# QUALITY AND ESTIMATED GLYCEMIC INDEX OF BREAD FORTIFIED WITH SOY PROTEIN ISOLATE

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**Title**

Quality and Estimated Glycemic Index of Bread Fortified with Soy Protein Isolate

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**MASTER OF SCIENCE**

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## ABSTRACT

More than 25 and 79 million individuals in the U.S. have diabetes and pre-diabetes, respectively. Many individuals are required to monitor blood glucose levels. Glycemic index (GI), a measurement of how a food affects the blood glucose level, is a method for selecting foods by diabetic populations. The effect of soy protein isolate (SPI) on the glycemic response of bread was determined, including the quality characteristics of bread made with various SPI percentages.

Lower loaf volumes, firmer texture and open grain structure were observed in breads as the level of SPI increased. Estimated GI (eGI) significantly decreased ($p<0.05$) with increased SPI levels and also a significant correlation ($p<0.05$) was found between these indices and SPI concentrations. This indicates that fortification of SPI in the bread can reduce eGI and possibly provide a health benefit to the diabetic population. This experiment provided useful insights into SPI effect on eGI and bread quality characteristics.

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## LIST OF ABBREVIATIONS

ANOVA ..... Analysis of Variance

AUC ..... Area Under Curve

CVD ..... Cardiovascular Disease

CRD ..... Completely Randomized Design

eGI ..... Estimated Glycemic Index

FDA ..... Food and Drug Administration

GI ..... Glycemic Index

HDL ..... High-Density Lipoprotein Cholesterol

LDL ..... Low-Density Lipoprotein Cholesterol

LSD ..... Least Significant Difference

PDCAAS ..... Protein Digestibility-Corrected Amino Acid Score

PDI ..... Protein Digestibility Index

pI point ..... Isoelectric point

RACC ..... Reference Amount Customarily Consumed

RCBD ..... Randomized Complete Block Design

RDS ..... Rapidly Digestible Starch

RS ..... Resistant Starch

RVA ..... Rapid Visco Analyzer

SDS ..... Slowly Digestible Starch

SPI ..... Soy Protein Isolate

TAG ..... Triacylglycerol

TS ..... Total Starch

# 1. INTRODUCTION

Tendency toward healthful eating and food selection have increased rapidly in the past few years. An increasing number of reports on functional foods and increased nutritional value of conventional food products illustrate the trend toward health promotion by the industry. In the U.S., however, lifestyle and eating habits have led to increased cardiovascular disease (CVD) and diabetes cases. The Center for Disease Control and Prevention (CDC) estimates that 25.6 million and 79 million individuals in the U.S. above the age of 20 have diabetes and pre-diabetes, respectively, with 1.9 million being diagnosed in 2010 (Anonymous, 2011).

Diabetes is a health condition characterized by the inability to make insulin (type 1 diabetes) or to utilize insulin properly (type 2 diabetes), resulting in a sudden spike of blood glucose upon consumption of food containing high carbohydrate levels (Anonymous, 2012). For such populations, measuring glycemic index (GI) is a useful way of managing the blood glucose level since GI indicates how the carbohydrates are digested. Furthermore, GI is calculated relative to white bread or glucose as the reference food with a value of 100 (Anonymous, 2012). The overall scale ranges from 0 to 100 where lower numbers indicate low GI. Low, medium and high GI are defined as GI of 55 or less, 56 – 69, and 70 or more, respectively (Anonymous, 2014). Examples of low GI food includes oatmeal, pasta and most fruits, medium GI includes whole wheat and brown rice and high GI includes white bread, potato and white rice. Since the time Jenkins et al. (1988) introduced the concept of GI, the relationship between GI and food, especially starch and glucose, has been studied (Wolver et al., 1991; Mann et al., 2004; Gellar and Nansel, 2009). However, multiple studies revealed that the effect of food on blood glucose is affected by many additional factors rather than carbohydrate or glucose alone *per se* (Jenkins et al., 1987; Goni et al., 1997).

Benefits of soy protein as a functional food have been widely studied, especially with regard to decreasing the risk of CVD. The Food and Drug Administration (FDA) approved a health claim in 1999 permitting the use of a statement on the food label that soy protein can help reduce the risk of coronary heart disease (FDA, 2012). The effect of soy protein on diabetes and obesity in animal studies recently has been reported, but few reports have been published using human subjects (Cederroth and Nef, 2009). Human studies are time-consuming and expensive, but they remain the acknowledged method for establishing how a particular food affects blood glucose level (Cederroth and Nef, 2009). In order to make the process easier and more convenient, a method to measure the estimated GI (eGI) was developed and the correlation between eGI and GI from clinical study was proven to be valid (Goni et al., 1997). However, no publications were found regarding the effects of soy protein on the eGI at various levels and how it affects the ability to reduce GI *in vitro*. In addition, the relationship between various levels of soy protein isolate (SPI) fortification in bread and its effect on quality characteristics has not been reported. Therefore, this study was conducted to determine the eGI of bread fortified with various levels of SPI and determine the effect of SPI on the end product quality characteristics.

## 2. HYPOTHESIS

Bread made with SPI is expected to have lower eGI than the bread made without SPI as an effect of increased protein content. Dilution of starch due to addition of protein, can account for decreased eGI since fewer carbohydrates will be present in finished product. Baking is expected to alter the composition of starch type, including rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), which should contribute to the lowered eGI value. The alternative hypothesis is that SPI will have no effect on eGI on bread quality.

## 3. OBJECTIVE

The objective of the study was to measure the expected glycemic index (eGI) of bread fortified with various levels of soy protein. In addition, the quality characteristics of bread were measured and the change in starch composition was determined.

# 4. LITERATURE REVIEW

## 4.1. Glycemic index (GI)

### 4.1.1. General concept of glycemic index (GI)

The concept of GI was initially developed as a need to quantify the glycemic response to different types of carbohydrates (Frost and Dornhorst, 2013). GI indicates the potential of carbohydrate-containing foods to raise the blood glucose level (Colombani, 2004). The scale of 0 to 100 is used where 100 is represented by white bread or glucose. Generally, GI of a food is calculated as the area under the blood glucose response curve (AUC) relative to the blood glucose response curve of white bread or glucose (Colombani, 2004). Therefore, the GI is only a relative comparison of the defined amount of carbohydrate called "available carbohydrate" (Colombani, 2004). Moreover, white bread is used as a reference; however, the validity of using bread has been questioned since the composition of white bread varies across the globe.

### 4.1.2. Factors affecting glycemic index (GI)

In general, there are a few factors that affect the GI of food including ripeness and storage time, processing and processing method, cooking method and variety of carbohydrate (Anonymous, 2014). For example, the more ripe fruits have the higher GI than not ripe fruits. The more processing or longer cooking the food go through, the higher the GI will be compared to less processed or shorter cooked food. Combining effect with the other food should be considered when planning a meal for diabetes. Other factors affecting diabetes are blood glucose, cholesterol, TAG levels, blood pressure and weight management and effects of food on these should be considered when making the meal plan for diabetes (Anonymous, 2014). Combination of carbohydrate counting and GI would be more effective than GI *per se*, as they are the better predictor of blood glucose response.

#### 4.1.3. Relationship between glycemic index (GI) and diabetes

The relationship between GI and diabetes is the most studied relationship, with the most evidence being from clinical studies. The blood glucose lowering effect of low GI foods has been documented for individuals with type 1 and 2 diabetes (Frost and Dornhorst, 2013). Furthermore, low GI food was found to be an effective control measure in youth with type 2 diabetes (Gellar and Nansel, 2009), which is a significant finding since increasing numbers of youth with type 2 diabetes are being diagnosed. Low GI food consumption also improved lipid and fibrinolytic profiles, i.e. the breakdown of blood clots (Frost and Dornhorst, 2013).

#### 4.1.4. Effects of glycemic index (GI) on cardiovascular disease (CVD)

Heart disease or CVD, is a leading cause of death in the world and hyperinsulinemia, caused by high GI food consumption, is a risk factor of the disease (Lukaczer et al., 2006). The intakes of food with high GI have been shown to increase CVD in women by 13 %, especially among overweight individuals (Ma et al., 2012). A strong association between GI and cholesterol, including low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) ratio, and triacylglycerol (TAG) has been documented and thus consumption of low GI foods could be a preventive measure for CVD (Ma et al., 2012).

Furthermore, SPI has been proven to reduce CVD risk by lowering the risk factors especially total cholesterol, LDL and TAG (Lukaczer et al., 2006). Lukaczer et al. (2006) suggested that SPI enhances the effects of a low GI diet by increasing HDL and decreasing TAG. The change in lipid profile by SPI is likely due to reduced hepatic cholesterol synthesis and increased LDL receptor production (Lukaczer et al., 2006). The combined effects of low GI food and SPI are beneficial, but the addition of SPI in foods as a means to reduce GI has not been documented.

### 4.1.5. Glycemic index (GI) determination methods

#### 4.1.5.1. *In vivo* method

*In vivo* GI measurement uses human subjects and GI is calculated by the blood glucose level from the actual blood sample drawn over a period of time. Therefore, selection of human subjects is one of the most important aspects of the test and will affect the test result. Subjects should include an equal number of men and women with body mass index (BMI) and blood glucose level in a normal range (Granfeldt et al., 1992). The subjects are required to fast before the test to prevent possible interference with test results, and all food and drink intakes are controlled during the test period. After collection of blood samples, blood glucose level is determined using glucose-oxidase peroxidase (GOPOD) assay; however, there are instruments on the market that provide instantaneous blood glucose level that are being used by diabetes patients and researchers (Granfeldt et al., 1992).

Variation of techniques and methodology used to measure GI greatly impacts results. Therefore, numerous studies were conducted to develop a reliable GI method. Jenkins et al. (1987) have investigated criticisms regarding the GI methodology. Differences in results caused by variable conditions of human subjects and mixed meal effects are the most criticized parameters (Jenkins et al., 1987; Wolver et al., 1991). Jenkins et al. (1988) indicated that the variations of results of early studies were related to different methodologies and suggested a standard method for GI analysis. For example, fasting blood glucose levels were not accounted for when absolute postprandial glucose was measured. This typically resulted in variable results and led to the recommendation that incremental (i.e. over time) glycemic response beused for GI (Jenkins et al., 1998). The validation process, based on the data analysis from numerous studies, increased the understanding and acceptance of the GI test.

4.1.5.2. *In vitro* method

A growing need to reduce the cost and time requirement and more efficiently measure GI has resulted in an alternative method for measuring the GI. This method is referred to as estimated GI (eGI) (Goni et al., 1997). This method involves imitating the *in vivo* starch digestion in test tubes (i.e. *in vitro*) using enzymes. Since starch hydrolysis method of eGI analysis is relatively new, experimental procedure to determine, validate and improve eGI is needed.

Validation of the methodology was the greatest obstacle regarding the use of this new method. Jenkins et al. (1987) first proposed that rate of starch digestion using human saliva and postprandial jejunal juice, discovering the glucose released *in vitro* had the similar response as the human subjects and thus the *in vitro* measurement of carbohydrate digestion could be a useful supplement to clinical study. The methodology was further developed to include commercially available enzymes (Snow and O'Dea, 1981). Factors that affect the rate of starch hydrolysis were investigated as means to develop an efficient protocol (Snow and O'Dea, 1981). Factors such as additional ingredients, physical form and processing methods were identified as affecting the rate of hydrolysis, hence the data analysis on multiple studies were used to identify the direct influence on these factors (Snow and O'Dea, 1981). Furthermore, correlations between *in vivo* and *in vitro* were investigated and good correlations were found in most studies (Holm et al., 1985; Bornet et al., 1989; Englyst et al., 1992). Even though modifications of the method and the investigation of factors that affect the rate of *in vitro* starch hydrolysis still continue today, the two most accepted *in vitro* methods are Englyst method and Goni method (Englyst et al., 1992; Goni et al., 1997).

4.1.5.3. Englyst method

Englyst et al. (1992) developed a method using small beads and guar gum to measure rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). Considering the fact that the actual chewing process would not break down the food structure as fine as milling, food samples were not milled at the sample preparation step, instead it was run through a mincer with a plate of 0.9 cm diameter holes (Englyst et al., 1992). However, the authors found that the particle size reduction was taking place after swallowing thus reducing particle size before hydrolysis by mincing the sample could falsely elevate the amount of RDS. This is important because the rate and extent of starch hydrolysis determine the eGI and these factors vary depending on the way a food sample is broken down (Englyst et al., 1992). In contrast, the authors reported that the glass balls with guar gum effectively facilitated the degradation of large particles thus increasing starch-enzyme contact in most types of food except legumes, in which the cell walls are relatively stronger than most food starches (Englyst et al., 1992).

Hoover and Zhou (2003) reported scanning electron microscope (SEM) images that showed little structural change in the native starch, which suggests that this starch is less susceptible to enzyme attack. SEM can measure the change of granular structure caused by the enzyme attack. The surface of degraded starch, due to enzyme attack, exhibits changes such as deformation and cracks (Hoover and Zhou, 2003). The degree of hydrolysis by enzyme differs depending on the factors such as state and composition of starch. Therefore, analysis using SEM could provide the supporting evidence on factors affecting the rate of hydrolysis.

4.1.5.4. Goni method

Goni et al. (1997) established the eGI using *in vitro* enzymatic starch hydrolysis and starch digestion rate over time. The method included treating the sample with pepsin to digest protein, α-amylase to hydrolyze starch and then amyloglycosidase to release glucose from starch (Goni et al., 1997). Results from eGI, GI from *in vivo* study and the reference GI were compared and a correlation of 0.894 was found between reference GI and eGI (Goni et al., 1997). The researchers found that legumes were digested at the slowest rate of all the food products measured. This could be due to dietary fiber and protein content, but the authors did not provide a valid explanation for the low GI of legumes (Goni et al., 1997).

**4.2. Soy protein isolate (SPI)**

SPI is prepared from dehulled soybean seeds after removal of most of the oil and water-soluble non-protein constituents and results in no less than 90 % protein (Berk, 1992). SPI has been acknowledged for its nutritional benefits as well as functionality for decades and multiple studies have been conducted regarding the quality characteristics of soy-fortified bread. SPI fortification in wheat-based products are common in the baking industry. Since soy protein has a low methionine content but is rich in lysine, and wheat flour is low in lysine, but is high in cysteine, which is a sulfur-containing amino acid that can be interconverted to methionine through metabolic process of human body, a complete protein is created when combined (Stauffer, 2005). Due to its required purity, which is more than 90%, the cost of SPI is usually five to seven times higher than the defatted soy flour (Berk, 1992). Therefore, bakers tend not to use SPI but instead use soy flour (Stauffer, 2005).

#### 4.2.1. Extraction method

Wet processing of SPI is the most common SPI extraction method (Figure 1). Protein from defatted soy flour or flakes is solubilized and separated from the rest of solid residue in alkali water, followed by precipitation from the solution at the isoelectric (pI) point and drying (Figure 1). Products obtained from precipitation at their pI point are called isoelectric soy protein. Neutralization of the precipitate with different bases, including calcium, sodium, potassium and ammonium, results in several types of SPIs (Berk, 1992). These products differ in their solubility as isoelectric soy protein and calcium proteinates have lower solubility than sodium, potassium and ammonium proteinates (Berk, 1992).

The different properties and functionalities of SPI come from the different bases used to extract protein. Different solution, such as water or alkali solution, or different drying methods alter the functionality of SPI (Chen and Rasper, 1982a). Water absorption and water retention capacity are higher with alkali-extracted SPI than extraction with water (Chen and Rasper, 1982a). High heat treatment during drying was found to decrease the reactive disulfide (SS) and sulfhydryl (SH) group, which affect the reaction with gluten in baking (Chen and Rasper, 1982a). Therefore, selection of the appropriate SPI is necessary for specific applications.

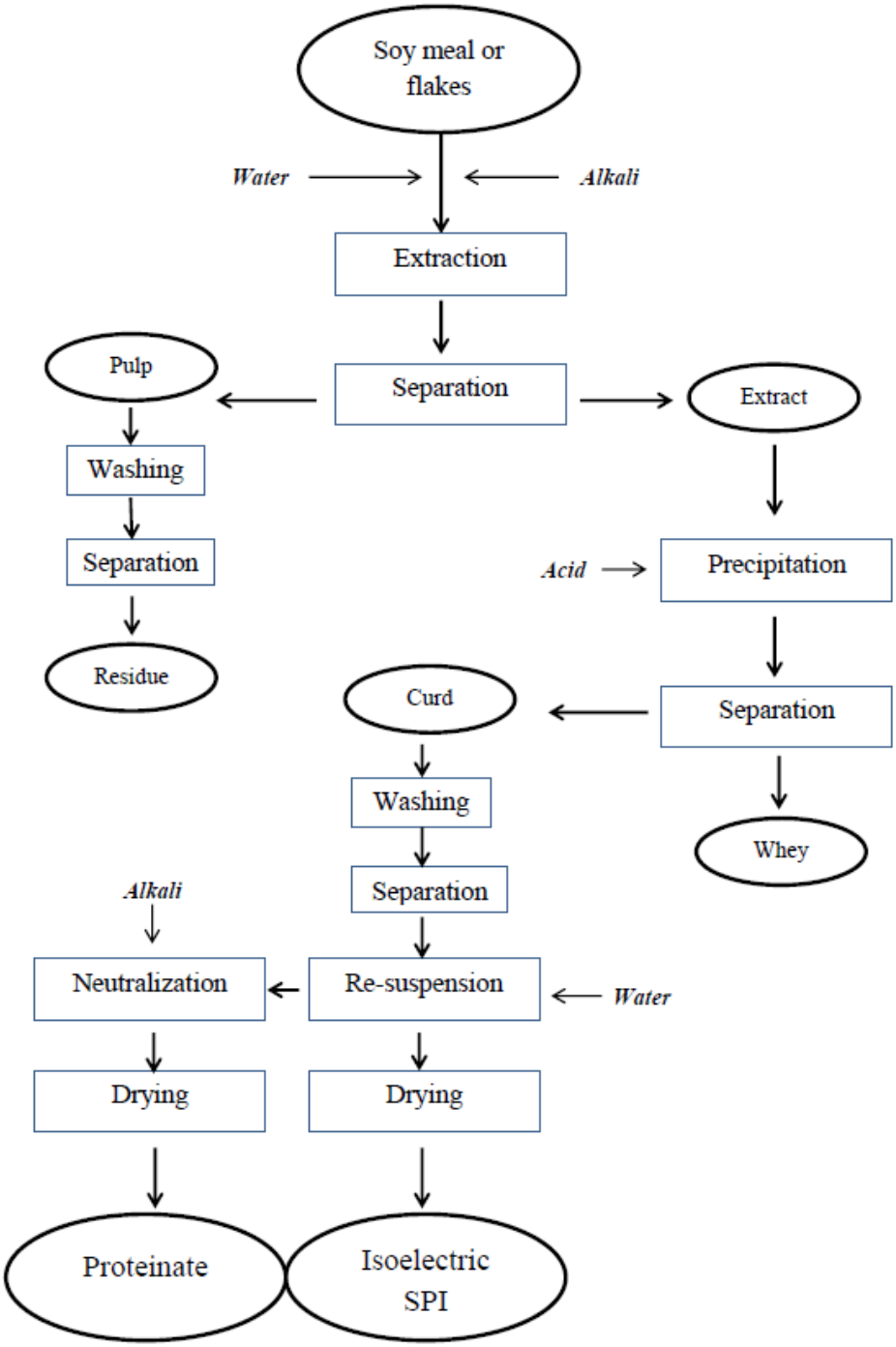


Figure 1. Schematic of soy protein isolate (SPI) extraction process (Adopted from Berk, 1992)

4.2.2. Advancements in soy protein isolate (SPI) production

Several technological developments became available in the process of SPI production in order to meet the unique functionality and nutritional requirements of the food industry. Modifications of pH, heat treatment and homogenization are the most important factors in altering the functionality of SPI (Egbert, 2004). A jet cooker, which uses the heat with shear caused by sonic velocity steam, enables the rapid elevation of temperature using steam, which improves gelling properties. In addition, flash cooling under vacuum is used for removing odor (Egbert, 2004). Some manufacturers use alcohol for washing the protein curd for the purpose of removing beany flavor. Use of alcohol in SPI was found to denature the proteins and greatly reduce the solubility (Egbert, 2004). Modification of pH at the precipitation process followed by heating of the slurry to around 110 – 140 $^{o}$C was found to restore the solubility (Egbert, 2004). Also, heating the precipitate at a lower temperature around 55 – 58 $^{o}$C was found to improve the color of SPI by making it lighter in color (Egbert, 2004).

4.2.3. Composition

Typical composition of SPI includes a protein content of 90 %, with 4 % fat and 6 % ash. The protein profiles in SPI vary depending on the soybean varieties, but in general soy protein consists of 80 to 90 % globulin and glycinin and the rest is the minor fraction of albumin and glutenin (Pyler and Gorton, 2010). SPI is the only complete protein of plant origin, due to the presence of nine essential amino acids (Table 1) (Hughes et al., 2011). Based on the protein digestibility-corrected amino acid score (PDCAAS), soy protein is complementary to protein from animal sources including milk, eggs and meat (Hughes et al., 2011).

Table 1. Amino acid profile (g) of soybeans, soy flour and different brands of soy protein isolate (SPI)

| Amino Acid profile | Raw soybeans [1] | Defatted soy flour [1] | SPI [1] | ProFam 781 [2] | GNC [2] |
|---|---|---|---|---|---|
| Alanine | 1.92 | 2.22 | 3.59 | 4.10 | 3.20 |
| Arginine | 3.15 | 3.65 | 6.67 | 7.70 | 6.37 |
| Asparatate | 5.11 | 5.91 | 10.20 | 11.50 | 9.77 |
| *Cysteine | 0.66 | 0.76 | 1.05 | 1.30 | 1.10 |
| Glutamine | 7.87 | 9.11 | 17.45 | 19.20 | 16.63 |
| Glycine | 1.88 | 2.17 | 3.60 | 4.10 | 3.43 |
| Histidine | 1.10 | 1.27 | 2.30 | 2.50 | 2.07 |
| *Isoleucine | 1.97 | 2.28 | 4.25 | 4.80 | 3.90 |
| *Leucine | 3.31 | 3.83 | 6.78 | 7.80 | 6.47 |
| *Lysine | 2.71 | 3.13 | 5.33 | 6.30 | 5.27 |
| *Methionine | 0.55 | 0.63 | 1.13 | 1.30 | 1.03 |
| *Phenlalanine | 2.12 | 2.45 | 4.59 | 5.00 | 4.37 |
| Proline | 2.38 | 2.75 | 4.96 | 5.20 | 4.37 |
| Serine | 2.36 | 2.73 | 4.59 | 5.00 | 4.37 |
| *Threonine | 1.77 | 2.04 | 3.14 | 3.70 | 3.03 |
| *Tryptophan | 0.59 | 0.68 | 1.12 | 1.60 | 0.97 |
| Tyrosine | 1.54 | 1.78 | 3.22 | 3.70 | 3.20 |
| *Valine | 2.03 | 2.35 | 4.10 | 4.80 | 3.80 |
| **Total** | **43.00** | **49.73** | **88.08** | **99.60** | **83.33** |

* Essential Amino Acid

[1] Adopted by USDA ARS National Agricultural Library Nutrient Data Laboratory

[2] Obtained from manufacturers

#### 4.2.4. Health benefits

The health benefit of soy protein is well acknowledged as the Food and Drug Administration (FDA) approved a health claim in 1999 permitting the use of a statement on food labels that soy protein can help reduce the risk of coronary heart disease (FDA, 2012). In order to use the claim, the product must meet the daily intake level of soy protein, which is 25 g per day or four daily servings of 6.25 g in addition to a low total fat intake (FDA, 2012). Furthermore,

the food must be low in cholesterol and saturated fat. Upon meeting the requirement, the following statement may be used on product packages; "25g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease, a serving of (name of food) provides (X) gram of soy protein" (FDA, 2012). The effect of lowering risk of heart disease is due to the effects of soy protein on lowering blood cholesterol levels, LDL and serum TAG, which are the preventative factors of CVD (Stauffer, 2005).

Isoflavones were found to be the main cause for the serum cholesterol-lowering effect and related health effects (Slack et al., 2006). Isoflavone in soybeans are well-known but the study revealed that isoflavones are not removed during the processing; thus, soy protein contains high amounts of isoflavones (Slack et al., 2006).

#### 4.2.5. Functionality

The most important functionalities of SPI include solubility, gelation, emulsification, viscosity, water binding and dispersibility (Egbert, 2004). Good solubility and dispersibility are especially important in beverage and food applications that are high in fat or contain diary. Good gelation, viscosity and water binding capacity are commonly seen in products that require textural contribution such as meat and dairy applications. In addition, water retention capacity, which functions by decreasing the fat absorption, is an ideal functionality in baking systems. The reason for this is that increased water absorption in the dough system prevents oil absorption, thus reducing oil uptake in fried foods such as donuts and batters (Stauffer, 2005).

##### 4.2.5.1. Gelation

Gelation of protein is caused by the formation of partially associated polypeptides and a three-dimensional network leading to increasing protein-protein interaction, which entraps water and causes structural rigidity (Egbert, 2004). Denaturation of protein is necessary for the gelation

to occur (Hermansson, 1986). Two categories of gels include those formed by random aggregation and those formed by more ordered association of molecules into strands (Hermansson, 1986). Structural order is an important factor to determine the functionality, and micro structure of gel is determined by the temperature that the gel was formed. For example, a heating temperature of 95 $^{o}$C creates coarser gel structure than heating at 75 $^{o}$C and the water holding capacity of such gel becomes poorer (Hermansson, 1986). In addition, pH and presence of salt greatly affect the gel properties due to pI point (Hermansson, 1986). Gelation happens only when the concentration of SPI is higher than 8 % (Sorgentini et al., 1995).

4.2.5.2. Water retention

Gel properties and water retention capacity of protein are related to each other since gelling of protein provides networks that absorb and hold water (Sorgentini et al., 1995). Increased aggregation of protein due to increased temperature or denaturation leads to reduction of water holding capacity. Two types of water held in the systems are bound water and retained water (Egbert, 2004). Bound water is not available as a solvent and depends on physiochemical properties of protein whereas retained water is trapped within the protein matrix. Bound water is physically held by hydrophilic interactions and is therefore difficult to remove. In contrast, trapped water retention is dictated by the structural integrity of that protein matrix. The greater the structural integrity, the greatly is the water retention (Egbert, 2004).

4.2.5.3. Emulsification

SPI functions as an emulsifier by reducing interfacial tension between two phases or by forming a physical barrier at the interface (Molina et al., 2001). Emulsifying activity and emulsifying stability are the two main parameters that affect the capability to improve an emulsion. Emulsifying properties vary depending on protein structure as surface hydrophobicity

and solubility influence the emulsifying activity whereas molecular flexibility affects the stability of emulsion (Molina et al., 2001). Generally, the higher the hydrophobicity and solubility, the better the emulsifying properties become (Petruccelli and Anon, 1994). Unfolding the protein alters the protein structure so that hydrophobic part of protein, which usually are imbedded into the inner structure, becomes exposed and causes increased hydrophobicity (Chen et al., 2011). This favors the interaction between oil phase and protein; therefore, enhances emulsification (Petruccelli and Anon, 1994). Heat and enzyme treatments are methods utilized to alter the emulsifying property of SPI. Change in emulsifying property affects emulsifying capacity and stability and it is measured by continuous addition of oil to a protein slurry (Egbert, 2004).

4.2.5.4. Solubility

Solubility of SPI is measured by determining the amount of protein that remains in suspension upon centrifugation (Egbert, 2004). Protein solubility largely depends on pH. SPI generally has the lowest solubility around their pI point, i.e. pH 4.5 (Egbert, 2004). Solubility is related to emulsifying property, water holding capacity and gelation as each functionality is interrelated to the structure of the protein. Heat treatment decreases the solubility because of denaturation and structural change caused by heat. Aggregation of protein caused by structural changes decreases solubility since aggregates are insoluble (Martins and Netto, 2006).

## 4.3. Bread system

### 4.3.1. Bread

Bread is one of the most popular and important sources of carbohydrate in the world. Food companies are trying to increase the nutritional value of bread, while maintaining the familiar appearance, by using white whole wheat or adding additives such as fiber and protein.

Soy protein has become a popular choice for bakers due to its functionality, availability and cost (Stauffer, 2005).

#### 4.3.2. Bread composition

Characteristics of bread are largely affected by ingredients and composition. The basic ingredients of bread are starch, protein, yeast and salt. Commercially available products contain additional ingredients to improve the sensory characteristics and shelf life. However, composition of food, especially starch, dietary fiber and protein, largely affects the rate of starch hydrolysis, which is one of the reasons why soy protein may alter eGI. Characteristics of key components of bread are discussed in more details in section 4.4-4.7.

#### 4.3.3. Physical interactions in bread

Physical reactions that occur in the dough system during mixing have a tremendous impact on bread characteristics. Dough becomes more resistant to force due to the formation of hydrogen bonds between glutenin and gliadin, resulting in formation of gluten with the presence of water (Visschers and de Jongh, 2005). As mixing continues, disulfide bonds (SS) between proteins are formed, resulting in protein polymers that have gas-retaining properties (Visscers and de Jongh, 2005). Disulfide bonds formed between polypeptide chains help stabilize the dough by immobilizing parts of protein chains as well as facilitating hydrogen bonds and hydrophobic interactions (Maforimbo et al., 2008). As mixing continues, dough is weakened by disrupted SS bonds as a result of continuous SS and sulfhydryl group (SH) interchange due to incorporation of oxygen; thus, increasing SH concentration and a breakdown of gluten occurs (Roccia et al., 2009).

### 4.4. SPI fortification in bread systems

#### 4.4.1. Rheological properties of bread

The quality characteristics of bread are affected upon addition of SPI in bread formulations. A negative correlation between SPI-fortification and imparted bread quality has been reported (Urade, 2011; Ribotta et al., 2005). SPI fortified bread was found to be firmer, drier, grainier, less tender and gummier than white bread (Urade, 2011). However, the variable effects depend on the SPI products and bread formulations. Therefore, additional changes in formulation beyond those published may create different effects on bread.

Changes in gluten network structure occur and dough system becomes diluted upon addition of SPI to bread formulations (Urade, 2011). As a result, change in Farinograph and Extensiograph profiles occurred, which includes reduction in the arrival time, stability and extensibility and increment of resistance (Urade, 2011). Ribotta et al. (2005) reported that addition of SPI affected the Farinograph development time and stability from 7.8 minutes to 5.8 minutes and 12.6 minutes to 8.5 minutes, respectively. SPI impacts gluten formation by tightly adhering to the gluten protein (Ryan and Brewer, 2007). SPI addition caused alteration in the viscoelastic characteristics of gluten system by reducing the availability of water in the dough system. SPI increases water absorption when added to a dough system, which results in increased water binding by reducing free water (Roccia et al., 2009). This reduces the lubrication function of free water in the gluten polymer; thus, affecting viscoelastic characteristics of dough (Urade, 2011).

Ribotta et al. (2005) reported that increasing the level of SPI from 3 % to 5 % changed the rheological properties by weakening the gluten and dough properties, which is caused by dilution of gluten. However, Extensiograph results indicated that SPI increased the dough

resistance to extension and reduced elasticity. The effect was enhanced at higher SPI levels (Roccia et al., 2009). The observed change in elasticity was due to increased water level. Higher SPI levels tend to increase water absorption capacity; thus, excess free water acts as a lubricant, making the dough more elastic but weaker (Roccia et al., 2009). In addition, increased level of SPI resulted in reduced loaf volume, which is related to weakened gluten properties (Ribotta et al., 2005).

Firmness of SPI-fortified bread was correlated with level of SPI fortification (Urade, 2011; Ribotta et al., 2005). Increased firmness was correlated to decreased volume of bread thus the firmness was not directly due to SPI but likely to be the result of SPI impact on gluten. Proper hydration of dough with additional water may solve the increased firmness and improve the texture since increased water level increased dough resistance but had no effect on extensibility (Urade, 2011).

#### 4.4.2. Loaf volume

Loaf volume of SPI-fortified bread was lower than the control bread (Ribotta et al., 2005). The SPI interruption of gluten likely caused lower volumes due to less gas retention capacity (Ribotta et al., 2005). In addition, there is an inverse relationship between loaf volume and crumb firmness and all of the effects increased as percentage of SPI fortification increased (Ribotta et al., 2005). Gluten yield also increased at higher SPI addition indicating that gluten network retains soy protein (Ribotta et al., 2005). The effect was further enhanced with unfolded soy protein; thus, the authors concluded that the state of protein, either as native or denatured, negatively affects the dough and end product properties (Ribotta et al., 2005).

Addition of SPI in bread alters the sensory characteristics. Beany flavor characteristic and bitterness, which are attributed to the phenolic compound present in SPI and soybeans, were

detected with SPI-fortified bread (Ivanovski et al., 2011). SPI-fortified bread crust color tends to be darker than the bread that does not contain SPI. The higher total amino acid and reducing sugar from SPI likely promoted Maillard reaction, and free sugars enhanced the caramelization reaction (Ivanovski et al., 2011). Also, SPI increases the firmness and stickiness of bread, which impart the good sensory characteristics compared to soy flour, leading to a conclusion that up to 20 % addition of SPI in the bread formulation is likely to prevent the negative effects of soy flour on the sensory characteristics of bread (Ivanovski et al., 2011).

## 4.5. Starch

### 4.5.1. Classification of starch

Carbohydrate can be largely classified into available and unavailable carbohydrate. Available carbohydrate can be digested and absorbed by the human body and represents starch and soluble sugars (Dewettinck et al., 2008). In contrast, unavailable carbohydrate cannot be digested and includes dietary fiber such as resistant starch, cellulose and soluble and insoluble fibers (Dewettinck et al., 2008).

Starch is categorized into three types based on the rate and extent of starch digestion. The categories based on types include rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst et al., 1992). RDS causes rapid increase in blood glucose due to conversion of starch to glucose, and it is usually measured within 20 minutes after consuming the food (Englyst et al., 1992). On the other hand, SDS tends to be digested slowly, where digestion lasts up to 120 minutes (Englyst et al., 1992). RS undergoes limited enzyme hydrolysis *in vitro* and thus it is considered to be beneficial for health since the RS can reach the large intestine without much structural disruption (Englyst et al., 1992).

RS can be classified into three groups, RS 1, 2 and 3, which are inaccessible to digestive enzyme, crystalline region of native starch granules and retrograded amylose, respectively (Hoover and Zhou, 2003). RS 3 is formed after heating due to the leaching of amylose, which upon cooling results in crystal formation and thus resists the enzymatic hydrolysis (Hoover and Zhou, 2003). Increased RS has been observed upon retrogradation of starch but the extent varies depending on property of starch, especially amylose content and processing steps and conditions (Ciliton et al., 2000). In general, the bread baking process does not substantially increase the amount of RS and generally contains about 3 % RS (Ciliton et al., 2000).

4.5.2. Starch properties

Cooking of starch causes structural changes and differences in functionality. General steps of starch cooking include gelatinization, swelling, pasting and retrogradation. The degree to which these steps occur depends on starch types and sources.

Gelatinization is the loss of molecular order within granules and is the result of heating starch granules in the presence of water (Whistler and Daniel, 2007). During baking, starch granules become disordered due to heating in presence of water (Dewettinck et al., 2008). Gelatinization temperature differs depending on the properties of starch including starch: water ratio, granule type, etc. (Whistler and Daniel, 2007). Higher temperature and longer processing time such as extrusion process increase the degree of gelatinization (Yong et al., 2011). Furthermore, an increased digestibility of starch has been reported in extruded snacks suggesting low levels of RS and SDS (Simon et al., 2012). Even though the bread processing does not employ as high a temperature as extrusion process, some degree of gelatinization does occur during the baking processing is expected to impact the starch digestibility.

Swelling of starch granules occurs when starch is heated in the presence of water under shear (Whistler and Daniel, 2007). Starch granules absorb water and, as a result of heating above gelatinization temperature, causes a rupture of the granules. The degree of swelling differs depending on the amylose/amylopectin ratio of starch, where amylopectin increases the swelling of starch granule due to hydrogen bonding with water while amylose is leached out of the granule.

Further heating of a starch slurry eventually cause the complete loss of starch granule integrity and produces a paste, which is the highly viscous starch mass (Whistler and Daniel, 2007). Viscosity of this starch mass continues to increase as heating and shear force continues until peak viscosity is reached. After peak viscosity is reached, the granule organization is completely lost and the viscosity decreases (Whistler and Daniel, 2007).

Retrogradation is a process whereby the starch polysaccharides become a more ordered state as starch cools, producing a rigid gel (Whistler and Daniel, 2007). This process occurs as soon as the bread comes out of oven and continues during the storage, increasing the crumb firmness called staling (Dewettinck et al., 2008). The structural change of starch significantly affects the digestibility of starch. Increased retrogradation was found as the percentage of protein increased in starch in both *in vitro* starch hydrolysis and rapid visco analyzer (RVA) results (Yong et al., 2011). This might be due to protein hindering the re-association of gelatinized starch molecules, lowering the starch digestion (Yong et al., 2011). Setback viscosity can be a direct measurement of retrogradation as it measures how much re-association of starch at the end of gel formation cycle (Figure 2) relative to the peak viscosity (Yong et al., 2011). Retrogradation is minimized in the solution where the starch is diluted due to the non-starch

substances hinders the re-association of molecules, consequently decreasing starch digestion and GI (Yong et al., 2011).

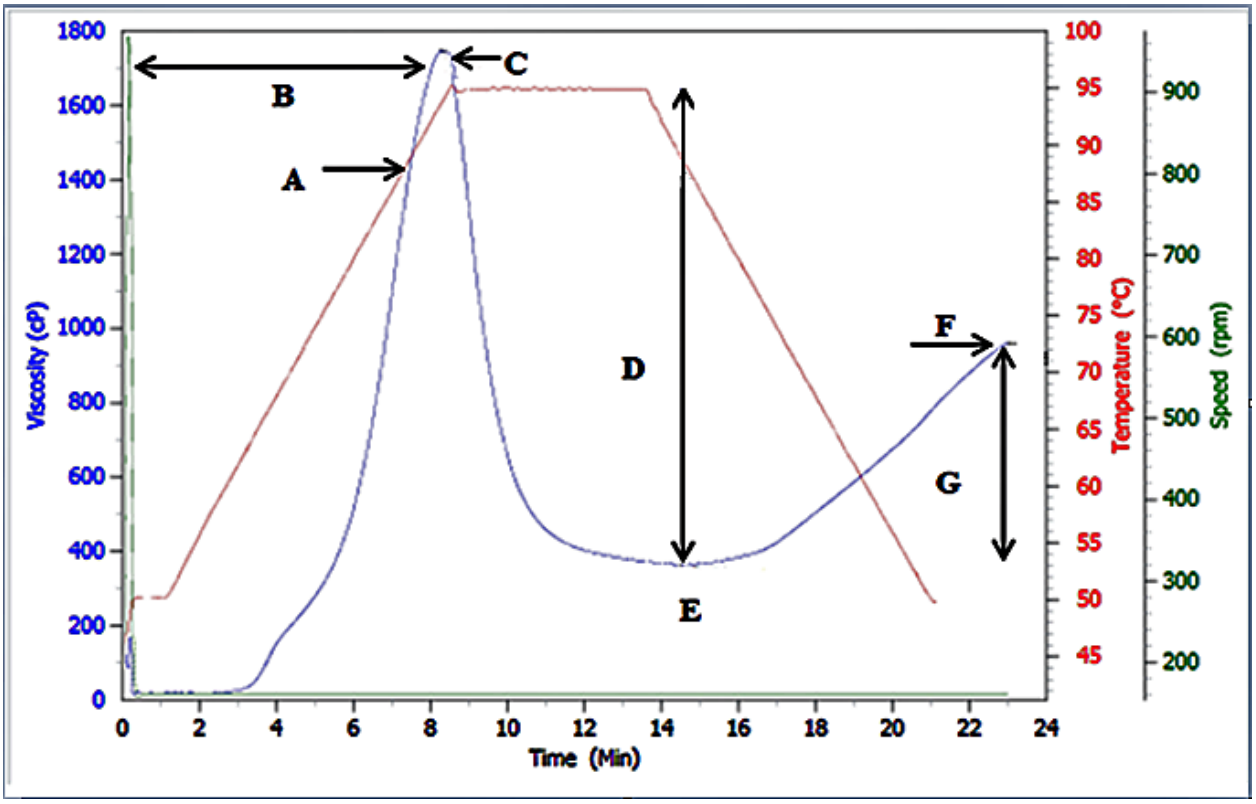


Figure 2. Rapid visco analyzer profile of spring wheat control flour;
A - Pasting temperature, B - Peak time, C - Peak viscosity, D - Breakdown, E - Holding strength, F - Final viscosity, G - Setback

4.5.3. Rapid visco analyzer (RVA)

RVA is useful to determine the gelatinization of starch. The typical RVA result provides peak viscosity, peak time, pasting temperature, holding strength, breakdown, final viscosity and setback (Figure 2). Peak viscosity is the maximum viscosity the starch reaches and peak time is the time it takes to get to peak viscosity (Anonymous, 2007). Pasting temperature is the temperature at which viscosity starts rising, and holding strength is the ability to withstand the continuous shear and heating at which viscosity of slurry is lowered (Anonymous, 2007).

Breakdown is the measure of viscosity reduction from peak viscosity. Setback is the difference between holding strength and final viscosity, indicating the ability of starch to retrograde (Anonymous, 2007).

## 4.6. Starch hydrolysis

### 4.6.1. Starch characteristics

Difference in starch properties causes difference in the rate and level of hydrolysis. One of the factors that cause major difference is amylose/amylopectin ratio. Hera et al. (2013) reported that the higher the amylose content the lower was the starch hydrolysis. Amylopectin has a larger molecular size, which makes it more susceptible to enzyme attack due to larger surface area (Singth et al., 2010). In addition, linear chains of amylose are more tightly bound to each other through hydrogen bonds than hydrogen-bonding between amylopectin, whose chains contain more branches (Singth et al., 2010). Therefore, increased amounts of amylose can increase the resistance to enzyme hydrolysis, favoring the slower rate of blood sugar release caused by decreased amounts of RDS (Hera et al., 2013).

### 4.6.2. Enzymes and enzyme inhibitors

Antinutrients such as phytic acid, tannin and polyphenol are naturally present in soybeans and other pulses and beans and negatively affect absorbance and digestion of nutrients (Lai et al., 2013). Phytates, tannins and polyphenol are also known as α-amylase inhibitors (Lai et al., 2013). These compounds decrease protein solubility and digestibility by interacting with protein by forming cross-linked proteins, which makes the protein less susceptible to proteolytic attack (Alonso et al., 2000). Extensive research has been conducted on the effect of phytic acid, the storage form of phosphorous in plants, on digestibility of protein (Lai et al., 2013). Extraction of SPI from soybean reduces the phytic acid content; however, the extraction condition does affect

the phytic acid content in the end product. Extraction solvent (water and potassium chloride) and pH affect the phytic acid content in the end product. Chemical solvent, such as potassium chloride, and pH between 9 and 10 decreased phytic acid in SPI (Lai et al., 2013). In addition, heat processing methods such as extrusion and boiling decrease these antinutrients due to degradation of molecular structure (Alonso et al., 2000).

#### 4.6.3. Particle size

Difference in the particle size affects the *in vitro* starch hydrolysis due to differences in susceptibility to enzyme attack. Hera et al. (2013) reported that the particle size significantly affects functional properties and starch features, including the rate of starch hydrolysis. Production of smaller particle size causes higher starch damage on some grains due to increased force and time of milling. However, this varies depending on the starch granule structure (Hera et al., 2013). Surface area of starch exposed to the enzyme attack increases as granule size becomes smaller; thus, increasing susceptibility to enzyme attack (Hera et al., 2013).

### 4.7. Estimated glycemic index (eGI) of different cereal products

#### 4.7.1. Biscuits

Marangoni and Poli (2008) fortified bread and biscuits with fiber and measured GI in human subjects and compared the result with non-enriched bread and biscuits. A 21 % and 41 % reduction of GI were observed when fiber-enriched bread and biscuits were consumed, respectively. The GI values of rye bread and mixed rye and wheat bread were significantly lower than the white bread (El, 1999). The author suggested the fiber in the flour delayed the rate of carbohydrate digestion.

#### 4.7.2. Bread with Chempedak flour

Zabidi and Aziz (2009) measured the eGI of bread using Chempedak (*Artocarpus integer*) flour. Chempedak belongs to Moraceae family and is native to Southeast Asia, and it is low in carbohydrate and fat and high in fiber, but has the same protein level as wheat flour. The rate of starch hydrolysis, as well as eGI, decreased as percentage of Chempedak flour increased (Zabidi and Aziz, 2009). The authors found that percentage of RS was inversely related to the rate of starch hydrolysis and thus eGI. According to the authors, the glycemic effect is strongly influenced by food texture and particle size, type of starch, ratio of amylose and amylopectin, physical entrapment of starch molecule within the food, food processing conditions and composition of food products.

As the amount of protein increases, digestibility of starch should decrease due to dilution of starch in the system and change in the composition of starch types such as RDS, SDS and RS. High protein addition can dilute the starch system, resulting in decreased starch digestibility and thus lowered eGI (Yong et al., 2011). Therefore, the impact of protein on the eGI should be determined.

### 4.8. Starch digestibility in the presence of different protein sources

#### 4.8.1. Whey protein

Whey protein, the soluble protein obtained by milk serum after coagulating casein at pH 4.6, is probably the most popular protein source due to its availability and functionality (Yong et al., 2011). Added whey protein in extruded products caused an increased eGI as well as total starch digestion, but the rate of starch digestion and glycemic load were decreased (Yong et al., 2011). Even though a clear conclusion could not be made, the authors mentioned two factors that affected the result, which included the experimental procedure of *in vitro* starch hydrolysis,

especially pepsin treatment used in the Goni method, and the presence of excess enzymes because of lower starch amounts (Yong et al., 2011). Pepsin treatment caused the higher rate of sample digestion resulting in higher GI, whereas replacing starch with protein causes a reduction in substrate and subsequently, an increase in enzyme: substrate ratio, leading to a higher digestion and GI (Yong et al., 2011).

Moreover, an inverse relationship between increased RDS and decreased rate of digestion was observed at high proportion of whey protein up to 50 %. Minimal retrogradation was found as percentage of protein increased in both *in vitro* starch hydrolysis and RVA results. This might be due to an increased amount of protein hindering the re-association of gelatinized starch molecules. Under this condition, starch molecules are physically apart from each other and tend to form complexes with protein rather than starch molecules, resulting in reduced starch digestion (Yong et al., 2011).

#### 4.8.2. Legumes

Increasing attention toward legumes as a functional food has been growing in the past years. Legumes generally contain high fiber, protein and antinutrient content, which are the main factors affecting starch digestibility. Legumes generally contain higher amylopectin than amylose contents, which are generally 70 % and 30 %, respectively (Thorne et al., 1983). Legumes with high amylose contents tend to be resistant to enzyme attack more than legumes with high amylopectin, due to difference in crystalline structure (Miao et al., 2011). High protein content promotes strong protein-starch association thus hinders the enzyme attack (Chung et al., 2008). Antinutrients, especially phytic acid, tannin and polyphenol content of legumes, affect the starch digestibility. These antinutrients are known as α-amylase inhibitor and will reduce starch digestibility (Thorne et al., 1983).

# 5. PRELIMINARY STUDY

## 5.1. Introduction

Since limited number of studies have been conducted specifically to investigate the relationship between amount of SPI and bread quality, the range of SPI that will produce an acceptable bread had to be determined. Furthermore, since different SPI produce great variance in end product quality (Chen et al., 1982a), properties of the specific SPI in the baking application needed to be determined. By conducting the bread baking in the same way as the actual experiment, the preliminary study enabled adjustment and a determination of the specific parameters, such as baking condition, bread storage and use of analytical equipment. Even though conducting a preliminary study provided additional outcomes, the main objectives were to evaluate and determine the acceptability of a specific SPI in bread baking, data collection was done only once and no statistical analyses was conducted.

## 5.2. Objectives

Objectives were to determine the amount of SPI in bread formulas and to determine the SPI levels best suited for bread baking.

## 5.3. Materials and methods

### 5.3.1. Materials

Wheat flour (Dakota Millers Choice, North Dakota Mill, Grand Forks, ND) was purchased from the local Food Service of America. SPI (Fitness Labs Nutrition Corporation, Torrance, CA) was purchased from a local pharmacy store. Instant yeast was purchased from Lesaffre Yeast Corporation (Milwaukee, WI). Shortening, salt and sugar were purchased from a local grocery store.

### 5.3.2. Methods

#### 5.3.2.1. Farinograph determination

Farinograph test was conducted to observe the characteristics of product in the dough system and measure the functionality of dough when different amounts of SPI were added. SPI percentages used ranged from 3 % to 20 %. Water absorption also was determined for each blend using the Farinograph.

#### 5.3.2.2. Test baking

Test baking was conducted using a 600 g batch size and straight dough method (Table 2). Water absorption obtained from Farinograph was used as a guideline; additional water was added to optimize the visco-elastic characteristic of dough. Consistent water temperature of 5.6 $^{o}$C was used and dough was mixed in a Hobart mixer (Hobart, Troy, OH) with a paddle attachment. Dough was mixed for 1 minute at low speed and further mixed at medium speed until optimal development of dough was achieved and fermented for 60 minutes at 35 $^{o}$C and 85 % relative humidity (RH) in a proofing chamber. Then, the dough was divided into 600 g by hand, molded in a Moline molder (PV1151, Moline, IL), placed into a pan and proofed for 60 minutes in the proofing chamber. Then, bread was baked at 200 $^{o}$C for 24 minutes, cooled and stored overnight in a bag until evaluations were completed.

#### 5.3.2.3. C-cell

C-cell instrument (Calibre Control International Ltd., Appleton, Warrington, UK) was used to capture the high definition images and bread crumb attributes such as average cell diameter, volume, cell wall thickness, crumb firmness and slice brightness.

Table 2. Formulations for 0, 3, 5, 8 and 10 % soy protein isolate fortified bread in grams

| Treatment | 0 % g | 3 % g | 5 % g | 8 % g | 10 % g |
|---|---|---|---|---|---|
| White Flour | 700 | 679 | 665 | 644 | 630 |
| SPI | 0 | 21 | 35 | 56 | 70 |
| Instant Yeast | 7 | 7 | 7 | 7 | 7 |
| Shortening | 42 | 42 | 42 | 42 | 42 |
| Salt | 10.5 | 10.5 | 10.5 | 10.5 | 10.5 |
| Sugar | 42 | 42 | 42 | 42 | 42 |
| Water | 482.3 | 492.1 | 496.3 | 504 | 514.5 |
| **Total** | **1283.8** | **1293.6** | **1297.8** | **1305.5** | **1316** |

5.3.2.4. Loaf volume

Loaf volume was measured after approximately 24 hours of baking. Loaf volume was measured by rapeseed displacement according to AACC International method 10-05 (AACC, 2002a).

5.3.2.5. Texture

Texture analysis was conducted according to AACC International method 74-09.01 (AACC, 2002b). Two slices with 12.5 mm thickness were used. Texture analyzer (TA-XT2, Stable Micro Systems, Godalming, UK) with 38 mm perspex cylinder probe along with a 50 kg load cell was used. The rate of 1.6 mm/s and the distance of 10 mm (40 % compression) were used for texture analysis. Readings (4-5) were taken from each sample and the average reading was recorded.

## 5.4. Results and discussions

### 5.4.1. Farinograph

Farinograph results showed that stability became shorter and development time extended with increased SPI levels (Table 3). Also, water absorption was substantially increased. The results agree with the literature except for development time, which increased instead of decreased (Urade, 2011). The results from the Farinograph were a good indication that high amounts of SPI in the dough may not yield acceptable bread quality. Therefore, treatments with 15 and 20 % of SPI were eliminated and rest of the formulations were used in the actual baking test.

Table 3. Farinograph dough properties of wheat flour fortified with 0 to 20 % soy protein isolate (SPI)

| SPI % | Absorption (%) | Dev. Time (min) | Breakdown (min) | Stability (min) | MTI* |
|---|---|---|---|---|---|
| 0 | 68.9 | 7:43 | 16:59 | 14:32 | 16 |
| 3 | 70.3 | 8:37 | 16:37 | 11:38 | 24 |
| 5 | 70.9 | 8:56 | 18:23 | 12:12 | 19 |
| 8 | 72.0 | 9:25 | 17:31 | 10:18 | 17 |
| 10 | 73.5 | 9:11 | 17:01 | 10:14 | 14 |
| 15 | 77.0 | 13:15 | 16:42 | 8:37 | 50 |
| 20 | 79.8 | 11:47 | 16:07 | 7:45 | 38 |

*MTI = Mixing tolerance index

### 5.4.2. Baking test

Increasing the amount of SPI deteriorates some of the baking quality indices (Table 4). Loaf volume decreased as the amount of SPI increased and this was confirmed by the C-cell test in which cell volume and number of cells decreased with higher levels of SPI (Table 5).

Table 4. Baking parameters and quality test results of wheat flour fortified with 0 to 10 % soy protein isolate (SPI)

| Treatment | 0 % | 3% | 5 % | 8 % | 10 % |
|---|---|---|---|---|---|
| Mixing L* | 1:00 | 1:00 | 1:00 | 1:00 | 1:00 |
| Mixing H** | 4:15 | 5:00 | 5:15 | 5:00 | 5:10 |
| Absorption | 69.4 | 75.3 | 74.9 | 76.0 | 77.5 |
| Bread weight (g) | 540.7 | 539.3 | 530.9 | 530.7 | 538.6 |
| Loaf volume (cc) | 2500 | 2237.5 | 2312.5 | 2050 | 1762.5 |
| Specific volume (cc/g) | 4.62 | 4.15 | 4.36 | 3.86 | 3.27 |

*Mixing at low speed
**Mixing at high speed

Table 5. C-cell parameters and firmness of wheat bread fortified with 0 to 10 % soy protein isolate (SPI)

| SPI % | Slice Brightness | Number of Cells | Wall Thickness / px | Cell Diameter / px | Cell Volume | Firmness (g) |
|---|---|---|---|---|---|---|
| 0 % | 141.2 | 4742.5 | 3.78 | 16.925 | 6.92 | 41.50 |
| 3 % | 139.25 | 5363.5 | 3.55 | 16.295 | 7.09 | 48.50 |
| 5 % | 129.6 | 4483.0 | 3.52 | 15.725 | 6.69 | 48.78 |
| 8 % | 122.75 | 4335.5 | 3.56 | 14.895 | 6.52 | 59.91 |
| 10 % | 150.55 | 6343.0 | 3.41 | 15.625 | 6.60 | 84.73 |

### 5.5. Conclusion and findings

Based on the Farinograph and bread baking results, the particular SPI used was assumed to provide unsatisfactory bread quality. Lower loaf volume, packed and therefore hard grain structure supported the poor baking test performance of SPI. Loaf volume decreased substantially with increasing amounts of SPI. In addition, C-cell test revealed the trend of increasing cell number, but decreasing cell diameter indicating the packed grain structure.

Texture analyzer results indicated firmness of the bread increased as SPI addition increased (Table 5).

Chen and Rasper (1982b) investigated the difference in rheological characteristics and baking performance using the SPI extracted from different methods. Alkali-extracted protein showed higher loaf volume depression effect than water-extracted protein and high correlations between loaf volume and Extensiograph (-0.765 and -0.936) and loaf volume and Farinograph water absorption (0.908 and 0.827) for each water- and alkali-extracted protein, respectively (Chen and Rasper, 1982b). From these findings, the particular SPI used in this preliminary study was determined to be non-functional; thus, another source of SPI, ProFam 781 (ADM, Decatur, IL) was selected to be used in the thesis study. The ProFam 781 is manufactured particularly for baking application and therefore was selected for use in the baking test.

In addition, change of dough characteristics at different levels of SPI is partially due to difference in water absorption. Water absorption was adjusted to achieve the optimal dough condition, but this caused difficulty in standardizing the test method and determining the real effect on bread quality caused by the SPI. The effect of SPI on crumb firmness and dough extensibility could not be assessed because water *per se* could supplement these effects. Therefore, the constant amount of water strictly following the Farinograph water absorption was used in the actual baking test.

# 6. MATERIALS AND METHODS

## 6.1. Materials

Commercial flour sample was obtained from local mill. SPI (ProFam 781) was purchased from ADM (Decatur, IL). Instant yeast was purchased from Lesaffre Yeast Corporation (Milwaukee, WI). Shortening, salt and sugar were purchased from a local grocery store.

## 6.2. Methods

### 6.2.1. Proximate analysis and basic dough tests

Proximate analysis was conducted on blends of flour at 5 different percentages of SPI (0, 3, 5, 8 and 10 %). Blending of flour and SPI was done by shaking 1000 g of flour : SPI mixtures in a plastic bag. Moisture was measured according to AACC International method 44-15.02 (AACC, 2002c). Ash was determined according to AACC International method 08-01.01 (AACC, 2002d). Protein (Protein Analyzer FP528, LECO, St. Joseph, MI) was measured according to AACC International method 46-30.01 (AACC, 2002e). Farinograph (C.W. Brabender, Hackensack, NJ) was conducted according to AACC International method 54-21.02 (AACC, 2002f). RVA (Newport Scientific, Jessup, MD) was determined according to AACC International method 76-21.01 (AACC, 2002g). Wet gluten and gluten index (Glutomatic, Perten, Pringfield, IL) were determined according to AACC International method 38-12.02 (AACC, 2002h).

### 6.2.2. Bread baking

Bread baking was conducted following the same method used in the preliminary study. The same ingredients and the same formulations (Table 6) were used except ProFam 781 (ADM, Decatur, IL) was used as the SPI source. Flour and SPI were blended prior to mixing with other ingredients in the mixer until appears to be blended.

Table 6. Formulations for 0, 3, 5, 8 and 10 % soy protein isolate fortified bread in grams

| Treatment | 0 % | 3 % | 5 % | 8 % | 10 % |
|---|---|---|---|---|---|
| White Flour | 1000 | 970 | 950 | 920 | 900 |
| SPI | 0 | 30 | 50 | 80 | 100 |
| Instant Yeast | 10 | 10 | 10 | 10 | 10 |
| Shortening | 60 | 60 | 60 | 60 | 60 |
| Salt | 15 | 15 | 15 | 15 | 15 |
| Sugar | 60 | 60 | 60 | 60 | 60 |
| Water | 628 | 653 | 660 | 669 | 688 |
| Total | 1773 | 1798 | 1805 | 1814 | 1833 |

Water absorption from Farinograph was used without any adjustment in order to eliminate difference caused by subjective judgment. Dough was mixed until optimal development using a Hobart mixer with a paddle attachment (Hobart, Troy, OH) and fermented for 60 minutes at 35 $^{o}$C and 85 % RH in a proofing chamber. Then, dough was divided into 600 g pieces, molded, placed into a pan and fermented for 60 minutes at 35 $^{o}$C and 85 % RH. Bread was baked at 200 $^{o}$C for 24 minutes.

6.2.3. Bread analyses

6.2.3.1. Loaf volume

Loaf volume was measured by rapeseed displacement method according to AACC International method 10-05.01 (AACC, 2002a). Measurement was taken on loaves approximately 24 hours after baking.

6.2.3.2. Texture

Texture analysis was conducted according to AACC International method 74-09.01 (AACC, 2002b). Two slices (12.5 mm thickness) from the 24 h old bread were used. Texture analyzer (TA-XT2, Stable Micro Systems, Godalming, UK) with 38 mm perspex cylinder probe along with a 50 kg load cell was used. The rate and distance parameters used were 1.6 mm/s and

10 mm (40 % compression), respectively. Readings (4-5) were taken from each sample and the average reading was recorded.

6.2.3.3. Water activity

Water activity meter (Aqualab, Pullman, WA) was used to measure water activity. The center of the crumb was cut out and placed in the machine.

6.2.3.4. C-cell

C-cell instrument (Calibre Control International Ltd., Appleton, Warrington, UK) was used to capture high definition images. The bread crumb attributes, such as average cell diameter, volume, cell wall thickness, crumb firmness and slice brightness, were determined from captured images using the computer software.

6.2.3.5. Color

Minolta color analyzer (CR-310, Minolta, Osaka, Japan) was used to determine the crumb color. Calibration was conducted daily using a white tile. L*, a*, b* value were used as L*, a* and b* represents brightness (0 = black and 100 = white), red and green color (+a = red and –a = green) and yellow and blue (+b = yellow and –b = blue), respectively.

6.2.3.6. Scanning electron microscope (SEM)

To prepare samples, a razor blade was used to cut from bread slices sections approximately 1 $cm^2$. These pieces then were attached to cylindrical aluminum mounts using colloidal silver paste (SPI Supplies, West Chester, Pennsylvania) and sputter coated (Model SCD 030, Balzers, Liechtenstein) with gold-palladium to make them electrically conductive. The samples were viewed and images obtained with a JEOL JSM-6490LV scanning electron microscope (JEOL USA, Peabody, Massachusetts) operating at an accelerating voltage of 15 kV.

6.2.4. Assay tests for starch and eGI characteristics of bread

6.2.4.1. Sample preparation

Bread loaves were sliced after 24 hours and immediately frozen using a blast freezer and freeze dried (FreeZone, Labconco, Kansas City, MO). The samples were milled using a coffee grinder into particles that pass through a 0.5 mm screen.

6.2.4.2. Total starch

Total Starch (TS) and Resistant Starch (RS) assay kits were purchased by Megazyme International (Brey, Ireland). Ethanol (0.2 ml) was added to samples (100 mg) in a glass test tube and α-amylase (3 ml) was added followed by incubation in a boiling water bath for 6 minutes. Then, amyloglucosidase (0.1 ml) was added and incubated for 30 minutes in a 50 $^{o}$C water bath. The solution was diluted to 100 ml with distilled water and centrifuged at 1500 g for 10 minutes (Multifuge X1R, Thermo Scientific, Waltham, MA). Glucose Oxidase-Peroxidase (GOPOD) reagent (3 ml) was added to the supernatant (0.1 ml) and incubated for 20 minutes at 50 $^{o}$C. D-glucose was used as a standard and analyzed along with blank. Spectrophotometer (GENESIS 10s UV-Vis, Thermo Scientific, Walthama, WA) was used to determine the color development by reading the absorbance against a blank at 510 nm. TS was calculated using an equation as follows:

$$TS\ (\%) = \Delta A \times F \times \frac{FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \qquad \text{(Eq. 1)}$$

Where $\Delta A$ = Absorbance, F = $\frac{100\ (\mu g\ of\ D-glucose-)}{absorbance\ of\ 100\ \mu g\ of\ glucose}$, FV = final volume, 0.1 = volume of sample analyzed, $\frac{1}{1000}$ = conversion from $\mu g$ to mg, $\frac{100}{W}$ = Factors to express starch as a

percentage of flour weight, W = dry sample weight in mg, $\frac{162}{180}$ = factors to covert from free D-glucose to anhydro D-glucose.

6.2.4.3. Resistant starch

Enzyme solution containing α-amylase (3 Ceralpha Units/mg) (1 g), amyloglucosidase (300 U/ml) (1 ml) and sodium maleate buffer (100 ml) was prepared. Bread sample (100 mg) and enzyme solution (4 ml) were placed in glass test tubes. Then the tubes were incubated in shaking water bath (200 strokes/min) at 37 $^{o}$C for 16 hours. After 16 hours, ethanol (4 ml) was added to the solution followed by centrifugation at 1500 g for 10 minutes. The pellet was removed and re-suspended in ethanol (8 ml) followed by centrifugation at 1500 g for 10 minutes. This procedure was repeated twice. The pellet was suspended in potassium hydroxide solution (2 ml) and stirred with a magnetic stirrer bar in ice water bath for 20 minutes. Then, sodium acetate buffer (8 ml) and amyloglucosidase (0.1 ml) were added to the solution and incubated for 30 minutes at 50 $^{o}$C water bath. GOPOD reagent (3 ml) was added to supernatant (0.1 ml) and incubated for 20 minutes at 50 $^{o}$C water bath and absorbance was read against a blank at 510 nm. Resistant starch was calculated using the equation as follows;

$$RS = \Delta E \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \quad \text{(Eq. 2)}$$

Where $\Delta E$ = Absorbance, F = conversion from absorbance to micrograms, $\frac{100}{0.1}$ = volume correction, $\frac{1}{100}$ = conversion from $\mu g$ to mg, W = dry sample weight in mg, $\frac{100}{W}$ = factor to present RS as a percentage of sample weight, $\frac{162}{180}$ = factor to convert from free D-glucose to anhydro D-glucose.

Consequently, non-resistant starch can be calculated using the equation below;

$$TS = RS + Non\text{-}resistant\ starch \quad \text{(Eq. 3)}$$

6.2.4.4. Estimated glycemic index (eGI) assay

eGI assay was conducted using 300 mg of ground sample obtained by passing through a 5 mm screen. Enzyme solution containing 9 ml, 6 ml and 81 ml of amyloglycosdiase (140 AGU/ml; Megazyme, Brey, Ireland), Ivertase (≥ 300 unites/mg; Sigma Aldrich, St. Louis, MO) and Pancreatin (8x USP; Sigma Aldrich, St. Louis, MO), respectively, was added to samples. The solution was prepared fresh daily and used immediately. Acetic acid buffer (pH 5.2) containing 4.1 g sodium acetate, 125 ml benzoic acid (0.5 g/100 ml) and 2 ml 1M calcium chloride was prepared. The buffer (20 ml) was added to a test tube containing sample (300 mg), guar gum (50 mg) (Sigma Aldrich, St. Louis, MO), five beads (1 cm diameter) and the enzyme solution mixture (5 ml). The mixture was incubated in a shaking water bath (200 strokes/min) (VWR International; Radnor, PA) for 180 minutes at 37 $^{o}$C. A sample was taken every 20 minutes into absolute ethanol (5 ml) and centrifuged. Supernatant (0.1 ml) was placed in a tube and GOPOD solution (3 ml) was added for glucose determination. The sample was incubated in a 50 $^{o}$C water bath for 20 minutes and color development was read in spectrophotometer against a blank at 510 nm.

Glucose standard curve was prepared from glucose solution at 6 different concentration levels (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg/ml) to obtain the equation and percent glucose released was calculated by plugging the absorbance into the equation. From that, area was obtained and hydrolysis index (HI) was calculated comparing the area under hydrolysis curve (AUC) with the AUC of white bread. The eGI was then calculated using the following equations:

$$AUC = \{C_\infty (t_f - t_0) - (\frac{C_\infty}{k}) [1 - exp [ -k (t_f - t_0)]]\} \quad \text{(Eq. 4)}$$

Where $C_\infty$ = equilibrium % of starch hydrolysis after X min, $t_f$ = final time, $t_0$ = initial time and k = kinetic constant.

$$Hydrolysis\ Index\ (HI) = \frac{AUC}{AUC\ with\ white\ bread\ control} \quad \text{(Eq. 5)}$$

$$eGI = 8.198 + (0.862)\ HI \quad \text{(Eq. 6)}$$

### 6.3. Experimental design

The objectives of the experiment were to determine the eGI of bread fortified with SPI and determine the effect of SPI on eGI and bread quality characteristics. To test these objectives, the following designs were used. A randomized complete block design (RCBD) containing 5 treatments (0, 3, 5, 8 and 10 % SPI), 3 replicates and 2 samplings, assuming each batch of bread makes 2 loaves, was used. For the statistical analysis, RCBD and regression analysis were conducted. Regression analysis was used to determine if there is any linear relationship between eGI at different levels of SPI. eGI was considered as a dependent variable and the different levels of SPI were considered as independent variables. RCBD was conducted using the mean of samplings as well as F-protected Least Significant Difference (LSD) at the *p* value of 0.05. $p < 0.05$ indicates the probability at 95 % of confidence level.

# 7. RESULTS AND DISCUSSIONS

## 7.1. Health claim requirement

Additional protein from SPI resulted in a substantial increase of crude protein (Table 7). Each serving of bread must contain at least 6.25 g of soy protein in order to use the health claim approved by the FDA (FDA, 2012). The FDA makes a recommendation of serving size known as reference amount customarily consumed (RACC). Based on the RACC, the serving size of bread is 50 g; thus, approximately 10 servings can be obtained from a loaf of bread. Theoretically, each serving of bread made from the 0, 3, 5, 8 and 10 % formulas contain approximately 0.0 g, 1.00 g, 1.66 g, 2.65 g, and 3.27 g of SPI, respectively, based on total batch weight and dough weight. This indicates that more than 10 % of SPI must be used to fulfill the health claim requirement. However, the calculation is based on only flour and SPI used in the formulation thus difference in the formulation such as additional ingredients and serving size could influence the calculated values.

## 7.2. Proximate analysis

Proximate analysis results indicated that there are no significant differences ($p<0.05$) between samples in ash and gluten index but there are significant differences in moisture, protein and wet gluten. Percentage of protein increased from 12.9 at 0 % SPI to 19.1 % at 10 % SPI, hence wet gluten was reduced from 32.7 % at 0 % SPI to 26.5 % at 10 % SPI (Table 7). Composition of soy protein differs greatly from wheat protein as SPI contains no gliadin and limited amount of glutenin, which are gluten forming proteins (Pyler and Gorton, 2010). Globulin, which is the major protein in SPI is soluble in salt solution; thus, this protein might have been lost during the washing process of the test and maybe the reason SPI had no effect on gluten index.

Reduction of wet gluten also indicates a change caused by SPI and protein functionality. Strong protein-protein association, which resulted in incorporating the SPI into gluten, may be responsible for the functionality change (Roccia et al., 2009). Consequently, dilution of gluten likely resulted, due to introduction of non-gluten protein into the gluten system (Urade, 2011). Both covalent and non-covalent interactions were reported for mixtures of wheat protein and SPI (Roccia et al., 2009). A disulfide bond (S-S) is a type of covalent bonding that stabilizes the dough through a thiol-disulfide interchange reaction. Disruption of disulfide bond results in weakening of the gluten network via an increase in sulfhydryl (SH) group concentration (Visschers and de Jongh, 2005).

Table 7. Moisture, ash, protein, wet gluten and gluten index of wheat flour fortified with soy protein isolate (SPI) on a 14 % basis

| SPI % | Moisture (%) | Ash (%) | Protein (%) | Wet gluten (%) | Gluten index |
|---|---|---|---|---|---|
| 0 | 12.5 ± 0.02 a* | 3.31 ± 0.04 a | 12.9 ± 0.1 ab | 32.7 ± 1.1 a | 93.8 ± 2.0 a |
| 3 | 12.4 ± 0.001 ab | 3.44 ± 0.2 a | 14.7 ± 0.07 b | 36.6 ± 4.5 ab | 86.4 ± 10.0 a |
| 5 | 12.3 ±0.08 b | 3.72 ± 1.1 a | 16.3 ± 0.2 c | 30.7 ± 0.4 bc | 90.1± 3.3 a |
| 8 | 12.1 ± 0.09 c | 2.69 ± 0.008 a | 17.8 ± 0.1 d | 27.6 ± 0.7 bc | 92.1 ± 1.2 a |
| 10 | 11.9 ± 0.003 d | 3.27 ± 0.1 a | 19.1 ± 0.2 e | 26.5 ± 0.3 c | 92.3 ± 0.1 a |

*The same letter in each column indicates there is no significant difference ($p$<0.05) between treatments.

Bonfil and Posner (2012) reported a high correlation between protein and wet gluten but not between protein and gluten index, indicating the increased protein affects the change in wet gluten but not the gluten index. Gluten index is not necessarily an indication of good baking quality because no correlation between gluten index and other parameter such as protein or SDS-

sedimentation test was found (Bonfil and Posner, 2012). While gluten index can provide a good estimation of baking properties for some types of wheat, there are exceptions especially when additional ingredients are incorporated into the flour.

Ribotta et al. (2005) reported gluten yield to increase due to gluten matrix trapping the SPI and thus interrupting the formation of the continuous matrix. This suggests that non-functional protein affects the gluten properties and therefore the end-product quality when mixed with gluten. The opposite result, than what was expected, was observed; possibly due to the state of protein, such as native or denatured, affecting the retention of SPI in the gluten matrix (Ribotta et al., 2005). Properties of SPI used in the experiment were not measured; thus, the difference in SPI source could be one of the reasons why the result did not agree with the hypothesis.

The gluten matrix phase becomes non-continuous as non-gluten protein becomes trapped in gluten matrix; thus, the structure causes poor gas retention capacity (Kim et al., 2003). This lead to bread with lower loaf volume and quality. Strong adhesion of gluten and starch enables the formation of a continuous network that supports the dough during fermentation (Kim et al., 2013). A more ordered cell structure of bread with and without SPI can be seen in SEM micrographs (Figure 3).

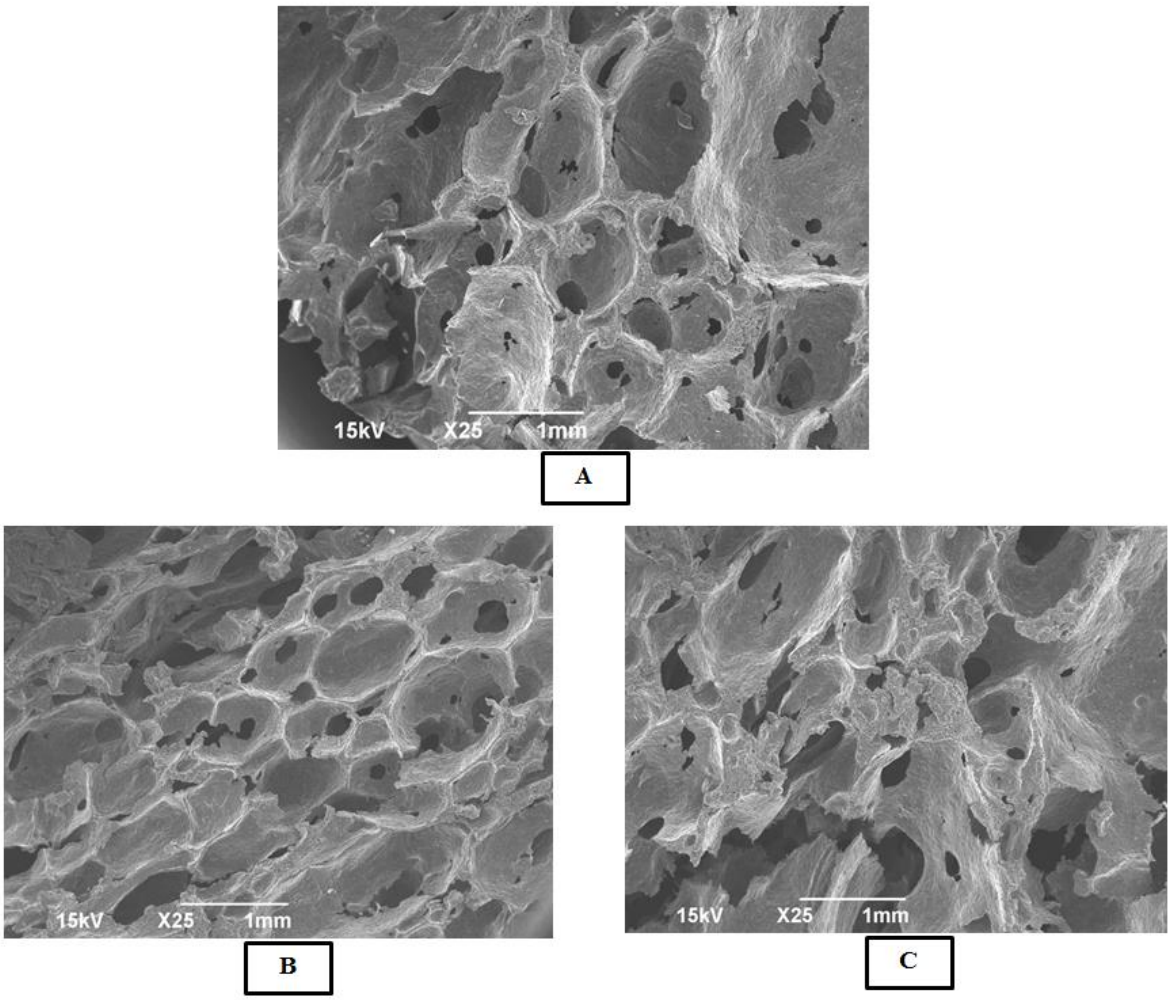


Figure 3. Scanning electron microscopic (SEM) images of bread cell structure at 25X magnification: A) Control, B) 5 % SPI and C) 10 % SPI

### 7.3. Rheological test

Farinograph was used to determine the water absorption as well as to estimate the bread quality. Water absorption increased from 62.8 % for 0 % SPI to 68.8 % for 10 % SPI and dough development time from 6.75 minutes to 9.93 minutes, respectively (Table 8). Stability was substantially decreased from 15.68 minutes for 0 % SPI to 4.13 minutes for 10 % SPI (Table 8).

Table 8. Farinograph dough properties of wheat flour fortified with soy protein isolate (SPI)

| SPI % | Water absorption (%) | Development time (min) | Stability (min) | MTI (tolerance index) | Breakdown (min) |
|---|---|---|---|---|---|
| 0 % | 62.8 | 6.75 | 15.68 | 12 | 18.69 |
| 3 % | 65.3 | 8.71 | 6.63 | 66 | 11.51 |
| 5 % | 66.0 | 8.40 | 4.18 | 89 | 10.11 |
| 8 % | 66.9 | 8.93 | 3.79 | 92 | 10.79 |
| 10 % | 68.8 | 9.93 | 4.13 | 89 | 11.70 |

Water absorption increased with increasing SPI, which is in agreement with literature and is likely due to hydrophilic properties of soy protein as it absorbs and interacts with more water (Maforimbo et al., 2008). Increased dough development time observed from 6.75 minutes for 0 % SPI to 9.93 minutes for 10 % SPI (Table 8) does not agree with the past studies where SPI caused faster development time (Urade, 2011; Ribotta et al., 2007). Ribotta et al. (2005) reported faster development time with increasing protein purity, where SPI had faster development time than soy flour. Krishnan and Darly-Kindelspire (2013) determined that soy protein concentrate (SPC), which contains at least 65 % protein, extended the mixing time at high ratio of SPC. In the case of the traditional bread system, starch absorbs water more quickly than protein and thus gluten takes longer to develop (Larsen, 1963).

In the case of bread system with non-traditional ingredients, such as SPI used in this experiment, the reason could be related to the availability and competition between protein and starch for water. Higher water absorption, as a result of SPI, was due to the ability of SPI to absorb more water, increasing competition with wheat protein, resulting in decreased water availability to gluten and longer time to fully develop the gluten network (Marchais et al., 2011). In addition, starch absorbs water at a much faster rate than gluten even though more gluten is present, thus the competition for the water availability becomes greater (Larsen, 1964).

Stability and breakdown decreased upon SPI addition, which agrees with the literature (Urade, 2011; Ribotta et al., 2005). The extent of the reduction on stability was especially notable, indicating the effect of SPI on gluten as discussed earlier. On the other hand, mixing tolerance index (MTI) and breakdown appear not to be affected by SPI (Table 8). MTI is an indication of flour's tolerance to mixing; the lower the value the better is the tolerance to mixing (Shuey, 1984). MTI changed extensively after SPI addition, indicating the weakened effects of dough for the mixing and mechanical handling, but the change in MTI was minimal between different levels of SPI.

Even though SPI fortification in bread was found to deteriorate the dough to some extent, use of oxidants and reductants could improve the dough properties. Maforimbo et al. (2008) observed that a combination of oxidants and reductants can help incorporate SPI into the gluten matrix and improve the dough stability. The reduction process breaks down the disulfide bonds and opens up the gluten polymer followed by the re-oxidation process in which soy protein can be incorporated into gluten network as the disulfide group is restored (Maforimbo et al., 2008). This supports that the oxidizing reaction improves the dough characteristics of SPI-fortified dough and the use of oxidants to improve functionality.

## 7.4. Starch properties

### 7.4.1. Rapid visco analyzer (RVA)

Pasting curve (Figure 3) was shifted down with higher SPI levels. Significant differences were found in peak viscosity, breakdown, setback and pasting temperature (Table 9). Peak viscosity and breakdown decreased from 1744 to 1405 cP and 1390 to 1075 cP, respectively. Setback decreased slightly from 580 to 447 cP while pasting temperature increased slightly from 76.8 to 79.2 $^{o}$C (Table 9).

Ribotta et al. (2007) and Lim and Narsimhan (2006) concluded that SPI increasing level (i.e. 0 % to 50 %) in SPI and wheat starch mixture increased peak viscosity, which was not observed in this experiment (Table 9). However, Bainy et al. (2010) reported reduction of peak viscosity at SPI levels of 0.5 % to 11 %. The authors assumed that higher concentration of solid contents at higher SPI level could be one reason (Lim and Narsimhan, 2006), while increase in aggregation of proteins as well as protein-protein interaction resulting from the formation of disulfide bonds caused an increase in viscosity at the peak viscosity (Bainy et al., 2010).

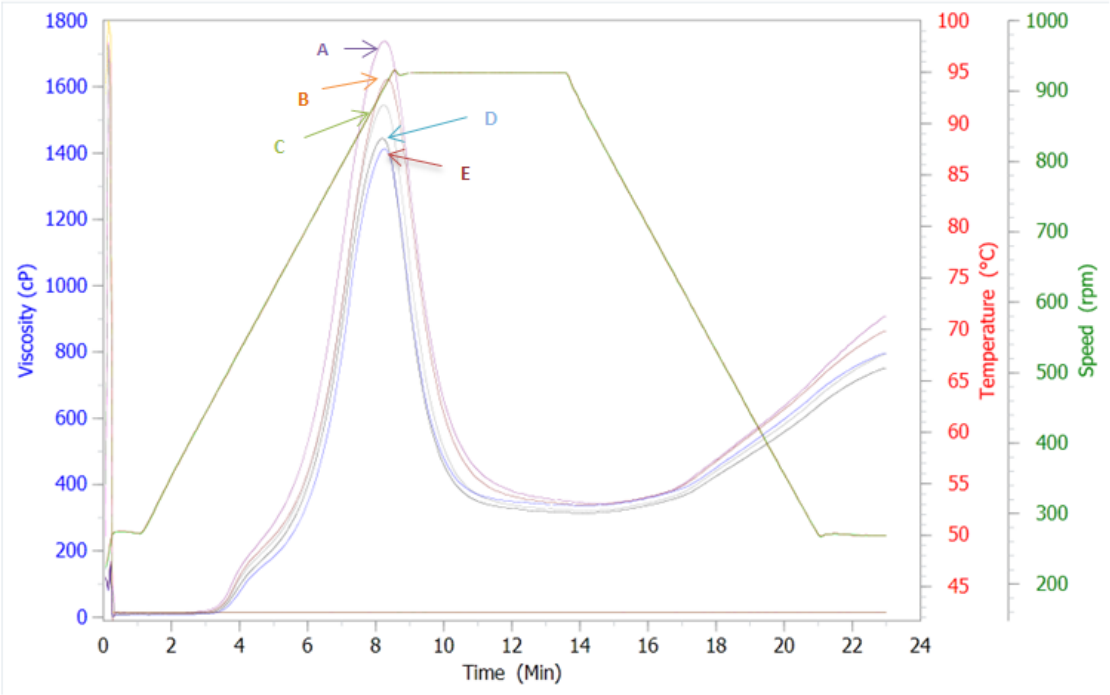


Figure 4. Rapid visco analyzer profile of wheat flour fortified with soy protein isolate (SPI): A- 0 % SPI, B- 3 % SPI, C- 5 % SPI, D- 8 % SPI, E-10 % SPI

Lower final viscosity and setback (Table 9) agrees with results of Bainy et al. (2010). However, the opposite effect was also observed (Lim and Narsimhan, 2006; Ribotta et al. 2007). Ribotta et al. (2007) reported increased cross-linking from protein-protein hydrophilic interaction as a reason for higher final viscosity while Lim and Narsimhan (2006) assumed greater starch-

protein hydrophobic interaction and formation of hydrogen bonds. The difference in experimental outcomes may be related to test parameters and SPI used to evaluate starch characteristics.

Table 9. Rapid visco analyzer characteristics of wheat flour fortified with soy protein isolate (SPI)

| SPI % | Peak (cP) | Trough (cP) | Breakdown (cP) | Final viscosity (cP) | Setback (cP) | Peak time (min) | Pasting temp ($^{o}$C) |
|---|---|---|---|---|---|---|---|
| 0 | 1744 a | 354 a* | 1390 a | 934 a | 580 a | 8.3 a | 76.8 a |
| 3 | 1574 b | 330 a | 1256 b | 828 a | 494 b | 8.3 a | 78.2 b |
| 5 | 1579 b | 335 a | 1244 b | 812 a | 494 b | 8.2 a | 78.2 b |
| 8 | 1466 c | 328 a | 1138 c | 779 a | 451 b | 8.2 a | 78.6 bc |
| 10 | 1405 c | 318 a | 1075 d | 777 a | 447 b | 8.2 a | 79.2 c |

*The same letter in each column indicates there is no significant difference ($p$<0.05) between treatments.

Interestingly, difference in the degree of denaturation did not cause the difference in starch properties but differences in globular protein subunit composition affected the RVA profile (Bainy et al., 2010). Higher percentage of cysteine residue found in some protein subunits could possibly enhance the formation of disulfide bridges and strengthen the network (Bainy et al., 2010).

Soy protein possibly restricted the swelling and gelatinization of starch, thus higher temperature was required for gelatinization and increased the onset of starch gelatinization temperature (Ribotta et al., 2005). Consequently, decreased peak viscosity and setback indicate the reduction of starch gelatinization or amount of starch available for gelatinization. Diluted starch in the system modifies the gelatinization profile and increases the viscosity from SPI. Lowered final viscosity and setback means decreased retrogradaton, which is favored in some

cases as it stabilizes the gel after cooling; thus, exhibits better storage property (Lim and Narsimhan, 2006). In addition, degree of retrogradation affects the degree of starch hydrolysis by changing the susceptibility of starch to enzyme attack and affecting the eGI (Yong et al., 2011).

7.4.2. Scanning electron microscopic (SEM)

Cell structure of bread containing low SPI level (0 % and 5 % SPI) is consistent and ordered (Figure 4) whereas bread with higher SPI (10 % SPI) appears more disrupted and inconsistent. In addition, the cell wall is rougher and more degraded with high SPI (Figure 4), even though no significant difference was observed from C-cell results. This further supports that continuous structure of bread matrix was interrupted by the presence of SPI. The image of SPI alone shows the small fragments of protein on the surface of large molecule (Figure 5). This might contribute to the discontinuous gluten matrix.

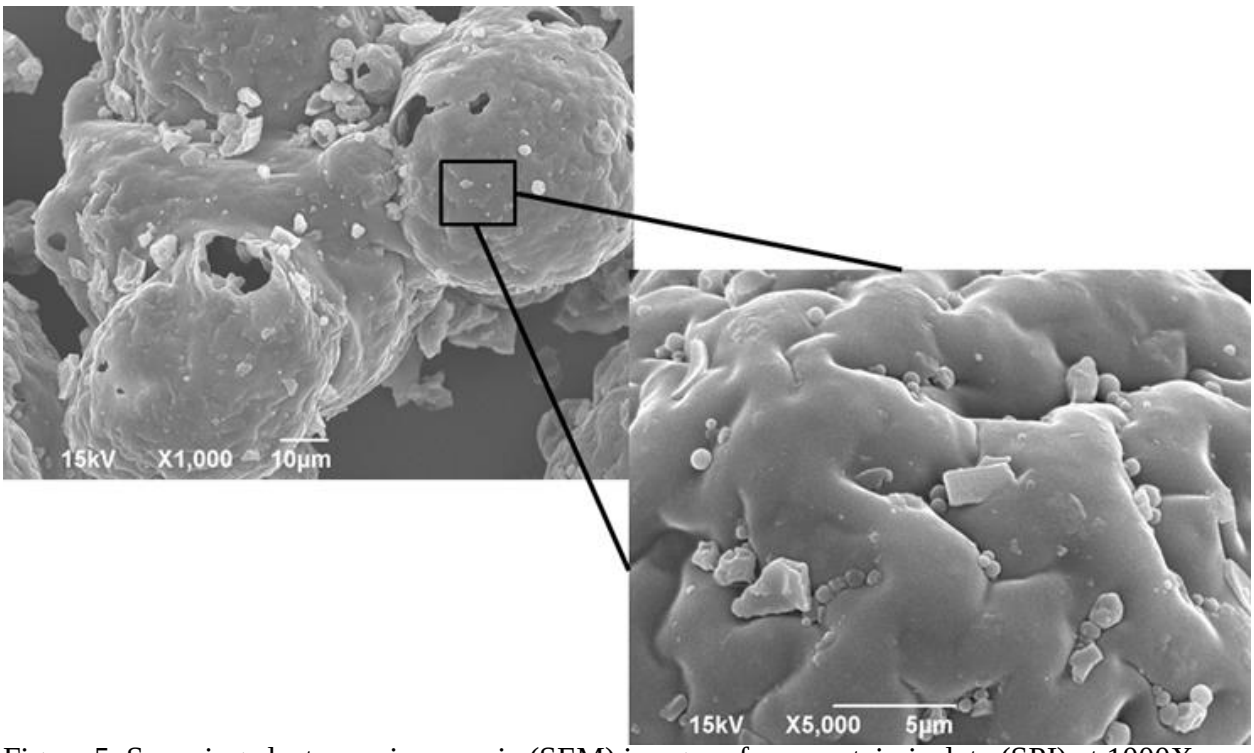


Figure 5. Scanning electron microscopic (SEM) images of soy protein isolate (SPI) at 1000X (left) and 5000X (right).

Microstructure of SEM images of bread shows holes with lower SPI levels (Figure 6), but the number of visible holes decreases with increased SPI percentage. These holes might be caused by disruption of gas bubbles (Kim et al., 2003). During the fermentation, swelling of starch and gluten provide the structure that entraps the carbon dioxide produced by yeast; therefore, making the gas bubbles. The lack of strong outer membranes that hold the gas has less ability to retain gas and can rupture more easily; therefore, the loaf volume decreases (Kim et al., 2003). Less gas cells observed in the higher SPI bread indicates less gas retention capacity during baking bread, which led to lowered loaf volume (Table 10).

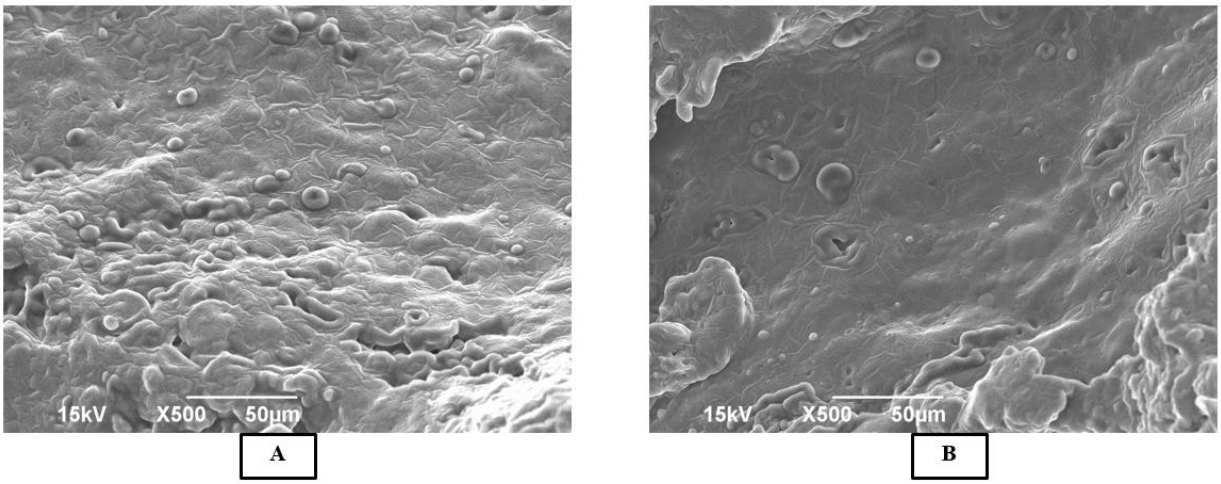


Figure 6. Scanning electron microscopic (SEM) images of bread at 500X magnification: A. 0 % SPI, B. 10 % SPI

Microstructure images (Figures 5 and 6) revealed that surface of higher SPI bread is flatter and smoother than that of 0 % SPI. Granule structure is clearly visible in 0 % SPI while 10 % SPI did not show any possible starch granules. This possibly indicates that starch granules have been disintegrated and structure is lost as a result of 10 % SPI addition compared to 0 % SPI. Heat treatment enhances starch-protein interaction by altering the protein structure thus

making it more available for starch granules to adsorb (Ryan and Brewer, 2007; Yong et al., 2011).

### 7.5. Bread baking tests

Whiteness of bread decreased, as measured by L* value and bread brightness of the C-cell test (Table 10), at higher SPI levels. Full fat soy flour and 90 protein digestibility index (PDI) soy flour is known to bleach flour and strengthen gluten when used in the baked products due to lipoxygenase activity (Stauffer, 2005). However, lipoxygenase activity in SPI is too low to affect the crumb color of bread. It is more reasonable to consider Maillard browning caused the darker color on both crumb and crust. The presence of lysine in SPI, which is an essential amino acid normally minited in wheat flour, has an available essential amino group that can readily react with reducing sugar resulting in browning color formation.

Table 10. Loaf volume, water activity, color and firmness of wheat bread fortified with soy protein isolate (SPI)

| SPI % | Loaf volume (cc) | Water activity | L* | a* | b* | Firmness (g) |
|---|---|---|---|---|---|---|
| 0 | 2406 ± 67.4 a** | 0.95 ± 0.015 a | 83.9 ± 0.93 a | -1.27 ± 0.08 a | 18.2 ± 0.47 a | 171.5 ± 24.7 a |
| 3 | 2250 ± 115.4 a | 0.96 ± 0.007 a | 82.0 ± 1.21 b | -0.72 ± 0.10 b | 19.5 ± 0.53 a | 189.9 ± 39.8 a |
| 5 | 2256 ± 171.3 a | 0.96 ± 0.004 a | 81.8 ± 1.29 b | -0.45 ± 0.09 c | 19.2 ± 0.49 a | 200.0 ± 48.3 a |
| 8 | 2250 ± 161.1 a | 0.96 ± 0.003 a | 80.5 ± 1.00 c | 0.0042 ± 0.15 d | 20.3 ± 0.52 b | 192.8 ± 42.5 a |
| 10 | 2047 ± 48.0 a | 0.96 ± 0.002 a | 79.3 ± 0.70 d | 0.47 ± 0.06 e | 21.0 ± 0.08 c | 219.1± 39.9 a |

** The same letter in each column indicates there is no significant difference ($p<0.05$) between treatments.

No significant difference in loaf volume and firmness was found between treatments (Table 10). Although the large differences in the results exist between 0 % and 10 % SPI, both

loaf volume and firmness have large standard deviation indicating there is a large variation between replicates (Table 10). This is also confirmed in the distribution of yield table from the statistical analysis (Figure 7). Thus, the differences in loaf volume obtained may not be of statistical difference but may be of practical significance due to visual changes (Figure 8).

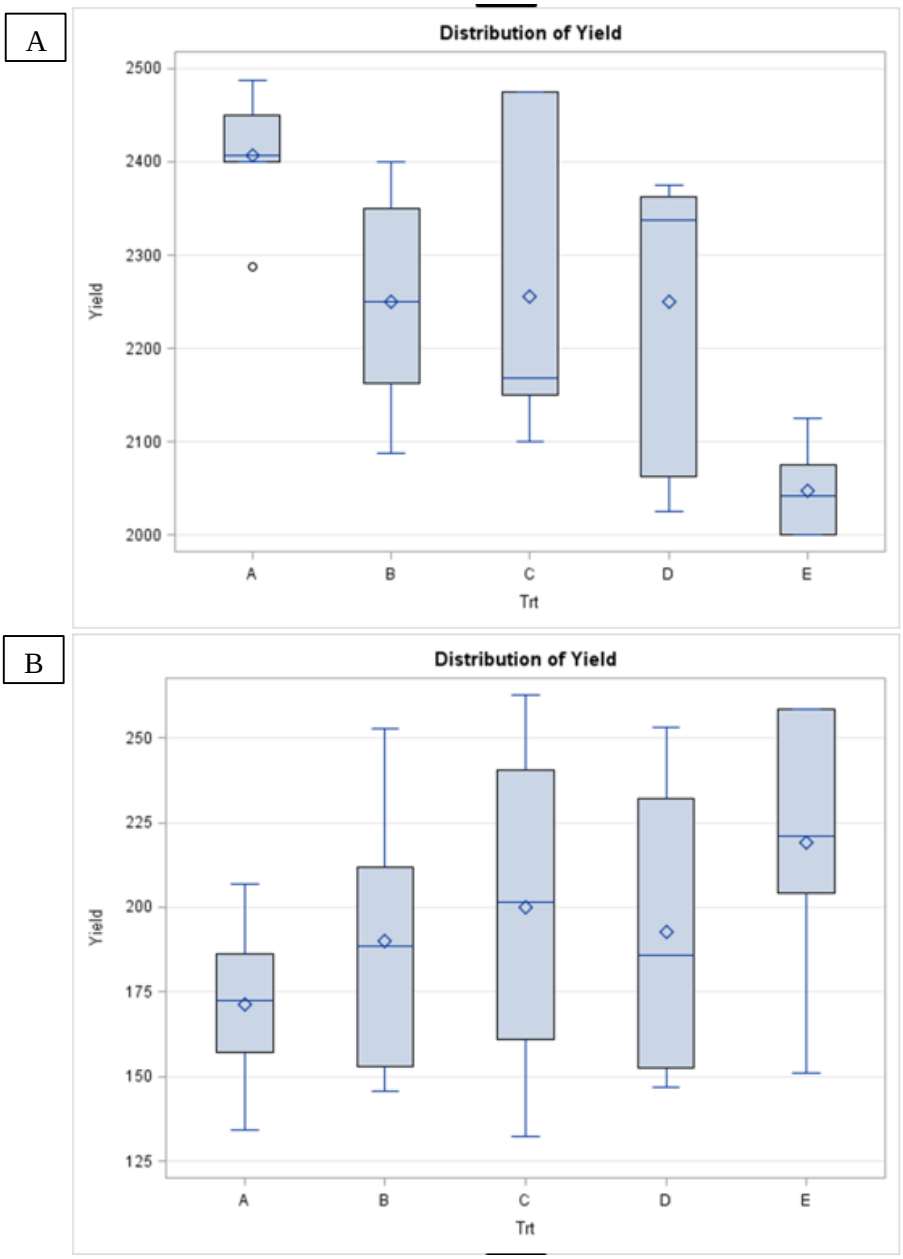


Figure 7. Distribution of yield table showing the variation of samples, A: Loaf volume, B: Firmness

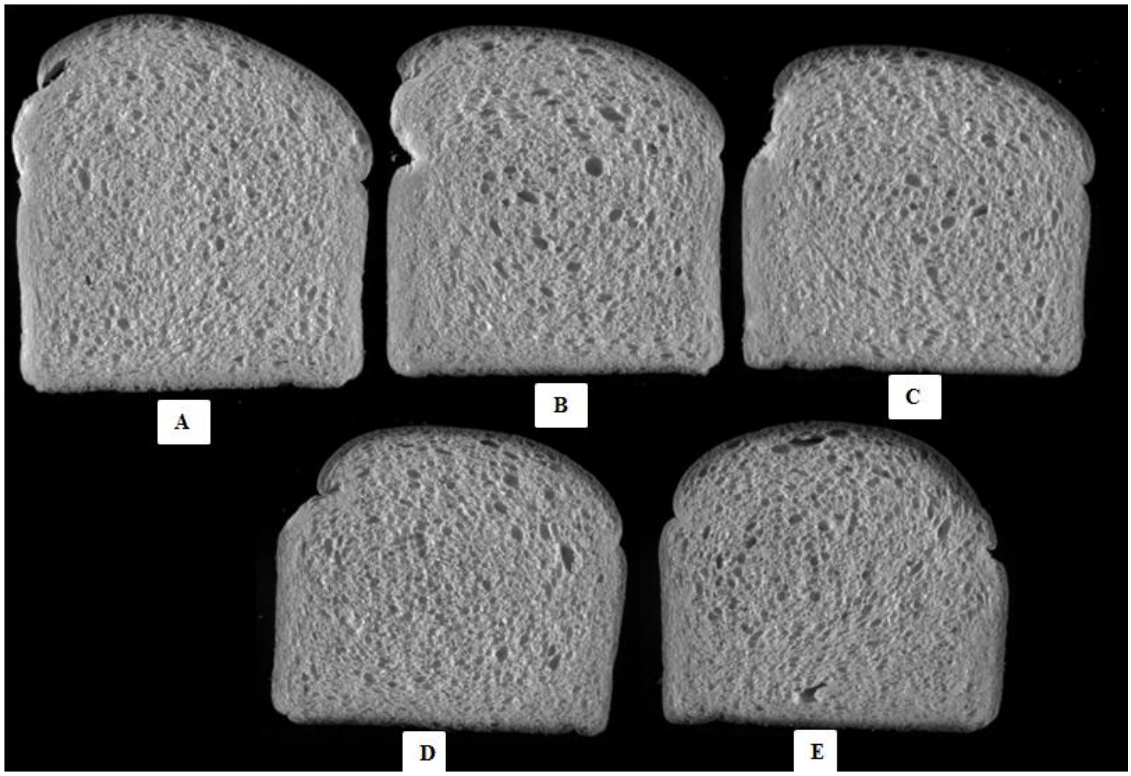


Figure 8. C-cell images of wheat bread fortified with soy protein isolate (SPI): (A) 0% SPI, (B) 3% SPI, (C) 5% SPI, (D) 8% SPI, (E) 10% SPI

Urade (2011) reported a trend of lowered bread volume and increased bread firmness at higher SPI levels. Loaf volume decreased causing bread to become firmer (Urade, 2011). In addition, lower loaf volume at higher SPI levels relates to C-cell results, reflecting the dilution of gluten. Reduction in the number of cells was observed at higher SPI levels along with a reduction of loaf volume (Table 11). However, average cell volume was not significantly different at different SPI levels (Table 11). This indicates that the cell structure of bread became more open as SPI increased, which was confirmed by the visual observation of crumb structure (Figure 8). Formation of cell structure happens strictly at the mixing stage and the number of cells formed does not change throughout the rest of the baking process, but only the size of cells change (Liu and Scanlon, 2003). High SPI levels caused dough to be more bulky and stiff, as

determined by visual observation, and this could have led to formation of a smaller number of cells.

Table 11. C-cell parameters of wheat bread fortified with 0 to 10 % soy protein isolate (SPI)

| SPI % | Slice brightness | Number of cells | Wall thickness | Cell diameter | Cell volume | Average cell elongation |
|---|---|---|---|---|---|---|
| 0 | 143 ± 1.6 a | 5901 ± 440 a* | 3.45 ± 0.1a | 15.88 ± 0.9 a | 6.77 ± 0.5 a | 1.58 ± 0.04 a |
| 3 | 138 ± 1.5 b | 5645 ± 265 a | 3.39 ± 0.1 a | 15.27 ± 0.9 a | 6.41 ± 0.5 a | 1.52 ± 0.03 a |
| 5 | 135 ± 0.8 b | 5299 ± 578 ab | 3.44 ± 0.2 a | 15.81 ± 1.3 a | 6.61 ± 0.5 a | 1.56 ± 0.07 a |
| 8 | 127 ± 3.7 c | 4959 ± 370 b | 3.47 ± 0.1a | 16.00 ±0.9 a | 7.01 ±0.7 a | 1.55 ± 0.03 a |
| 10 | 123 ± 1.8 d | 4684 ± 272 b | 3.48 ± 0.1a | 15.82 ± 1.4 a | 6.98 ± 0.7 a | 1.52 0.04 a |

*The same letter in each column indicates there is no significant difference ($p$<0.05) between treatments.

Gluten strength affects the ability of dough to retain gas within cells. Weak gluten tends to cause coalescence of gas cells during baking resulting in larger cell volume (Liu and Scanlon, 2003). This might be one of the reasons why the number of cells decreased as SPI increased, in turn causing cell volume to increase even though there was a reduction of loaf volume. However, the differences were not statistically significant, which indicates that SPI does not affect the crumb structure significantly.

**7.6. Assay tests on starch and estimated glycemic index (eGI) characteristics of bread**

Both eGI and TS of bread were statistically different ($p$<0.05) while RS was not. A 6 % reduction in TS was found as SPI increased from 0 % to 10 % (Table 12). The eGI decreased from 109 to 97 as SPI increased, which supports the hypothesis of the study.

### 7.6.1. Factors affecting estimated glycemic index (eGI)

The eGI of the SPI fortified breads were higher than 100 (Table 12). Assumption can be made that the commercial bread sample, which was used as a reference bread in the calculation had completely different properties. Commercial bread formulation tends to contain more additives such as dough strengtheners, conditioners and preservatives for extended shelf life; thus, the amount of available starch might have been less than the control bread prepared in the laboratory. In addition, commercial bread undergoes retrogradation the longer it stays on the shelf. This might have affected the digestibility of bread resulting in larger value than the reference bread. As an alternative, known amount of glucose or control white bread prepared in-house could be used as a reference sample and this problem might eliminate the issue related to store-bought bread. In fact, eGI using AUC of 0 % SPI bread instead of store-bought white bread was calculated. Result showed the trend of decreasing eGI of 0 %, 3 % and 5 % SPI as 100, 93 and 87, respectively. This further supports the idea; therefore, experimental procedure should be modified and further investigated in the future.

Increased starch-protein interaction might be partially responsible for lowered eGI. In wheat flour, starch granules are surrounded by a protein network, in which starch is protected from enzymatic hydrolysis and a reduced rate of starch hydrolysis (Jenkins et al., 1987). Upon incorporation of SPI to wheat flour, SPI is entrapped in the protein matrix hindering the function of gluten as network forming (Ribotta et al., 2005).

Table 12. Total starch (TS), resistant starch (RS) and estimated glycemic index (eGI) of wheat bread fortified with soy protein isolate (SPI)

| SPI % | TS | RS | eGI |
|---|---|---|---|
| 0 | 68.1 ± 1.5 a | 1.25 ± 0.07 a* | 109 ± 4.5 a |
| 3 | 65.9 ± 1.6 b | 1.31 ± 0.06 a | 105 ± 7.1 ab |
| 5 | 64.7 ± 2.2 bc | 1.29 ± 0.06 a | 103 ± 5.3 bc |
| 8 | 63.2 ± 1.0 cd | 1.27 ± 0.07 a | 100 ± 3.3 cd |
| 10 | 61.9 ± 2.3 d | 1.29 ± 0.03 a | 97 ± 3.0 d |

*The same letter in each column indicates there is no significant difference ($p$>0.05) between treatments.

Even though RDS, SDS and RS were not statistically different, RDS was 25.2 % for 0 % SPI and 24.1 % for 10 % SPI, whereas 11 % lower SDS was observed in the bread with 10 % SPI (Figure 9). It should be noted that RS was measured using Megazyme assay kit for higher accuracy but RDS and SDS were calculated by percent hydrolysis as a part of eGI calculation according to Englyst et al. (1992). The Englyst method states that all the fractions are inter-related as the sum of RDS, SDS and RS equals TS. However, since TS results obtained from Englyst method and Megazyme kit differed greatly, the Megazyme assay was used to obtain TS due to the accuracy that the assay kit has provided, thus the equation of TS = RDS + SDS + RS was ignored. This means that the sum of RDS, SDS and RS shown in Figure 9 does not add up to TS shown in Table 12.

Increased SDS provides the health effect of slow release of glucose over time avoiding spikes of blood glucose level (Lehmann and Robin, 2007). The trends observed in the experiment were the opposite. Higher SPI and heat treatment were expected to increase SDS; consequently, decreasing RDS since RDS and SDS are inversely related (Englyst et al., 1992). In

addition, low GI is an indication of slower blood glucose release thus lower GI should indicate increased SDS. Even though the Englyst method proved to provide relevant measurement of RDS, SDS and RS, the result might vary depending on multiple factors such as modifications and enzymes used, thus further study is necessary to investigate the accuracy of the starch fraction following the starch hydrolysis method.

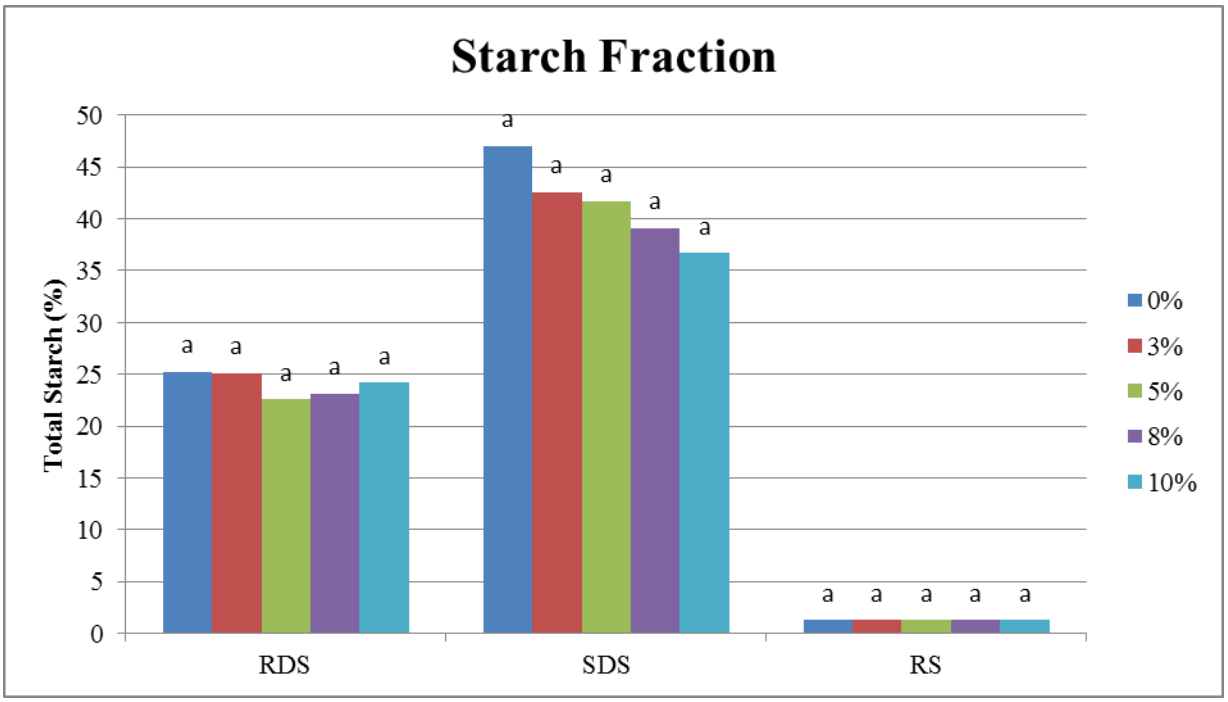


Figure 9. Rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) relative to total starch (TS). The same letter in each column indicates there is no significant difference ($p>0.05$) between treatments.

### 7.6.2. Adjusted amount of starch hydrolysis

The method to obtain eGI from starch hydrolysis involves digestion of starch. In order to determine the formulation, it is important to determine if difference of starch content in the sample affects the degree of starch digestion and therefore the eGI. In order to determine if reduction of eGI was caused by decreasing amounts of starch, the amount of starch was calculated based on the total starch content and the adjusted starch amount was calculated so that

equal amount of starch was used in the Englyst assay (Table 13). Sample size increased from 300 mg at 0 % SPI to 343.6 mg at 10 % SPI for that assay.

Table 13. Difference in the amount of starch among wheat samples fortified with soy protein isolate and adjusted amount of starch present in the samples

| SPI % | Total starch (%) | Starch amount in sample (mg) | Difference (%) | Additional starch required (mg) | Adjusted amount of sample (mg) |
|---|---|---|---|---|---|
| 0 | 66.37 | 199.11 | 0.00 | 0.00 | 300.00 |
| 3 | 66.20 | 198.60 | 0.17 | 0.77 | 300.77 |
| 5 | 63.97 | 191.91 | 2.40 | 11.26 | 311.26 |
| 8 | 62.35 | 187.05 | 4.02 | 19.34 | 319.34 |
| 10 | 57.95 | 173.85 | 8.42 | 43.58 | 343.58 |

The Englyst assay was conducted using the sample amount specified in order to determine if difference in starch amount produces the different results. Based on the eGI obtained by the Englyst assay, eGI decreased even though the starch amount was adjusted based on TS content (Table 14). This indicates that it is not starch content that affects the result but different factors that cause the lower GI. Although the relationship the between amount of starch available in the sample and digestibility of starch has been reported, Yong et al. (2011) indicated that there was no consistent trend when a fixed amount of starch was used.

Table 14. Estimated glycemic index (eGI) of wheat bread fortified with soy protein isolate (SPI) obtained after the sample were adjusted for the same starch content

| SPI % | eGI |
|---|---|
| 0 | 103 ± 0.932 |
| 3 | 102 ± 3.199 |
| 5 | 101 ± 1.566 |
| 8 | 98 ± 1.939 |
| 10 | 99 ± 0.215 |

### 7.7. Regression analysis

Correlation between SPI % and eGI was investigated using regression analysis. $R^2$ value of 0.9952 indicates that there was a good linear relationship between SPI % and eGI (Figure 10). Therefore, further regression analysis was conducted using simple linear correlation. Significant correlations ($p$<0.05) between SPI and eGI and TS were found but not between SPI and RS (Table 15). This clearly supports the hypothesis that additional SPI reduces eGI and therefore may provide health benefits.

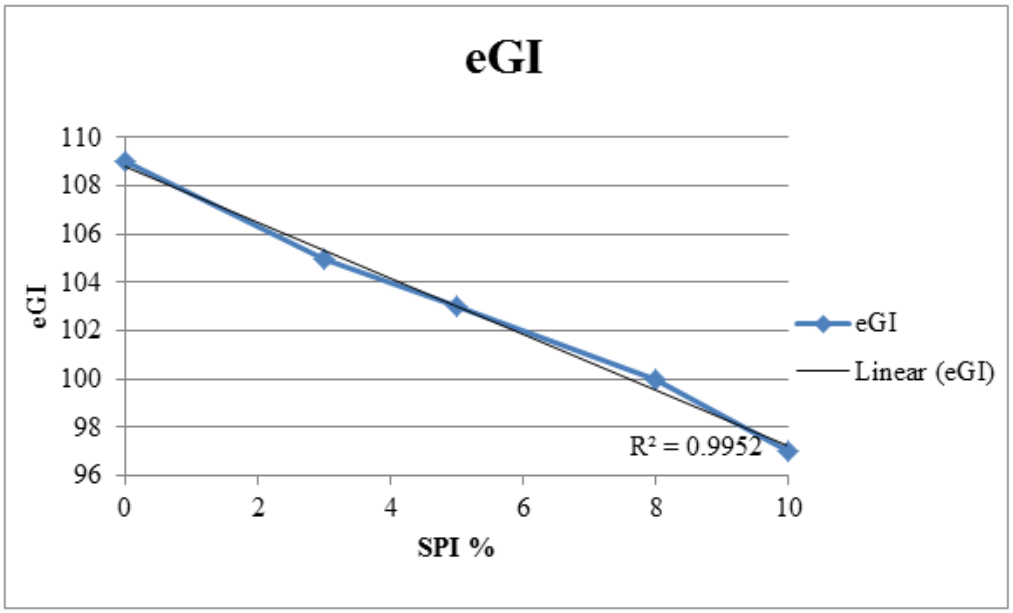


Figure 10. A simple linear correlation between percent soy protein isolate (SPI) and estimated glycemic index (eGI)

Table 15. Summary of Analysis of Variance (ANOVA) of estimated glycemic index (eGI), total starch (TS) and resistant starch (RS) obtained by regression analysis

| Source | Pr > F |
|---|---|
| Estimated glycemic index (eGI) | <.0001 ** |
| Total starch (TS) | <.0001 ** |
| Resistant starch (RS) | 0.8091 |

# 8. SUMMARY AND CONCLUSIONS

Increased SPI levels from 0 % to 10 % had a minimal effect on bread quality. Statistical analysis revealed that increased SPI levels significantly ($p<0.05$) affected wet gluten, probably due in part to the dilution of gluten by introducing non-functional proteins to the gluten matrix. However, bread quality analysis revealed that loaf volume and firmness of bread were not significantly ($p<0.05$) different, indicating SPI up to 10 % had minimal effect on bread quality. Effects of SPI differ depending on the type of SPI used, probably because of processing methods and protein composition of SPI changes the structure and functionality of protein. In order to improve bread quality further, bread conditioner or dough strengthener could be used to aid gluten strength. Furthermore, significant correlation was found between SPI and eGI supports the hypothesis that SPI aids in reducing eGI; therefore, possible health benefits may be achieved.

# 9. FUTURE WORK

Optimizing bread formula using additional ingredients such as dough conditioner and determine how it affects the ability of SPI to reduce eGI is required to commercialize bread. In addition, comparing the nature of SPI and investigating the effects on eGI will be important since understanding physiochemical properties of SPI could provide tremendous information about bread characteristics. Furthermore, developing a standard procedure for eGI analysis will be necessary to increase the use and popularity of eGI. Increasing reproducibility of the test will be the main focus on developing standard procedure, and using glucose or bread containing 0 % SPI in place of standard white bread should be tested to avoid variance caused by difference of ingredients and baking procedure. Furthermore, acceptance of bread sample should be tested in the sensory test with trained panelists.

## REFERENCES

AACC International. 2002a. Approved Methods of Analysis, 11th Ed. Method 10-05. Guideline for measurement of volume by rapeseed displacement method. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002b. Approved Methods of Analysis, 11th Ed. Method 74-09.01. Measurement of bread firmness by universal testing machine. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002c. Approved Methods of Analysis, 11th Ed. Method 44-15.02. Moisture - Air-oven method. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002d. Approved Methods of Analysis, 11th Ed. Method 08-01.01. Ash - Basic method. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002e. Approved Methods of Analysis, 11th Ed. Method 46-30.01. Crude protein - combustion method. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002f. Approved Methods of Analysis, 11th Ed. Method 54-21.02. Rheological behavior of flour by Farinograph: Constant flour weight procedure. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002g. Approved Methods of Analysis, 11th Ed. Method 76-21.01. General pasting method for wheat or rye flour or starch using the rapid visco analyzer. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

AACC International. 2002h. Approved Methods of Analysis, 11th Ed. Method 38-12.02. Wet gluten, dry gluten, water-binding capacity, and gluten index. Approved November 3, 1999. AACC International, St. Paul, MN, USA.

Alonso, R., Aguiirre, A., Marzo, F. 2000. Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. Food Chemistry 68, 159-165.

Anonymous. American Diabetes Association. 2014. Glycemic index and diabetes. Retrieved from http://www.diabetes.org/food-and-fitness/food/what-can-i-eat/understanding-carbohydrates/glycemic-index-and-diabetes.html (Accessed March 31, 2014).

Anonymous. Center for disease control and prevention (CDC). 2011 National diabetes fact sheet. Retrieved from http://www.cdc.gov/diabetes/pubs/factsheet11.htm (Accessed September 30, 2013).

Anonymous. Rapid visco analyzer operation manual. 2007. Newport Scientific Pty Ltd., Warriewood NSW, Australia.

Anonymous. University of Sydney. 2012. About Glycemic Index. Retrieved from http://www.glycemicindex.com/about.php (Accessed September 23, 2013).

Bainy, E., Corredig, M., Poysa, V., Eoodrow, L., Tosh, S. 2010. Assessment of the effects of soy protein isolates with different protein composition on gluten thermosetting gelation. Food Research International 43, 1684-1691.

Berk, Z., 1992. Isolated Soy protein. In: Food and agriculture organization. Technology of production of edible flours and protein products from soybeans. Food and Agriculture Organization by United Nations Agricultural Service Bulletin No. 97.

Bonfil, J., Posner, S. 2012. Can bread wheat quality be determined by gluten index? Journal of Cereal Science 56, 115-118.

Bornet, F., Fontvieille, A., Rizkalla, S., Colonna, P., Blayo, A., Mercier, C., Slama, G. 1989. Insulin and glycemic response in healthy humans to native starches processed in different ways: correlation with in vitro α-amylase hydrolysis. Journal of Clinical Nutrition 50, 315-323

Cederroth, C., Nef, S. 2009. Soy, phytoestrogens and metabolism: A review. Molecular and Cellular Endocrinology 304, 30-42.

Chen, L., Chen, J., Ren, J., Zhao, M. 2011. Modifications of soy protein isolates using combined extrusion pre-treatment and controlled enzymatic hydrolysis for improved emulsifying properties. Food Hydrocolloids 25, 887-897.

Chen, S., Rasper, V. 1982a Functionality of soy protein in wheat flour/soy isolate doughs. I. Characterization of isolates prepared using different isolation procedure. Canadian Institute of Food Science and Technology Journal 15, 203-210.

Chen, S., Rasper, V. 1982b Functionality of soy protein in wheat flour/soy Isolate doughs. II. Rheological properties and bread making potential. Canadian Institute of Food Science and Technology Journal 15, 211-220.

Chung, H., Liu, Q., Hoover, R., Warkentin, T., Vandenberg, B. 2008. In vitro starch digestibility, expected glycemic index, and thermal and pasting properties of flours from pea, lentil and chickpea cultivars. Food Chemistry 111, 316-321.

Colombani, P. 2004. Glycemic index and load – Dynamic dietary guidelines in the context of disease. Physiology and Behavior 83, 603-610.

Dewettinck, K., Van Bockstaele, F., Kuhne, B., Van de Walle, D., Courtens, T., Gellynck, X. 2008. Nutritional value of bread: Influence of processing, food interaction and consumer perception. Journal of Cereal Science 48, 243-257.

Egbert, W. 2004. Isolated soy protein: Technology, properties, and applications. In: Liu, K. (Eds.), Soybeans as functional foods and ingredients. American oil chemist's society (AOCS) Press, Champaign, IL, pp. 134-162.

El, N. 1999. Determination of glycemic index for some breads. Food Chemistry 67, 67-69.

Englyst, H., Kingman, S., Cummings, J. 1992. Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition 46, 33-50.

Englyst, K., Vinoy, S., Englyst, H., Lang, V. 2003. Glycaemic index of cereal products explained by their content of rapidly and slowly available glucose. British Journal of Nutrition 89, 329-339.

Food and Drug Administration (FDA). 2012. Code of federal regulations 21, 101.82. Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.82 (Accessed October 29, 2013).

Food and Drug Administration (FDA). Code of federal regulation Title 21. Reference amount customarily consumed per eating occasion. Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.12 (Accessed October 29, 2013).

Frost, G., Dornhorst, A. 2013. Glycemic Index. In: Caballero, B., Allen, L., Prentice, A., (Eds.), Encyclopedia of human nutrition volume 2. Academic Press, MA, pp. 393-398.

Gellar, L., Nansel, R. 2009. High and low glycemic index mixed meals and blood glucose in youth with Type 2 diabetes or impaired glucose tolerance. Journal of Pediatrics 154, 455-458.

Cilion et al. 2000. In: Gibson, G., Williams, C. Functional foods. CRC Press LLC. Boca Raton, FL. pp. 315-364.

Goni, I., Garcia-Alonso, A., Saura-Calixto, F. 1997. A Starch hydrolysis procedure to estimate glycemic index. Nutritional Research 17, 427-437.

Granfeldt, Y., Bjorck, I., Drews, A. 1992. An in vitro procedure based on chewing to predict metabolic response to starch in cereal and legume products. European Journal of Clinical Nutrition 46, 649-660.

Hera, E., Gomez, M., Rosell, C. 2013. Particle size distribution of rice flour affecting the starch enzymatic hydrolysis and hydration properties. Carbohydrate Polymers 98, 421-427.

Hermansson, A. 1986. Soy protein gelation. Journal of American oil chemists' society 63, 658-666.

Hoover, R., Zhou, Y. 2003. In vitro and in vivo hydrolysis of legume starches by α-amylase and resistant starch formation in legumes – A review. Carbohydrate Polymers 54, 401-417.

Hughes, G., Ryan, D, Mukherjea, R., Schasteen, C. 2011. Protein digestibility-corrected amino acid scores (PDCAAS) for soy protein isolate and concentrate: Criteria for evaluation. Journal of Agriculture and Food Chemistry 59, 12707-12712.

Ivanovski, B., Seetharaman, k., Duizer, M. 2011. Development of soy-based bread with acceptable sensory properties. Journal of Food Science 71, 71-76.

Jenkins, D., Thorne, M., Wolever, T., Jenkins, A., Rao, V., Thompson, L. 1987. The effect of starch-protein interaction in wheat on the glycemic response and rate of in vitro digestion. American Journal of Clinical Nutrition 45, 946-951.

Jenkins, D., Wolver, T., Jenkins, A. 1988. Starchy foods and glycemic Index. Diabetes Care 11, 149-159.

Kim, H., Morita, N., Lee, S., Moon, K. 2003. Scanning electron microscopic observations of dough and bread supplemented with gastrodia elata blume powder. Food Research International 36, 387-397.

koksel, F., Scanlon, M. 2012. Effects of composition on dough development and air entrainment in doughs made from gluten-starch blends. Journal of food science 56, 445-450.

Krishnan, P., Darly-Kindelspire, J. 2013. Food product innovations using soy ingredients. Journal for Human Nutrition and Food Science 1, 1011.

Lai, Y., Mondor, M., Moresoli, C., Drolet, H., Gros-Louis, M., Ippersiel, D., Lamarche, F., Arcand, Y. 2013. Production of soy protein isolate with low phytic acid content by membrane technologies: Impact of the extraction and ultrafiltration/diafiltration condition. Journal of Food Engineering 114, 221-227.

Larsen, R. 1963. Hydration as a factor in bread flour quality. Journal of Cereal Chemist 41, 181 - 187.

Larson, N., Greenwood, D. 1990. Water addition and the physical properties of mechanical dough development doughs and breads. Journal of Cereal Science 13, 195-205.

Lehmann, U., Robin, F. 2007. Slowly digestible starch - its structure and health implications: A review. Trends in Food Science and Technology 18, 346-355.

Lim, H., Narsimhan, G. 2006. Pasting and rheological behavior of soy protein-based pudding. LWT 39, 343-349.

Liu, Z., Scanlon, G. 2003. Predicting mechanical properties of bread crumb. Food and Bioproducts Processing 81, 224-238.

Lukaczer, D., Liska, D., Lerman, R., Darland, G., Schiltz, B., Tripp, M., Bland, J. 2006. Effect of a low glycemic index diet with soy protein and phytosterol on CVD risk factors in postmenopausal women. Nutrition 22, 104-113.

Ma, X., Liu, J., Song, Z. 2012. Glycemic load, glycemic index and risk of cardiovascular disease: meta-analyses of prospective studies. Atherosclerosis 223, 491-496.

Maforimbo, E., Skurray, G., Uthayakumran, S., Wringley, C. 2008. Incorporation of soy proteins into the wheat-gluten matrix during dough mixing. Journal of Cereal Science 47, 380-385.

Mann, J., Leeuw, D., Hermansen, K., Karamanos, B., Karlstrom, B., Katsilambros, N., Riccardi, G., Rivellese, A., Rizkalla, S., Slama, G., Toeller, M., Uusitupa, M., Vessby, B. 2004. Evidence-based nutritional approaches to the treatment and prevention of diabetes mellitus. Nutrition, Metabolism, Cardiovascular Disease 14, 373-394.

Marangoni, F., Poli, A. 2008. The glycemic index of bread and biscuits is markedly reduced by the addition of a proprietary fiber mixture to the ingredients. Nutrition, Metabolism, and Cardiovascular Disease 18, 602-605.

Marchais, L., Foisy, M., Mercier, S., Villeneuve, S., Mondor, M. 2011. Bread-making potential of pea protein isolate produced by a novel ultrafilitration/diafiltration process. Procedia Food Science 1, 1425-1430.

Martins, V., Netto, F. 2006. Physiochemcial and functional properties of soy protein isolate as a function of water activity and storage. Food Research International 39, 145-153.

Miao, M., Zhang, T., Mu, W., Jiang, B. 2011. Structural characteristics of waxy maize starch residue following in vitro pancreatic and amyloglucosidase synergistic hydrolysis. Food Hydrocolloids 25, 214-220.

Molina, E., Papadopoulou, A., Ledward, D. 2001. Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. Food Hydrocolloids 15, 263-269.

Petruccelli, S., Anon, M. 1994. Relationship between the method of obtention and the structural and functional properties of soy protein isolates. 2. Surface properties. Journal of Agriculture and food chemistry 42, 2170 - 2176.

Pyler, J., Gorton, A. 2010. Baking Science and Technology Volume I: Fundamentals and ingredients Forth ed. Sosland Publishing Company. Kansas City, MO.

Ribotta, P., Arnulphi, S., Leon, A., Anon, M. 2005. Effect of soybean addition on the rheological properties and bread making quality of wheat flour. Journal of the Science of Food and Agriculture 85, 1889-1896.

Ribotta, R., Colombo,A., Leon, A., Anon, M. 2007. Effects of soy protein on physical and rheological properties of wheat starch. Starch 59, 613-624.

Roccia, P., Ribotta, P., Perez, G., Leon, A. 2009. Influence of soy protein on rheological properties and water retention capacity of wheat gluten. LWR- Food Science and Technology 42, 358-362.

Ryan, J., Brewer, S. 2007. In situ examination of starch granule-soy protein and wheat protein interactions. Food Chemistry 104, 619-629.

Shuey, W. 1984. Interpretation of Farinograph. In: D'Appolonia. B., Kunerth, W. (Eds.), 1984. The Farinograph handbook third ed. American Association of Cereal Chemist Inc. St. Paul, MN, pp. 31-37.

Simon, C., Hall, C., Tulbek, M. 2012. Effects of extruder screw speeds on physical properties and in vitro starch hydrolysis of precooked pinto, navy, red, and black bean extrudates. Cereal Chemistry 89, 176-181.

Singth, J., Dartois, A., Kaur, L. 2010. Starch digestibility in food matrix: A review. Trends in Food Science and Technology 21,168-180.

Slack, F., Lichtenstein, A., Van Horn, L., Harris, W., Kris-Etherton, P., Winston, M. 2006. Soy protein, isoflavones, and cardiovascular health: An American heart association science advisory for professional from the Nutrition Committee. Circulation 113, 1034-1044.

Snow, P., O'Dea, K. 1981. Factors affecting the rate of hydrolysis of starch in food. American Journal of Clinical Nutrition 34, 2721-2727.

Sorgentini, D., Wagner, J., Anon, M. 1995. Effects of thermal treatment of soy protein isolate on the characteristics and structure - Function relationship of soluble and insoluble fractions. Journal of Agriculture Food Chemistry 43, 2471-2479.

Stauffer, C. 2005. Soy protein baking second edition. Retrieved from http://www.asaim-europe.org/BACKUP/pdf/Soy%20Protein%20in%20Bakery%20%20update.pdf (Accessed November 13, 2013).

Thorne, M., Thompson, L., Jenkins, D. 1983. Factors affecting starch digestibility and the glycemic response with special reference to legumes. American Journal of Clinical Nutrition 38, 481-488.

Urade, R. 2011. Fortification of bread with soy proteins to normalize serum cholesterol and triacylglycerol levels. In: Preedy, V., Watson, R., Patel, B., Flour and Breads and their Fortification in Health and Disease Prevention. Academic Press, Waltham, MA, pp. 417-427.

United states department of agriculture (USDA). 2013. National Agricultural Library Nutrient Data Laboratory. Retrieved from http://ndb.nal.usda.gov/ndb/foods/show/4721?fg=&man=&lfacet=&count=&max=25&sort=&qlookup=soybeans&offset=&format=Full&new= (Accessed October 9, 2013).

Visschers, R., de Jongh, H. 2005. Disulphide bond formation in food protein aggregation and gelation. Biotechnology Advances 23, 75-80.

Whistler, R., Daniel, J. 2007. Carbohydrates. In: Fennema, O., (Ed.), Food Chemistry Forth edition. Marcel Dekker, Inc. New York, pp. 69-137.

Wolver, T., Jenkins, D., Jenkins, A., Hosse, R. 1991. The glycemic index: Methodology and clinical implications. American Journal Clinical Nutrition 54, 846-854.

Yong, Z., Chan, H., Garcia, C., Sopade, A. 2011. Weighing up whey fortification of foods: Implications for kinetics of starch digestion and estimated glycemic index of model high-protein-low-carbohydrate food systems. Carbohydrate Polymers 84, 162-172.

Zabidi, A., Aziz, A. 2009. *In vitro* starch hydrolysis and estimated glycemic index of bread substituted with different percentage of chempedak (*Aetocarpus integer*) seed flour. Food Chemistry 117, 64-68.

# APPENDIX

## A.1. Englyst, RDS, SDS and RS assay procedures

i. Materials (To analyze 14 tubes/day, if more tubes are to be analyzed, change the amount of buffer and enzymes)

a. Sodium acetate buffer (pH 5.2)

b. Saturated benzoic acid (Stable at RT)

- Add 2 g of benzoic acid to 400 ml dd water and stir for 8 hours.

c. 1M Calcium Chloride solution (Stable for 2 weeks at RT)

- Weigh 14.7 g $CaCl \cdot 2H_2O$

- Bring volume to 100 ml\

d. 0.1M sodium acetate buffer (pH 5.2) – **Stable for 48 HOURS**

- Dissolve 4.1 g sodium acetate (anhydrous) in 125 ml saturated benzoic acid.

- Bring volume to 500ml with dd water.

- Adjust the pH to 5.2 with 0.1 M acetic acid (5.8 mL/L) - apprx. 50ml

- Add 2 ml CaCl solution per 500 ml

e. Glucose standard solution (5mg/ml)

- Weight 100 mg glucose.

- Bring volume to 20 ml with sodium acetate buffer.

f. Amyloglucosidase solution: 140 AGU/ml (Megazyme)

- Bring 1.07 ml concentrated AMG to 25 ml with dd water.

g. Invertase (sigma I-4504)

- Weigh 45 mg invertase.

- Add 6 ml dd water.

- Stir for 5 minutes with magnetic stirrer.

h. Pancreatin solution (sigma P-7545) – **Prepare fresh every day**.

- Weigh 3g pancreatin into each of 5 centrifuge tubes.
- Add 20 ml dd water to each of tubes.
- Stir for 10 minutes at 4 $^{o}$C.
- Centrifuge for 10 minutes at 3000g

i. Enzyme solution mix – **Use IMMEDIATELY after preparation**.

- Mix 9 ml AMG solution + 81 ml pancreatin solution + 6 ml invertase solution.

j. Glucose standard ( 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg/ml) – Stable for 1-2 weeks

- Weight 125, 50, and 25 mg of dry glucose.
- Bring the volume to 25 ml to make the 1.0, 2.0, and 5.0 mg/ml solution.
- Dilute a portion of each solution by 10x to make 0.1, 0.2 and 0.5 mg/ml solution.

k. Absolute ethanol

l. Guar gum (sigma G-4129)

m. GOPOD kit (Megazyme K-GLUC)

n. Glass or marble beads (1.5 cm diameter)

ii. Procedure

1. Pour 5 ml of absolute ethanol in 15ml centrifuge tube (a day before).
2. Make buffer if necessary.
3. Weigh out samples and guar gum.
4. Make enzyme solution.

iii. Readily Digetible Starch (RDS) and Slowly Digestible starch (SDS)

1. Weigh 300 mg sample into duplicate tubes and 500 mg of bread standard (2x).
2. Prepare glucose standard tubes and one blank tube (acetate buffer).
3. Add 20 ml sodium acetate buffer.

- For not-cooked samples

  Place in boiling water bath for 30 minutes.

  Place in 37 $^{o}$C water bath and wait to equilibrate.

- For cooked samples including bread samples

4. Add 50 mg guar gum and 5 glass beads to each tube.
5. Shake (200 strokes/min) tubes in water bath (37 $^{o}$C) for 10 min to disperse contents.
6. Add 5 ml enzymes to each tube at 1 minute intervals timing from each addition. (see timetable)
7. Every 20 min, remove 0.5 ml of each sample into a tube containing 5 ml absolute ethanol and shake (G20, 40, 60, 80, 100, 120, 140, 160 and 180).
8. Centrifuge the tubes at 1500 g for 10 minutes and save the supernatant.

iv. Glucose determination

9. Add 0.1 ml water for blank and 0.1 ml for glucose standards (6 tubes x 2) to test tubes.
10. Take 0.1 ml from each tube and add to test tubes.
11. Add 3 ml GOPOD.
12. Incubate at 50 $^{o}$C for 20 min.
13. Read absorbance at 510 nm.

v. Calculation

1. Corrected std. abs. = (Std. abs.) – (std. blank abs.)
2. Corrected sample abs. = (sample abs.) – (sample blank abs.)
3. Create glucose std. curve and obtain equation. Use the equiation to calculate Glu (mg/ml) for each sample and blank.
4. Y=mx+b → m(corrected sample abs.) + b= y = Glc (mg/ml)

   Use y = corrected sample abs. - b) / m
5. Corrected Glu = Glu x 5.5 x 25
6. (CGlu) / (Sample wt) = % Glucose released
7. (% Glu released) x 0.9 x 100= % starch digested*

*DO not use this if using the 0.9 multiplier in the following step.

% Rapidly digested starch = (G20) x 0.9

% Slowly digestible starch = (G120 – G20) x 0.9

% Resistant starch = (G0 x 0.9) – (RDS + SDS) or (TS) – (RDS + SDS)\

**A.2. Total starch assay procedure**

1. Add 100 mg of samples to a glass test tube and add 0.2 ml of ethanol to wet the sample. Stir in vortex.
2. Add 3 ml of α-amylase and incubate the tube in a boiling water bath for 6 min.
3. Place the tubes in 50 $^{o}$C water bath and add 0.1 ml of AMG. Incubate for 30 min.

4. Transfer the solution to 100 ml volumetric flask and adjust the volume to 10 ml with DI water.
5. Centrifuge an aliquot at 3000 rpm for 10 min. Take 1 ml of solution and diluted to 10 ml with DI water.
6. Transfer 0.1 ml (X2) of solution to glass test tubes and control and blank.

   Control – 0.1 ml of D-glucose std

   Blank – 0.1 ml of water
7. Add 3.0 ml of GOPOD reagent to each tube and incubate the tubes for 20 min at 50 ºC.
8. Read Absorbance at 510 nm.

### A.3. Supplemental information of baking and starch assay

**Table A.1. Bread formulation in baker's percent**

| Treatment | 0% % | 3% % | 5% % | 8% % | 10% % |
|---|---|---|---|---|---|
| White Flour | 100 | 97 | 95 | 92 | 90 |
| SPI | 0 | 3 | 5 | 8 | 10 |
| Instant Yeast | 1 | 1 | 1 | 1 | 1 |
| Shortening | 6 | 6 | 6 | 6 | 6 |
| Salt | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Sugar | 6 | 6 | 6 | 6 | 6 |
| Water | 68.9 | 70.3 | 70.9 | 72 | 73.5 |
| Total | 183.4 | 184.8 | 185.4 | 186.5 | 188 |

**Table A.2. eGI result with adjusted starch and comparison**

| Adjusted starch | 1 | | 2 | |
|---|---|---|---|---|
| | eGI | SD | eGI | SD |
| 0% | 103 | ± 0.392 | 94 | ± 0.190 |
| 3% | 102 | ± 3.199 | 90 | ± 1.170 |
| 5% | 101 | ± 1.566 | 88 | ± 0.152 |
| 8% | 98 | ± 1.939 | 83 | ± 2.706 |
| 10% | 99 | ± 0.215 | 84 | ± 0.890 |

## A.4. ANOVA results of regression analysis

**Table A.3. ANOVA results of regression analysis between soy protein isolate and estimated glycemic index (eGI)**

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Sum of Squares** | **Mean Square** | **F Value** | **Pr > F** |
| **Model** | 1 | 170.70460 | 170.70460 | 23.19 | <.0001** |
| **Error** | 28 | 206.09540 | 7.36055 | | |
| **Corrected Total** | 29 | 376.80000 | | | |

**Table A.4. ANOVA results of regression analysis between soy protein isolate and total starch (TS)**

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Sum of Squares** | **Mean Square** | **F Value** | **Pr > F** |
| **Model** | 1 | 239.77935 | 239.77935 | 49.00 | <.0001** |
| **Error** | 28 | 137.02065 | 4.89359 | | |
| **Corrected Total** | 29 | 376.80000 | | | |

**Table A.5. ANOVA results of regression analysis between soy protein isolate and resistant starch (RS)**

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Sum of Squares** | **Mean Square** | **F Value** | **Pr > F** |
| **Model** | 1 | 0.75261 | 0.75261 | 0.06 | 0.8091 ns |
| **Error** | 23 | 289.78739 | 12.59945 | | |
| **Corrected Total** | 24 | 290.54000 | | | |