<td>(Guan et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(80 – 300 MPa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcalase</td>
<td></td>
<td>(10 h) hydrolysis time</td>
<td>EAQRLLF, PSLRSYLAE, PDRSIHGRQLAE, FITAFR, and RGQVLS.</td>
<td>(Hanafi et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td></td>
<td>(Alcalase, pH 8.0, 60°C; Bromelain, pH 6.5, 45°C; Flavourzyme, pH 8.0, 55°C; Papain, pH 6.5, 70°C).</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavourzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromelain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defatted young soybean</td>
<td></td>
<td>Antihypertensive</td>
<td></td>
<td>IRHFNEDVLVPGPVYPY, IRHFNEDVLVPPGVYPYW, IYNFREGDLIAVPTG, VSIIDTNSLENQLDQMPRR, and YRAELSEQDIFVIPAG.</td>
<td>(Coscueta et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean meal protein isolate</td>
<td>Corolase PP</td>
<td>Antihypertensive</td>
<td>Proteins isolated at (70°C, 1 h; 90°C, 30 min) at (30 min to 24 h) hydrolysis time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antioxidant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 2: Examples of soybean protein derived bioactive peptides and their different bioactive properties (Continued).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme</th>
<th>Bioactive properties</th>
<th>Hydrolysis conditions</th>
<th>Peptide sequence identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted soybean flour</td>
<td>Peptidases from latex of <em>Maclura</em></td>
<td>Antioxidant</td>
<td>45°C for different hydrolysis times (10–180 min)</td>
<td>Fourteen theoretical peptide sequences containing antioxidant amino acids at &gt;60%</td>
<td>(Jara et al., 2018)</td>
</tr>
<tr>
<td></td>
<td><em>pomifera</em> fruit</td>
<td></td>
<td></td>
<td>(Jara et al., 2018)</td>
<td></td>
</tr>
<tr>
<td>Full-fat soybean flakes</td>
<td>Alcalase</td>
<td>Antioxidant</td>
<td>pH 8.5, 55°C for 3 h</td>
<td>ALPEEVQH, VIPGVPY, VVPGHPF, TILLPH, and NEPWWPK</td>
<td>(Zhang et al., 2018b).</td>
</tr>
<tr>
<td>Fermented soybean extract</td>
<td>Fermented with <em>Aspergillus</em></td>
<td>Antihypertensive</td>
<td>-</td>
<td>Leu-Val-Gln-Gly-Ser</td>
<td>(Rho et al., 2009)</td>
</tr>
<tr>
<td></td>
<td><em>oryzae</em></td>
<td></td>
<td></td>
<td>(Rho et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Germinated soybean</td>
<td>Pepsin</td>
<td>Anticancer</td>
<td>Gastrointestinal digestion</td>
<td>Twelve peptides of different lengths from 7 to 13 amino acids</td>
<td>(González-Montoya et al., 2018b)</td>
</tr>
<tr>
<td></td>
<td>Pancreatin</td>
<td>Anti-inflammatory</td>
<td>Gastric (37°C, 1 h)</td>
<td>(González-Montoya et al., 2018b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intestinal (37°C, 2 h)</td>
<td>(González-Montoya et al., 2018b)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Examples of soybean protein derived bioactive peptides and their different bioactive properties (Continued).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzyme</th>
<th>Bioactive properties</th>
<th>Hydrolysis conditions</th>
<th>Peptide sequence identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy protein isolate</td>
<td>Alcalase</td>
<td>Hypocholesterolemic</td>
<td>DH 18% (pH 8.0, 60°C)</td>
<td>WGAPSL</td>
<td>(Zhong et al., 2007b)</td>
</tr>
<tr>
<td>β-Conglycinin purified from soybean defatted flour</td>
<td>Alcalase</td>
<td>Fatty acid synthase inhibitory peptides</td>
<td>-</td>
<td>KNPQLR, EITPEKNPQLR and RKQEEDEDEEQQRE</td>
<td>(Martinez-Villaluenga et al., 2010)</td>
</tr>
<tr>
<td>Soy protein powder</td>
<td>Alkaline proteinase</td>
<td>Antidiabetic</td>
<td>Alkaline proteinase (pH 9, 50°C)</td>
<td>Antidiabetic (α-glucosidase)</td>
<td>(Wang et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td>Antihypertensive</td>
<td>Papain (pH 6.5, 60°C)</td>
<td>LLPLPVLK, SWLRL, and WLRL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>Antioxidant</td>
<td>Trypsin (pH 7, 37°C). (For 8 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean peptides</td>
<td>-</td>
<td>Hypocholesteremic</td>
<td>-</td>
<td>IAVPGEVA, IAVPTGVA, and LPYP</td>
<td>(Lammi et al., 2015)</td>
</tr>
</tbody>
</table>
1.3.3 Characterization of soybean protein hydrolysates

1.3.3.1 Degree of hydrolysis

Degree of hydrolysis (DH) is the main parameter that monitors the extent of the protein hydrolysis when carried out by specific proteolytic enzymes (Nielsen et al., 2001). The characterization of the protein hydrolysates which includes the determination of their degree of hydrolysis and their peptides distribution according to the chain length, is essential when developing new food products or dietary supplements (Morais et al., 2013). There are several methods for determining the degree of hydrolysis of proteins and each method has a different principle. The first principle is the determination of the hydrolytic release of nitrogen, where nitrogen becomes soluble in the presence of precipitating agents. The second principle is the determination of the free $\alpha$-amino-groups. The third principle is the titration of the protons that released upon cleavage of peptide bonds. The last principle is the measurement of the changes in the freezing point of the protein solution by osmometry (Morais et al., 2013).

The parameters that are mostly used in the DH of soy protein hydrolysis are the average peptide chain length and the average molecular weight, and the DH reported for soy protein hydrolysates is usually between 1 – 39.5% (Sun, 2011). Hrckova et al. (2002), have indicated that the highest DH in soybean protein hydrolysates was 39.5% obtained by flavourzyme at 480 min. In addition, Wang et al. (2019) estimated the DH and the maximum degree after 8 h hydrolysis was (36.84%) using alkaline proteinase. Vallabha & Tiku, (2014) obtained a DH (22%) after the fermentation of the soy protein by lactic acid bacteria for 36 h. Coscueta et al. (2016) prepared the soy protein hydrolysates with corolase PP under two temperature
conditions (70°C, 1 h; 90°C, 30 min), and reached a maximum degree of hydrolysis 18.9% and 20.4% at 70°C and 90°C, respectively at 10 h of hydrolysis. Corolase PP has been also used in preparing soy protein hydrolysates under high hydrostatic pressure, and the highest DH obtained was 30.6% at 4 h of hydrolysis and 200 MPa (Guan et al., 2018). Furthermore, the highest degree of hydrolysis in soy protein hydrolysates obtained using a proteolytic extract prepared from a *Maclura pomifera* (plant-protease) latex after 180-min (3 h) was 36.2% (Jara et al., 2018). These studies indicated that DH in soy protein hydrolysates varies and it depends on the type of enzymes used, condition of hydrolysis applied as well as composition of protein substrate.

### 1.3.3.2 Electrophoretic techniques for separation of proteins

Electrophoresis is defined as the movement and separation of charged ionic particles by the effect of an electric field. The mobility of the ionic particles is estimated according to the particle size, shape, charge, and temperature during the separation. Several types of separation by electrophoresis can be used such as polyacrylamide gel electrophoresis, isoelectric focusing, and capillary electrophoresis (Fritsch & Krause, 2003).

Polyacrylamide gel electrophoresis (PAGE) method has been extensively used for the analysis of soybean proteins and their hydrolysates. PAGE combined with the ionic detergent sodium dodecyl sulfate (SDS) is applied to separate the protein subunits according to their size. In the characterization protocols, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method is used to estimate the proteins composition of their subunits and their molecular mass (Smith, 2010). The SDS-desaturated proteins are separated mainly by sieving effects. For instance, the
lower molecular weight proteins will migrate faster through the gel, and to obtain a fairly accurate estimation, the migration rate of the sample protein can be compared to the standard proteins with a known molecular weight (Fritsch & Krause, 2003).

For characterization of soybean-derived proteins and protein hydrolysates, Mujoo et al. (2003) analyzed the total protein profile for different varieties of soybean using SDS-PAGE and it was found that the protein bands were similar among all the varieties. β-conglycinin (7S) subunits were (α', α, and β) with molecular weight of around 80,000, 75,000, and 50,000, respectively. The acidic subunits of glycinin (11S) were found to have a molecular weight around 36,000 to 10,000, while the basic subunits were approximately 15,000. Jara et al. (2018) used the tricine-SDS-PAGE to separate the hydrolysates of the soybean protein isolate into their molecular masses. They obtained several β-conglycinin subunits (α, α', and β) with molecular masses between 47 and 67 kDa. Moreover, the acidic and basic subunits of glycinin with molecular masses of 36 and 22 kDa, respectively were also reported. Coscueta et al. (2016) have also used the tricine-SDS-PAGE to characterize the soybean protein hydrolysates of different molecular weight profiles. The gel electrophoresis clearly displayed separate bands for subunits for glycinin and β-conglycinin for both soybean hydrolysates, which were prepared at 70°C for 1 h and 90°C for 30 min. For soybean protein hydrolysate produced at 70°C (SPH70), glycinin subunits were intact until 4 h, while β-conglycinin bands were hydrolyzed and nearly disappeared at 4 h. Glycinin subunits of soybean hydrolysate produced at 90°C (SPH90) remained intact and nearly disappeared at 4 h, and β-conglycinin bands started to disappear at 30 min. Besides, the author has reported that the hydrolysates SPH70 and SPH90 have revealed new bands at 25 kDa and below 20 kDa up to small peptides at the bottom of the gel (Coscueta et al., 2016).
Furthermore, Hrckova et al. (2002) investigated the effect of the hydrolysis of soybean proteins by different enzymes by using SDS-PAGE. It was noticed that the glycycin consist of acidic and basic subunits and β-conglycinin subunits (α, α’, and β). The protein hydrolysates were subjected to breakdown by all the enzymes used (novozyme, flavourzyme, and alcalase), and ultimately the low molecular weight proteins have concentrated only at the bottom of the gel. Glycinin exhibits higher resistance to the enzymatic hydrolysis, whereas β-conglycinin degrades easily. The basic glycycin fraction breaks down slower than the acidic glycycin fraction, which could be due to the fact that the basic glycycin peptides tend to form large complexes and thus becomes less subjected to the enzymatic hydrolysis (Hrckova et al., 2002).

1.3.3.3 Chromatographic techniques

Chromatographic techniques which are based on partitioning or distribution of sample molecules between the mobile phase and stationary phases have been applied to separate different organic components. The fixed or stationary phase can be liquid, or frequently a solid, while the moving or mobile phase can be a gas, liquid, or supercritical fluid. Chromatographic techniques are further divided into the different techniques applied, such as gas chromatography, liquid chromatography, paper chromatography, and column liquid chromatography (Ismail & Nielsen, 2010). The high-performance liquid chromatography (HPLC) method is now being used extensively for analyzing protein hydrolysates and peptides due to the effective separation, versatility, better resolution, speed (short times for analysis), and suits the automation procedures (de Llano & Sánchez, 2003). Chromatographic separation of peptides based on the molecule size (gel permeation chromatography), charge (ion-exchange chromatography), hydrophobicity (reversed-phase and interaction
chromatography), and with combinations of all these methods (de Llano & Sánchez, 2003) is being used for characterizing protein hydrolysates and peptides.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is widely used for separating peptides according to their hydrophobic properties (Rizzello et al., 2016). Reversed-phase columns that are employed for the separation of small peptides are with pore sizes of 6 – 10 nm. Large peptides with molecular weights of 4000 kDa are recommended to be separated using a column with pore sizes of 30-50 nm (de Llano & Sánchez, 2003). RP-HPLC has been largely used for the analysis of plant proteins (Reuhs & Rounds, 2010). An example of a study where five steps of purification procedures were conducted to produce a novel ACE-inhibitory peptide from fermented soybean extract. The purification procedure involved ultrafiltration and other consecutive chromatographic methods, which included the RP-HPLC, cation exchange chromatography, and gel permeation chromatography. The author reported that the novel ACE-inhibitory peptide which was separated showed a 66-fold inhibitory activity as compared to the fermented soybean extract (Rho et al., 2009).
Chapter 2: Methods

2.1 Enzymes and reagents

Alcalase from *Bacillus licheniformis* (EC 3.4.21.62), bromelain from pineapple stem (EC 3.4.22.32), flavourzyme from *Aspergillus oryzae* (EC 232-752-2), pepsin from gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), α-amylase from porcine pancreas (EC 3.2.1.1), DPP-IV human (EC 3.4.14.5), porcine pancreatic lipase (EC 3.1.1.3), cholesterol esterase enzyme from porcine pancreas, (EC 3.1.1.13) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

Reagents like petroleum ether, sulfuric acid, Kjeldahl catalyst, bile salts (Oxgall), Folin-Ciocalteu reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroom-2-carboxylic acid (Trolox), o-phthalaldehyde (OPA), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), p-nitrophenyl-α-D-maltohexaoside (pNPM), monosodium phosphate monobasic, disodium phosphate dibasic, Gly-Pro-p-nitroanilide, Trizma base, Acarbose, Diprotin (Ile-Pro-Ile), p-nitrophenyl butyrate, Orlistat, sodium acetate, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, potassium persulphate, methanol, ethanol, hexane, sodium hydroxide, sodium dodecyl sulphate (SDS), sodium tetraborate, β-mercaptoethanol were all purchased from BDH middle east or Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

2.2 Sample preparation

Mature soybean (MS) seeds were procured from the local supermarket of Al Ain, Abu Dhabi, United Arab Emirates (UAE). Young soybean (YS) seed powder was procured from Kimuraya Company, Japan. The dried MS seeds were crushed using a
commercial mixer grinder (Moulinex, France) and sieved through a mesh (20 µm) to obtain a fine powder. The resultant powders of MS and YS were stored at -20°C under airtight conditions until used.

2.3 Proximate composition analysis

The proximate composition of YS and MS was estimated using the standard procedures of Association of Official Analytical methods (AOAC, 2000). Moisture content was estimated by hot air oven method at 70°C for three days. Fat content was determined by Soxhlet apparatus using petroleum ether as an organic solvent at 80°C for 1 h. Total protein content was analyzed by Kjeldahl method and ash content was determined by weighing the amount of residues present after burning the samples in the muffle furnace at 550°C for 18 – 24 h. Carbohydrate content was then estimated using subtraction method.

2.4 Phase 1: Analysis of bioactive properties of young and mature soybean subjected to simulated gastrointestinal digestion (SGID)

2.4.1 In-vitro simulated gastrointestinal (SGID) of young and mature soybean powder

A static model that simulates digestion in stomach and intestine was adopted according to a method as described by Ahmad et al. (2019), involving, simulated gastric juice (SGJ) for gastric digestion and simulated intestinal juice (SIJ) to mimic intestinal digestion. Briefly, SGJ was prepared by addition of 3 g/l of pepsin and sodium chloride (NaCl) 9 g/l in sterile deionized water and the pH was adjusted to 2.0 using 1.0 mol/l hydrochloric acid (HCl). Similarly, SIJ was prepared by mixing 10 g/l bile salts (Oxgall) and 3 g/l of pancreatin in sterile phosphate-buffered saline and the pH was adjusted to 8.0 with 0.1 M NaOH solution. To proceed with simulated gastrointestinal digestion (SGID), 5 g of YS and MS powdered samples were
individually mixed with 50 ml of SGJ and incubated for 2 h to digest the samples under simulated gastric conditions. Thereafter, an aliquot of 20 ml from each sample was taken to represent the gastric digested sample (SGD) and to the remaining portion, 50 ml of SIJ was added and incubation was performed at 37°C for 3 h under constant stirring to obtain a complete SGID sample. The obtained samples were then heated at 100°C for 10 min to stop the enzymatic reaction and centrifuged at 10,000 × g for 10 min at 4°C. All the supernatants (SGD and SGID) thus obtained were stored at -20°C until further analysis which was carried out within one month.

2.4.2 Total phenolic content (TPC)

The total phenolic contents of the digested YS and MS samples were determined using Folin-Ciocalteu assay (El Sohaimy, 2013). Briefly, 0.5 ml of the digested soybean samples were mixed with 2.5 ml of Folin-Ciocalteu reagent followed by addition of 2 ml of sodium carbonate (Na₂CO₃) (75 g/l). The mixtures were then incubated for 5 min at 50°C, 200 µl volume was transferred to a 96 well microplate and the absorbance of the samples was measured at 760 nm a microplate reader (Epoch 2, BioTek, VT, USA), and the phenolic content was expressed as mg gallic acid equivalents per gram of soybean (mg GAE/g).

2.4.3 Inhibition of key enzymes related to diabetes

2.4.3.1 Inhibition of α-amylase (AA)

The AA inhibition was evaluated for the digested samples of YS and MS as described by Mudgil et al. (2019c). Approximately, 25 µl of the samples were mixed with 50 µl of p-nitrophenyl-α-D-maltohexaoside (pNPM; 5mM) (substrate) and 50 µl of porcine pancreatic AA enzyme (5 mg/ml) in 96-well microplate. The total reaction
volume was set at 250 µl using sodium phosphate buffer (0.02 M; pH 6.9) and incubated at 37°C for 90 min. A control reaction (without soybean samples) was included and prepared in a similar way to represent a 100% enzyme activity. The absorbance of the produced p-nitrophenyl was measured at 405 nm on a microplate reader (Epoch 2, BioTek, VT, USA). To eliminate the background absorbance produced from samples blank reactions (samples with substrate and buffer without enzyme) were conducted for each sample. Acarbose was used as a positive inhibition control. Each sample was analyzed in triplicate and the percentage of AA inhibition was calculated using equation:

\[
\% \text{ Enzyme inhibition} = \left[ 1 - \left( \frac{C - D}{A - B} \right) \right] \times 100,
\]

where A (control), B (control blank), C (sample), and D (sample blank) refer to the absorbance values of reaction vials containing enzyme and buffer, No enzyme only buffer, enzyme and sample, and No enzyme only sample, respectively. Substrate was present in all reactions.

The inhibitory concentration values that inhibit 50% (IC\text{50}) of AA activity were measured by plotting the percentage inhibition as a function of the test compound concentration. The IC\text{50} values were expressed as µg of phenolic equivalent/ml.

2.4.3.2 Inhibition of dipeptidyl peptidase IV (DPP-IV)

The inhibitory activity of DPP-IV was determined as described by Kamal et al. (2018) for the YS and MS. Soybean samples (25 µl) were added to 50 µl of the reaction substrate Gly-Pro-p- nitroanilide (0.5 mM), 25 µl of DPP-IV (8 U/ l), 100 µl of 0.1 M of TRIS-HCl buffer (pH 8.0) and the mixture was incubated at 37°C for 90 min. The absorbance of p-nitroanilide released was measured at 405 nm using the microplate reader (Epoch 2, BioTek, VT, USA). A control reaction (without soybean
samples) was included and prepared in a similar way to represent a 100% enzyme activity. Blank reactions were also prepared as described above in Section 2.4.3.1. Diprotin (Ile-Pro-Ile), was used as a positive inhibition control. The percentage of DPP-IV inhibitory activity was measured using the equation as described above in Section 3.4.3.1. The inhibitory concentration values that inhibit 50% (IC$_{50}$) of DPP-IV activity were measured by plotting the percentage inhibition as a function of the test compound concentration. The IC$_{50}$ values were expressed as μg of phenolic equivalent/ml.

### 2.4.4 Inhibition of key enzymes related to obesity

#### 2.4.4.1 Inhibition of pancreatic lipase (LIP)

The LIP inhibitory activity assay was estimated according to method conducted by Ahmad et al. (2019). Briefly, samples (25 μl) were mixed with 25 μl of pancreatic LIP solution (5 mg/ml), 50 μl of p-nitrophenyl butyrate (5 mM) as substrate, and sodium phosphate buffer (0.1 M, pH 7.2) in a 96-well microplate and incubated at 37°C for 90 min. A control reaction (without soybean samples) was included and prepared in a similar way to represent a 100% enzyme activity. The absorbance of the released p-nitrophenyl was monitored at 405 nm in a microplate reader (Epoch 2, BioTek, VT, USA). Blank reactions were also prepared as described above in Section 3.4.4.1. Orlistat was used as positive inhibition reaction control and the percentage of LIP inhibition was measured using the similar equation as described in Section 2.4.3.1. The inhibitory concentration values that inhibit 50% (IC$_{50}$) of LIP activity were measured by plotting the percentage inhibition as a function of the test compound concentration. The IC$_{50}$ values were expressed as μg of phenolic equivalent/ml.
2.4.4.2 Inhibition of cholesterol esterase (CE)

The CE inhibition was determined using a method described by Mudgil et al. (2019b). Briefly, in a 96-well microplate, 25 μl of test samples, 50 μl of substrate containing 5 mM p-nitrophenyl butyrate in 100 mM sodium phosphate buffer and 100 mM NaCl (pH 7.2) were incubated with 50 μl of porcine pancreatic CE (5 μg/ml) at 37°C for 30 min. The released p-nitrophenol upon the enzymatic hydrolysis of p-nitrophenyl butyrate was measured at 405 nm on the microplate reader. Orlistat was used as positive inhibition control and the percentage of the inhibitory activity against CE was calculated using the equation described in Section 2.4.3.1. The inhibitory concentration values that inhibit 50% (IC\textsubscript{50}) of CE activity were measured by plotting the percentage inhibition as a function of the test compound concentration. The IC\textsubscript{50} values were expressed as μg of phenolic equivalent/ml.

2.4.5 Antioxidant activities

2.4.5.1 Ferric reducing/antioxidant power (FRAP) assay

The ferric reducing ability of MS and YS upon SGID was evaluated using the ferric reducing/antioxidant power (FRAP) as described by Benzie and Strain, (1996) and further modified by Al-Shamsi et al. (2018). Working solution of FRAP reagent was prepared by addition of sodium acetate buffer (300 mM; pH 3.6), FeCl\textsubscript{3}.6H\textsubscript{2}O (20 mM) and 2,4,6-tripyridyl-s-triazine (TPTZ; 10 mM prepared in 40 mM HCl) in a ratio of 10:1:1 v/v. The FRAP assay was carried out in 96 well microplate where, 10 μl of test samples were mixed with 200 μl of the freshly prepared FRAP reagent and the incubation was carried out at 37°C for 30 min in the dark. The absorbance of the resultant blue product (ferrous tripyridyltriazine complex) was read at 593 nm in a microplate reader (Epoch 2, BioTek, VT, USA). For sample blank preparation FRAP
reagent devoid of FeCl3.6H2O was used instead. A standard curve of Trolox ranging from 50 – 600 μM was prepared and were the FRAP power of samples was expressed as Trolox equivalent antioxidant activity (TEAC) μg of phenolic equivalent/ ml.

2.4.5.2 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The antioxidant activity (scavenging of the ABTS radical) was measured as according to Al-Shamsi et al. (2018). The stock solutions used in the essay were 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. To prepare the working solution, an equal quantities of the two stock solutions were mixed and left to react for 12 h in the dark at room temperature to generate ABTS radicals. Approximately, 20 μl of sample with a concentration range of 0.5–10 mg/l was mixed with 180 μl of ABTS solution and made to stand for 30 min at room temperature in dark. The absorbance was then read at 734 nm the microplate reader (Epoch 2, BioTek, VT, USA). A sample blank at each concentration was prepared in the same manner except that methanol was used instead of ABTS solution. A standard curve using Trolox ranging from 50 to 600 μM was made and the activity was expressed as Trolox equivalent antioxidant activity (TEAC) μg of phenolic equivalent/ ml.

2.4.6 Anti-inflammatory (AI) activities

AI activity was conducted according to the method described by Kamal et al. (2018). Briefly, in a 96-well microplate, the reaction mixture was prepared by mixing 100 μl of sample, 50 μl of egg albumin (from fresh hen egg), and 100 μl of phosphate buffer saline (PBS) (pH 6.4). The control was prepared by replacing sample volume with same amount of PBS with distilled water. Incubation of the reaction mixture was set at 37°C for 15 min and then heated at 70°C for 5 min to terminate the reaction. The
absorbance of the reaction mixture was measured at 600 nm in a microplate reader (Epoch 2, BioTek, VT, USA). The standard curve was prepared with diclofenac sodium at a final concentration of 100 to 2,500 µg/ml that was used as a reference drug. The results were expressed as Diclofenac sodium equivalent capacity (DSEC) of µg phenolic equivalent/ ml.

2.5 Phase 2: Analysis of bioactive properties of young and mature soybean protein hydrolysates upon enzymatic hydrolysis

2.5.1 Isolation of proteins

YS and MS proteins were isolated according to a method described by Aluko and Monu, (2003) and modified by Mudgil et al. (2019d). The YS and MS seeds were initially grinded and then sieved through a sieve (3 µm pore size) to obtain a fine powder as described earlier in Section 2.2. Then, YS and MS flours (YS = 500 g, MS = 1000 g) were defatted in hexane for 2 h and centrifuged at (4255 × g, 15 min, 4°C). After centrifugation, deionized water was added at a ratio of 1:5 w/v, and the pH was adjusted to (10–11) with 20% NaOH to facilitate solubilization of proteins. The mixtures thus obtained were stirred at 400 rpm for 4 h at room temperature (24 ± 2°C) and the obtained slurries were then stored overnight at 4°C to promote better separation of proteins. The slurries were then homogenized using IKA ultra turrax T25 basic homogenizer at 15,000 rpm for 5 min and centrifuged two times at 4700 × g for 15 min at 4°C and then again at 10,000 × g for 15 min at 4°C. The resultant supernatants were collected and filtered through Whatman no. 1 filter paper to remove any remaining fat. Proteins were then precipitated using pH drop method, for which pH was lowered to 4.0 using 6M HCl. The precipitated proteins were then separated by centrifugation at 10,000 × g; 10 min, 4°C and washed twice using a deionized water. The obtained pellets were neutralized using 1.0 M NaOH to pH 7.0, stored at −20°C
and then freeze dried using LyoAlfa freeze dryer (Telstar, Barcelona, Spain). The protein content of YS and MS was determined according to the Kjeldahl method (Kjeldahl, 1883), and their values are displayed in Table 3.

Table 3: Protein content of young and mature soybean seeds and their protein isolates (%).

<table>
<thead>
<tr>
<th>Protein content</th>
<th>Seed (%)</th>
<th>Protein isolate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young soybean</td>
<td>36.62</td>
<td>68.85</td>
</tr>
<tr>
<td>Mature soybean</td>
<td>41.38</td>
<td>75.71</td>
</tr>
</tbody>
</table>

2.5.2 Production of protein hydrolysates

The freeze dried soybean protein concentrates were subjected to hydrolysis using different proteolytic enzymes (alcalase, bromelain and flavourzyme) as described by Aluko and Monu, (2003) and modified by Mudgil et al. (2019d). First, the YS and MS protein concentrates were dispersed in distilled water to a protein content of 4% v/v and the pH was adjusted to (7.0). YS and MS proteins were then divided into three equal proportions in Schott bottle for the enzymatic hydrolysis using the three different enzymes in triplicate as per the plan depicted in Table 4. A portion of 60 ml of each YS and MS protein was kept separately to be used as control. Then, the pH was adjusted for both soybeans slurries to (pH 8.0 for alcalase and pH 7.0 for bromelain and flavourzyme) and monitored by a digital pH meter (Starter 3100, Ohaus, Parsippany, NJ). Enzyme: substrate (E:S) ratio at 1:100 was prepared by dissolving a pre-calculated amount of enzymes in 1 ml of deionized water and then added to their particular YS or MS protein concentrates. Temperature-controlled water bath was used to carry out the enzymatic hydrolysis at 50°C, at speed of 100 ramps/min for 6 h. YS
and MS hydrolysates were taken after every 2 h of incubation, and the enzymes were
deactivated by heating the hydrolysate samples at 100°C for 5 mins and cooled down
immediately in ice bath. Hydrolysate samples were centrifuged at 10,000 × g, 5 min,
4°C, and the protein content was determined using bicinchoninic acid assay and
resultant supernatants were stored at -20°C for further analysis.

Table 4: Hydrolysis conditions for producing the young and mature soybean
hydrolysates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme: Substrate ratio</th>
<th>pH</th>
<th>Time (h)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td>1:100</td>
<td>8.0</td>
<td>2, 4, 6</td>
<td>50 - 60°C</td>
</tr>
<tr>
<td>Bromelain</td>
<td>1:100</td>
<td>7.0</td>
<td>2, 4, 6</td>
<td>50°C</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>1:100</td>
<td>7.0</td>
<td>2, 4, 6</td>
<td>50 - 55°C</td>
</tr>
</tbody>
</table>

2.5.3 Characterization of young and mature soybean protein hydrolysates upon
enzymatic hydrolysis

2.5.3.1 Degree of hydrolysis

Degree of hydrolysis (DH) was analyzed using the o-phthaldialdehyde (OPA)
method as described by Church et al. (1983) and modified by Jafar et al. (2018). To
prepare OPA reagent, 40 mg of OPA was dissolved in 1 ml of methanol and then mixed
with 2.5 ml of sodium dodecyl sulphate (SDS) (20% w/w), 25 ml of 100 mM sodium
tetraborate buffer (pH 9.3), and 100 µl of β-mercaptoethanol. Milli Q water was then
used to raise the final volume to 50 ml. Hydrolysate samples (20 µl) were added to 200
µl of OPA reagent in a 96-well plate. After 1 h of incubation at room temperature in
the dark, the absorbance was monitored at 340 nm in a microplate reader (Epoch 2,
BioTek, VT, USA). The free amino nitrogen (FAN) content was determined using a
standard curve of tryptone and % DH was determined by using equation below, where unhydrolysed MS and YS were used as control samples.

\[
DH\% = \left( \frac{FAN_{\text{hydrolysates}} - FAN_{\text{intact protein}}}{FAN_{\text{intact protein}}} \right) \times 100
\]

2.5.4 Inhibition of key enzymes related to diabetes and obesity

Enzyme inhibitory assays were carried out as described in Sections 2.4.3 and 2.4.4 above. The half-maximal inhibitory concentration (IC\textsubscript{50}) values were determined by plotting the percentage inhibition as a function of the test compound concentration expressed in \(\mu\)g of protein equivalents/ ml.

2.5.5 Antioxidant activities

2.5.5.1 2,2’-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

DPPH radical-scavenging activity was determined by DPPH assay as described by Al-Shamsi et al. (2018). Briefly, samples (100 \(\mu\)l) were diluted with 100 \(\mu\)l of 0.15 mM 2,2’-diphenyl-1-picrylhydrazyl (DPPH) in 95% ethanol. The mixture was shaken and made to stand at room temperature for 30 min in the dark. The absorbance was monitored at 517 nm using the microplate reader (Epoch 2, BioTek, VT, USA). (The preparation of the blank was done in the same manner except that the distilled water was used instead of the sample) Preparation of standard curve of Trolox was made in the range of 10–60 \(\mu\)M and the activity. The results were expressed as Trolox equivalent antioxidant activity (TEAC) \(\mu\)g of protein equivalent/ ml.
2.5.5.2 FRAP and ABTS antioxidant activities

The FRAP and ABTS assays were performed as described in Section 2.4.5 above and their values were expressed as Trolox equivalent antioxidant activity (TEAC) μg of protein equivalent/ ml.

2.5.6 Anti-inflammatory (AI) activities

The AI activities performed as described in Section 2.4.6 above. The results were expressed as Diclofenac sodium equivalent capacity (DSEC) of μg protein equivalent/ ml.

2.6 Statistical analysis

All experiments were carried out in three independent replicates and each replicate was analyzed twice. To determine statistically significant differences between the samples (P<0.05), the data were subjected to Analysis of Variance (ANOVA) and appropriate means separation was conducted using Tukey’s multiple range test using a statistical software program (SPSS version 25 for Windows, 2012, Chicago, IL, USA).
3.1 Proximate composition

The proximate composition of YS and MS flour has been expressed in g/100g. As shown in Figure 1, moisture content was higher in YS (7.28 ± 0.17 g/100g) than MS (5.71 ± 0.17 g/100g). Ash content was slightly higher in MS (5.28 ± 0.05 g/100g) than YS (4.67 ± 0.02 g/100g). Similarly, fat content was slightly higher in MS than YS with values of 15.91 ± 1.56 g/100g and 15.004 ± 0.22, respectively. Moreover, MS contained a higher content of protein (41.38 ± 0.006 g/100g) compared to the YS (36.58 ± 0.006 g/100g). However, YS showed high carbohydrate value than MS with values of 36.46 g/100g and 31.72 g/100g, respectively.

Figure 1: Proximate composition of young and mature soybean. Values are represented as (g/100 g). Keynotes: YS-young soybean, MS-mature soybean.
3.2 Phase 1: Effect of simulated gastrointestinal digestion (SGID) on total phenolic content and bioactive properties of young and mature soybeans

3.2.1 Total phenolic compounds (TPC)

The results of the total phenolic content (TPC) which are expressed as gallic acid equivalent (µg GAE /ml) are presented in Figure 2. TPC was analysed for YS and MS samples after simulated gastric and intestinal stages. The results showed that all of the samples of YS and MS contained phenolic compounds and their concentrations increased after the samples were subjected to gastrointestinal phase (SGID). TPC increased after the gastric and the SGID phase of digestion in both YS and MS. YS samples displayed a higher concentration of TPC when compared to MS, except that for the control sample of YS before SGID (55.66 ± 13.83 µg GAE /ml) which showed less concentration than control (before SGID) of MS (176.05 ± 15.96 µg GAE /ml). TPC of YS has increased significantly after subjecting the sample to gastric and SGID phases of digestion when compared to the control sample. After complete SGID treatment, YS showed the highest TPC (689.27 ± 12.06 µg GAE /ml), followed by YS subjected to gastric phase (300.10 ± 19.35 µg GAE /ml), and the control YS samples (55.66 ± 13.83 µg GAE /ml). Similar observations were found for MS. TPC of MS increased significantly after subjecting it to gastric digestion and SGID (211.47 ± 63.95 and 376.98 ± 42.76 µg GAE /ml) compared to the control sample (176.05 ± 15.96 µg GAE /ml).
Figure 2: Total phenolic content of young and mature soybean after simulated gastric and gastrointestinal digestion (SGID). Data is presented as µg of gallic acid equivalent/mg of ml of sample (µg GAE /ml). Different small letters on each bar indicates significant difference between different samples analysed. Keynotes: YS-young soybean, MS-mature soybean.

3.2.2 Inhibition of key enzymes related to diabetes by young and mature soybean subject to SGID

3.2.2.1 α-amylase (AA) inhibitory activity

The AA inhibitory activity of YS and MS samples subjected to simulated gastric and gastrointestinal digestion (SGID) is depicted in Figure 3 and is represented in AA inhibitory-IC$_{50}$ (µg/ml). As indicated, all samples of YS and MS inhibited the AA enzyme effectively compared to control soybean samples (before SGID). Overall, the strongest inhibitory activity of AA was shown by MS at gastric digestion (IC$_{50}$: 25.95 ± 0.84 µg/ml) while the lowest was shown by the control samples of MS and YS (IC$_{50}$: 35.01 ± 0.47 and 34.85 ± 2.3 µg/ml, respectively). For YS, the highest inhibition of AA enzyme was display after the gastric digestion phase (IC$_{50}$: 28.23 ± 2.13 µg/ml) compared to the control and samples subjected to complete SGID with
IC$_{50}$ values of 34.85 ± 2.3 µg/ml and 30.83 ± 2.23 µg/ml, respectively. Furthermore, a similar observation was shown by MS extract, where gastric digested samples showed higher inhibition compared to control and complete SGID samples. Overall, gastric digested MS showed the highest AA inhibition with an IC$_{50}$ value of 25.95 ± 0.84 µg/ml, followed samples subjected to complete SGID (IC$_{50}$ value 30.1 ± 1.08 µg/ml) and control sample (IC$_{50}$: 34.85 ± 2.3 µg/ml). MS and YS showed an insignificant difference of AA enzyme inhibition for control and SGID samples, and a significant difference was observed for the gastric digested samples where MS was slightly lower (P<0.05) in the IC$_{50}$ value compared to YS.

![Figure 3: AA inhibition of young and mature soybean upon subjecting to gastric and gastrointestinal digestions (SGID). Values are represented in IC$_{50}$ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed. Keynotes: YS-young soybean, MS-mature soybean.](image-url)
3.2.2.2 Dipeptidyl peptidase IV (DPP-IV) inhibitory activity

The ability of YS and MS subjected to simulated gastric and SGID treatments to inhibit the DPP-IV enzyme is shown in Figure 4. YS and MS samples were able to inhibit DPP-IV activity at different levels when subject to SGID. In general, YS and MS after simulated gastric digestion exhibited the highest DPP-IV inhibition with a DPP-IV inhibitory IC₅₀ value of 22.34 ± 0.28 and 22.7 ± 0.85 µg/ml, respectively (P>0.05). While YS and MS after complete SGID exhibited lowest inhibition of DPP-IV.

![Figure 4: DPP-IV inhibition of young and mature soybean upon subjecting to gastric and gastrointestinal digestions (SGID). Values are represented in IC₅₀ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed. Keynotes: YS-young soybean, MS-mature soybean.](image)

Thus, in case of YS, the simulated gastric digested samples showed the highest DPP-IV inhibition with an IC₅₀ value of 22.34 ± 0.28 µg/ml, followed by the control YS samples with DPP-IV inhibitory IC₅₀ value of 28.56 ± 0.64 µg/ml, and those
subjected to complete SGID with DPP-IV inhibitory IC₅₀: 33.45 ± 0.69 µg/ml. A similar pattern was observed for the MS samples, where the sample at gastric digestion showed the highest inhibitory activity, then the control sample and the least inhibitory activity was shown by the sample subjected to complete SGID with IC₅₀ values of 22.7 ± 0.85 µg/ml, 27.08 ± 1.16 µg/ml, and 33.43 ± 1.79 µg/ml, respectively. Overall, the differences between the YS and MS samples when subjected to gastric, SGID, and the control were insignificant (P>0.05).

3.2.3 Inhibition of key enzymes related to obesity by young and mature soybean subject to SGID

3.2.3.1 Pancreatic lipase (LIP) inhibitory activity

LIP inhibitory activity of the simulated gastric digested and SGID samples from YS and MS is shown in Figure 5. As can be seen from the figure, all YS and MS samples have exhibited LIP inhibitory activities with IC₅₀ values ranging from 88.68 ± 8.66 to 36.94 ± 1.84 µg/ml. The highest inhibitory activity of the LIP enzyme was observed by the gastric digested YS (36.94 ± 1.84 µg/ml) and the least inhibition was presented by the YS control samples sample (IC₅₀: 88.68 ± 8.66 µg/ml). YS displayed significant increase in the LIP inhibition upon gastric digestion (IC₅₀: 36.94 ± 1.84 µg/ml) and complete SGID (IC₅₀: 46.94 ± 1.63 µg/ml). On the other hand, MS displayed no change in inhibition of LIP when the samples were subjected to simulated gastric digestion and to the inhibition displayed by control sample (P>0.05). The control of MS showed LIP inhibitory IC₅₀ value of 45.53 ± 1.66 µg/ml, and IC₅₀ of the gastric digested samples was 43.36 ± 0.31 µg/ml, and SGID treated MS possessed an IC₅₀ value of 46.54 ± 3.05 µg/ml. In general, the IC₅₀ value of YS control sample was significantly higher than MS control sample, and the gastric digested sample of YS
was significantly lower than the IC$_{50}$ value of MS. However, the IC$_{50}$ values of YS and MS when subjected to SGID insignificantly increased (P>0.05).

![Figure 5: LIP inhibition of young and mature soybean upon subjecting to gastric and gastrointestinal digestions (SGID). Values are represented in IC$_{50}$ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed. Keynotes: YS-young soybean, MS-mature soybean.](image)

3.2.3.2 Cholesterol esterase (CE) inhibitory activity

The inhibition of the CE enzyme by YS and MS extracts is depicted in Figure 6. YS and MS samples subjected to simulated gastric and SGID have shown enhanced CE inhibitory activity when compared to control samples (P>0.05). Overall, YS after simulated gastric digestion showed the strongest inhibitory activity of CE enzyme while YS control showed the lowest inhibition (P<0.05). YS samples subject to simulated gastric digestion showed a CE inhibition with IC$_{50}$ value of 29.82 ± 0.71 µg/ml, followed by the samples subjected to SGID in which the CE inhibition decreased with an IC$_{50}$ value of 43.32 ± 1.75 µg/ml, and then the control YS showed the lowest CE inhibition with IC$_{50}$ value of 70.26 ± 2.81 µg/ml. On the other hand, the CE inhibitory activity of MS control samples was significantly higher (IC$_{50}$: 30.3 ± 1.2
μg/ml) than the SGID of MS samples where the inhibition decreased with IC₅₀ values of (35.08 ± 0.76 μg/ml) and gastric digested samples with IC₅₀ value of (39.53 ± 0.73 μg/ml) (P<0.05). Generally, the control of MS was more potent to CE enzyme compared to YS, and the gastric digested sample of YS displayed a higher CE inhibition than that of MS. While the SGID sample of MS significantly higher in inhibiting the CE enzyme than that of YS.

![Figure 6: CE inhibition of young and mature soybean upon subjecting to gastric and gastrointestinal digestions (SGID). Values are represented in IC₅₀ (μg/ml). Different small letters on each bar indicates significant difference between different samples analysed. Keynotes: YS-young soybean, MS-mature soybean.](image)

3.2.4 Antioxidant activities by young and mature soybean subject to SGID

### 3.2.4.1 Ferric-reducing antioxidant power (FRAP)

FRAP of simulated gastric digested and SGID samples from YS and MS are presented in Table 5. Generally, all samples have shown an ability to reduce ferric iron
to ferrous iron and the activity ranged from $151.5 \pm 12.19$ to $585.981 \pm 92.71$ TEAC µg/ml. The ferric reducing ability of YS and MS samples were improved after the gastric digestion and complete SGID. In the YS samples, FRAP activity insignificantly increased from an initial value of $151.5 \pm 12.19$ in the control YS to $160.12 \pm 40.18$ and $203.54 \pm 28.08$ TEAC µg/ml ($P>0.05$) in simulated gastric and SGID samples, respectively. MS samples showed a significant increase in FRAP activity from an initial value of $166.94 \pm 11.94$ in the control MS samples to $453.72 \pm 72.06$ and $585.98 \pm 92.71$ TEAC µg/ml for simulated gastric and SGID samples, respectively ($P<0.05$). The highest FRAP activity was obtained in MS after SGID ($585.981 \pm 92.71$ TEAC µg/ml), and the least activity was recorded by YS control sample ($151.5 \pm 12.19$ TEAC µg/ml). The FRAP values of control sample of YS were lower than that of MS ($151.5 \pm 12.19$ and $166.944 \pm 11.94$ TEAC µg/ml, respectively) ($P>0.05$). The reducing power of the gastric digested samples from MS increased significantly by 1.5-folds ($453.722 \pm 72.06$ TEAC µg/ml) compared to undigested control, and for YS the FRAP activity increased insignificantly ($151.5 \pm 12.19$ to $160.129 \pm 40.18$ TEAC µg/ml. Overall, MS samples showed higher reducing power compared to YS.

3.2.4.2 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

ABTS radical scavenging activity of YS and MS subjected to simulated gastric and SGID is displayed in Table 5 and expressed in TEAC µg/ml. Both YS and MS displayed effectiveness in scavenging ABTS radicals. The ABTS radical scavenging activity increased upon simulated gastric and SGID of both YS and MS samples. However, the increase in radical scavenging activity in case of YS was insignificant ($P>0.05$), while there was a significant increase in case of MS samples ($P<0.05$). The highest ABTS radical scavenging activity was shown by the MS after SGID (2614.63
± 50.03 TEAC µg/ml) while the lowest activity was shown by MS control (2086.89 ± 50.59 TEAC µg/ml). The control sample of YS showed a higher ABTS radical scavenging activity compared to the MS (2600.15 ± 60.62 vs 2086.89 ± 50.59 TEAC µg/m, respectively). Moreover, gastric digested samples of YS (2638.1 ± 38.93 µg/ml) showed higher ABTS radical scavenging activity compared to MS (2256.05 ± 47.77 TEAC µg/ml). However, subjecting YS to complete SGID resulted in the decrease of ABTS radical scavenging activity from 2600.15 ± 60.62 to 2587.73 ± 27.43 TEAC µg/ml (P>0.05). After complete SGID, relatively similar ABTS radical scavenging activity was obtained for YS (2587.73 ± 27.43 µg/ml) and MS (2614.63 ± 50.03 µg/ml) (P>0.05). The overall results suggest that YS has proved to be a better scavenger of ABTS radicals compared to the MS, although the MS showed an improved ABTS radical scavenging activity after simulated gastric and SGID.

3.2.5 Anti-inflammatory (AI) inhibitory activities

The AI activity of YS and MS subjected to simulated gastric and SGID expressed in diclofenac sodium equivalent capacity (DSEC µg/ml) is shown in Table 5. As can be seen from the table, all the YS and MS samples displayed varied AI activity ranging from 163.12 ± 20.5 to 4879.75 ± 226 DSEC µg/ml. A significant (P<0.05) increase in the AI activity was recorded after the YS and MS samples were subjected to simulated gastric and SGID. The SGID MS showed the highest AI activity (4879.75 ± 226 DSEC µg/ml) while the control of YS showed the lowest activity (163.12 ± 20.5 DSEC µg/ml). For YS, the AI activity of the control sample (151.5 ± 12.19 DSEC µg/ml) have increased significantly after the gastric digestion (519.25 ± 75.8 DSEC µg/ml), and further increase was obtained upon SGID (2427.5 ± 70.6 DSEC µg/ml). A similar trend was also observed for MS, where the control sample
showed the lowest AI activity ($712.75 \pm 49.8 \text{ DSEC } \mu g/ml$), and then sharply increased after digesting the sample through the gastric and SGID ($2486.87 \pm 175.2$ and $4879.75 \pm 226. \text{ DSEC } \mu g/ml$, respectively). In general, the values of the AI activity were higher for MS compared to the YS ($P<0.05$).

Table 5: Ferric-reducing antioxidant power (FRAP), 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and anti-inflammatory (AI) activity of young and mature soybeans after the gastric and simulated gastrointestinal digestion (SGID).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABTS</th>
<th>FRAP</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2600.15±60.62</td>
<td>151.50±12.19</td>
<td>163.12±20.53</td>
</tr>
<tr>
<td>Young soybean</td>
<td>2638.10±38.93</td>
<td>160.12±40.18</td>
<td>519.25±75.8</td>
</tr>
<tr>
<td>Gastric digestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2587.73±27.43</td>
<td>203.54±28.08</td>
<td>2427.50±70.63</td>
</tr>
<tr>
<td>SGID</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature soybean</td>
<td>2086.89±50.59</td>
<td>166.94±11.94</td>
<td>712.75±49.86</td>
</tr>
<tr>
<td>Gastric digestion</td>
<td>2256.05±47.77</td>
<td>453.72±72.06</td>
<td>2486.87±175.26</td>
</tr>
<tr>
<td>SGID</td>
<td>2614.63±50.03</td>
<td>585.98±92.71</td>
<td>4879.75±226.02</td>
</tr>
</tbody>
</table>

Data represents mean ± SD. Different small alphabets in the same column represent significant difference between the samples.

ABTS is represented in Trolox Equivalent Antioxidant Capacity (TEAC) \( \mu g/ml \).
FRAP is represented in Trolox Equivalent Antioxidant Capacity (TEAC) \( \mu g/ml \).
AI is represented in Diclofenac Sodium Equivalent Capacity (DSEC \( \mu g/ml \)) \( \mu g/ml \).

3.3 Phase 2: Effect of the enzymatic hydrolysis on bioactive properties of young and mature soybean protein hydrolysates

In the phase 2, as previously mentioned young and mature soybean proteins were isolated and then hydrolysed using three different enzymes. The generated hydrolysates were analysed for different bioactive properties as described below.
3.3.1 Characterization of young and mature soybean protein hydrolysates upon enzymatic hydrolysis

Young and mature soybean protein hydrolysate were characterized for the extent of hydrolysis using degree of hydrolysis (DH) assay. DH for each hydrolysate were expressed in %.

3.3.1.1 Degree of hydrolysis

The degree of hydrolysis (DH) of YS and MS protein hydrolysates generated by each enzyme was analyzed and expressed in terms of percentage (%) of hydrolysis as displayed in Figure 7. Overall, the DH of YS and MS protein hydrolysates produced by each enzyme showed a similar pattern in which the DH increases with increasing the time of hydrolysis (P<0.05). DH of YS hydrolysates produced by each enzyme (alcalase, bromelain, and flavourzyme) at 2, 4 and 6 h, were in a range of 31.67 ± 4.57 to 72.39 ± 2.06 %, respectively. Alcalase derived hydrolysate at 2 h showed the lowest DH (31.67 ± 4.57 %) and bromelain derived hydrolysate at 6 h showed the highest DH (72.39 ± 2.06%). In contrast, MS protein hydrolysates showed overall higher DH compared to YS protein hydrolysates with values ranging from 55.22 ± 3.01 to 80.83 ± 2.2% (P<0.05). Among the MS, the highest DH (80.83 ± 2.2%) was displayed by alcalase derived hydrolysate at 6 h hydrolysis and the lowest DH obtained was shown by flavourzyme derived hydrolysate (55.22 ± 3.01%) at 2 h of hydrolysis. Among all the hydrolysates of YS, DH of bromelain generated hydrolysates were significantly higher (P<0.05) than alcalase and flavourzyme generated hydrolysates when generated at 4 and 6 h of hydrolysis. Similarly, among MS hydrolysates, bromelain generated hydrolysates showed a significantly higher DH compared to the alcalase and flavourzyme generated hydrolysates when generated at 2 and 4 h of hydrolysis. Bromelain derived YS and MS protein hydrolysates showed higher DH than alcalase. 
and flavourzyme derived hydrolysates. It is evident from this study that bromelain is the most effective enzyme for generating both YS and MS protein hydrolysates, having shown higher DH than alcalase and flavourzyme derived hydrolysates. Overall, YS hydrolysates were found to be less responsive to hydrolysis in comparison to MS hydrolysates as indicated by lower percentage of DH throughout the varying time of enzymatic hydrolysis, except for flavourzyme-generated hydrolysates that showed a higher percentage of DH. It is noteworthy that MS hydrolysates were more responsive to enzymatic hydrolysis compared to YS derived hydrolysates.

![Figure 7: Degree of hydrolysis (DH %) of young and mature soybean protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis time. Different small letters on each bar indicates significant difference between different samples analysed.](image)

3.3.2 Inhibition of key enzymes related to diabetes by young and mature soybean protein hydrolysates

3.3.2.1 α-amylase (AA) inhibitory activity

AA inhibitory properties of young and mature soybean protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis is
shown in Figure 8 and represented as AA-inhibitory-IC$_{50}$ (µg/ml). The intact protein of young soybean showed higher AA-inhibitory properties with an IC$_{50}$ of 79.37 ± 1.45 µg/ml compared to the intact protein of mature soybean with AA-inhibitory IC$_{50}$ value of 87.68 ± 1.41 µg/ml. All hydrolysates prepared from alcalase, bromelain, and flavourzyme after the hydrolysis for 2, 4, and 6 h resulted in a significant reduction in their AA-inhibitory IC$_{50}$ values as compared to their intact proteins (P<0.05) indicating that hydrolysis improved the AA inhibitory properties. The AA-inhibitory properties demonstrated by alcalase-generated YS hydrolysates were slightly lower (P>0.05) than those shown by MS hydrolysates at 2 h hydrolysis time (IC$_{50}$: 42.66 ± 0.86 and 42.05 ± 0.33 µg/ml), respectively. However, at 4 h there was no significant difference (P>0.05) in the IC$_{50}$ values between the two samples. At 6 h of hydrolysis, the IC$_{50}$ values of both YS and MS hydrolysates increased, indicating lower AA inhibition. Alcalase derived hydrolysates of MS showed enhanced AA inhibition compared to the YS hydrolysates. Bromelain-derived protein hydrolysates of YS displayed a significant reduction of AA inhibition after 2, 4 and 6 h of hydrolysis. AA-inhibitory IC$_{50}$ value of 35.05 ± 0.5 µg/ml was obtained for bromelain generated YS hydrolysates at 2 h followed by the hydrolysates generated at 4 h and 6 h, with IC$_{50}$ values of 41.3 ± 0.36 µg/ml and 48.12 ± 0.29 µg/ml, respectively. On the other hand, bromelain-generated MS hydrolysates at 2 and 6 h of hydrolysis exhibited insignificant increase in the AA inhibition (P>0.05) as indicated by their lower (IC$_{50}$: 41.26 ± 0.72 and 40.73 ± 0.49 µg/ml) compared to the IC$_{50}$ of the hydrolysates generated at 4 h (IC$_{50}$: 44.54 ± 0.5 µg/ml) (P<0.05). The bromelain-generated hydrolysates of YS produced at 2 h showed a higher inhibition of AA than MS hydrolysates. However, the AA inhibition then decreased continuously for YS hydrolysates to 6 h compared to those of MS (P<0.05). Flavourzyme-digested hydrolysates of YS displayed a significantly higher
AA inhibition at 2 h hydrolysis with IC₅₀ value of 26.57 ± 0.29 µg/ml followed by a decreased in AA inhibitory activity with the prolonged hydrolysis from 4 and 6 h (P<0.05). While the flavourzyme produced hydrolysates of MS showed higher inhibition at 4 h (IC₅₀: 36.79 µg/ml), followed by 2 h and 6 h (IC₅₀: 40.12 µg/ml) and (IC₅₀:42.15 µg/ml), respectively. The flavourzyme-generated hydrolysates of YS showed a significantly lower IC₅₀ value than MS at 2 h and 4 h of hydrolysis. However, an insignificant decrease was found in AA inhibitory activity of both YS and MS hydrolysates at 6 h of hydrolysis.

Figure 8: AA inhibitory activity of young and mature soybean protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis time. Values are represented in IC₅₀ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed.

### 3.3.2.2 Dipeptidyl peptidase IV (DPP-IV) inhibitory activity

DPP-IV inhibitory activity of YS and MS hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4, and 6 h of hydrolysis is shown in Figure 9. All the protein hydrolysates displayed higher DPP-IV inhibitory activities compared to their
intact proteins. The IC$_{50}$ value of the intact protein of YS (IC$_{50}$: 137.5 ± 3.71 µg/ml) was significantly higher than MS protein (IC$_{50}$: 80.788 ± 4.4 µg/ml) indicating lower DPP-IV inhibitory activity (P<0.05). Upon hydrolysis, 2 to 3 times increase in inhibition of DPP-IV was observed for both YS and MS protein hydrolysates. In general, MS hydrolysates produced using alcalase, bromelain, and flavourzyme at hydrolysis times 2, 4, and 6 h presented a similar trend in which their DPP-IV inhibition increased with increase in the hydrolysis time from 2 to 6 h (P<0.05), except for flavourzyme-generated hydrolysates at 6 h which decreased slightly in DPP-IV inhibition (P>0.05). For alcalase-generated hydrolysates, DPP-IV inhibition of both MS and YS significantly (P<0.05) increased with increased hydrolysis time. The highest DPP-IV inhibitory activity was recorded at 6 h hydrolysis as indicated by lower IC$_{50}$ value for YS (39.44 ± 0.28 µg/ml) and MS (50.2 ± 0.77 µg/ml). For bromelain and flavourzyme produced MS protein hydrolysates, the highest DPP-IV inhibitory activity was recorded at 4 and 6 h of hydrolysis compared to the hydrolysates produced at 2 h. Overall, amongst the hydrolysates from MS protein hydrolysates, the highest DPP-IV inhibitory activity was displayed by flavourzyme-generated hydrolysate at 4 h (IC$_{50}$: 32.63 ± 0.18 µg/ml) compared to alcalase and bromelain generated hydrolysates (P<0.05). Similarly, amongst all YS protein hydrolysates, flavourzyme-generated hydrolysates at 2 h demonstrated the highest DPP-IV inhibition with an IC$_{50}$ value of 29.26 ± 0.57 µg/ml (P<0.05). Bromelain-produced YS hydrolysates demonstrated an insignificant change in the DPP-IV inhibition when hydrolysis time increased from 2 to 6 h (P>0.05). The DPP-IV inhibitory activity of alcalase-derived hydrolysates of YS significantly increased when the hydrolysis was prolonged up to 6 h (P<0.05). The alcalase-generated hydrolysates of YS displayed lower IC$_{50}$ values in comparison to MS generated hydrolysates (P<0.05) at different times of hydrolysis.
Similarly, for bromelain-generated hydrolysates, the IC$_{50}$ value of YS hydrolysates was significantly lower (P<0.05) than MS hydrolysates. However, at 6 h of hydrolysis, an insignificant change in the IC$_{50}$ value was observed between MS and YS hydrolysates (P>0.05). The IC$_{50}$ value of flavourzyme-generated hydrolysates of YS at 2 h was significantly lower than that of MS derived hydrolysates. Flavourzyme-generated hydrolysates of YS showed an opposite trend to those of MS, where the IC$_{50}$ values of MS hydrolysates decreased compared to YS hydrolysates when the time of hydrolysis increased from 2 to 6 h. Overall, the results obtained revealed that flavourzyme generated YS hydrolysates at 2 h and MS hydrolysates at 4 h hydrolysis showed the most potent DPP-IV inhibitory activity.

![Figure 9](image)

Figure 9: DPP-IV inhibitory activity of young and mature soybean protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis time. Values are represented in IC$_{50}$ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed.
3.3.3 Inhibition of key enzymes related to obesity by young and mature soybean protein hydrolysates

3.3.3.1 Pancreatic lipase (LIP) inhibitory activity

Pancreatic LIP inhibitory displayed by YS and MS protein hydrolysates is displayed in Figure 10. As indicated LIP inhibitory activity was significantly enhanced after the hydrolysis by different enzymes when compared to their intact soybean proteins (P<0.05). The unhydrolyzed protein of YS (IC$_{50}$: 100.55 ± 1.44 µg/ml) was more effective in inhibiting the LIP activity in comparison to the unhydrolyzed MS protein (IC$_{50}$: 137.9 ± 9.1 µg/ml) (P<0.05). Alcalase-produced YS hydrolysate showed higher LIP inhibitory properties compared to MS protein hydrolysates counterpart (P<0.05). Alcalase 4 and 6 h generated YS hydrolysates exhibited the highest LIP inhibition with an IC$_{50}$ value of 38.21 ± 1.8 and 35.75 ± 0.38 µg/ml, respectively (P>0.05) compared to the IC$_{50}$ values of the hydrolysates produced at 2 h (43.33 ± 2.17 µg/ml) (P<0.05). Bromelain-produced YS protein hydrolysate showed highest inhibition of LIP at 2 h with IC$_{50}$ value of 30.04 ± 0.15 µg/ml, which decreased when the hydrolysis was extended to 4 and 6 h (IC$_{50}$: 38.76 ± 0.25 and 37.96 ± 0.88 µg/ml), respectively (P<0.05). Flavourzyme-digested YS protein hydrolysate at 2 h displayed insignificantly higher LIP inhibition with IC$_{50}$ value of 30.41 ± 0.18 µg/ml compared to YS hydrolysates generate at 4 and 6 h of hydrolysis (IC$_{50}$: 33.04 ± 0.18 and 36.94 ± 0.23 µg/ml), respectively (P>0.05). In contrast, MS hydrolysate produced by flavourzyme showed a higher inhibition at 4 h with IC$_{50}$ value of 65.15 ± 3.52 µg/ml, followed by 2 h (IC$_{50}$: 80.15 ± 3.78 µg/ml), and 6 h (IC$_{50}$: 85.52 ± 7.57 µg/ml) generated hydrolysates (P<0.05). Similar trend was observed for the MS hydrolysates produced by bromelain which shows highest LIP inhibition at 4 h of hydrolysis with IC$_{50}$ value of 92.71 ± 6.14 µg/ml, followed by 2 h (IC$_{50}$: 99.51 ± 8.23 µg/ml) and 4 h
(IC\textsubscript{50}: 111.8 ± 5.18 µg/ml) generated hydrolysates, respectively. Alcalase-produced hydrolysates of MS displayed non-significant difference in LIP inhibition at different times of hydrolysis (P>0.05), except that the inhibition was slightly enhanced at 4 h of hydrolysis. Overall, the LIP inhibitory activity demonstrated by YS hydrolysates produced by alcalase, bromelain, and flavourzyme throughout the different hydrolysis time periods were significantly higher compare to the MS derived hydrolysates (P<0.05).

Figure 10: LIP inhibitory activity of young and mature soybean protein hydrolysates produced by the alcalase, bromelain, and flavourzyme at 2, 4, and 6 h of hydrolysis time. Values are represented in IC\textsubscript{50} (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed.

3.3.3.2 Cholesterol esterase (CE) inhibitory activity

The inhibition of CE by YS and MS protein hydrolysates generated by alcalase, bromelain, and flavourzyme at 2, 4, and 6 h of hydrolysis is shown in Figure 11. YS and MS hydrolysates exhibited a higher CE inhibition than their intact proteins (P<0.05), and MS protein (IC\textsubscript{50}: 79.8 ± 2.36 µg/ml) displayed a significantly lower IC\textsubscript{50} value than YS protein (IC\textsubscript{50}: 88.23 ± 2.85 µg/ml). YS protein hydrolysates
produced by all three enzymes showed a similar trend for CE inhibition, where the highest inhibition of CE was observed at 2 h hydrolysis, and then a decrease in CE inhibition was noticed with prolonged hydrolysis for 4 and 6 h (P<0.05). It is observed that among the YS protein hydrolysates produced at 2 h of hydrolysis time, flavourzyme-produced hydrolysates displayed a significantly higher CE inhibitory activity with an IC$_{50}$ value of 27.08 ± 0.43 µg/ml compared to those produced by alcalase and bromelain (P<0.05). YS protein hydrolysate generated by alcalase and bromelain at 2 h displayed CE inhibitory IC$_{50}$ values of 44.98 ± 1.24 µg/ml and 60.95 ± 1.99 µg/ml, respectively. In case of MS protein hydrolysate, flavourzyme generated hydrolysates displayed the most potent CE inhibitory activity followed by alcalase, and bromelain. CE inhibitory activity of alcalase-generated MS hydrolysates decreased when hydrolysis time increased from 2 to 4 h (P<0.05) followed by no significant change at 6 h of hydrolysis (P>0.05). The CE-inhibitory IC$_{50}$ value of 36.25 ± 0.59 µg/ml and 34.73 ± 0.29 µg/ml were reported for alcalase-generated MS hydrolysates at 4 and 6 h of hydrolysis (P>0.05), respectively, which increased significantly to 46.7 ± 1.22 µg/ml at 2 h hydrolysis (P<0.05). While bromelain-produced hydrolysates exhibited a highest inhibition of CE at 2 h (IC$_{50}$: 47.33 ± 5.07 µg/ml) followed by reduction in the inhibition at 4 h (IC$_{50}$: 53.43 ± 4.67 µg/ml) and 6 h (IC$_{50}$: 61.37 ± 8.57 µg/ml) (P<0.05). Despite the fact that flavourzyme-produced MS protein hydrolysates were most efficient in inhibiting CE compared the alcalase and bromelain, however, no significant increase in CE inhibition with increase of hydrolysis time was noticed at 4 and 6 h (P>0.05). In general, alcalase-generated YS hydrolysates at 2 h showed no significant difference from alcalase generated MS hydrolysate (P>0.05). Furthermore, an opposite trend was observed in the CE inhibitory activity of both MS and YS alcalase generated hydrolysates as the
hydrolysis duration progresses. For YS hydrolysates, a continuous decrease in their CE inhibitory activity with prolonged hydrolysis time was recorded, while those of MS inhibitory activity were significantly enhanced with increase of hydrolysis time from 2 to 6 h. The IC₅₀ value of bromelain-generated hydrolysates of MS was significantly lower than YS hydrolysates (P<0.05). However, YS hydrolysate at 2 h were not significantly different (P>0.05) from MS hydrolysate at 6 h. Flavourzyme-generated hydrolysates of YS at 2 h were significantly lower in IC₅₀ than that of MS at the same hydrolysis time and was the best among all hydrolysates in inhibiting CE. This result suggested that flavourzyme-generated YS hydrolysates at 2 h demonstrated the most potent CE inhibitory activity. Although the IC₅₀ values of flavourzyme-generated MS hydrolysates at 4 and 6 h were significantly lower compared to those of YS hydrolysates (P<0.05).

Figure 11: CE inhibitory activity of young and mature soybean protein hydrolysates generated by the alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis time. Values are presented as IC₅₀ (µg/ml). Different small letters on each bar indicates significant difference between different samples analysed.
3.3.4 Antioxidant properties of young and mature soybean protein hydrolysates

3.3.4.1 Ferric-reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) of YS and MS protein hydrolysates is displayed in Table 6 and expressed as TEAC µg/ml. The FRAP varied significantly (P<0.05) between the hydrolyzed and unhydrolyzed samples of YS and MS generated by alcalase, bromelain, and flavourzyme at 2, 4, and 6 h of hydrolysis except for all 2 h hydrolysates from YS. YS and MS unhydrolyzed protein samples displayed the lowest reducing power (211.8 ± 12.65 and 587.37 ± 100.09 TEAC µg/ml, respectively) compared to the hydrolyzed samples. Unhydrolyzed MS protein exhibited a stronger FRAP activity than YS protein (P<0.05). The highest reducing power was shown by flavourzyme-generated MS at 6 h hydrolysis (1502.93 ± 71.79 TEAC µg/ml) while the lowest was shown by flavourzyme-generated YS at 2 h (229.88 ± 24.98 TEAC µg/ml). For YS, the bromelain-generated YS hydrolysate at 6 h showed a significantly higher reducing power with values of 823.29 ± 49.25 TEAC µg/ml compared to the alcalase and flavourzyme generated hydrolysates (P<0.05). YS hydrolysates generated from bromelain enzyme demonstrated the most effective reducing power, followed by alcalase, and flavourzyme. It is evident from this study that flavourzyme-generated YS hydrolysates displayed an increase in FRAP when hydrolysis progressed from 2 to 4 h (229.88 ± 24.98 to 572.12 ± 24.6), and then decreased at 6 h (396.32 ± 51.88 TEAC µg/ml). On the other hand, MS hydrolysates generated by flavourzyme showed a significant increase in the FRAP values (1113.27 ± 46.14 to 1502.93 ± 71.79 TEAC µg/ml) with the increase of hydrolysis time from 2 to 6 h. Alcalase-generated YS and MS protein hydrolysates both displayed increase in FRAP with the increase in hydrolysis time from 2 to 6 h with values ranging from
280.59 ± 25.83 to 704.12 ± 37.7 and 993.39 ± 55.56 to 1400.83 ± 70.55 TEAC µg/ml, respectively. However, MS hydrolysates displayed stronger FRAP (P<0.05) than the YS hydrolysates. Moreover, bromelain-generated YS hydrolysates also displayed an increase in FRAP with the increase in hydrolysis time from 2 to 6 h (285.83 ± 30.79 to 823.29 ± 49.25 TEAC µg/ml), however, for MS hydrolysates generated by bromelain, an increase in FRAP from 2 to 4 h (1190.8 ± 52.92 to 1294.47 ± 95.89 TEAC µg/ml) was followed by a slight decrease (P>0.05) at 6 h of hydrolysis (1284.75 ± 48.36 TEAC µg/ml). In general, FRAP of alcalase, bromelain, and flavourzyme-generated hydrolysates of MS were higher than those of YS throughout the hydrolysis time, and all these hydrolysates displayed stronger reducing powers as the hydrolysis time increased (P<0.05), except for flavourzyme-generated hydrolysates of YS.

Table 6: Ferric-reducing antioxidant power (FRAP) of young and mature soybeans protein hydrolysates generated by alcalase, bromelain, and flavourzyme at 2, 4, 6 h of hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Young Soybean</th>
<th>Mature Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>211.8 ± 12.65 a</td>
<td>587.37 ± 100.09 c</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>280.59 ± 25.83 a</td>
<td>993.39 ± 55.56 f</td>
</tr>
<tr>
<td>Alcalase</td>
<td>4 h</td>
<td>427.7 ± 51.8 b</td>
<td>1041.32 ± 13.31 fg</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>704.12 ± 37.7 d</td>
<td>1400.83 ± 70.55 ij</td>
</tr>
<tr>
<td>Bromelain</td>
<td>2 h</td>
<td>285.83 ± 30.79 a</td>
<td>1190.8 ± 52.92 hi</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>604.53 ± 31.03 cd</td>
<td>1294.47 ± 95.89 ij</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>823.29 ± 49.25 e</td>
<td>1284.75 ± 48.36 i</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>2 h</td>
<td>229.88 ± 24.98 a</td>
<td>1113.27 ± 46.14 gh</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>572.12 ± 24.60 c</td>
<td>1252.56 ± 6.00 i</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>396.32 ± 51.88 b</td>
<td>1502.93 ± 71.79 k</td>
</tr>
</tbody>
</table>

Data represents mean ± SD. Different small alphabets in the table represents significant difference between the samples. FRAP is represented in Trolox Equivalent Antioxidant Capacity (TEAC) µg/ml.
3.3.4.2 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

ABTS radical scavenging activity of YS and MS protein hydrolysates generated by alcalase, bromelain, and flavourzyme for 2, 4, and 6 h is present in Table 7 and expressed as Trolox Equivalent Antioxidant Capacity (TEAC) µg/ml. The unhydrolyzed samples of YS and MS showed the lowest ABTS radical scavenging activity (1602.02 ± 144.98 and 1810.94 ± 105.96 TEAC µg/ml), respectively, and MS unhydrolyzed protein displayed insignificantly higher activity than YS unhydrolyzed protein (P>0.05). Upon hydrolysis, all YS derived hydrolysates exhibited a significantly (P<0.05) higher ABTS radical scavenging activity compared to control YS soybean protein samples, except alcalase 2 h generated hydrolysate (P>0.05). The highest ABTS radical scavenging activity among YS and MS was found in alcalase generated hydrolysate at 6 h of hydrolysis (2538.38 ± 44.65 TEAC µg/ml and 2428.07 ± 194.78 TEAC µg/ml, respectively). Alcalase-generated hydrolysates of MS displayed insignificantly (P>0.05) higher ABTS radical scavenging activity than flavourzyme and bromelain generated hydrolysates. In addition, alcalase-generated hydrolysates of MS at 2 h were insignificantly higher in scavenging of ABTS radicals than that of YS (P>0.05). However, at 4 h of hydrolysis, an insignificant increase was observed (P>0.05) for both MS and YS hydrolysates, and at 6 h of hydrolysis, ABTS radical scavenging activity of YS hydrolysates insignificantly increased compared to MS hydrolysates (P>0.05). For bromelain-generated hydrolysates at 2 h of hydrolysis, ABTS radical scavenging activity of YS hydrolysates (2077.47 ± 63.7TEAC µg/ml) was slightly higher (P>0.05) than MS hydrolysates (2063.08 ± 399.14TEAC µg/ml). An insignificant increase in the ABTS radical scavenging activity was observed
(P>0.05) in both YS and MS hydrolysates at 4 and 6 h of hydrolysis. Flavourzyme generated hydrolysates of YS and MS showed an insignificant increase in ABTS radical scavenging and ranged from (2143.64 ± 79.58 to 2476.77 ± 90.34 TEAC µg/ml) and (1827.47 ± 137.87 to 2166.84 ± 423.64 TEAC µg/ml), respectively. Flavourzyme-generated hydrolysates of YS showed an insignificant ABTS radical scavenging activity (P>0.05) compared to MS hydrolysates when produced at 2, 4, and 6 h of hydrolysis. The overall results suggest that YS and MS hydrolysates generated by different enzymes at various hydrolysis times improved ABTS radical scavenging activity than their intact protein samples.

Table 7: 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of young and mature soybeans protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 h of hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Young Soybean</th>
<th>Mature Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>1602.02 ± 144.98 a</td>
<td>1810.94 ± 105.96 ab</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>1994.49 ± 84.09 abc</td>
<td>2147.01 ± 317.74 bdef</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>2293.47 ± 53.83 cdef</td>
<td>2239.78 ± 120.37 cdef</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>2538.38 ± 44.65 f</td>
<td>2428.07 ± 194.78 def</td>
</tr>
<tr>
<td>Alcalase</td>
<td>2 h</td>
<td>2077.47 ± 63.7 bcde</td>
<td>2063.08 ± 399.14 bcd</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
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<td>2120.84 ± 141.49 bcde</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>2287.89 ± 81.84 cdef</td>
<td>2253.15 ± 251.73 cdef</td>
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<tr>
<td>Bromelain</td>
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<td>2143.64 ± 79.58 bcdef</td>
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<td></td>
<td>4 h</td>
<td>2203.01 ± 61.14 bcdef</td>
<td>2068.49 ± 251.19 bcd</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>2476.77 ± 90.34 ef</td>
<td>2166.84 ± 423.64 bcdef</td>
</tr>
</tbody>
</table>

Data represents mean ± SD. Different small alphabets in the table represents significant difference between the samples. ABTS is represented in Trolox Equivalent Antioxidant Capacity (TEAC) µg/ml.
3.3.4.3 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity of YS and MS protein hydrolysates is depicted in Table 8. The data shows that all the hydrolysates generated by different enzymes at various hydrolysis times possess significant DPPH radical scavenging potential. The unhydrolyzed (control) samples of YS and MS proteins showed the lowest DPPH radical scavenging activity with values of 827.69 ± 40.9 and 1079.69 ± 90.63 TEAC µg/ml, respectively. During the progression of hydrolysis, the DPPH radical scavenging activity of YS and MS protein hydrolysates increased with the increase in the hydrolysis time from 2 to 6 h. The highest DPPH radical scavenging activity was displayed by the MS protein hydrolysate generated with bromelain at 6 h (3658.02 ± 86.5 TEAC µg/ml), followed by the YS hydrolysates generated with alcalase at 6 h with value of 2471.79 ± 82.57 TEAC µg/ml. Among YS hydrolysates, DPPH radical scavenging activity of the alcalase generated hydrolysates displayed a significant increase (P<0.05) with the increase in time of hydrolysis from 2 h to 6 h (1105.25 ± 89.36 to 2471.79 ± 82.57 TEAC µg/ml). While the YS hydrolysates generated by bromelain and flavourzyme showed an insignificant (P>0.05) increase of DPPH radical scavenging activity from 2 h to 4 h of hydrolysis and followed by a significant increase at 6 h of hydrolysis with values of 2450.51 ± 42.81 and 2247.94 ± 33.65 TEAC µg/ml, respectively. Bromelain generated hydrolysates of MS showed a remarkable increase in DPPH radical scavenging activity from 2188.46 ± 152.45 to 3658.02 ± 86.5 TEAC µg/ml when the hydrolysis time increased from 2 to 6 h compared to those of YS (1763.44 ± 53.96 to 2450.51 ± 42.81 TEAC µg/ml) (P<0.05). Overall, the results showed that DPPH radical scavenging activity exhibited by MS hydrolysates was significantly higher than those exhibited by YS hydrolysates. Interestingly, bromelain was most effective in generating YS and MS hydrolysates.
with potential DPPH radical scavenging activities. The flavourzyme generated hydrolysates of YS displayed an insignificant increase in DPPH radical scavenging activity when time of hydrolysis increased from 2 to 4 h of hydrolysis (P>0.05), which was followed by a significant increase when time of hydrolysis increase to 6 h in case of both YS and MS (P<0.05).

Table 8: 2,2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of young and mature soybeans protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 hours of hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Young Soybean</th>
<th>Mature Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>827.69 ± 40.9 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1079.69 ± 90.63 &lt;sup&gt;b&lt;/sup&gt;</td>
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<td>2 h</td>
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<td>1596.71 ± 91.48 &lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Alcalase</td>
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<td>1744.83 ± 92.95 &lt;sup&gt;cde&lt;/sup&gt;</td>
<td>1705.64 ± 94.22 &lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>2471.79 ± 82.57 &lt;sup&gt;h&lt;/sup&gt;</td>
<td>2034.35 ± 96.82 &lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>1763.44 ± 53.96 &lt;sup&gt;cde&lt;/sup&gt;</td>
<td>2188.46 ± 152.45 &lt;sup&gt;fg&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bromelain</td>
<td>4 h</td>
<td>1864.02 ± 36.07 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>2316.69 ± 37.33 &lt;sup&gt;gh&lt;/sup&gt;</td>
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<td></td>
<td>6 h</td>
<td>2450.51 ± 42.81 &lt;sup&gt;h&lt;/sup&gt;</td>
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<td>1638.15 ± 75.83 &lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>4 h</td>
<td>1838.97 ± 78.66 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>1861.78 ± 68.19 &lt;sup&gt;e&lt;/sup&gt;</td>
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<td>2465.25 ± 77.18 &lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represents mean ± SD. Different small alphabets in the table represents significant difference between the samples. DPPH is represented in Trolox Equivalent Antioxidant Capacity (TEAC) µg/ml.

3.3.5 Anti-inflammatory (AI) properties of young and mature soybean protein hydrolysates

The ability of YS and MS protein hydrolysates to inhibit the inflammation in the *in-vitro* conditions is displayed in Table 9 and presented as diclofenac sodium
equivalent capacity (DSEC µg/ml). All the generated YS and MS hydrolysates showed an AI activity that varied when different enzymes were used for various times of hydrolysis. Unhydrolyzed YS and MS protein isolate samples displayed the lowest AI activities with values of 3355.71 ± 42.93 and 3595.08 ± 74.85 DSEC µg/ml, respectively, and both unhydrolyzed samples displayed insignificant difference amongst each other (P>0.05). Upon hydrolysis, AI activity increased in all the hydrolysates with the highest activity reported in bromelain-generated YS hydrolysates at 4 h (7527.83 ± 164.18 DSEC µg/ml) while the lowest activity was shown by the flavourzyme generated YS hydrolysate at 2 h (3777.58 ± 214.68 DSEC µg/ml) (P<0.05). For MS, alcalase-generated hydrolysates at 4 h showed the highest AI activity (5912.08 ± 30.92 DSEC µg/ml), followed by bromelain 4 h (5674.66 ± 71.45 DSEC µg/ml) and alcalase 6 h (5347.5 ± 27.55 DSEC µg/ml) generated hydrolysates which did not different significantly from each other (P>0.05). Alcalase and bromelain generated MS hydrolysates showed a similar trend where significantly higher (P<0.05) AI activity was displayed by the hydrolysates produced at 4 h hydrolysis time (5912.08 ± 30.92 and 5674.66 ± 71.45 DSEC µg/ml), respectively followed by a decline in the activity at 6 h. While for flavourzyme-generated MS hydrolysates, those produced at 2 h hydrolysis time showed a higher AI activity (4787.16 ± 72.29 DSEC µg/ml) than the hydrolysates produced at 4 h (4573.74 ± 37.25 DSEC µg/ml), and then the activity increased significantly at 6 h (4857.24 ± 59.33 DSEC µg/ml) compared to 4 h but was not significantly different to the hydrolysates generated at 2 h of hydrolysis (P>0.05). While for YS, hydrolysates produced by alcalase and bromelain showed higher AI activity than flavourzyme generated hydrolysates (P<0.05). Alcalase-generated hydrolysates of YS showed a significant increase in the AI activity when the time of hydrolysis increased from 2 h (5639.58 ±
314.95 DSEC µg/ml) to 4 h (5907.33 ± 138.63 DSEC µg/ml) (P>0.05) and further to 6 h (7185.08 ± 13.68 DSEC µg/ml) (P<0.05). Bromelain-generated hydrolysates of YS and MS displayed a similar trend where the hydrolysates produced at 4 h showed a maximum AI activity (7527.83 ± 164.18 and 5674.66 ± 71.45 DSEC µg/ml, respectively) which further declined at 6 h of hydrolysis time (6868.16 ± 208.82 and 4191.66 ± 173.38 µg/ml, respectively). On the other hand, the AI activity of the flavourzyme-generated YS hydrolysates showed an increase with the increase in time of hydrolysis from 2 to 6 h (3777.58 ± 214.68 to 5050.5 ± 134.08 DSEC µg/ml, respectively) (P<0.05). When comparing between alcalase generated YS and MS hydrolysates, alcalase-generated hydrolysates of YS at 2 h were effective against inflammation in comparison to those of MS (P<0.05). MS and YS hydrolysates exhibited an insignificant increase against inflammation at 4 h of hydrolysis (P>0.05). However, YS hydrolysates produced at 6 h of hydrolysis showed a significant AI activity than MS hydrolysates. For bromelain-generated hydrolysates, the AI activity of YS was significantly higher (P<0.05) than those of MS. At 4 h of hydrolysis, both YS and MS hydrolysates displayed higher activity against inflammation than those produced at 2 h of hydrolysis, however, the hydrolysates of YS were more potent compared to those of MS (P<0.05). Further hydrolysis up to 6 h showed a significant reduction in the AI activity for both MS and YS hydrolysates, although, hydrolysates of YS were more effective than MS (P<0.05). On the other hand, flavourzyme-generated hydrolysates of MS were significantly higher against inflammation than YS at 2 h of hydrolysis, although no significant difference (P>0.05) was observed in both MS and YS produced at 4 h. Furthermore, the AI activity of YS hydrolysates increased significantly compared to MS hydrolysates at 6 h hydrolysis. Overall, the results indicated that alcalase generated MS hydrolysates showed higher AI activities, while
bromelain-generated YS hydrolysates produced at 4 h were more effective against inflammation than alcalase and flavourzyme generated hydrolysates.

Table 9: Anti-inflammatory activity of young and mature soybeans protein hydrolysates produced by alcalase, bromelain, and flavourzyme at 2, 4 and 6 hours of hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Young soybean</th>
<th>Mature soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 h</td>
<td>3355.71 ± 42.93 a</td>
<td>3595.08 ± 74.85 a</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>5639.58 ± 314.95 f</td>
<td>5036 ± 54.69 e</td>
</tr>
<tr>
<td>Alcalase</td>
<td>4 h</td>
<td>5907.33 ± 138.63 f</td>
<td>5912.08 ± 30.92 f</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>7185.08 ± 13.68 g</td>
<td>5347.5 ± 27.55 f</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>4638.16 ± 69.23 d</td>
<td>4553.25 ± 166.88 c</td>
</tr>
<tr>
<td>Bromelain</td>
<td>4 h</td>
<td>7527.83 ± 164.18 h</td>
<td>5674.66 ± 71.45 f</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>6868.16 ± 208.82 g</td>
<td>4191.66 ± 173.38 b</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>3777.58 ± 214.68 b</td>
<td>4787.16 ± 72.29 d</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>4 h</td>
<td>4235.33 ± 241.79 c</td>
<td>4573.74 ± 37.25 c</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>5050.5 ± 134.08 e</td>
<td>4857.24 ± 59.33 d</td>
</tr>
</tbody>
</table>

Data represents mean ± SD. Different small alphabets in the same column represents significant difference between the samples. Anti-inflammatory is represented in Diclofenac Sodium Equivalent Capacity (DSEC µg/ml) µg/ml.
4.1 Proximate composition

Proximate analysis determines the major components of food including moisture, fat, ash (total minerals), protein, and carbohydrate contents (Nielsen, 2010). The moisture content of dried YS seeds was higher than MS seeds and is in agreement with previous studies (Redondo-Cuenca et al., 2007). Overall, the values for ash content of MS (5.28%) corresponds well with those reported by Li et al. (2012) and Redondo-Cuenca et al. (2007), while ash content of YS (4.67%) was slightly lower than 4.89% as reported by Ren et al. (2012). The protein content of MS was found to be higher than YS and is in corroborations with the results obtained by (Redondo-Cuenca et al., 2007) and (Ren et al., 2012). Although the fat content of YS (14.96%) agrees with previous literature (Ren et al., 2012), the values for MS were similar to those reported by Li et al. (2012) and slightly lesser than 18.56% reported by Redondo-Cuenca et al. (2007). Results obtained for carbohydrate contents of YS and MS are also in agreement with previous research where YS was found to possess higher carboydrates content than MS and is related to the fact that YS contains significantly higher proportions of starches (13% and 50%) in cotyledons at the beginning of maturation and then falls to approximately 1% in MS (Güzeler & Yildirim, 2016; Redondo-Cuenca et al., 2007).

4.2 Phase 1: Effect of simulated gastrointestinal digestion (SGID) on total phenolic content and bioactive properties of young and mature soybeans

4.2.1 Total phenolic content (TPC)

Phenolic compounds are group of small molecules having a structure that consist of at least one phenol unit and can be classified based on their chemical
structure including, phenolic acids, tannins, quinones, flavonoids, coumarins, and curcuminoids (Gan et al., 2019). Phenolic compounds are widely distributed among the plant kingdom and mostly found in plant tissues such as seeds, leaves, fruits, and roots (de la Rosa et al., 2019).

Various studies have reported the TPC of soybean seeds (Malenčič et al., 2007; Tan et al., 2017), germinated soybean (Guzmán-Ortiz et al., 2017), soymilk (Rodríguez-Roque et al., 2013), fermented soybeans (Ketnawa & Ogawa, 2019), and soybean by-products (Tyug et al., 2010). Rodríguez-Roque et al. (2013) have reported the changes in TPC of soymilk following in-vitro gastrointestinal digestion. It was observed that TPC increased significantly upon gastric and intestinal digestions compared to the non-digested soymilk (70.3 ± 1.8 and 69.8 ± 1.7, and 61.4 ± 1.2 mg GAE/100 ml, respectively). The author indicated that the gastric digestion influences the release of phenolic compounds from the soymilk matrix (Rodríguez-Roque et al., 2013). These findings are in agreement with the results of the present study that gastric and intestinal digestions prompted the release of phenolic compounds from both YS and MS.

The phenolic content of MS and YS, however, was found to be lesser than those reported by other researchers and could be attributed to differences in soybean variety. For instance, Liu and Xu (2015) reported the TPC content of yellow and black soybean as 2.75 mg GAE/g and 9.75 mg GAE/g, respectively. Whereas green soybean TPC was found in the range of 1.01-1.39 mg GAE/g (Kumar et al., 2014). It is also reported the reproductive stage also has a profound effect on the phenolic content of soybean. Kumar et al. (2009) evaluated the TPC of different reproductive stages of soybean seed and found that maximum TPC was found at the R5 stage and then decreased continuously till maturity.
4.2.2 Inhibition of key enzymes related to diabetes by young and mature soybean subject to SGID

4.2.2.1 α-amylase (AA) inhibitory activity

The enzymes that catalyze the hydrolysis of oligosaccharides and polysaccharides into absorbable units are AA and α-glucosidase that contribute to almost 70% of carbohydrate digestion in humans (Ahmad et al., 2019). The inhibition of both of these enzymes is considered as a useful approach for the treatment of non-insulin dependent diabetes (Ercan & El, 2016). The present study investigated the effect of SGID on the release of bioactive compounds from YS and MS with capability to inhibit AA. Results obtained in the present study depicted a strong enhancement in the α-amylase inhibitory activities of both YS and MS subjected to simulated gastric and intestinal digestion.

Although there are no direct comparative studies existing in the literature that identified the effect of simulated digestion on the AA inhibitory potential of soybeans, some researchers have reported the inhibitory potential of other legumes. For instance, Tan et al. (2017), reported the lowest AA-IC50 value of black soybean as 0.25 mg/ml, while 0.67 mg/ml for black turtle bean. Crude and semi purified phenolic extract obtained from black soybean also reported an AA-IC50 of 2.25 mg/ml and 1.60 mg/ml, respectively. Similarly, Ademiluyi and Oboh, (2013) investigated the ability of phenolic-rich extracts from soybean against AA with IC50 values as 526.32 µg/ml and 320.51 µg/ml, respectively. These studies are in agreement with findings of the present study that YS and MS extracts can inhibit the AA enzyme. Apart from these, a multitude of studies have reported the AA inhibitory activity from different grains and plants such as jute leaf (Corchorus olitorius) (Oboh et al., 2012), black chokeberry (Aronia melanocarpa L.) (Worsztynowicz et al., 2014), and quinoa (Hemalatha et al.,
2016; Herrera et al., 2019). For instance, Erkan and El (2016) have determined the AA inhibitory activity of chickpea and Tribulus terrestris upon in-vitro digestion and reported IC$_{50}$ values of 343 µg/ml and 167 µg/ml, respectively. Similarly, phenolic compounds obtained from quinoa showed effective inhibition of AA with IC$_{50}$ values of 108.68 µg/ml from quinoa bran and 148.23 µg/ml from quinoa hull, respectively (Hemalatha et al., 2016). Comparison of the obtained findings with the previously reported literature indicated that IC$_{50}$ values obtained in the present study are lower and more potent than reported by other researchers. The results obtained further suggests the role of soybean towards their potential in the management of diabetes and its related complications. However, further studies in animal model systems are needed to provide strong evidence for these outcomes.

4.2.2.2 Dipeptidyl peptidase IV (DPP-IV) inhibitory activity

DPP-IV inhibitors are known to be as front-line treatment for the management of hyperglycemia in diabetic patients and in a quest to extract and identify DPP-IV inhibitors from natural resources. Various plant and food sources have been screened by many scientists globally. However, the existing literatures lack the investigations into the effect of SGID on the DPP-IV inhibitory action of YS and MS. Therefore, an attempt was made in the present study to understand the effect of simulated digestion process on DPP-IV inhibitory activity. The results obtained suggested an improvement in DPP-IV inhibitory potential of both YS and MS upon SGID treatment which might be attributed to the release of bioactive components like phenolics and peptides from soybean upon digestion.

A previous study conducted on berry wine anthocyanins and flavonoids upon SGID showed DPP-IV-IC$_{50}$ values of 0.07 to >300 µM and the soy isoflavone
genistein possess DPP-IV-IC\textsubscript{50} of 0.48 µM which is far much lower than those obtained in the present study (Fan et al., 2013). The results obtained in the present study could be compared to the results obtained by Di Stefano et al. (2019) in which, different methods may influence the DPP-IV inhibitory activities. The authors investigated the effect of germination, heat treatment, and solid-state fermentation on the DPP-IV inhibitory activity of faba beans and chickpeas and indicated tremendous improvement in DPP-IV inhibitory activity as suggested by decline in their IC\textsubscript{50} values. Similarly, anthocyanin-rich water extracts obtained from maize also showed DPP-IV activity with IC\textsubscript{50} values ranging from 65.5 to 702.7 µg/ml and are comparable to those obtained in the present study (Zhang et al., 2019). Moreover, a Brazilian native Xylopia aromatica plant showed a DPP-IV-IC\textsubscript{50} value of 0.71 mg/ml (Oliveira et al., 2018), and a marine seaweed Turbinaria ornata showed DPP-IV-IC\textsubscript{50} values of 55.2 µg/ml and is equivalent to the results of the present study (Unnikrishnan et al., 2014). Therefore, the data suggest that YS and MS flour when subjected to SGID displayed potential inhibition of the DPP-IV enzyme.

4.2.3 Inhibition of key enzymes related to obesity by young and mature soybean subject to SGID

4.2.3.1 Pancreatic lipase (LIP) inhibitory activity

LIP enzyme mainly hydrolyzes the triacylglycerols into monoacylglycerols and free fatty acids, so its inhibition is considered as a potential treatment for obesity and its related metabolic diseases (Herrera et al., 2019). Scientists worldwide have screened a number of resources for the existence of pancreatic LIP inhibitors. However, studies related to the ability of YS and MS before and after SGID against pancreatic LIP are still non-existent in literature. The results obtained suggested the presence of potent pancreatic LIP inhibitory compounds among both YS and MS. The
results were found to be superior in terms of lower IC₅₀ values when compared with those reported by other researchers. The LIP inhibitory activity of YS and MS in the present study were higher compared to those obtained for phenolic extracts of lentils (Zhang et al., 2015), black chokeberry extracts (Worsztynowicz et al., 2014), quinoa and fenugreek extracts (Herrera et al., 2019). However, they were similar to those obtained by Tan et al. (2017), where LIP inhibitory IC₅₀ values of the phenolic extracts fractions of black soybean and black turtle bean were found to be 81 µg/ml and 76 µg/ml, respectively.

The effect of SGID on the LIP inhibitory activities of soybean was evident and as explained by Liu and Xu, (2015), changes in the pancreatic LIP inhibitory potential was found to be related to the increased concentration of saponins among other phenolic compounds. In another study, the extracts of Xylopia aromatica were reported to inhibit pancreatic LIP with an IC₅₀ value of 0.71 mg/ml which is significantly higher than the results obtained in the present study (Oliveira et al., 2018). Whereas, LIP inhibitory IC₅₀ values of chickpea (9.74 µg/ml) and Tribulus terrestris phenolics (15.3 µg/ml) were found to be significantly lower than those obtained in present study (Ercan & El, 2016). The wide variation between IC₅₀ values obtained from different extracts could be due to the variation in their phenolic content and other bioactive compounds. Hence, further investigation into the identification and characterization of major inhibitory fractions with potent LIP inhibition must be undertaken.

4.2.3.2 Cholesterol esterase (CE) inhibitory activity

Pancreatic cholesterol esterase (CE) is responsible for the hydrolysis of dietary cholesterol esters which releases the free cholesterol in the lumen of the small intestine.
In addition, CE promotes in combining the cholesterol into the mixed micelles and supports the transportation of free cholesterol to the enterocyte (Ngamukote et al., 2011). Very few studies have investigated the cholesterol-lowering effects of plants and phenolics through the inhibition of CE enzyme. There are no previous literatures on the inhibitory activity of YS and MS upon SGID against CE enzyme. The results obtained from the current study are first in this direction that reports that SGID of YS and MS resulted in improvement of CE inhibitory activity. The improvement in CE inhibitory potential could be due to the release of bound phenolics upon digestion that might have resulted in increased inhibition of CE. A significant decline in IC$_{50}$ values was observed upon gastric digestion which then increased upon further intestinal digestion and might be due to the degradation of the compounds which were released during simulated gastric digestion and might have not been able to interact with the active site of CE, thus resulting in a decrease in CE inhibition.

Although there is no direct study related to the impact of SGID on CE inhibitory activities of soybean derived phenolic extract, Adisakwattana et al. (2010), reported a similar CE-IC$_{50}$ value from the grape seed extract (27.27 µg/ml) that not only displayed stronger inhibition of CE under in-vitro condition but also successful in decreasing serum triglyceride and cholesterol levels in high fat fed rats governed through inhibition of lipid digestion and absorption. In addition, Ngamukote et al. (2011) reported a concentration dependent CE inhibition from major polyphenolic compounds such as gallic acid, catechin, and epicatechin from grape seeds. This study is the first report on the CE inhibitory potential of soybean when subjected to SGID, but further investigations are required in order to identify and elucidate the mechanism of action of potent CE inhibitory compounds originating from YS and MS upon gastrointestinal transit.
4.2.4 Antioxidant activities by young and mature soybean subject to SGID

4.2.4.1 Ferric-reducing antioxidant power (FRAP)

All samples of YS and MS extracts upon SGID showed significant improvement in ferric reducing antioxidant power (FRAP) as indicated by higher FRAP values in Table 5. Overall, MS was found to possess better antioxidant activity in terms of FRAP value than YS and could be attributed to the levels, types, and concentration of different phenolics at different stages of maturity. As indicated by Mtololo et al. (2017), the antioxidant activity of cowpeas seeds in terms of FRAP is significantly impacted due to the action of processing such as boiling, pressure cooking, and in-vitro digestion. The results obtained were similar to the present study where an improvement in FRAP activity was observed upon simulated digestion indicating the role of digestive enzymes and condition in the release of bioactive components capable of reducing ferric ions leading to higher FRAP values.

Numerous studies determined the antioxidant activities of MS and YS extracts by measuring their reducing powers. For instance, Kumar et al. (2014) evaluated the reducing power of 16 immature (young) soybean genotypes, and their FRAP values range between (3.95 - 10.94) μM/g with a positive correlation between total isoflavone content and FRAP activity. The obtained results are in stark contrast to those obtained by Kumar et al. (2010) where they reported stronger FRAP activity among green soybean in comparison to black or yellow soybean due to the difference in carotenoid and lutein contents among yellow and black soybean. The lower FRAP values obtained in the present study could be attributed to variety differences between MS and YS. As FRAP values could be affected by the stage of maturation it could be considered as another reason for differences in FRAP values between YS and MS. A recent study
conducted into the impact of maturation stage on the reducing power of soybean concluded that vegetative state of soybean contains higher FRAP activity than the reproductive stages (Peiretti et al., 2019). The higher antioxidant activities obtained by ABTS assay compared to FRAP assay are attributed to the fact that ABTS assay is useful to the hydrophilic and lipophilic antioxidants due to the reagent, which is soluble in both aqueous and organic solvents, while FRAP assay is used particularly to water-soluble antioxidants (hydrophilic antioxidants) (Guzmán-Ortiz et al., 2017).

4.2.4.2 2′2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The application of ABTS radicals scavenging assay has largely been employed to determine the scavenging abilities, in which their color reduce due to the presence of antioxidant-containing samples (Seo et al., 2017). In addition, ABTS radicals are associated with the scavenging capacities of chain breaking and hydrogen donating antioxidants (Lee et al., 2019). Table 5 depicts that YS and MS extracts after the gastrointestinal digestion has shown better antioxidant activity in terms of scavenging of ABTS radicals. Similar results on the effect of gastrointestinal digestion were reported by Apea-Bah et al. (2016) for porridges prepared with cereals and cereal–legume composites with significantly higher ABTS scavenging activity among digested samples of porridges when compared with the unhydrolyzed sample and was attributed to the release of extractable phenolics bound to proteins (Apea-Bah et al., 2016). In addition, the obtained results also indicated a significant increase in scavenging activity after the complete gastrointestinal digestion compared to the gastric digestion. Apea-Bah et al. (2016) reported a similar observation and stated that in simulated duodenal digestion, the protein digestion is more effectively carried out by the pepsin and pancreatic protease (trypsin) rather than pepsin alone in the
simulated gastric digestion, which consequently led to the release more flavonoids bound to the proteins during the simulated duodenal digestion than in the simulated gastric digestion.

Although there is a scarcity in the literature about the impact of gastric and the intestinal digestions of YS and MS on the antioxidant activities, a number of studies have reported the antioxidant activities of soybeans with possible correlation to existence of phenolic compounds such as isoflavones and varies from cultivar to cultivar and also between different substrates. For instance, Han and Baik, (2008) reported the ABTS radical scavenging activity of soybean (10.2 µmol TEAC g⁻¹) and it was higher than those of chickpeas, yellow peas, green peas but lower than the two lentils (cv. Pardina and cv. Crimson). Similarly, a wide variation in ABTS radical scavenging was observed upon germination of soybean by Guzmán-Ortiz et al. (2017) which was attributed to additional synthesis of newer phenolic compounds and isoflavones during germination. Similar results were obtained by Lee et al. (2019), where solid-state fermentation of germinated soybean with Tricholoma matsutake remarkably increased the ABTS radicals scavenging activity. Hence, the present study suggests that gastrointestinal digestion could have also led to either release of bound phenolics or some other bioactive components (e.g. bioactive peptides and vitamins) that displayed more efficient scavenging of ABTS radicals.

4.2.5 Anti-inflammatory (AI) activities

Inflammation is a physiological process that occurs due to the body’s response to harmful stimuli such as infection, irritants, or damaged cells (Bouhlali et al., 2020). Therefore, suitable compounds capable of inhibiting the inflammatory events are much sought after in form of anti-inflammatory drugs. However, synthetic drugs currently
in use for the treatment of inflammation are widely considered unsafe due to their possibility to cause iatrogenic reactions, drug-related toxicity, and other adverse reactions that could complicate the progression of the treatment on long-term use (Bouhlali et al., 2020). Therefore, natural substances that can inhibit the protein denaturation of the tissues can be useful as safer and much convenient AI drugs (Kamal et al., 2018).

In the present study, an in-vitro model of inflammation as denaturation of egg albumin protein was used to determine the AI activity of YS and MS extracts. The results were expressed as diclofenac sodium equivalent capacity and results obtained depicted a stronger AI activity among YS and MS extracts after the gastric and intestinal digestions. The results were found to be comparable to those obtained by Ezzat et al. (2018), where ginger extracts also showed higher AI activity (66%) by the inhibition of the heat induced albumin denaturation. Similarly, Moroccan date fruit seeds were also found to have an effective AI activity by inhibition of protein denaturation with IC\textsubscript{50} values ranging from 90.34 - 193.71 \(\mu\text{g}/\text{ml}\) which is comparable to the IC\textsubscript{50} value of the diclofenac sodium (IC\textsubscript{50} = 225.04 \(\mu\text{g}/\text{ml}\)) (Bouhlali et al., 2020). The results obtained were ascribed to the protein-polyphenolic interactions and their positive impact on the thermal stability of proteins. It is known that the interactions of phenols with proteins cause changes in the secondary structure of protein leading to variation in their heat stability (Bouhlali et al., 2020). Hence, variations of the AI activity of YS and MS could be related to the phenolic composition and their interactions with egg albumin proteins.

Plant extracts have shown different modes of AI activity other than their ability to prevent protein denaturation. Kao et al. (2007) determined the AI activity of the soybean cake isoflavones in animal model of inflammation and found that isoflavone
powders and genistein standard were effective in lowering leukocyte number and lipopolysaccharide (LPS)-induced inflammation (Kao et al., 2007). Similarly, Purple maize genotypes were also reported to have an AI activity primarily corresponding to the quercetin, luteolin, and rutin compounds (Zhang et al., 2019). Moreover, white kidney beans and round purple beans extracts have displayed high AI activity by decreasing the production of Nitric oxide (NO) and cytokine mRNA expression of LPS stimulated macrophages (García-Lafuente et al., 2014). All these studies have correlated the AI activities with the presence and action of phenolic compounds, which cannot be ruled in this study where upon SGID increase in TPC of YS and MS was observed.

4.3 Phase 2: Effect of the enzymatic hydrolysis on bioactive properties of young and mature soybean protein hydrolysates

The last two decades have seen increased scientific interest in the production, identification, and characterization of bioactive peptides from plant sources of proteins (Chatterjee et al., 2018; Maqsood et al., 2021). These bioactive peptides which usually remain hidden in their parent sequence become active upon their release in the gut due to the action of gut proteases and are also generated during food processing, fermentation, or through enzymatic hydrolysis (Singh et al., 2014). The main focus of the research directed towards bioactive peptides is the elucidation of their beneficial physiological functions and mechanism of action. Till today, immense number of soy-derived peptides have been identified by various researchers globally with a multitude of biological functions including but not limited to lipid-lowering, anti-diabetic, anti-cancerous, anti-hypertensive, AI, and antioxidant (Chatterjee et al., 2018). However, no such study has been extended towards the generation of bioactive peptides from YS
proteins, and therefore the present study was undertaken to elucidate the antidiabetic, anti-obesity, and antioxidant function of YS proteins in comparison to MS proteins.

4.3.1 Characterization of protein hydrolysates obtained from young and mature soybean proteins

4.3.1.1 Degree of hydrolysis (DH%)

Small peptides and free amino acids which are generated upon enzymatic hydrolysis of the food proteins are known to enhance the nutritional value and bioactive properties of food proteins. However, sometimes higher level of hydrolysis leads to the development of bitter peptides that might then obstruct the application of these protein hydrolysates in development of new food products owing to their negative impact on sensorial characteristics of food products (Sun, 2011). Similarly, it is also important to monitor the extent of hydrolysis in order to control the hydrolysis process due to its probability of affecting the functional and bioactive properties of the hydrolysates (Jafar et al., 2018). Therefore, degree of hydrolysis (DH%), a basic parameter used to depict the extent of the hydrolysis and the properties of the hydrolysates by exhibiting the percentage of the cleaved peptide bonds must be analyzed (Chabanon et al., 2007). In addition, the DH% is also an indicator of the ability of a protease to degrade the proteinaceous substrate (Hanafi et al., 2018). Currently, the protein hydrolysates with high DH% (>10%), which are produced by the food industry are used as nutritional supplements for medicine and sport due to their ability to give a high solubility and optimal intestinal absorption (Marinova et al., 2008).

As per the results obtained in present study, the bromelain-generated YS protein hydrolysates showed significantly (P>0.05) higher DH values (72.39 % ± 2.06) at 6 h than the hydrolysates generated by alcalase and flavourzyme. These observations
are in agreement with those reported by Hanafi et al. (2018), where bromelain generated hydrolysates from green soybean showed higher DH values of around 61.60% after 10 h of hydrolysis in comparison to alcalase, papain, and flavourzyme. Alcalase generated hydrolysates of both YS and MS (2 h, 4 h, 6 h) also displayed high DH values, and are attributed to the fact that alcalase being an endopeptidase has a range of specificity of peptide bonds during hydrolysis (Sbroggio et al., 2016). Moreover, results of the present study in Figure 7 indicates that the DH values increases with the hydrolysis time, which is similar to the results of Mudgil et al. (2018) where the DH of the camel milk protein hydrolysates increased with increasing hydrolysis time from 3 h to 9 h using alcalase, bromelain, and papain. Furthermore, the hydrolysis rate of quinoa protein hydrolysates generated by bromelain, chymotrypsin, and protease also showed similar increment in DH values during hydrolysis time progression (Mudgil et al., 2019d).

Wang et al. (2019) have also observed that DH values of soy protein hydrolysates generated with alkaline protease, papain, and trypsin increased with the increase in hydrolysis time. However, the results obtained in present study report significantly higher hydrolysis of soybean proteins in comparison to those of Hrckova et al. (2002), where DH values upon hydrolysis of defatted soy flour using flavourzyme after 480 minutes showed only an increment of 3.2% to 5.1%. In general, these differences in DH values obtained in the present study and those obtained by other researchers are attributed to the differences in the reaction rate, enzyme specificity, and substrate affinity (Jafar et al., 2018). Moreover, alcalase is a serine protease, bromelain and papain are cysteine protease and flavourzyme contains a mixture of exo and endopeptidases with unique flavor generation and debittering benefits. Alcalase is reported to possess a very broad substrate specificity and hence is
considered as one of the most efficient enzymes in the generation of hydrolysates from a variety of proteins (Jung et al., 2016). Even papain and bromelain differ in their specificity towards proteins as papain hydrolyses peptide bonds contributed by an adjacent amino acid lysine, arginine and phenyl-alanine while bromelain preferred amino acid next to lysine, arginine, phenylalanine, and tyrosine (Wan Mohtar et al., 2014). Therefore, variation observed in degree of hydrolysis when different enzymes are used for hydrolysis is because of various factors discussed above.

4.3.2 Inhibition of key enzymes related to diabetes by young and mature soybean protein hydrolysates

A number of previous studies have correlated the consumption of soybean and their action in the maintenance of glucose homeostasis in diabetic patients (Chatterjee et al., 2018). Generally, in humans glucose homeostasis is regulated through combine actions of two incretin hormones (Glucose-Dependent Insulinotropic Polypeptide (GIP) and Glucagon-Like Peptide-1 (GLP-1)) that governs the secretion of insulin from pancreatic β-cells that in turn regulates the cellular uptake of blood glucose keeping their level in check (Silveira et al., 2013). However, the action of these incretin hormones is negatively impacted by the action of a human serine protease, dipeptidyl peptidase-IV (DPP-IV) causing the degradation of incretin hormones and thus reduced insulin secretion. Similarly, α-amylase (AA), an enzyme that is responsible for the hydrolysis of starch carbohydrates in the digestive system increases the levels of blood glucose upon food consumption causing postprandial hyperglycemia (Garza et al., 2017). Blocking the hydrolysis of complex starches to simple sugars in the small intestine thus could lead to decreased rate of carbohydrate digestion and reduced absorption of glucose in the blood (Vileacundo et al., 2017). Therefore, inhibition of AA and DPP-IV is considered as a prime therapeutic strategy for controlling the
menacing effects of diabetes. Owing to the side effects associated with synthetic AA inhibitor Acarbose (Sun et al., 2017), natural alternatives devoid of any side effects are needed. Numerous studies have indicated the antidiabetic roles of soy-derived peptides with mechanism unknown and it is therefore, anticipated that they could be exerting their antidiabetic action through inhibition of these enzymes (Sanjukta & Rai, 2016).

4.3.2.1 α-amylase (AA) inhibitory activity

Although there are some existent studies that report the AA inhibitory potential of mature soybean hydrolysates. Till date, there are no previous studies regarding the AA inhibitory potential of YS hydrolysates. Keeping this in consideration, a comparative investigation into the AA inhibitory activities of YS and MS protein hydrolysates have been investigated using in-vitro assays.

The results of the current study suggest that YS hydrolysates are better inhibitors of AA in comparison to MS hydrolysates. In addition, it was also observed that flavourzyme and bromelain derived hydrolysates displayed better inhibition of AA compared to alcalase generated hydrolysates. With the change in time of hydrolysis, a variable effect was observed where hydrolysates generated at 2 h displayed higher inhibitory activity against AA than those obtained after 4 and 6 h. These results could be attributed to the fact that inhibition of AA enzymes is strictly regulated by amino acid sequences present in the peptides and therefore, it is anticipated that peptides generated at 2 h were more potent inhibitor to AA and could have been further degraded to less inhibitory peptides upon further hydrolysis (Mudgil et al., 2019a; Nongonierma & FitzGerald, 2019). Overall, the results obtained in the present study were found to be superior than those obtained by González-Montoya et
al. (2018a), where an IC$_{50}$ value of 1.70 mg/ml was reported for germinated soybean protein digest upon SGID against salivary AA. Similarly, Ademiluyi et al. (2014) have reported AA inhibitory activity of fermented soybean condiment in diabetic rats both under *in-vitro* and *in-vivo* conditions in a concentration dependent manner with AA-IC$_{50}$ value of 1063.8 μg/ ml.

Legumes in general are considered a rich source of peptides with significant antidiabetic activities. In one such similar study Ngoh and Gan (2016), reported the AA inhibitory action of pinto bean protein hydrolysates produced by protamex hydrolysis. However, the AA-IC$_{50}$ values obtained in the present study are far higher than those obtained from moringa seeds protein hydrolysates with an AA inhibitory IC$_{50}$ value of 0.123 μg/ ml (Garza et al., 2017). Overall, the obtained results shows that the bromelain and flavourzyme derived YS protein hydrolysates displayed excellent AA inhibitory properties and thus could have positive implication in management of diabetes.

4.3.2.2 Dipeptidyl peptidase IV (DPP-IV) inhibitory activity

The inhibition of DPP-IV has been considered as a novel strategy for managing Type 2 diabetes mellitus (Mada et al., 2019). The inhibitors of DPP-IV were one of the first classes of oral antidiabetic drugs to be prospectively designed as anti-hyperglycemic agents. DPP-IV inhibitory protein hydrolysates are widely being explored in various food products such as plant (Deacon & Lebovitz, 2016) and dairy proteins (Nongonierma et al., 2014), camel skin gelatin (Mudgil et al., 2019c), egg yolk protein by-product (Zambrowicz et al., 2015), amaranth (Velarde-Salcedo et al., 2013), quinoa (Mudgil et al., 2020), soybean and lupin (Lammi et al., 2018). Results obtained regarding DPP-IV inhibitory activities of YS and MS protein hydrolysates
revealed a significant increase in DPP-IV inhibitory activities upon hydrolysis. Intact proteins also showed inherent DPP-IV inhibitory activity which is in line with previous reports (Chatterjee et al., 2018; Sanjukta & Rai, 2016). Interestingly, among intact proteins, the DPP-IV-IC\(_{50}\) value was found to be higher for YS protein in comparison to MS protein. However, upon hydrolysis, it was observed that YS protein hydrolysates were more potent inhibitor than MS protein hydrolysates. The DPP-IV IC\(_{50}\) values obtained are comparable to the existing literature.

For instance, González-Montoya et al. (2018a) reported that the SGID derived samples of germinated soybean proteins were found to inhibit DPP-IV with an IC\(_{50}\) value of 1.49 ± 0.14 mg/ml. It was also reported that fractionation of germinated soybean digest through 5 and 10 kDa membrane displayed more effective DPP-IV inhibition with IC\(_{50}\) values 0.91 ± 0.17 and 1.18 ± 0.15 mg/ml, respectively. Furthermore, Wang et al. (2019) reported an inhibitory activity of DPP-IV with an IC\(_{50}\) value of 2.73 mg/ml when soy protein hydrolysates were produced by alkaline protease. These results also suggested that the bioactivity of YS and MS protein hydrolysates could be further enhanced after gastrointestinal digestion. In another study conducted by Nongonierma & FitzGerald, (2015), soy hydrolysates generated upon in-vitro SGID exhibited DPP-IV inhibitory activity with IC\(_{50}\) values of 1.85 mg/ml. Overall, from the results obtained for AA and DPP-IV inhibition, it can be suggested that both YS and MS proteins demonstrated significant anti-diabetic effects with former being more potent. Thus, both YS and MS protein-derived bioactive protein hydrolysates may help in glycemic management strategy without any side effects. However, further work is needed to fully determine the mechanism of inhibitory action, their efficacy under in-vivo conditions and their bioavailability in as well as identification of the peptide sequence in the most potent hydrolysates.
4.3.3 Inhibition of key enzymes related to obesity by young and mature soybean protein hydrolysates

Anti-obesity activities of MS derived bioactive peptides have been among the best explored bioactive properties both *in-vitro* and *in-vivo* only after their antihypertensive effects. The mechanistic evaluation into anti-obesity action of soy peptides has been directed to their inhibition of enzyme related to cholesterol biosynthesis known as 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) which is linked to their hypolipidemic effect (Kasim *et al.*, 1992; Singh *et al.*, 2021; Wang *et al.*, 2018). It is reported that peptide LPYP from the glycinin subunit, and IAVPGEVA and IAVPTGVA are potent inhibitors of HMGCoAR (Pak *et al.*, 2005). Similarly, KNPQLR, EITPEKNPLR, and RKQEEDEEQQRE obtained from glycinin subunit of soy protein were found to be inhibitory to the action of enzyme fatty acid synthase activity. Therefore, these studies indicate that peptides obtained from the YS and MS could also lead to inhibition of the enzymes that are related with fats digestion and absorption in the human body such as pancreatic lipase (LIP) and cholesterol esterase (CE).

4.3.3.1 Pancreatic lipase (LIP) inhibitory activity

Lipases are the enzymes that digest fats such as triacylglycerol- and phospholipases. Gastric and pancreatic lipases are enzymes that catalyze the hydrolysis of dietary triacylglycerols which is essential for their absorption by enterocytes, to enable the absorption of dietary fat in the body (Mukherjee, 2003). Pancreatic LIP (triacylglycerol acyl hydrolase) is the main lipolytic enzyme that is synthesized and secreted through the pancreas and plays a major role in the hydrolysis of 50–70% of total dietary fats (Birari & Bhutani, 2007). Therefore, the inhibitory
activity of pancreatic LIP to delay and inhibit the digestion and absorption of fat, have been suggested for the development of anti-obesity agents (Seyedan et al., 2015).

Similarly, cholesterol is important for the synthesis of steroid hormones, bile acids, and vitamin D in the body. Despite that, hypercholesteremia causes the formation of plaques in arteries which results in arteriosclerosis and subsequent hypertension (Mada et al., 2019). Pancreatic CE is an enzyme, which get secreted from pancreas into the intestinal track, is responsible for the hydrolysis of multiple substrates such as cholesterol esters, fat-soluble vitamins, triglycerides, and phospholipids. CE can regulate the serum cholesterol levels and is involved in lipoprotein metabolism and can cause a harmful effect in atherosclerosis processes (John et al., 2011). Bioactive peptides of different food products have been demonstrated to decrease cholesterol levels. Although LIP and CE inhibitory activity of camel milk protein hydrolysates (Jafar et al., 2018; Mudgil et al., 2019b), fermented milk (Gil-Rodríguez & Beresford, 2019; Kim & Lim, 2020), and millet grains (Jakubczyk et al., 2019) have been studied so far. There are no previous studies on the inhibitory activity of LIP and CE by MS and YS protein hydrolysates.

Results obtained from the present study indicated significant improvement in LIP and CE inhibitory activity upon hydrolysis of YS and MS proteins. Specifically, flavourzyme generated hydrolysates were more efficient in inhibiting both LIP and CE with IC$_{50}$ values in the range of 65.15 and 32.96 µg/ml protein equivalent for MS soybean protein hydrolysates, whereas 30.41 and 27.08 µg/ml protein equivalent for YS soybean protein hydrolysates, respectively. Significantly lower LIP and CE-IC$_{50}$ values were also observed for alcalase and bromelain generated hydrolysates when compared to intact proteins. These results are also in agreement with previously published results. For instance, yellow field pea (*Pisum sativum* L.) hydrolysates
produced through alcalase and trypsin hydrolysis showed potent LIP inhibitory activities as displayed with their low IC$_{50}$ values of 3.95 mg/ml and 3.98, respectively (Awosika & Aluko, 2019). Similarly, Ngoh et al. (2017) reported that the pinto bean-derived hydrolysates have inhibited LIP activity ranging from ~23 to ~87%, whereas cumin-seed derived hydrolysates and peptides displayed LIP inhibitory potential in the range of 50.1 to 54.6% (Siow et al., 2016). The aforementioned studies also indicated that the hydrolysates derived from plant proteins could exhibit the inhibitory activity of LIP, which also agrees with the obtained results of the YS and MS hydrolysates. Not only plant proteins but proteins from dairy stream have also been reported to exert potent LIP inhibitory actions (Mudgil et al., 2018). In a study conducted on alcalase, bromelain and papain generated hydrolysates from camel milk, displayed a pattern similar to the YS hydrolysates where the hydrolysates presented lower IC$_{50}$ values with the increase in hydrolysis time (Mudgil et al., 2018). In silico analysis into the inhibitory mechanism of these hydrolysates indicated that the generated peptides exert their inhibitory mechanism against LIP through binding to substrate binding unit and catalytic binding sites of LIP (Mudgil et al., 2018).

### 4.3.3.2 Cholesterol esterase (CE) inhibitory activity

CE is a pancreatic enzyme present in bile that catalyzes the release of cholesterol and free fatty acids from dietary cholesterol esters. CE plays an important role in cholesterol metabolism by various mechanism such as regulation of cholesterol transportation and conversion of lysine to lysolecithin that is required for micelle formation. As such, inhibition of CE can slowdown cholesterol uptake by human body indirectly by decreasing the level of cholesterol produced from dietary lipids (Heidrich et al., 2004). Although no direct study on the CE inhibitory effects of plant seed
proteins has been reported previously, results obtained by Zhong et al. (2007a) demonstrated that the hypocholesterolemic effect of soy-derived peptides on mice indicated that alcalase derived soy hydrolysate showed a cholesterol micellar solubility inhibitory rate of 48.6% which was suggested as a potential hypocholesterolemic effect. Similarly, Kobayashi et al. (2012) also reported that fermented soymilk can modulate cholesterol metabolism in rats fed with high cholesterol diet. It was demonstrated that upon the ingestion of fermented soymilk, the liver weight and fat mass of the rats were reduced, lowered cholesterol levels, hepatic triglyceride, and plasma total cholesterol level were observed (Kobayashi et al., 2012). Furthermore, it was observed that high-protein soymilk has a significant cholesterol-lowering effect when it was replaced with protein from cow’s milk completely (Sirtori et al., 1999). Another example of different plant proteins is cumin seed derived peptides demonstrated to inhibit cholesterol micelle formation showing an inhibitory effect up to 80% (Siow et al., 2016). These studies are in agreement with the results of the present study of YS and MS which has shown potential to inhibit cholesterol esterase to a significant level.

CE enzyme inhibition was also demonstrated by camel milk whey protein hydrolysates generated by gastric and pancreatic enzymes (Jafar et al., 2018). Furthermore, Jemil et al. (2017) reported that the fermented sardinelle protein hydrolysates contain bioactive peptides, which significantly reduced hyperlipidemia. Similarly, camel milk protein hydrolysates were found to be significantly inhibited the activities of CE enzyme under in-vitro conditions (Mudgil et al., 2019b). Similarly, various other peptides such as lactostatin and soystatin (Nagaoka, 2018) from β-lactoglobulin and soy, respectively as well as peptide WGAPSL (Zhong et al., 2007b) were reported to exert hypocholesterolemic effects via mechanisms like CE inhibition.
Overall, as the information related to LIP and CE inhibitory action of protein hydrolysates is quite limited, thus more research needs to be performed to elucidate the LIP and CE inhibitory effects of such peptides.

4.3.4 Antioxidant activity by young and mature soybean protein hydrolysates

Enzymatic hydrolysis has been known to boost the functional and biological properties of proteins including antioxidant activities. Antioxidant properties of the hydrolysates mainly rely on different factors including the composition of the native protein, method of protein isolation, peptide sequence, structure, molecular weight, and the degree of hydrolysis (Ajibola et al., 2011). In addition, the antioxidant activities of these peptides are also dependent on the sequence of amino acids, and that in turn is determined by the specificity of proteases used for hydrolysis that could positively affect their antioxidant activities (Coscueta et al., 2016). Bioactive peptides exert their antioxidant activities using several mechanisms such as radical scavenging, lipid peroxidation inhibition, chelation of metal ion, reduction of free radicals, etc. (Sarmadi & Ismail, 2010). Therefore, it is important to use different kinds of antioxidant assays in order to elucidate the total antioxidant power of generated hydrolysates. The value of Trolox Equivalent Antioxidant Capacity (TEAC) is used as an expression of the antioxidant activity that can enable comparing antioxidant capability between different laboratories and therefore, results obtained in this study are also expressed as TEAC (µg/ml of protein equivalent) (Jara et al., 2018). Overall, to estimate the total antioxidant capacity of YS and MS hydrolysates three different antioxidant assays with different mechanisms were used.
4.3.4.1 Ferric-reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) assay determines the ability of test compounds to reduce ferric ion ($\text{Fe}^{3+}$)–TPTZ complex to ferrous ($\text{Fe}^{2+}$)–TPTZ complex that generate a blue color product with absorbance maxima at 593 nm. The intensity of blue color developed is directly correlated with reducing capability of test compounds (Sbroggio et al., 2016). Intact proteins of YS and MS displayed significant antioxidant power and are in-line with previous findings from other researchers. There are different plant seed protein hydrolysates that showed FRAP capacities such as African yam bean seed (Ajibola et al., 2011), cowpea seed (Olusegun & Emmanuel), rice bran (Thamnarathip et al., 2016), mung bean ((Lapsongphon & Yongsaawatdigul, 2013; Sonklin et al., 2018), and cumin seeds (Siow & Gan, 2017).

The results obtained in the present study indicated that MS protein hydrolysates have significantly higher FRAP activity in comparison to YS protein hydrolysates. These distinct differences in the FRAP between YS and MS protein hydrolysates could be attributed to various factors such as sequence of peptides, position of particular amino acids in the sequence, and their accessibility to react with ferric ions (Elías et al., 2008). In addition, sequence and presence of hydrophobic/aromatic amino acids within the peptides are also reported to affect FRAP activities of hydrolysates (Salami et al., 2011). Moreover, the reason could be due to the presence of peptides with different molecular sizes and amino acids sequences, which could also result in different structural functionality and thus reducing capacity of the peptides within the hydrolysates (Meza-Espinoza et al., 2018). Overall, hydrolysis of both types of soybean proteins significantly improved the FRAP activities of hydrolysates.
Additionally, there are several researches that are in-line with the findings of the present study which show that soybean protein hydrolysates can act as antioxidant compounds by displaying reducing power. Zhang et al. (2019) have reported that soybean peptides generated upon alcalase hydrolysis (VVFVDRD, VIYVVDDL, IYVVDDL, and IYVFVR) possess FRAP activities in the range of 54.7-79.0 mM. Another study reported that the soy crude hydrolysates produced with proteases derived from Bromelia pinguin and Bromelia karatas fruits also displayed high FRAP values of 8.57- and 8.89-mM TE/g protein, respectively (Meza-Espinoza et al., 2018). Furthermore, okara protein hydrolysates also showed increase in FRAP activity with the increase in time of hydrolysis and corresponded directly with DH% values. The range of TEAC values for FRAP activity were comparative to the values obtained in the present study (Sbroglio et al., 2016).

Nonetheless, several other studies have also reported higher reducing power appearing from the low molecular weight hydrolysates (smaller peptides) rather than the high molecular weight. For instance, Meza-Espinoza et al. (2018) reported a higher FRAP capacity from the low molecular weight fractions (≤1 kDa and ≤5 kDa) of soy protein crude hydrolysates. In addition, the smallest fraction of pinto bean protein hydrolysates (=3 kDa) that was generated by protamex have shown the highest FRAP (0.81 mM) (Ngoh & Gan, 2016). Ajibola et al. (2011) have also reported that the smallest fraction of the African yam bean seed protein hydrolysate (1 kDa) has significant reducing power compared to the fractions with higher molecular weight peptides (1–3, 3–5, 5–10 kDa). The results of YS and MS hydrolysates are highly indicating that these hydrolysates can be an advantageous ingredient to be utilized as antioxidants in nutraceutical and functional food formation.
4.3.4.2 2′2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

ABTS radical scavenging activity method is based on measuring the discoloration of the ABTS radicals as a result of the presence of antioxidant compounds (Coscueta et al., 2016). The results obtained in the present study indicated that hydrolyzed YS and MS proteins showed a higher ABTS radical scavenging activity compared to their intact proteins. The increase in ABTS radical scavenging of hydrolysates correlated well with increase in DH values and are in agreement with study conducted by Guan et al. (2018) where a similar positive relationship between DH of the soy protein isolate hydrolysates and ABTS radical scavenging activity was observed. These results are also in accordance with Sbroggio et al. (2016) where increased hydrolysis of okara (soybean by-product) protein hydrolysate produced upon alcalase hydrolysis was found to significantly increase the ABTS radical scavenging activity from 68.6% to 99.5%. Similarly, flavourzyme induced hydrolysis was also reported to increase the ABTS radical scavenging of hydrolysates from 67.2 to 88.2% (Sbroggio et al., 2016). The result of the present study also suggests that that hydrolysis of YS and MS protein led to an increased in ABTS radical scavenging activity when the DH increased.

Furthermore, previous research on soy protein hydrolysates prepared with the peptidases from latex of Maclura pomifera fruit also showed an increase in ABTS radical scavenging activity with a TEAC value of 157.6 μmoles TE/g of protein and are well in agreement with the present results (Jara et al., 2018). Similar ABTS radical scavenging activity was also reported by Song et al. (2020) for cottonseed proteins and their hydrolysates with values ranging between 568.87 – 1086.76 μM TEAC/mg protein equivalent, respectively. The higher ABTS radical scavenging effect of
hydrolysates is usually correlated with higher content of positively charged amino acids and lower content of negatively charged amino acids (Song et al., 2020). Therefore, more investigations are needed towards the identification and characterization of individual peptides and their physicochemical properties and their mechanistic evaluation towards their inherent antioxidant powers.

4.3.4.3 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The method of DPPH radical scavenging activity is based on the ability of the antioxidant compounds to donate a hydrogen atom to the free radical (Sbroggio et al., 2016). The oil-soluble free DPPH radicals turn into a stable product after accepting hydrogen or an electron from the antioxidant generating a non-toxic species that in turn is supposed to inhibit the propagation phase of lipid oxidation (Amadou et al., 2011). The results obtained pertaining to DPPH radical scavenging activity of YS and MS protein hydrolysates are represented in Table 8 and depicted that bromelain generated hydrolysates showed the highest DPPH radical scavenging activity followed by flavourzyme generated hydrolysates. In addition, it was observed that there was a positive correlation between time of hydrolysis and DPPH radical scavenging, which is similar to the results obtained for ABTS radical scavenging activity. Although intact protein of MS was found to be a better scavenger of DPPH radicals, hydrolysates from YS protein possessed a better DPPH radical scavenging compared to their intact protein indicating that they may possess peptide sequences with potent DPPH radical scavenging activities. The inherent radical scavenging capacity of intact proteins from YS and MS is related with the capacity of their terminal amino acids residues like Phe, Trp, His, Tyr, and Cys, for their capacity to donate electron to DPPH radical and is
also reported well in literature that both hydrolyzed and unhydrolyzed proteins have the capacity to scavenge DPPH radicals (Kumar et al., 2016).

Overall, all three enzymes used in generating YS and MS protein hydrolysates in this study were able to generate peptides with potent DPPH radical scavenging activities. The obtained results were found to be similar to Zhang et al. (2010) where soy protein hydrolysates produced by validase and neutral protease were found to be a significantly more potent scavengers of DPPH radicals than those produced by alkaline protease. Similar trend was also observed by Zheng et al. (2019) in black bean (Phaseolus vulgaris L.) protein hydrolysates prepared with bromelain and alcalase which exhibited significantly enhanced DPPH scavenging activity compared to hydrolysates produced by ficin. The obtained DPPH-TEAC values are also similar to those reported for alcalase derived soy sauce protein hydrolysates (Yang et al., 2011). Furthermore, fermented soy protein meal hydrolysates obtained after fermentation with lactobacillus plantarum LP6 also reported to possess enhanced DPPH radical scavenging effects and are attributed to the release of peptides upon hydrolysis of proteins upon degradation by microbial proteases (Amadou et al., 2011). The positive correlation has been observed between DH values and DPPH radical scavenging activity that is similar to those observed for alcalase generated okara protein hydrolysates where a progressive increase in DH% also corresponded to increased DPPH radical scavenging (Sbroggio et al., 2016). Similarly, Jamdar et al. (2010) also reported that as the DH increase, the DPPH radical scavenging activity increased for peanut protein hydrolysates. Overall, the results suggest that plant proteins especially YS proteins could be used as a significant source of antioxidant peptides.
4.3.5 Anti-inflammatory (AI) activities

Inflammation is considered as one of the prime risk factors for the development of various ailments and chronic diseases such as obesity, diabetes, and cancer progressions. Generally, infected cells produce various pro-inflammatory cytokines that lead to DNA damage and promote or initiate tumor (Kamal et al., 2018). Few reports suggest that peptides derived from food products can control these inflammatory events by the protection of protein against inflammation induced by protein and DNA damage (Iskandar et al., 2013). These reports prompted us to investigate the AI properties of soybean-derived protein hydrolysates.

As indicated in the results section, all YS and MS protein hydrolysates displayed significant AI properties through albumin anti-denaturation effect. In addition, it was noticed that MS hydrolysates produced by alcalase and bromelain and YS hydrolysate produced by bromelain have displayed an increase in AI activity at 2 and 4 h of hydrolysis and then declined significantly at 6 h. A similar observation was recorded by Mudgil et al. (2019a) where alcalase and papain-generated camel milk protein hydrolysates showed an increase in AI activities from 3 to 6 h before showing a decline at 9 h of hydrolysis. The phenomenon was attributed to the high DH and degradation of proteins as a result of papain and alcalase which might have eventually degraded the potent AI peptides (Mudgil et al., 2019a).

Although not direct AI studies have been reported previously but few related studies have shown inhibition of pro-inflammatory mediators by soybean-derived protein hydrolysates (Martinez-Villaluenga et al., 2009; Vernaza et al., 2012). Martinez-Villaluenga et al. (2009) studied the AI effect of soybean genotypes enriched in β-conglycinin protein hydrolysates produced by alcalase on LPS-induced macrophages RAW 264.7 cell line. It was reported that 5 µM of the soy protein
hydrolysates have reduced the production of NO (18–35%) and PGE\(_2\) (47–71%), and also reduced the expression of inducible nitric oxide synthase (iNOS) (31–53%) and cyclooxygenase-2 (COX-2) (30–52%). In addition, Vernaza et al. (2012) reported that the alcalase hydrolysis of the Brazilian soybean protein have shown an AI activity by inhibiting the inflammatory markers such as NO, PGE\(_2\), COX-2, iNOS, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) in LPS-induced RAW 264.7 (cell line) macrophages. Since the direct evidence related to AI activities of soybean protein hydrolysates are scarce, this study highlights the AI potential of soybean protein hydrolysates that could be generated at the commercial level through enzymatic hydrolysis.
Chapter 5: Conclusion

Soybean has been known for the potential health benefits and for its high protein content that could be considered as an alternative to animal proteins. Soybean (yellow dried seed) bioactive compounds such as protein hydrolysates, peptides, and phenolic compounds were explored and reported previously in the literature for their different biological activities. However, the data regarding the biological activities of YS bioactive compounds and precisely their protein hydrolysates are scarce. Therefore, this research project aimed to generate protein hydrolysates from YS and MS proteins via enzymatic hydrolysis in-vitro with different enzymes (alcalase, bromelain, and flavourzyme). Moreover, the effect of simulated gastric and intestinal digestion (SGID) of YS and MS flours on their potential bioactive properties was investigated.

Both YS and MS flours upon in-vitro SGID and their protein hydrolysates generated via enzymatic hydrolysis exerted different biological activities. Higher antioxidant and AI activities were demonstrated by soybean flours as well as protein hydrolysates upon SGID and enzymatic hydrolysis, respectively. The concentration of the TPC of YS increased significantly after the simulated gastric digestion and the SGID. In addition, it was evident that YS and MS flours have exhibited a variation in the inhibitory activity against the diabetes and obesity related enzymatic marker (AA, DPP-IV, CE, and LIP) upon simulated gastric digestion followed by a decrease in inhibitory activity when subjected to simulated intestinal digestion (P<0.05). While for antioxidant activities, MS flour showed a higher FRAP, ABTS radical scavenging activity and AI activity compared to YS flour.
Protein hydrolysates generated from YS and MS also displayed higher inhibitory activity against AA, DPP-IV, CE, and LIP enzymes compared to their intact proteins, and the IC$_{50}$ values of YS and MS hydrolysates varied. The inhibitory activity of LIP and DPP-IV enzymes were significantly higher by YS hydrolysates compared to MS hydrolysates, while the opposite was evident for MS hydrolysates in inhibiting CE enzyme. The DH of MS hydrolysates is higher than YS hydrolysates. Similarly, the antioxidant properties such as FRAP, ABTS, and DPPH radical scavenging activities as well as AI activities of YS and MS hydrolysates were enhanced upon enzymatic hydrolysis (P<0.05). YS hydrolysates were higher in ABTS radical scavenging activity and AI activity than MS hydrolysates. Furthermore, MS hydrolysates displayed higher FRAP, as well as the hydrolysates produced by flavourzyme and bromelain have also displayed higher DPPH radical scavenging activity than YS hydrolysates. Overall, the present study showed that the YS protein hydrolysates could be an important source of bioactive peptides with multifunctional bioactive properties and could be utilized as a potential functional ingredient for food fortification. While MS has been extensively studied for the production of protein hydrolysates and their application as functional food ingredients have also been explored. Further studies on YS, and its compositional analysis and bioactive properties should be investigated in detail using different model systems. Furthermore, isolation, characterization, fractionation, and identification of the potential bioactive peptides in the hydrolysates specifically for the YS protein hydrolysates should be carried out. For validation, \textit{in-vivo} experiments on animal models are also essential to confirm the findings of this research.
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